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**EPIGENETICALLY REGULATED
MICRO-RNA-193B TARGETS *CCND1*
IN PROSTATE CANCER**

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ABSTRACT

Kirsi Kaukoniemi: Epigenetically regulated microRNA-193b targets *CCND1* in prostate cancer

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Prostate cancer (PC) is the most commonly diagnosed cancer and one of the leading causes of cancer-related deaths in Europe. While the majority of patients are cured, some cases progress to advanced stages for which no curative treatments are available. Although current diagnostic methods are effective in detecting PC, they struggle to efficiently distinguish indolent tumors from aggressive ones, often leading to overtreatment. Consequently, new biomarkers are needed for both diagnostic and therapeutic purposes.

Epigenetic modifications regulate gene expression without affecting the underlying DNA sequence. Among these, DNA hypermethylation of gene promoters is the most extensively studied epigenetic alteration, typically associated with transcriptional silencing.

MicroRNAs (miRNAs) are small RNA molecules that regulate gene expression post-transcriptionally and are integral components of the epigenetic regulatory network. Like protein-coding genes, miRNA genes are subjected to regulation at both genetic and epigenetic levels. In PC and other cancers, many miRNAs are differentially expressed.

The aim of this study was to validate the previously observed hypermethylation and reduced expression of miR-193b in PC and investigate whether miR-193b targets *CCND1* in prostate cancer. *CCND1* encodes for cyclin D1 protein, a key regulator of G1/S transition in the cell cycle. The results revealed that miR-193b is hypermethylated in PC samples compared to benign prostatic hyperplasia (BPH), and its expression is lower in more advanced pT3-stage prostatectomy tumors compared to pT2-stage tumors.

The interaction between miR-193b and *CCND1* was investigated using various methods. Expression studies in 22Rv1 cells, which exhibit low endogenous miR-193b and high *CCND1* levels, showed that forced overexpression of miR-193b led to reduced *CCND1* mRNA and cyclin D1 protein levels. Additionally, the phosphorylation of RB, a target of the cyclin D1-CDK4/6 complex, was reduced. Luciferase assays demonstrated that miR-193b binds to the *CCND1* 3'UTR and this result was validated through rescue assay.

Cyclin D1 expression in clinical samples was evaluated using immunohistochemistry (IHC) on tissue microarrays. IHC analysis showed that cyclin D1 expression is elevated in castration-resistant prostate tumors compared to hormone-naïve tumors. Furthermore, a CDK4/6 inhibitor was applied to PC cell lines with varying endogenous levels of miR-193b and *CCND1* expression. Growth of 22Rv1 and VCaP cells, which exhibit low endogenous miR-193b and high *CCND1* expression, was significantly reduced following inhibitor treatment. In contrast, the inhibitor had no effect on the growth of PC-3 and DU145 cells, which have high endogenous miR-193b and low *CCND1* expression.

Altogether, the data presented in this thesis demonstrates that miR-193b targets cyclin D1 in prostate cancer and that *MIR193B* promoter is hypermethylated and its expression is reduced in PC tumors.

Keywords: Prostate cancer, microRNA (miRNA), miR-193b, *CCND1*, cyclin D1, hypermethylation

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TIIVISTELMÄ

Kirsi Kaukoniemi: *CCND1* on epigeneettisesti säädellyn mikroRNA-193b:n kohdegeeni eturauhassyövässä

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Eturauhassyöpä on miesten eniten diagnosoitu syöpä ja yksi yleisimmistä kuolemaan johtavista syöpätyypeistä Euroopassa. Suurin osa sairastuneista potilasta paranee, mutta joissain tapauksissa tauti etenee tilanteeseen, johon ei ole parantavaa hoitoa tarjolla. Nykyiset diagnostiset menetelmät ovat tehokkaita toteamaan eturauhassyövän, mutta ne eivät pysty erottamaan hitaasti kasvavia syöpiä aggressiivisista, nopeasti leviävistä syövästä, mikä voi johtaa vähäoireisten syöpien liialliseen hoitamiseen. Tämän vuoksi on suuri tarve löytää tehokkaampia merkkiaineita syövän diagnosointiin ja hoitoon.

Epigeneettiset muutokset säätelevät geenien ilmenemistä muuttamalla DNA-sekvenssiä. Eniten tutkittu epigeneettinen muutos on geenien promootorialueella tapahtuva DNA:n hypermetylaatio, joka tyypillisesti johtaa geenin ilmenemisen hiljentymiseen.

Mikro-RNAt (miRNAt) ovat lyhyitä RNA-molekyylejä, josta säätelevät geenien ilmenemistä transkription jälkeen sitoutumalla kohdegeeniin lähetti-RNA-molekyylin. Mikro-RNAt ovat osa epigeneettistä säätelyjärjestelmää ja niitä säädelään samalla tavoilla geneettisesti ja epigeneettisesti kuin proteiinia koodavia geenejäkin. Eturauhassyövässä ja muissa syövässä on havaittu useita miRNA-molekyylejä, joiden ilmeneminen tai toiminta on normaalista poikkeavaa.

Tämän tutkimuksen tavoite oli varmistaa aiemmin julkaistussa tutkimuksessa todettu *MIR193B*:n hypermetylaatio ja vähentynyt ilmeneminen eturauhassyövässä sekä tutkia, onko *CCND1* miR-193b:n kohdegeeni eturauhassyövässä. *CCND1* koodaa sykliini D1 proteiinia, joka on merkittävä säätelijä solusyklin etenemisessä G1-vaiheesta S-vaiheeseen. Tulosten perusteella *MIR193B* on hypermetyloitunut eturauhassyövässä verrattuna hyvänlaatuiseseen eturauhasen liikakasvuun. Lisäksi miR-193b:n ilmeneminen on vähäisempää edenneen pT3-tason kasvaimissa kuin pT2-tason prostatektomiakasvaimissa.

miR-193b:n ja *CCND1*:n vuorovaikutusta tutkittiin usealla menetelmällä. miR-193b:n, *CCND1*:n ja sykliini D1:n ilmenemistä tutkittiin 22Rv1 soluissa, joissa miR-193b ilmenee luonnostaan hyvin matalalla tasolla ja *CCND1* korkealla tasolla. Näissä soluissa miR-193b:n yliekspressio aiheutti *CCND1*:n ilmenemisen vähenemisen sekä RNA- että proteiinitasolla. Lisäksi sykliini D1-CDK4/6 -kompleksin kohdeproteiinin RB:n fosforylaatiotaso laski. Lusiferaasireportterikokeella todettiin, että miR-193b sitoutuu *CCND1*-geenin 3'UTR-alueelle ja tulos validoitiin rescue-kokeella. Molemmissa kokeissa käytettiin 22Rv1-soluja.

Sykliini D1 -proteiinin ilmenemistä kliinisissä eturauhassyöpänäytteissä tutkittiin immunohistokemiallisella analyysillä. Analyysin tulosten perusteella sykliini D1 -proteiini ilmenee enemmän kastroatioresistenteissa tuumoreissa kuin prostatektiomiatiuumoreissa. Lisäksi eri eturauhassyöpäsoluja käsiteltiin CDK4/6-inhibiittorilla. Inhibiittorin vaikutukset eri solulinjoihin riippui niiden luontaisesta miR-193b- ja *CCND1*-ilmenemistasoista. Esimerkiksi 22Rv1- ja VCaP-soluissa, joissa on luonnostaan matala miR-193b-taso ja korkea *CCND1*-taso, inhibiittori aiheutti merkittävän kasvuhidastuman. Sen sijaan luonnostaan korkean miR-193b:n ja matalan *CCND1*:n ekspression omaavien PC-3- ja DU145-solujen kasvuun inhibiittorikäsittelyllä ei ollut vaikutusta.

Tulosten perusteella *CCND1* on miR-193b:n kohdegeeni eturauhassyövässä. Lisäksi *MIR193B*:n promootorialue on hypermetyloitunut ja sen ekspressio on vähentynyt eturauhassyövässä.

Avainsanat: Eturauhassyöpä, mikro-RNA (miRNA), miR-193b, *CCND1*, cyclin D1, hypermetylaatio

Tämän julkaisun alkuperäisyys on tarkastettu Turnitin OriginalityCheck -ohjelmalla.

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PREFACE

This work was carried out in the Molecular Biology of Prostate Cancer research group at Tampere University in the Faculty of Medicine and Health Technology and the former University of Tampere in the Faculty of Medicine and Life Sciences, BioMediTech, and Institute of Biotechnology.

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LIST OF SYMBOLS AND ABBREVIATIONS

5hmC	5-hydroxymethylcytosine
5mC	5-methylcytosine
ADT	androgen deprivation therapy
AR	androgen receptor
BPH	benign prostate hyperplasia
CCND1	cyclin D1
CDK	cyclin-dependent kinase
CGI	CpG islands
CIP	CDK-interacting protein
CKI	cyclin-dependent kinase inhibitor
CpG	cytidine-guanidine dinucleotide
CRPC	castration-resistant prostate cancer
DHT	5 α -dihydrotestosterone
DNA	deoxyribonucleic acid
DNMT	DNA methyltransferase
DRE	digital rectal examination
DSB	DNA double-stranded break
FDA	U.S. Food and Drug Administration
G0	non-proliferative (quiescent) phase of cell cycle
G1	Gap 1 phase of cell cycle
G2	Gap 2 phase of cell cycle
GS	Gleason score
GSTP1	Glutathione S-transferase pi 1
HAT	histone acetyltransferases
HDAC	histone deacetylases
IHC	immunohistochemistry
ISUP	International Society of Urological Pathology
KIP	kinase inhibitory protein
KMT	histone lysine methyltransferases
lncRNA	long non-coding RNA
MBD	methyl-binding domain
mCRPC	metastatic castration-resistant prostate cancer
mCSPC	metastatic castration-sensitive prostate cancer
miRNA	micro-RNA
MRI	magnetic resonance imaging
PC	prostate cancer
PCR	polymerase chain reaction
PD0332991	CDK4/6 inhibitor palbociclib
PIN	prostatic intraepithelial neoplasia
PMD	partially methylated domains
Pol II	RNA polymerase II
Pol III	RNA polymerase III
PRC2	Polycomb repressive complex 2
pri-miRNA	primary miRNA
PRMT	protein arginine methyltransferases
PSA	prostate-specific antigen
PTM	post-translational modification
qRT-PCR	quantitative reverse transcription polymerase chain reaction
RNA	ribonucleic acid
S	DNA synthesis
TMA	tissue microarray

TNM	primary tumor/regional lymph node/distant metastasis
TURP	transurethral resection of prostate
UTR	untranslated region

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The authorship was shared because Hanna Rauhala gave the original idea for the publication. Kirsi Kaukoniemi participated in the study design with the supervisors. Kirsi Kaukoniemi designed and performed the majority of the laboratory work and data analyses. Kirsi Kaukoniemi was responsible for writing the first draft of the manuscript, performing the additional work requested by the reviewers, and finalizing the manuscript for submission.

1. INTRODUCTION

Cancer arises through a series of genetic and epigenetic alterations that accumulate in cells over time, eventually allowing them to circumvent growth-regulatory mechanisms and invade surrounding tissues (Hanahan, 2022). These alterations predominantly affect tumor suppressor genes and oncogenes. In normal cells, proto-oncogenes control processes such as proliferation, apoptosis, and differentiation. However, when mutated, proto-oncogenes can convert into oncogenes, leading to aberrant protein expression that drives uncontrolled cell growth and survival. In contrast, tumor suppressor genes function to inhibit cell proliferation, and their dysregulation allows cells to evade growth suppression. Typically, oncogenic mutations involve gain-of-function changes, whereas tumor suppressor genes undergo loss-of-function mutations.

Genomic aberrations arise through a range of mechanisms that either activate or silence gene expression. These mechanisms include large-scale alterations in chromosome structure and number, such as translocations, duplications, insertions, inversions, and deletions, as well as single-nucleotide mutations that affect individual DNA bases (Fröhling & Döhner, 2008). Besides genetic changes, epigenetic modifications such as DNA hypermethylation, hypomethylation, and histone modifications can influence gene expression without altering the underlying DNA sequence. MicroRNAs (miRNAs) are integral to epigenetic regulation, modulating gene expression post-transcriptionally by promoting target mRNA degradation or inhibiting translation. Furthermore, miRNAs themselves are subject to genetic and epigenetic regulation (Gujrati et al., 2023).

Prostate cancer (PC) is the most frequently diagnosed cancer and the third leading cause of cancer-related mortality in men across Europe (Dyba et al., 2021). In Finland, PC is the most frequently diagnosed cancer in men, with 5,514 new cases reported in 2022 (Pitkäniemi et al., 2022). After lung cancer, it is the second leading cause of cancer-related deaths among Finnish men. Prostate cancer predominantly affects older men, with the risk increasing significantly with age. Currently, more than 85% of newly diagnosed patients are over 60 years of age.

While some prostate tumors are indolent and may not require immediate treatment, others are aggressive, grow rapidly, and resist available therapeutic options. At present, there are no reliable biomarkers or methods to distinguish between indolent from aggressive tumors in their early stages, emphasizing the need for novel biomarkers (Rebello et al., 2021). miRNAs hold promise as such potential biomarkers.

2. REVIEW OF THE LITERATURE

2.1 Prostate cancer

2.1.1 Prostate gland anatomy and histology

The prostate is an exocrine gland that is part of the male reproductive system, and it secretes fluid that contains different products to nourish semen (Rebello et al., 2021). Normal prostate development and function are dependent on functional androgen receptor (AR). AR is a ligand dependent transcription factor that is activated upon binding androgens such as testosterone and 5 α -dihydrotestosterone (DHT). Activated AR is transported into nucleus where it dimerizes and binds its DNA target sequences together with its coregulatory proteins. AR induces transcription of its target genes which are important in maintaining cellular homeostasis and normal prostate function. Probably the best known examples of AR target genes are transmembrane protease serine 2 (*TMPRSS2*) and *KLK3* which encodes prostate-specific antigen (PSA) (Rebello et al., 2021; Tan et al., 2015).

The prostate is located under the bladder surrounding the urethra and ejaculatory tracks (Figure 1A). It can be divided into three histological zones, central, transition, and peripheral zones, first described by McNeal in 1981. Moreover, the periurethral zone is located within the transition zone and all these are surrounded by the fibromuscular region. The peripheral zone makes up about 70 % of the normal prostate tissue. Prostate cancer arises mainly from the peripheral zone, as almost 80 % of the tumors originate from this area. The transition zone is located near the prostatic urethra. In elderly men, the transition zone is often enlarged to benign prostate hyperplasia, BPH. The central zone is located between the peripheral and transition zones surrounding the ejaculatory ducts (Ittmann, 2018; Rebello et al., 2021).

The normal prostate gland is composed of ducts and small glands (acini) embedded in the stroma (Figure 1B). Duct and acini epithelium consist of a single layer of luminal cells, surrounded by one layer of basal and a small number of neuroendocrine cells. Basal cells are adjacent to the basal membrane that is anchored to stromal cells with smooth muscle cells and fibroblasts (Ittmann, 2018; Rebello et al., 2021). Luminal epithelial cells

are secretory cells that produce and extract various substances (proteins, enzymes, lipids, etc.) that facilitate and enhance sperm motility, nourish the sperm, and protect sperm from an acidic environment in the urethra. For example, luminal cells extract PSA to liquefy the ejaculate and prostatic acidic phosphatase that hydrolyses phosphorylcholine into choline to nourish sperm. The prostatic fluid makes up 20-30% of the seminal fluid volume (Aalberts et al., 2014). In addition, luminal cells also express high levels of AR. Instead, basal cells are non-secreting cells characterized by oval nuclei, a small amount of cytoplasm, and low levels of AR and PSA expression. The number of neuroendocrine cells in the prostate is small, they comprise only about 1% of the prostate epithelial cells. These cells are scattered between luminal and basal cells (Ittmann, 2018; Rebello et al., 2021).

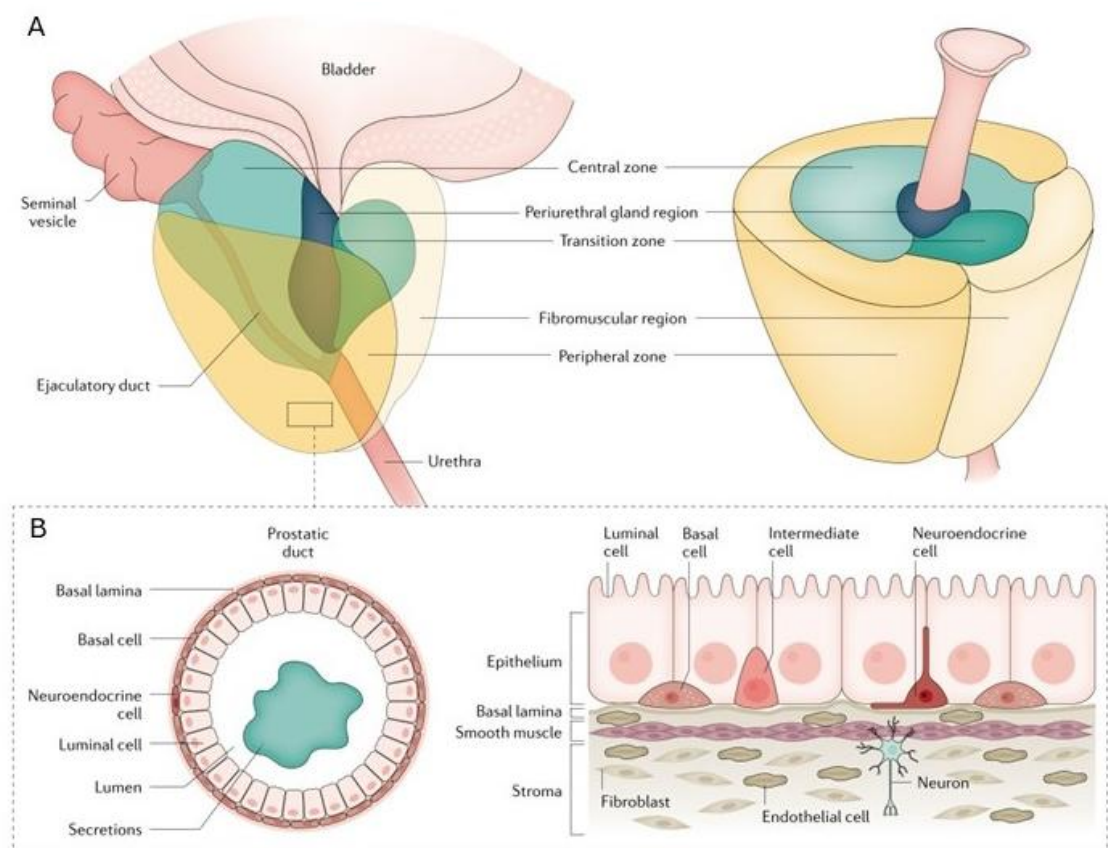


Figure 1. Anatomy of the prostate. A) Anatomic zones of the prostate B) Cellular organization of the prostate epithelium. Figure is adapted from Rebello et al. (2021), reproduced with permission from Springer Nature.

2.1.2 Basics of PC origin and progression

Most PCs are adenocarcinomas that arise from luminal and basal epithelial cells whereas only a small fraction of tumors originate from neuroendocrine cells (Rebello et al., 2021). In BPH the amount of both stromal and epithelial components is increased, whereas the epithelial layer may flatten as the stromal section increases (Foster, 2000). High-grade prostatic intraepithelial neoplasia (PIN) lesions are considered precursors to prostatic carcinoma and are usually found adjacent to tumor lesions. Typically in PIN, luminal epithelial cells are proliferated within the benign prostatic gland (Brawer, 2005; Rebello et al., 2021). In turn, nuclear atypia (enlarged nuclei and prominent nucleoli) is the major difference between PC adenocarcinoma and benign glands. Furthermore, infiltrative growth pattern and disappeared or unidentifiable glandular structures are typical for malignant lesions as the disease progresses (Magi-Galluzzi, 2018). Moreover, in advanced castration-resistant prostate cancer (CRPC), the normal glandular structures are unrecognizable and cancer cells are dispersed.

Prostate cancer metastases are often found in lymph nodes and/or bones. Metastatic tumors include both *de novo* metastatic castration-sensitive prostate cancer (mCSPC) and metastatic castration-resistant prostate cancer (mCRPC). CRPC is a cancer that progresses during or after androgen deprivation therapy (ADT), that is used to treat advanced disease. As the disease progresses from organ-confined to metastatic, the dysregulation of the AR signaling pathway plays a key role. *AR* alterations are rare in the early stage of prostate cancer, but they are the major drivers of CRPC and mCRPC (Fujita & Nonomura, 2019; Rebello et al., 2021; Sandhu et al., 2021).

2.1.3 Somatic gene alterations affecting prostate cancer development and progression

In PC, genomic rearrangements are common. However, specific genetic alterations that distinguish indolent from aggressive tumors have not been found. This is most likely due to the multifocal nature and intra-tumor heterogeneity of the PC. Coding somatic point mutations are relatively rare in PC tumorigenesis. The most prevalent mutations in early PC are those in *SPOP* (5-15%) and *FOXA1* (3-5%) (Figure 2). Mutations in *PTEN* and *TP53* occur in 10-20% of localized PC and over 40% in metastatic CRPC (mCRPC). Furthermore, also the fusion between *TMPRSS2* and ETS-group transcription factor

ERG (*TMPRSS2::ERG*) fusion is common, occurring in 40-60% of early and advanced tumors (Tomlins et al., 2005; Rebello et al., 2021; Sandhu et al., 2021).

Progression to CRPC is driven by dysregulation of additional genes. For example, *MYC* overexpression is detected in 20-30% and *RB* loss in 20-50% of mCRPC cases. Although *AR* alterations are rare in the early stages of prostate cancer, they are the major drivers of CRPC and mCRPC and are detected in 60-70% of mCRPC cases (Sandhu et al., 2021). Overexpression of *AR* is caused by either *AR* amplification (Visakorpi et al., 1995) or point mutations, ligand-independent *AR* splice variants, alterations in androgen biosynthesis, or androgen cofactors (Fujita & Nonomura, 2019).

Also, genomic instability and large-scale genomic alterations are typical for PC. Copy number alterations, kataegis (regions of localized gene hypermutations), chromothripsis (regions of chromosome shattering), and chromoplexy (high-frequency, genome-wide gene structural rearrangements) have been detected in about 30% of tumors in patients with localized PC and they are associated with disease progression (Rebello et al., 2021; Rubin & Demichelis, 2018). In addition to genetic changes, also epigenetic modifications play an important part in prostate tumorigenesis. Epigenetic changes are introduced in chapter 2.2.

This text focused solely on somatic mutations; however, it is important to highlight that prostate cancer is one of the most heritable cancers in men, with heritability estimated at 5-15% of all diagnosed cases. Therefore, germline mutations also have a significant impact on PC development (Vietri et al., 2021).

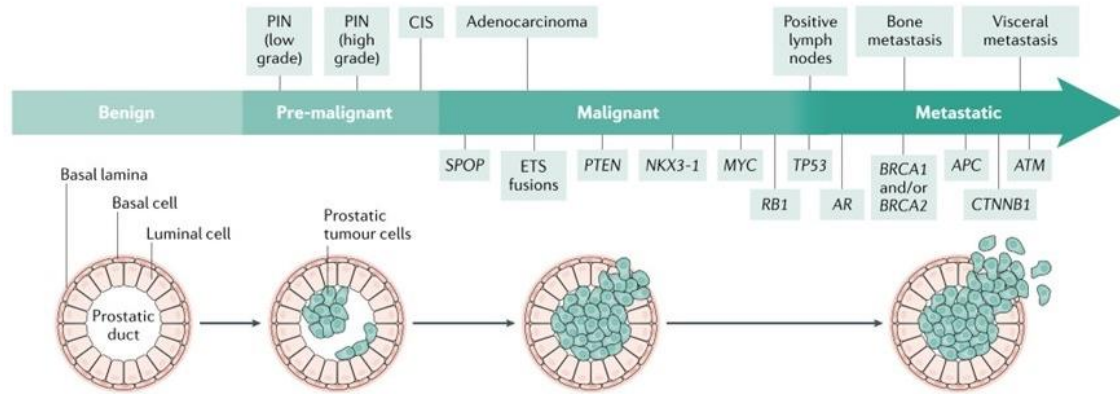


Figure 2. Gene mutations linked to the progression of prostate cancer. Common mutations according to their enrichment at different disease stages. PIN, prostatic intraepithelial neoplasia; CIS, adenocarcinoma in situ. Figure is adapted from Rebello et al. (2021), reproduced with permission from Springer Nature.

2.1.4 Diagnostics

The two primary methods usually used to discover PC are digital rectal examination (DRE) of the prostate and measurement of PSA (ng/ml) from the blood (Rebello et al., 2021; M. C. Wang et al., 1979). Neither DRE nor PSA are specific for PC alone so they both can give false positive or negative results. In addition to cancer, BPH and prostatitis increase the PSA levels and physical palpation might be positive or negative despite the cancer (Rebello et al., 2021). Due to specificity issues, PSA screening easily leads to overdiagnosis and is not recommended as a population-level screening test. However, PSA testing is used in detecting recurrence after treatment, although in the future multiple factors, including biomarkers and gene expression profiles might be used to better evaluate e.g survival after treatment (Duffy, 2020).

If risk assessment (DRE, PSA, life expectancy, family history) indicates PC, the diagnosis is confirmed by an ultrasound-guided transperineal or transrectal core needle biopsy. Nowadays magnetic resonance imaging (MRI) is recommended before the biopsy and in some centers, it is also possible to take MR-targeted biopsies. Also, systematic biopsies are used, especially if the risk assessment shows high risk (EAU, 2023). The biopsies are evaluated histopathologically and graded using Gleason grading system (Gleason, 1966). After diagnosis is confirmed, additional imaging can be performed to assess if and how the cancer has spread. Finally, all results are reviewed

together to estimate the stage and prognosis of the cancer and determine the treatment options (EAU, 2023).

Gleason grading

Gleason grading is the most commonly used system to grade the PC at diagnosis by assessing the glandular differentiation pattern of the tumor from hematoxylin and eosin (H&E) stained histological tissue samples. This system identifies five distinct glandular patterns, with Gleason pattern 1 representing the most differentiated and Gleason pattern 5 being the least differentiated. The Gleason score (GS) is calculated as the sum of the primary and secondary patterns observed in the sample. The primary pattern represents the most dominant, while the secondary pattern reflects either the second most prevalent pattern in prostatectomy samples or any high-grade pattern in systematic biopsies. If only one pattern is identified, the secondary pattern is considered identical to the primary (Gleason, 1966; van Leenders et al., 2020).

Gleason grading serves as an important prognostic indicator for PC, with higher Gleason scores correlating with worse prognoses. The currently used grading system is based on the International Society of Urological Pathology (ISUP) consensus conferences held in 2005 and 2014. In this system, there are 5 different prognostically different grade groups which are based on Gleason scores (Table 1) (Epstein et al., 2016). To reduce variability due to subjective interpretation by pathologists, Gleason grading is often integrated with other prognostic markers (EAU, 2023).

Table 1. Gleason grading. Table formatted from Epstein et al. (2016).

Grade Group	Gleason score
1	≤ 6
2	$3+4 = 7$
3	$4+3 = 7$
4	$4+4 = 8$ $3+5 = 8$ $5+3 = 8$
5	9-10

TNM staging

The TNM staging is a standardized classification scheme for describing the anatomical progression of cancers. The clinical TNM classification is based on DRE and it describes the size and possible extension of the primary tumor (T), if it has spread to the nearby lymph nodes (N), and the presence of distant metastases (M) (EAU, 2023). There are four distinct clinical T-stages: T1, a clinically inapparent tumor that is not palpable; T2, a tumor that is palpable and confined within the prostate; T3, tumor that extends through the prostatic capsule; and T4, tumor is fixed or invades adjacent structures other than seminal vesicles (Brierley et al., 2017). Furthermore, pathological TNM (pTNM) staging closely aligns with the clinical TNM. pTNM assesses histopathologically the extent of tumors within the prostate and surrounding tissues from radical prostatectomy samples. Organ-confined PCs are pathological stage pT2 tumors (Cheng et al., 2012; Brierley et al., 2017).

2.1.5 Treatment

The selected treatment option for PC is based on the stage of the cancer, the patient's own opinion, life expectancy, general health, and co-morbidity (EAU, 2023). Patients are divided into different risk groups (low, intermediate, high) to choose the most appropriate treatment. These risk groups are defined by the TNM stage, Gleason score, and diagnostic PSA level (D'Amico et al., 1998; EAU, 2023).

A life expectancy of ten years is typically used as the threshold to determine the potential benefit of localized treatment. For patients with small, localized tumors and a low-risk of progression, watchful waiting or active surveillance is a viable strategy to avoid overtreatment and mitigate side effects associated with early intervention. In some cases also low-risk patients can be treated. For patients with localized tumors at intermediate or high risk of progression, active treatment is favored over watchful waiting or active surveillance. The primary treatment options for localized disease are prostatectomy and radiation therapy. Prostatectomy involves the surgical removal of the entire prostate gland and seminal vesicles, often accompanied by the removal of regional lymph nodes (EAU, 2023).

For high-risk patients with locally advanced PC or metastatic disease, radiation therapy, and prostatectomy might not be sufficient, and additional therapies are required. Additional therapy includes androgen deprivation therapy (ADT) which inhibits androgen function, as prostate cancer growth is androgen-dependent. ADT is used either alone or in combination with other drugs such as chemotherapy agent docetaxel and AR signaling inhibitors enzalutamide, apalutamide, and darolutamide or CYP17 inhibitor abiraterone acetate (EAU, 2023).

Prostate cancer is defined as castration-resistant when the disease progresses during ADT. CRPC can be either non-metastatic or metastatic. Although there are currently no curative treatments for CRPC, patients can be treated with several drugs. For example, PARP (poly (ADP-ribose) polymerase) inhibitors olaparib, talazoparib, and niraparib have been approved to treat mCRPC patients. These drugs are used for patients who have DNA repair gene deficiency (EAU, 2023).

2.2 Epigenetics

The term epigenetics means “on top of genes” and it is used to describe changes in chromatin that do not affect the sequence of DNA. There has been discussion about the definition of epigenetics but a consensus definition is still lacking (Lu et al., 2020). Conrad Waddington first introduced the term in 1942, describing how changes in a cell’s phenotype could be transmitted across generations without modifications to the DNA sequence (Waddington, 2012). Probst et al. proposed in 2009 that epigenetic changes should be defined as “the heritable changes in phenotype or gene expression not caused by the alterations in the underlying DNA sequence”. However, others, such as Bird (2007) suggested that the definition of epigenetics should include both heritable and transient DNA and chromatin marks.

Epigenetic changes are reversible, allowing for more rapid regulation than permanent alterations in the genomic sequence (Lu et al., 2020). These changes include DNA and RNA methylation, histone modifications, chromatin remodeling, and non-coding RNAs including microRNAs (miRNA), long non-coding RNAs (lncRNA), and circular RNAs. The enzymes responsible for these epigenetic changes on DNA and histones are classified into four categories: writers, erasers, readers, and movers. Writers, DNA

methyltransferases (DNMTs), histone lysine methyltransferases (KMTs), and histone acetyltransferases (HATs), introduce modifications to DNA and histones. Erasers, DNA demethylases (ten to eleven translocation, TET) perform active DNA demethylation whereas histone lysine demethylases (KDMs) and histone deacetylases (HDACs) remove these post-translational modifications (PTMs) from histones. Readers, i.e. proteins with bromodomain and chromodomain, recognize and bind to acetylated or methylated histone residues. Movers refer to a group of chromatin-remodeling proteins that move nucleosomes thus allowing gene transcription. Importantly, DNA methylation and histone modifications do not function in isolation but interact with each other to regulate gene expression (Bates, 2020; Tao et al., 2024; Gu et al., 2024).

Dysregulation of epigenetic modifications has been closely linked to the initiation and progression of various cancers. Aberrant epigenetic changes can activate oncogenes, silence tumor suppressor genes, and disrupt critical signaling pathways, contributing to cancer development (Tao et al., 2024). Indeed, alterations in epigenetic regulation are recognized as a hallmark of cancer (Hanahan, 2022).

2.2.1 Chromatin

Chromatin is a highly organized structure composed of DNA and histone proteins, enabling the efficient packaging of DNA within the nucleus while also regulating its accessibility. The basic unit of chromatin is a nucleosome, in which 145-147 bp DNA segment is tightly wrapped around a histone octamer. This histone octamer is composed of two H2A/H2B dimers and one H3/H4 tetramer, which together form the histone core. Each histone protein has an N-terminal tail that extends outward from the nucleosome core, frequently undergoing various PTMs. Adding or removing PTMs to the N-terminal tails of histone plays a key role in altering chromatin structure and thus DNA accessibility (Chen et al., 2021; Lu et al., 2020; McGinty & Tan, 2015).

Chromatin can be classified into two distinct types based on its level of compaction: euchromatin and heterochromatin. Euchromatin is loosely packed and contains the majority of transcriptionally active genes. Heterochromatin, in contrast, is more densely packed, gene-poor, and typically contains genes that are transcriptionally inactive. Heterochromatin is further divided into constitutive and facultative types. Constitutive heterochromatin is composed primarily of repetitive DNA elements and remains stably condensed, playing critical roles in maintaining chromosome integrity, suppressing

transposon activity, and preventing recombination. On the other hand, facultative heterochromatin forms dynamically in response to specific developmental cues or during processes such as tissue differentiation. Facultative heterochromatin is detected for example in inactivated X-chromosome (Nishibuchi & Déjardin, 2017).

Repetitive DNA elements make up nearly 50% of the human genome, with various types of repetitive sequences distributed throughout. In primates, centromeric regions are composed primarily of α -satellite DNA, a class of 171bp long sequences that can span up to several megabases and constitute up to 10% of the genome. Pericentromeric areas in humans consist of different satellite families depending on the chromosome. These satellite families vary in repeat length and GC content (Thakur et al., 2021). Telomere regions, located at the very end of all chromosomes consist of tandem repeats “TTAGGG”, which can extend over 100kb, depending on the organism, cell type, and age (Nishibuchi & Déjardin, 2017).

Transposable elements (TEs) represent the most prevalent class of repetitive sequences in mammalian genomes, scattered throughout the genomic landscape. When active, TEs can insert into coding or regulatory elements thereby contributing to genetic variation and genomic instability. Transposable elements are broadly categorized into DNA transposons and RNA transposons (retrotransposons), with the latter further divided into long terminal repeat (LTR) retrotransposons and non-LTR retrotransposons. Non-LTR retrotransposons include long-interspersed nuclear elements (LINEs) and short-interspersed nuclear elements (SINEs). (Nishibuchi & Déjardin, 2017).

2.2.2 Histone modifications

The N-terminal tails of nucleosome core histone proteins undergo the most diverse array of epigenetic modifications. The most common and widely studied chemical post-translational histone modifications are methylation and acetylation. Aberrations of histone methylation and acetylation have been strongly linked to cancer. In addition to methylation and acetylation, histones can also undergo a wide range of other modifications, such as phosphorylation, ubiquitination, sumoylation, and other atypical modifications, including adenylation, ADP-ribosylation, butyrylation, citrullination, crotonylation, deamination, formylation, hydroxylation, lactylation, O-GlcNAcylation, propionylation, and proline isomerization all mediated by specific enzymes. Unlike histone methylation and acetylation, the impact of aberrations in these other chemical

modifications of histones on cancer development requires further studies (Tao et al., 2024). Histone modifications are named after the histone protein, amino acid residue, and the modification. For example, H3K4me3 stands for histone 3 lysine 4 trimethylation.

Histone methylation

Histone methylation occurs at specific arginine and lysine residues in the N-terminal tails of core histones. Lysine methylation is catalyzed by histone methyltransferases (KMTs), while arginine methylation is mediated by protein arginine methyltransferases (PRMTs). KMTs have been more extensively studied than PRMTs, likely due to their earlier discovery and distinctive regulatory roles. Lysine residues can undergo mono-, di-, or trimethylation, while arginine residues are restricted to mono- or dimethylation (Gu et al., 2024; Tao et al., 2024).

The transcriptional impact of histone lysine methylation depends on both the location of the modified lysine residue and its methylation state. Although lysine residues on various histones can be methylated, the most well-studied ones are H3K4, H3K9, H3K26, H3K27, H3K36, H3K79, H4K5, H4K12, and H4K20. Methylation of H3K4, H3K36, and H3K79 is typically associated with active transcription, whereas methylation of H3K9, H3K27, and H4K20 correlates with transcriptional repression. Furthermore, the functional consequences of methylation can vary depending on the degree of modification. For instance, monomethylation of lysine 9 in histone 3 (H3K9me1) is generally linked to active transcription, while trimethylation of the same residue (H3K9me3) is found in transcriptionally inactive regions (Gu et al., 2024; Tao et al., 2024).

Dysregulation of KMT activity has been implicated in the initiation and progression of various cancers, including prostate cancer. KMTs are also being explored as therapeutic targets. One example is EZH2 (Enhancer of Zeste Homolog 2), a core component of the Polycomb repressive complex 2 (PRC2), which is responsible for catalyzing the methylation of histone 3 lysine 27 (H3K27). This modification promotes heterochromatin formation and transcriptional silencing (Park et al., 2021). In prostate cancer, the expression *EZH2* is elevated in aggressive carcinomas and associated with disease progression (Varambally et al., 2002). In addition to its enzymatic activity, EZH2 has also polycomb-independent functions. EZH2 is an AR coactivator (Xu et al., 2012) as it binds

to AR promoter to activate its transcription to enhance AR signaling (Kim et al., 2018). Currently, there is ongoing research to identify compounds that promote EZH2 degradation to inhibit both enzymatic and non-enzymatic functions of EZH2 (Park et al., 2021).

Histone acetylation

Acetylation of nucleosome core histones leads to a more open chromatin structure, facilitating the binding of regulatory proteins, such as transcription factors to their target sequences on DNA. As a result, histone acetylation is commonly associated with transcriptionally active chromatin. This modification is mediated by HATs, which transfer an acetyl group from acetyl coenzyme A to lysine residues on the histone N-terminal tail. Aberrations in histone acetylation levels have a significant impact on tumorigenesis. Hyperacetylation leads to the activation of oncogenes whereas hypoacetylation is associated with the silencing of tumor suppressor genes (Tao et al., 2024). For example, highly homologous HATs CBP and p300 are coactivators of AR and have oncogenic activities in PC (Fu et al., 2000, p. 3; Zhong et al., 2014). Androgen deprivation therapy causes upregulation of CBP and p300 and it is suggested that this enhances disease progression by increasing AR activity (Comuzzi et al., 2004; Heemers et al., 2007; Sardar et al., 2024). Furthermore, CBP/p300 have been suggested as key mediators of DNA damage repair in CRPC (Sardar et al., 2024).

Hypoacetylation is frequently driven by the overexpression of histone deacetylases (HDACs), which remove acetyl groups, leading to reduced histone acetylation and increased chromatin compaction (Tao et al., 2024). The HDAC family is divided into four classes: I, IIa, IIb, and IV. Notably, only class I HDACs are involved in deacetylating nuclear proteins, while the other classes also target cytoplasmic non-histone proteins. In PC, increased activity of HDACs is correlated with elevated serum PSA levels and with increased tumor cell invasion (Nowacka-Zawisza & Wiśnik, 2017).

Due to their role in chromatin regulation, HATs and HDACs are promising therapeutic targets in cancer. Currently, four HDAC inhibitors have been approved by the FDA for use in other cancers, such as cutaneous T-cell lymphoma and multiple myeloma (Bates, 2020). However, in prostate cancer, the safety and efficacy of HDAC inhibitors are still under investigation (Biersack et al., 2022). There are ongoing PC clinical trials for

therapies with CBP and p300 inhibitor that represses the acetyltransferase activity of these proteins (Sugiura et al., 2021).

2.2.3 DNA methylation

DNA methylation is the most widely studied epigenetic chromatin modification and it is associated with chromatin condensation and gene silencing. DNA methylation is predominantly found in transcriptionally inactive regions of the genome, such as telomeres, centromeres, repetitive DNA sequences, and the inactive X chromosome. DNA methylation plays a crucial role in processes like X-chromosome inactivation, genomic imprinting, and the maintenance of genomic stability (Yang et al., 2023).

DNA methylation occurs when a methyl group is added to the 5-carbon position of cytosine, forming 5-methylcytosine (5mC), in CpG dinucleotides (Yang et al., 2023). Most CpG dinucleotides in the human genome are methylated and located in the repetitive areas of the DNA that make up almost half of the genome. Methylation of the repeat elements is considered a “safeguard” of the genome, as the repetitive regions are embedded into protective heterochromatin, hence maintaining the stability of the genome and blocking the transcription of the repeats (Lu et al., 2020; Pappalardo & Barra, 2021).

Unlike methylated CpGs scattered through the genome, unmethylated CpGs reside mostly in clusters called CpG islands (CGIs). CGIs comprise only about 1% of the genome and are mainly located in the promoter regions of genes. Genes with unmethylated promoter CGIs are considered transcriptionally active genes (Lu et al., 2020; Pappalardo & Barra, 2021). CpG islands are flanked by CpG island shores (\pm 2kb around islands) and shelves (2-4kb around islands) (Timp et al., 2014) that are also normally hypomethylated and mark gene regulatory loci such as enhancers (Skvortsova et al., 2019).

The regulation of DNA methylation is mediated by enzymes known as 5mC writers and erasers - specifically, DNA methyltransferases (DNMTs) and ten-eleven translocation (TET) enzymes (Shin et al., 2023). In humans, the *de novo* methyltransferases, DNMT3A and DNMT3B, establish new methylation marks during early development. These enzymes target unmethylated DNA independently of replication (Jones, 2012; Lu et al., 2020). DNMT1 maintains methylation patterns by preferentially targeting

hemimethylated DNA, which is common after DNA replication when the template strand is methylated and the newly synthesized strand is unmethylated (Sharif & Koseki, 2018). DNA demethylation is facilitated by the TET enzymes TET1, TET2, and TET3, which convert 5mC into unmethylated cytosine through a series of oxidation steps, starting with 5-hydroxymethylcytosine (5hmC) and progressing through intermediate forms such as 5-formylcytosine and 5-carboxylcytosine before reverting to cytosine. In addition, 5hmC does not only function as a transitory stage in the demethylation pathway but it also persists as a stable epigenetic modification and reflects TET3 enzyme activity (Gu et al., 2024). 5hmC is associated with both transcriptional activation and repression (Yang et al., 2023).

Aberrant DNA methylation patterns typically involve both global DNA hypomethylation and localized hypermethylation of CGIs at gene promoters, which often coexist in tumor cells (Nishiyama & Nakanishi, 2021). In prostate cancer, recurrent genome-wide and locus specific DNA methylation changes have been known for decades. Over the years, there has been increasing evidence that multiple aberrant methylation marks could together possibly serve as biomarkers, at least separating benign and cancerous tissue but potentially also aid in distinguishing indolent tumors from more aggressive ones. Furthermore, components of epigenetic regulation are an intriguing target for cancer treatment (Conteduca et al., 2021; Massie et al., 2017).

DNA hypomethylation in prostate cancer

Global DNA hypomethylation is a common event for all tumor types (Ehrlich, 2009). It was the first epigenetic phenomenon described in 1983 through three seminal studies by Feinberg & Vogelstein a, Gama-Sosa et al., and Feinberg & Vogelstein b. Global DNA hypomethylation is often observed during the early stages of tumorigenesis with its extent often increasing as the disease progresses. While some debate remains regarding the precise role of DNA hypomethylation in cancer development, it is now widely accepted that aberrant hypomethylation of repetitive elements, such as those found in centromeric and telomeric regions, disrupts chromatin structure, thereby increasing genomic instability (Pappalardo & Barra, 2021).

In prostate cancer, global DNA hypomethylation increases as the disease progresses. Primary tumors typically exhibit lower methylation levels compared to BPH, and metastatic tumors show even greater hypomethylation than primary tumors

(Yegnasubramanian et al., 2008; Zhao et al., 2020). Notably, Yegnasubramanian et al., (2008) suggested that hypomethylation of repetitive LINE-1 sequences occurs relatively late in the progression of prostate cancer. However, recent findings by Guo et al., (2023) identified small, partially methylated domains (PMDs), referred to as core PMDs, which consistently exhibited hypomethylation in tumors ranging from indolent, low-grade (GS6) localized cancers to metastatic disease. Similarly to others, they also detected that the DNA hypomethylation spreads to larger genomic areas as the disease progressed from early cancer to metastatic disease.

In addition to the genome-wide hypomethylation observed in prostate cancer, gene-specific hypomethylation events have also been identified. Promoter hypomethylation has been associated with increased gene expression. For instance, Zhao et al., (2020) reported that the increased expression of key androgen-responsive genes *AR* and *KLK3* was associated with promoter hypomethylation. Furthermore, they described several expression-related hypomethylated regions adjacent to *AR* promoter, *AR* enhancer, and one locus downstream of *AR*, all of which were associated with increased *AR* expression in metastatic prostate cancer. These regions exhibited hypomethylation exclusively in metastatic samples, but not in BPH, while hypomethylation of the *AR* promoter was already present in BPH. These results suggest that the AR-pathway is regulated partly by DNA hypomethylation in prostate cancer.

DNA hypermethylation in prostate cancer

In addition to global DNA hypomethylation, also DNA hypermethylation is an important factor in prostate cancer initiation and progression. DNA hypermethylation often targets gene regulatory elements such as promoters and enhancers, leading to the silencing of tumor suppressor genes (Nishiyama & Nakanishi, 2021). Promoter hypermethylation can prevent transcription factors from binding to their target sites or recruit methyl-binding domain proteins (MBDs), which, in concert with histone modifications, can restructure chromatin to suppress gene expression (Lu et al., 2020).

Focal promoter DNA hypermethylation, which leads to gene downregulation is a common event in PC (Kim et al., 2011; Kobayashi et al., 2011; Lin et al., 2013; Zhao et al., 2020). For example, genes such as *APC* (adenomatous polyposis coli) involved in apoptosis, cell migration, and cell adhesion, and *RASSSF1* (Ras association domain family member

1) involved in cell cycle regulation are hypermethylated and their expression is reduced in advanced PC tumors (Jerónimo et al., 2004; Kuzmin et al., 2002; Liu et al., 2002).

However, probably the most well-known example of promoter hypermethylation-mediated gene silencing is the Glutathione S-transferase pi 1 (*GSTP1*) gene, which plays a role in drug metabolism and DNA protection from oxidative stress. *GSTP1* was the first gene identified as hypermethylated and downregulated in PC, as reported by Lee et al. in 1994. Its promoter hypermethylation is seen in all stages of the disease (Yegnasubramanian et al., 2004) and represents an early event in prostate cancer development, being detectable in PIN lesions (Nakayama et al., 2003), primary tumors, and CRPC samples (Yegnasubramanian et al., 2004) but not in normal prostate tissue or BPH (Nakayama et al., 2003). Subsequent studies have consistently confirmed these findings across multiple sample sets (Kim et al., 2011; Kobayashi et al., 2011; Lin et al., 2013; Yegnasubramanian et al., 2004; Zhao et al., 2020). Given its high detection frequency, *GSTP1* promoter hypermethylation is regarded as a recurrent event in PC.

GSTP1 hypermethylation can also be detected in body fluids, such as serum and urine (Henrique & Jerónimo, 2004; Woodson et al., 2008), making it an intriguing biomarker candidate. However, despite its frequent detection in PC, the prognostic value of *GSTP1* hypermethylation remains controversial. For instance, among 15 studies evaluating the prognostic significance of *GSTP1* hypermethylation in PC, seven found no correlation with biochemical recurrence (Lam et al., 2020).

Several reports suggest that promoter hypermethylation rates generally rise from prostate cancer primary tumors to CRPC (Kim et al., 2011; Kobayashi et al., 2011; Lin et al., 2013; Yegnasubramanian et al., 2004; Zhao et al., 2020). Interestingly, there is emerging evidence that instead of only inhibiting transcription, promoter hypermethylation could also enhance gene expression, as reviewed by Smith et al. (2020). Furthermore, aberrant hypermethylation within the gene bodies has been associated with increased, rather than decreased, gene expression (Wang et al., 2022). For example, Su et al., (2018) suggested that hypermethylation within methylation canyons contributes to the overexpression of homeobox (HOX) genes in prostate cancer. Moreover, *AR* gene body hypermethylation is associated with elevated *AR* expression (Zhao et al., 2020).

2.2.4 microRNAs

MicroRNAs (miRNAs) are small, ~22 nucleotides long, single stranded, noncoding RNA molecules that were first discovered in *Caenorhabditis elegans* in the early 1990s (R. C. Lee et al., 1993). Since their discovery, miRNAs have been extensively studied for their roles in various diseases, including cancer, cardiovascular disease, and neurological disorders (Condrat et al., 2020). According to the latest version of the miRNA database, miRBase (v22), over 2,500 mature miRNA sequences have been identified in *Homo sapiens* (Kozomara et al., 2019).

The primary function of miRNAs is to act as post-transcriptional regulators of gene expression. They modulate gene expression by binding to their target mRNA molecules, which either leads to mRNA degradation or inhibition of translation (Gujrati et al., 2023). A single miRNA can target multiple mRNAs, and conversely, a single mRNA can be regulated by several miRNAs, adding the complexity to the miRNA regulatory network (Condrat et al., 2020). As miRNAs regulate gene expression without altering the DNA sequence, they play a part in the epigenetic regulatory network. Numerous studies implicate dysregulated miRNAs in the pathogenesis of various diseases, including cancers such as prostate cancer (Gujrati et al., 2023).

In addition to their intracellular roles, miRNAs are also involved in intercellular signaling. While most miRNAs are located within cells, they can also be detected in various body fluids, including blood, urine, and saliva. Circulating miRNAs hold potential as biomarkers for cancer detection, monitoring disease progression, and assessing treatment response, as they can be easily measured through non-invasive liquid biopsies. However, further research is needed to validate the use of miRNAs as clinical biomarkers (Condrat et al., 2020). The complex regulatory network of miRNAs, where one miRNA may target multiple genes and one gene can be regulated by multiple miRNAs, complicates the interpretation of their role as biomarkers (Sempere et al., 2021). Despite these challenges, ongoing clinical studies aim to identify reliable miRNA biomarkers, such as those for prostate cancer diagnosis and prognosis (Gujrati et al., 2023).

Biogenesis of miRNAs

Genes encoding miRNAs can exist either as individual genes or clustered together in the genome, with a single cluster containing multiple miRNA genes. MicroRNAs are classified into two groups based on their genomic location: intragenic and intergenic. Intragenic miRNAs are predominantly processed from introns, and in some cases, from noncoding exons of their host genes. These miRNAs are transcribed with their host gene but are processed separately. Conversely, intergenic miRNAs are located in noncoding regions of the genome and are regulated by their own promoters, independent of any host gene. MicroRNAs within clusters are transcribed as polycistronic transcripts, which are then processed into individual mature miRNAs (Conti et al., 2020; O'Brien et al., 2018; Treiber et al., 2019).

MicroRNA biogenesis occurs via two main pathways: canonical and non-canonical, with the canonical pathway being the predominant mechanism (O'Brien et al., 2018). For most miRNAs, the canonical biogenesis begins in the nucleus with the transcription of a primary miRNA (pri-miRNA) by RNA polymerase II (Pol II) (Figure 3). However, miRNAs containing Alu sequence repeats are transcribed by RNA polymerase III (Pol III) (Gujrati et al., 2023). In addition, Pol II processes pri-miRNAs by adding 5' guanosine-cap and a 3' polyadenylated tail (Conti et al., 2020; Gujrati et al., 2023; S. Lin & Gregory, 2015). Pri-miRNA molecules can be several thousand kilobases in length and typically contain one or more RNA hairpin structures. In the canonical pathway, the pri-miRNA is cleaved by the Microprocessor complex, consisting of the RNA-binding protein DiGeorge Syndrome Critical Region 8 (DGCR8) and the RNase III enzyme Drosha, into precursor miRNAs (pre-miRNAs) of approximately 80 base pairs (Bofill-De Ros & Vang Ørom, 2024). The pre-miRNA is then exported from the nucleus to the cytoplasm by the nuclear transport receptor protein Exportin 5 (XPO-5)/Ran GTP complex (Conti et al., 2020).

In the cytoplasm, pre-miRNAs are further processed by the RNase III enzyme DICER, along with the cofactor TAR RNA-binding protein 2 (TRBP2), into 21-25 nucleotide-long double-stranded mature miRNAs (Gujrati et al., 2023). The strands of the duplex are designated based on the orientation of the pre-miRNA: the 5p-miRNA originates from the 5' end, and the 3p-miRNA from the 3' end of the pre-miRNA (O'Brien et al., 2018). After Dicer cleavage, one of the miRNA strands, known as the guide strand, is loaded onto the Argonaute (AGO) protein to form an RNA-induced silencing complex (RISC) (Gujrati et al., 2023). Both the 5p and 3p strands can act as guide strands depending on cellular conditions, but typically, the strand with lower 5' stability or a 5' uracil is favored

for AGO loading, while the other strand, called the passenger strand, is degraded (Bofill-De Ros & Vang Ørom, 2024; O'Brien et al., 2018).

Within the RISC complex, the guide strand binds to its target mRNA based on base pairing through a short, 2-8 nucleotide-long 'seed' sequence that recognizes the miRNA Response Element (MRE) located in the 5' or 3' UTR of the target mRNA (Bofill-De Ros & Vang Ørom, 2024; Conti et al., 2020). The extent of complementarity between the miRNA and the mRNA determines the fate of the target: full complementarity leads to mRNA degradation by AGO2 endonuclease, while partial complementarity inhibits AGO2 activity, resulting in translational repression or mRNA deadenylation by blocking ribosome binding and activity (Conti et al., 2020).

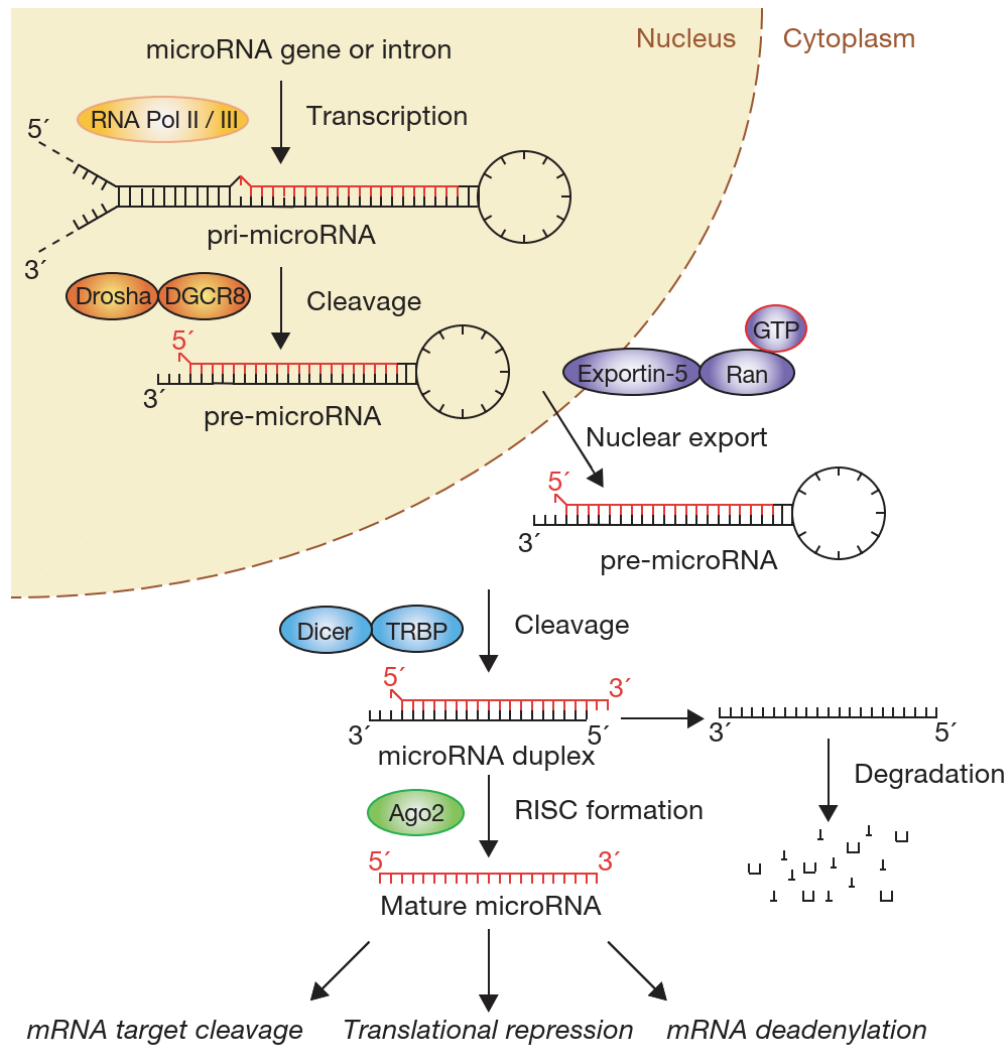


Figure 3. The canonical miRNA biogenesis pathway. In the nucleus, miRNA genes are transcribed by RNA polymerase II or III into primary miRNA (pri-miRNA), which is cleaved into shorter precursor-miRNA (pre-miRNA). The pre-miRNA is exported to the cytoplasm via Exportin 5-Ran GTP complex, where it is further processed into mature miRNA. The functional guide strand of the mature miRNA is then incorporated into the RNA-induced silencing complex (RISC) along with Argonaute (AGO2) protein. The target mRNA is recognized by the guide RNA which leads to mRNA silencing through mechanisms such as mRNA cleavage, translational repression, or mRNA deadenylation. Meanwhile, the passenger strand (black) is degraded. Figure is adapted from Winter et al., (2009), reproduced with permission from Springer Nature.

MicroRNA biogenesis can be disrupted through the same mechanisms affecting protein coding gene expression, namely gene copy number alterations, chromosomal aberrations, changes in DNA methylation or histone modifications, and transcriptional regulation. Additionally, disruptions in RNA processing and stability can affect miRNA production and function. Furthermore, mechanisms that influence miRNA activity without significantly altering miRNA expression, such as post-translational modifications of

miRISC components and the shortening of the 3' UTR of target mRNAs, also impact the function of mature miRNAs (Sempere et al., 2021).

microRNAs in prostate cancer

Various studies have identified a wide range of miRNAs that are deregulated in prostate cancer. Some of these dysregulated miRNAs are overexpressed (oncogenic miRNAs) while others are downregulated (tumor suppressor miRNAs). Different miRNAs are associated with PC pathogenesis as their abnormal function disrupts cell cycle control by promoting cell proliferation, survival, invasion, metastasis, and immune evasion (Gujrati et al., 2023).

Several miRNAs have been implicated in PC, either through overexpression or underexpression. For example, miR-17-5p is overexpressed in PC and is associated with an aggressive disease and biochemical recurrence (Hoey et al., 2019). MiR-17-5p targets the *PTEN* tumor suppressor gene by inhibiting *PTEN* activity, thereby driving tumor growth and invasion (Stoen et al., 2021). Similarly, miR-96-5p is significantly overexpressed in PC and targets *FOXO1* (forkhead box O1), facilitating cell proliferation (Haflidadóttir et al., 2013). In contrast, miR-29b and miR-30c are downregulated in PC, playing roles in regulating cell differentiation, proliferation, and apoptosis. Their reduced expression is linked to higher Gleason scores and increased metastasis to lymph nodes and bones (Zhu et al., 2018). Additionally, Porkka et al. (2007) identified 37 downregulated and 14 upregulated miRNAs in hormone-refractory advanced cancer and 22 downregulated and 8 upregulated miRNAs in untreated early-stage PC.

From an epigenetic regulation perspective, micro-RNA-145 is one notable miRNA among those downregulated in PC. Interestingly, miR-145 downregulates *DNMTB* expression and when downregulated, *MIR145* expression is increased due to promoter hypomethylation which suggests a crosstalk between miR-145 and *DNMT3B* in prostate cancer (Xue et al., 2015). In addition, miR-193b and miR-132 have been identified as hypermethylated and silenced tumor suppressors in prostate cancer (Formosa et al., 2013; Rauhala et al., 2010). Furthermore, *MIR34B* is significantly downregulated due to promoter hypermethylation, functioning as a tumor suppressor in PC (Majid et al., 2013). MiR-34b directly targets DNMTs and HDACs leading to partial demethylation and active chromatin modifications, and also inhibits epithelial-to-mesenchymal transition by targeting Akt and its downstream proliferative genes.

Multiple miRNAs are also associated with the regulation of the AR pathway. For example, Östling et al. (2011) identified 71 miRNAs that regulate AR protein expression, with 52 miRNAs downregulating and 19 miRNAs upregulating AR levels. They found an inverse correlation between AR and miR-34a expression in PC, where decreased miR-34a expression was associated with increased AR levels, suggesting that miR-34a could be a key regulator of AR. MiR-34a also targets genes such as *MET*, *BCL2*, and *MYC* among others to regulate e.g. cell cycle, cell proliferation, and invasion (Gujrati et al., 2023). AR itself regulates miRNAs, as demonstrated for example Jalava et al. (2012), who showed that miR-32 is AR-regulated and overexpressed in CRPC. Forced expression of miR-32 protects cells from apoptosis, and the gene *BTG2* (B-cell translocation gene 2), involved in e.g. cell cycle and apoptosis, was identified as a putative target for miR-32. The expression of *BTG2* was reduced in PC and almost entirely abolished in CRPC, linking its loss to disease progression.

Beyond understanding their role in cancer progression, miRNAs are being explored as therapeutic targets and biomarkers in prostate cancer. MiRNA-based therapies aim to restore the expression of tumor-suppressive miRNAs or inhibit oncogenic miRNAs to suppress tumor growth (Gujrati et al., 2023). The miRNA biogenesis pathway can be interfered with in several ways: at the nuclear or cytoplasmic level (small-molecule inhibitors), miRNA replacement therapy (miRNA mimics), and functional inhibition of the mature miRNA (AntimiRs, miRNA sponges, and miRNA masks), or disruption of miRNA-target mRNA interactions (Winkle et al., 2021).

Ongoing clinical trials are investigating the potential of miRNA profiling for diagnosing and monitoring prostate cancer progression. While no direct miRNA-targeting therapies are currently available for prostate cancer treatment, several promising miRNA-based therapeutic approaches are under investigation. For example, MRX34, a liposomal miR-34a mimic, has shown efficacy in treating PC-3 and DU145 xenografts in preclinical studies but has not yet been tested in clinical trials for PC patients (Gujrati et al., 2023).

Moreover, clinical trials aim to determine the prognostic and therapeutic relevance of miRNAs in PC. These include assessments of miRNA expression levels as indicators of clinical outcomes, whether specific miRNA expression profiles are related to high-grade PC, and the association of circulating miRNA expression profiles with lymph node metastases. One trial, for example, uses a panel of six miRNAs (miR-21, miR-141, miR-200a, miR-200c, miR-375, and miR-3687) to predict responses to enzalutamide in patients with mCRPC (Gujrati et al., 2023).

miR-193b in prostate cancer

The *MIR193B* gene, which encodes hsa-miR-193b, is located on the short arm of chromosome 16 (16p13.2). *MIR193B* is clustered with *MIR365a* and forms the miR-193 family together with *MIR193A*. In prostate cancer, hsa-miR-193b-3p has been identified as an epigenetically regulated putative tumor suppressor (Rauhala et al., 2010). This study demonstrated that miR-193b is silenced in 22Rv1 prostate cancer cell line due to hypermethylation. In clinical prostate tumors, increased methylation and decreased expression of miR-193 were observed compared to BPH samples. Additionally, miR-193b was shown to reduce the proliferation of prostate cancer cells in the S-phase of the cell cycle. Subsequent studies by Torres-Ferreira et al., (2017) and Mazzu et al., (2019) further confirmed the miR-193 hypermethylation in clinical samples and PC cell lines. Notably, Torres-Ferreira et al., (2017) suggested that promoter hypermethylation of miRNA-193b, miR-129-2, and miR-34b/c could together serve as potential biomarkers for PC detection and prognosis in tissue and urine samples. Additionally, Mazzu et al., (2019) identified several possible target genes for miR-193b, of which they validated *FOXM1* and *RRM2* genes as direct targets of miR-193b in prostate cancer.

2.3 The cell cycle

The cell cycle has been widely studied since Walther Flemming's first drawings of different cell stages leading to cell division in 1882. The cell cycle is a structured process that cells must undergo in the correct sequence to divide. In the mitotic cell cycle, the cell gives rise to two genetically identical daughter cells. This process is highly conserved and precisely controlled to govern the genome duplication and formation of the two daughter cells (Uzbekov & Prigent, 2022). Controlled cell proliferation is an essential part of tissue homeostasis and therefore it is unsurprising that any alterations in its regulation can lead to various diseases, such as cancer. One of the defining characteristics of cancer is the continuous stimulation of cell proliferation, as Hanahan (2022) reviewed. For instance, mutations that disturb the cell cycle control may allow continuous cell division by impairing the cells' ability to exit the cell cycle. Therefore, the normal and aberrant function of cell cycle in multiple cancers is widely studied to produce new insights into cell cycle control mechanisms that could be utilized in cancer treatment (Matthews et al., 2022).

2.3.1 Overview of the cell cycle

There are two main stages in the cell cycle, an interphase followed by an M phase (Figure 4). The cellular content is duplicated during interphase and the duplicated content is divided into two daughter cells in the M phase (Matthews et al., 2022). The interphase stage is divided into three orderly phases: G1 (Gap 1), S (DNA synthesis), and G2 (Gap 2). During G1, the cell grows, makes a new set of organelles and proteins for the daughter cells, and prepares for DNA synthesis. Following the G1 phase, the cell cycle progresses to S phase, where DNA replication is initiated. In the subsequent G2 phase, the cell undergoes further growth, completes DNA replication, and prepares for mitosis in M phase. Upon completion of the G2 phase, the cell enters the M phase, which includes both mitosis and cytokinesis (Matthews et al., 2022; Uzbekov & Prigent, 2022; Vermeulen et al., 2003). Mitosis is subdivided into five phases: prophase, prometaphase, metaphase, anaphase, and telophase. During mitosis, the long interphase DNA molecules condense into a highly compact form and the sister chromatids of compacted chromosomes are segregated into two distinct sets. At the end of mitosis, two fully functional nuclei are formed around each set of chromosomes (McIntosh, 2016). Mitosis is followed by cytokinesis, wherein the entire cellular content is divided, resulting in the formation of two genetically identical daughter cells (Matthews et al., 2022).

Throughout the cell cycle, several different checkpoints detect possible defects in DNA synthesis and chromosome segregation. If such defects are detected, the checkpoints are activated, and they induce cell cycle arrest. Cell cycle arrest allows cells to repair the defects, thus preventing their transmission to the resulting daughter cells (Malumbres & Barbacid, 2009). Based on the severity of the damages, the arrested cells can either re-enter the cell cycle, permanently exit the cell cycle and become senescent, or undergo apoptosis (Matthews et al., 2022).

During interphase, possible DNA double-stranded breaks (DSBs) activate the DNA damage checkpoint, and if necessary, the cell cycle is arrested until the breaks have been repaired. Before committing to DNA synthesis, at the restriction checkpoint, the cell decides whether it stays in G1, proceeds to S phase, or exits the cell cycle into the non-proliferative G0 (quiescent) phase. The majority of fully differentiated cells in an adult body rest in G0, sometimes even for years, waiting for a signal to transit back to G1 to start the cell cycle again (Matthews et al., 2022; Uzbekov & Prigent, 2022; Vermeulen et al., 2003).

The intra-S phase checkpoint in turn controls the replication process and ensures the accuracy of DNA replication (Iyer & Rhind, 2017). If necessary, intra-S checkpoint arrests the cell cycle to block entry to G2 (Matthews et al., 2022). Furthermore, in G2, a critical DNA damage checkpoint monitors the integrity of the replicated DNA, permitting entry into mitosis only if the DNA is undamaged (Thu et al., 2018; Uzbekov & Prigent, 2022).

In addition to G1, S, and G2 checkpoints, there is an important mitotic checkpoint in metaphase /anaphase transition as well. This mitotic checkpoint monitors the attachment of chromosome kinetochores to mitotic spindle microtubules. If for example unattached kinetochores or two kinetochores attached to the same microtubule are detected, the cell cannot progress from metaphase to anaphase and the cell cannot exit mitosis (Uzbekov & Prigent, 2022). Mitosis is followed by cytokinesis, wherein the entire cellular content is divided, resulting in the formation of two genetically identical daughter cells (Matthews et al., 2022; Vermeulen et al., 2003).

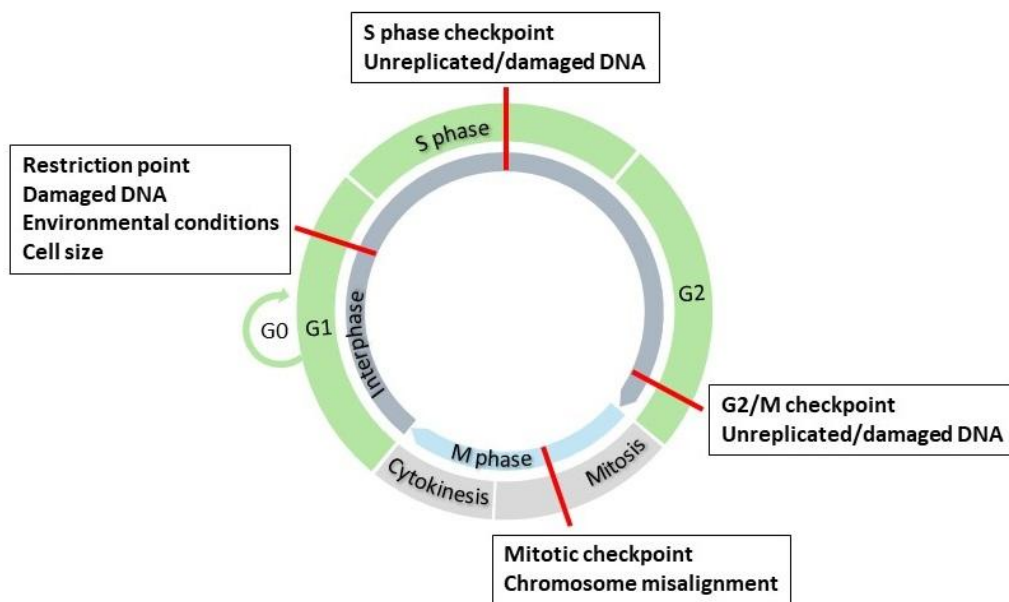


Figure 4. Cell Cycle. Throughout the cell cycle, a eukaryotic cell replicates all of its cellular content during interphase. The cell's DNA is replicated in the S phase and replicated DNA is segregated during mitosis in the M phase of the cycle. Segregation of the cellular content happens during cytokinesis at the end of the M phase. After cytokinesis, the cell can either stay in the G1 phase and enter a new round of cell division or exit the cell cycle into a non-proliferative G0 (quiescence) state. Cell cycle is controlled in different checkpoints before and after DNA replication and also in mitosis at the metaphase-anaphase transition. Figure is based on illustrations by Matthews et al., (2022) and Thu et al., (2018).

2.3.2 Interplay of cyclins and CDKs regulate cell cycle progression

The cell cycle progression is driven by cyclin-dependent kinase (CDK) activity. CDKs are serine-threonine kinases that complex with cyclin proteins to phosphorylate their target proteins (Malumbres, 2014). Cyclins function as the regulatory subunits of the CDK/cyclin complex. CDKs respond to the extracellular mitogenic signals and inhibitory intracellular cell cycle checkpoint signals from e.g. damaged DNA to regulate cell division. (Ding et al., 2020).

During G1 phase, mitogenic signals induce the expression of D-type cyclins D1, D2, and D3 that bind and activate CDKs 4 and 6 (Figure 5). Activated cyclin D-CDK4/6 complex initiates the phosphorylation of retinoblastoma (RB) protein that functions as the inhibitor of transcription factor E2F and keeps the E2F-dependent transcription inactive during G1. The phosphorylation of RB by cyclin D-CDK4/6 complex results in pRB dissociation from E2F, thus initiating the E2F-dependent transcription (Kato et al., 1993; Lundberg & Weinberg, 1998). This results in the transcription of G1/S target genes such as cyclins E and A. In late G1, E-type cyclins bind and activate CDK2, and these complexes further phosphorylate RB, leading to its complete inactivation. The fully released E2F can function in transcription initiation at its full capacity. Following this, the increased expression of cyclin A and the increased activity of cyclin A-CDK2 complex results in S phase entry (Ding et al., 2020; Malumbres & Barbacid, 2009; Matthews et al., 2022). During the late stages of S phase, the accumulated cyclin A-CDK2 complex drives the transition from S phase to G2 (Malumbres & Barbacid, 2009; Thu et al., 2018). At the end of interphase in G2, cyclin A activates CDK1 to facilitate the onset of mitosis. In the early stages of mitosis, the A-type cyclins are degraded which induces the formation of the cyclin B-CDK1 complexes. Cyclin B-CDK1 complexes drive cells through mitosis (Malumbres & Barbacid, 2009; Matthews et al., 2022).

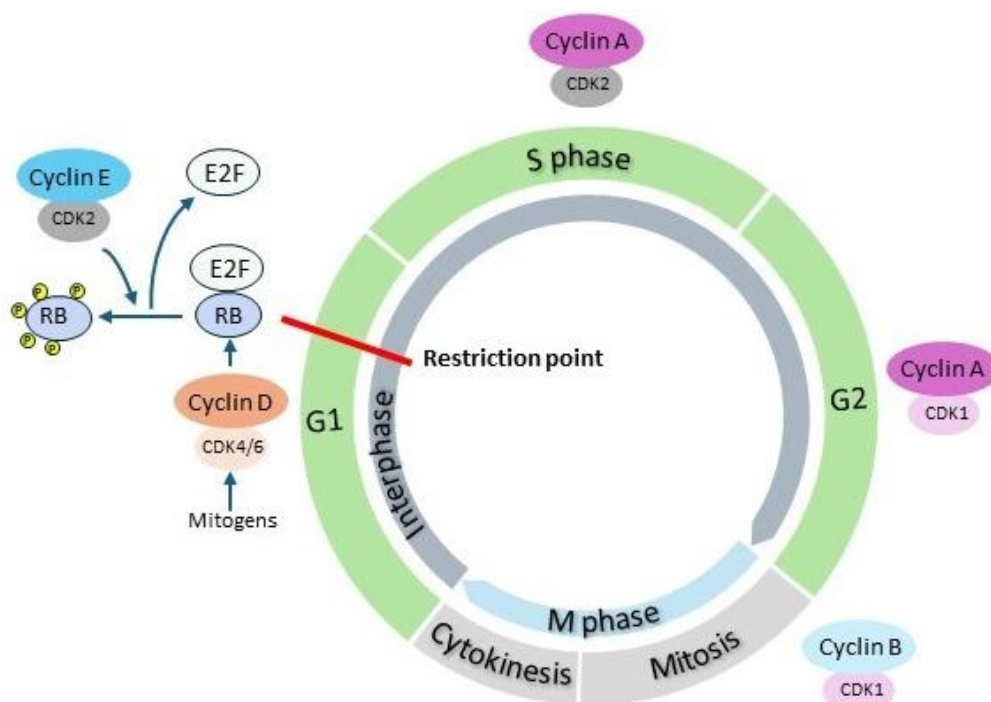


Figure 5. Cyclin-dependent kinase (CDK)-cyclin complexes regulate the progression of cell cycle. Cyclins activate cyclin-dependent kinases (CDKs). Once activated, CDKs phosphorylate specific target proteins to regulate cell cycle progression. The accumulation of the cyclin D-CDK2/4 complex initiates cell cycle entry by promoting the E2F-dependent transcription of e.g. cyclins E and A which then associate with CDK2. The accumulation of cyclin A/CDK2 complex is crucial for overcoming the restriction point at the G1/S transition. During late S phase, in increasing activity of cyclin A-CDK2 facilitates the transition from S phase to 2. Subsequently, at the end of G2, cyclin A activates CDK1, triggering the onset of mitosis. In the early mitosis, cyclin A is degraded, and cyclin B complexes with CDK1 which is needed for successful completion of mitosis. Figure is based on illustrations by Matthews et al., (2022) and Thu et al., (2018).

While cyclin-CDK complexes are central to regulating cell cycle progression, their activity is also tightly regulated by various factors, including cyclin-dependent kinase inhibitors (CKIs). CKIs are categorized into two main families: the CDK-interacting protein/kinase inhibitory (CIP/KIP) family, which includes proteins p21^{CIP1}, p27^{KIP1}, and p57^{Kip2}, and the inhibitor of CDK4 (INK4) family, consisting of p16^{INK4a}, p15^{INK4b}, p18^{INK4c}, and p19^{INK4d}. The CIP/KIP proteins can bind to cyclins A, B, D, and E, along with their respective CDKs (CDK1, CDK2, CDK4, and CDK6), modulating the activity of these cyclin-CDK complexes. Depending on their post-translational modifications, CIP/KIP proteins can either inhibit or increase the activity of the associated cyclin-CDK complexes. In contrast, INK4 family proteins specifically bind to CDK4 and CDK6, preventing the phosphorylation of the RB protein, which in turn represses the transcription of genes required for S phase entry (Csergeová et al., 2024).

Given the complexity of the cell cycle and its control mechanisms, there are multiple possibilities for malfunction which together with other genetic and epigenetic alterations can contribute to cancer development. For instance, mutations in signaling pathways that control the entry and exit of the S phase may promote uncontrolled cell cycle progression. When irreversible DNA damage is detected, the DNA damage checkpoint can trigger cellular responses such as quiescence, senescence, or apoptosis through p53-dependent pathways. Consequently, p53 is crucial in cancer prevention, and it is not surprising that the *TP53* gene, which encodes the p53 protein, is the most frequently mutated gene in various cancers (Matthews et al., 2022).

2.3.3 Cyclin D1 in prostate cancer

Of the three cyclin D family proteins—D1, D2, and D3—cyclin D1 is uniquely and significantly overexpressed in solid tumors, where it exerts functions independent of CDK4/6 and RB1, promoting tumorigenesis. Cyclin D1 is the most extensively studied member of the D-type cyclins in cancer development and progression (Tchakarska & Sola, 2020). The cyclin D1 protein, encoded by the *CCND1* gene located on the long arm of chromosome 11 (11q13), is expressed in most human cells, excluding bone marrow stem cells (Montalto & De Amicis, 2020). The *CCND1* gene is one of the most frequently mutated genes across various tumor types and, often found to be amplified (Aaltonen et al., 2020). While cyclin D1 is primarily known for regulating cell cycle progression, it also plays critical roles in DNA damage response, chromosome stability, cellular senescence, autophagy, mitochondrial function, cell migration, metabolism, and immune surveillance (Tchakarska & Sola, 2020).

Dysregulation of cyclin D1 leads to uncontrolled cellular proliferation, establishing *CCND1* as an oncogenic driver in several malignancies. Cyclin D1 dysregulation can occur for example via genetic or epigenetic mutations in the *CCND1* gene or through alterations in mitogen signaling pathways. Additionally, also loss of microRNAs, such as miR-15a and miR-16 in prostate cancer, can contribute to *CCND1* dysregulation (Montalto & De Amicis, 2020; Pluta et al., 2024; Qie & Diehl, 2016).

A notable genetic variation in *CCND1* is the G870A polymorphism at the exon 4 splice donor site, leading to alternative splicing and the formation of two protein isoforms (Betticher et al., 1995) which are now known as cyclin D1a and cyclin D1b. Cyclin D1a is the canonical, full-length protein encoded by 5 exons. Due to a splicing error in the

exon 4/intron 4 boundary, cyclin D1b is missing the exon 5 encoded amino acids and has gained 33 novel amino acids at the C-terminus due to translation into a stop codon in intron 4. Cyclin D1b lacks domains responsible for its degradation and ligand-independent interactions with nuclear receptors (Knudsen, 2006). Cyclin D1a shuttles between the nucleus and cytoplasm whereas D1b variant is considered constitutively nuclear (Solomon et al., 2003). The D1b isoform has been detected in cancers such as prostate, breast, and esophagus (Burd et al., 2006; Lu et al., 2003; Millar et al., 2009). Both isoforms can bind CDK4/6 (Dulińska-Litewka et al., 2022) though the D1b-CDK4/6 complex shows reduced phosphorylation of RB compared to the D1a-CDK4/6 complex (Lévêque et al., 2007; Solomon et al., 2003).

Despite the lower capacity to inactivate RB, cyclin D1b has an increased tumor-promoting activity. Increased expression levels of cyclin D1b have been observed in PC (Comstock et al., 2009), where it plays a distinct role compared to cyclin D1a by driving cellular transformation and promoting tumor growth (Augello et al., 2015). It has been shown that both cyclin D1a and D1b bind directly to AR. By binding to AR, cyclin D1a prevents the formation of an active conformation and thereby suppresses ligand-dependent AR activity (Burd et al., 2006; Petre-Draviam et al., 2005). Although cyclin D1a downregulates AR-dependent transcription, cyclin D1b does not suppress AR activity in the same manner. For instance, cyclin D1b is less effective at repressing the AR target gene PSA compared to D1a. Moreover, unlike cyclin D1a, cyclin D1b promotes androgen-dependent proliferation of prostate cancer cells (Burd et al., 2006). In addition to AR regulation, cyclin D1 also plays a role in DNA double-strand break repair mechanisms. According to Marampon et al., (2015), silencing of cyclin D1 impairs DSB repair in androgen-independent prostate cancer cells both *in vitro* and *in vivo*, sensitizing them to radiotherapy.

2.3.4 Cell cycle as a target of cancer treatment

The critical role of cyclins and CDKs in the regulation of the cell cycle makes them attractive targets for cancer treatment (Matthews et al., 2022). As cyclins are not enzymatically active, they are challenging therapeutic targets. However, their catalytic partner CDKs can be targeted (Qie & Diehl, 2016).

The development of effective CDK inhibitors has been challenging due to specificity issues, as these inhibitors can impact multiple protein kinases, leading to significant

biological side effects in patients. Despite these challenges, the CDK4/6 inhibitors palbociclib (PD0332991), ribociclib (LEE011), and abemaciclib (LY2835219) have been approved by the U.S. Food and Drug Administration (FDA) for the treatment of hormone receptor-positive advanced breast cancer in combination with endocrine therapies. Additionally, ribociclib and abemaciclib are currently being evaluated in combination with other drugs and in the treatment of various other cancer types (Pluta et al., 2024). These CDK4/6 inhibitors function by preventing the phosphorylation of the RB protein, thereby blocking the transition from the G1 phase to the S phase of the cell cycle (Matthews et al., 2022).

Besides CDK4/6 inhibitors, other cell cycle-targeting therapies exist for different cancers. For instance, docetaxel, a chemotherapy agent that disrupts mitotic spindle formation, has been approved for use in a broad range of cancers, including prostate cancer. (Matthews et al., 2022).

3. AIMS OF THE STUDY

A previous study had identified miR-193b as an epigenetically regulated microRNA in prostate cancer but its target genes in PC were unknown. Because miRNAs regulate the expression of protein-coding genes, it was important to identify the miR-193b targets in prostate cancer. There were several suggested target genes for miR-193b in other cancers, such as *CCND1* in hepatocellular carcinoma, pancreatic carcinoma, and melanoma. *CCND1* is known to encode cyclin D1 protein which has a significant role in modulating the cell cycle transition from G1 to S phase. Interestingly, a previous study showed that miR-193b reduced the number of prostate cancer cells in the S-phase of the cell cycle. Therefore, the two specific aims of this study were:

1. To confirm the hypermethylation and expression pattern of miR-193b in clinical prostate cancer samples
2. To investigate whether miR-193b targets *CCND1* in prostate cancer

4. MATERIALS AND METHODS

4.1 Clinical samples, xenografts and cell lines

Clinical samples were obtained from Tampere University Hospital (TAUH, Tampere, Finland). To study miR-193b methylation, fresh-frozen tissue samples including 10 BPH), 26 untreated prostatectomy specimens (PC), and 9 castration resistant tumors (CRPC) were used. Instead, 78 hormonally untreated PC prostatectomy specimens (Table 2) were used to study the expression miR-193b. BPH samples were obtained from transurethral resection of prostate (TURP) or (cysto) prostatectomies from patients with BPH or bladder cancer. Untreated cancer samples were obtained from prostatectomies and CRPC samples from TURP. Histological analysis confirmed that the samples used in this study contained >70% cancerous or hyperplastic tissue.

Table 2. Description of clinical samples used to study miR-193b expression.

	Number of samples	%
Prostatectomy specimens	78	
Gleason score		
<7	33	42
7	32	41
>7	13	17
pT stage		
pT2	46	59
pT3	32	41
Mean PSA ng/mL	11.8 (range: 3.15-51.5)	
Mean age at diagnosis	62.1 years (range: 47.4-71.8)	

For cyclin D1 immunohistochemical analysis, tissue microarrays (TMAs, Table 3) were constructed of 267 formalin-fixed prostate cancer specimens (198 prostatectomies and 69 CRPC samples). The Ethics Committee of Tampere University Hospital and the National Authority for Medicolegal Affairs have approved the use of clinical tumor material.

Table 3. Description of clinical samples used in immunohistochemistry.

	Number of samples	%
Prostatectomy specimens	198	
Gleason score		
<7	70 (35)	35
7	98 (50)	50
>7	30 (15)	15
pT stage		
pT2	142 (72)	72
pT3	53 (27)	27
Mean age at diagnosis	63 years (median 63, range 44-74 yrs)	
Median follow-up time	76 months (range 4.9-218.7 mo)	
Locally recurrent CRPCs	69	
Mean time from the beginning of treatment to the TURP	43 months (range 3-144 mo)	

Dr. RL Vessella provided the 17 PC LuCaP xenografts. The prostate cancer cell lines 22Rv1, PC- 3, LNCaP, and DU145 were purchased from the American Type Cell Collection (Manassas, VA). LAPC- 4 and VCaP cell lines were provided by Dr. Charles Sawyers (University of California at Los Angeles, Los Angeles, CA) and Dr. Jack Schalken (Radboud University Nijmegen Medical Center, Nijmegen, the Netherlands), respectively. All cell lines were cultured under recommended conditions.

4.2 DNA and RNA extraction

For the methylation analysis from clinical samples, freshly frozen tissue blocks were cut into 10 × 20-micrometer sections using a cryotome. DNA was isolated using an AllPrep RNA/DNA Minikit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Trizol® reagent (Invitrogen, Life Technologies Corporation, Carlsbad, CA) was used according to the manufacturer's protocol to isolate total RNA from cell lines as well as for miR-193b and *CCDN1* expression analysis in 22Rv1 cells. For microarray from xenograft samples, the RNA was extracted using the *mirVana*TM miRNA isolation kit (Thermo Fischer/Ambion, Austin, TX) according to the manufacturer's instructions.

4.3 miR-193b methylation analysis

miR-193b methylation in clinical samples was studied using the Methyl Binding Domain (MBD) qPCR method. For this, the MethylMiner™ Methylated DNA Enrichment Kit (Invitrogen) was used according to the manufacturer's instructions. Briefly, 2 µg of genomic DNA was fragmented by sonication using Bioruptor® (Diagenode, Denville, NJ) to an average length of ~250 bp. Methylated DNA was then enriched by binding to magnetic beads coated with the methyl-CpG-binding domain of the human MBD2 protein (Methyl-CpG Binding Domain Protein 2). The enriched DNA was eluted as a single fraction using 2 M NaCl. Finally, the DNA was ethanol-precipitated and resuspended in 50 µL of DNase-free water. For q-PCR, iQTM SYBR® Green supermix and CFX96 q-RT-PCR detection system (Bio-Rad Laboratories Inc.) were used. Each sample was run in duplicate. The methylation status of miR-193b was calculated from the elution-fraction signal in relation to the total q-PCR signal. The sequences of the primers used in the q-PCR were:

miR-193b_DMR_f1 (for) 5'-TGGCGTTTCTGGTTTCTCTT-3'

miR-193b_DMR_r2 (rev) 5'-CGCACCTTTTCTCCTCATTT-3'

4.4 Cell transfection and co-transfection

To determine the effect of miR-193b expression on cyclin D1, RB, and pRB protein levels, 22Rv1 cells were transfected with miRNA precursors purchased from Ambion (Applied Biosystems/Ambion, Austin, TX). Cells were transfected with a final concentration of 5 nmol/L pre-miR-193b or pre-miR-scrambled control using INTERFERin transfection reagent (Polyplus-transfection, Illkirch, France) according to the manufacturer's instructions. The cells were collected 3 days after transfection. Cotransfection of 22Rv1 cells for the cyclin D1 rescue was performed using jetPRIME transfection reagent (Polyplus-transfection). 22Rv1 cells were transfected with 10 µg of plasmid, pCMV-CCND1 lacking a 3'UTR (Plasmid 19927; Addgene, Cambridge, MA), or the control plasmid pCMV6- AC-GFP (OriGene Technologies, Rockville, MD) along with either pre-miR-193b or pre-miR-scramble at a final concentration of 10 nmol/L. The cells were collected 2 days after transfection.

4.5 Protein and RNA expression analysis

4.5.1 Western Blot

Nuclear and cytoplasmic proteins for RB and pRB western blots were extracted separately from cells growing on a 10-cm plate using Dignam's method (Dignam et al., 1983). The cells were harvested using a scraper. Briefly, harvested cells were first suspended in Hypotonic buffer (10 mM Hepes, pH 7.9, 1.5 mM MgCl₂, 10 mM KCl, 0.2 mM PMSF, 0.5 mM DTT), and cytoplasmic proteins were collected from supernatant. The remaining pellet was then resuspended with low-salt buffer (20 mM HEPES, pH 7.9, 25% glycerol, 1.5 mM MgCl₂, 20 mM KCl, 0.2 mM EDTA, 0.2 mM PMSF, 0.5 mM DTT). Nuclear proteins were collected using a high-salt buffer (as a low-salt buffer, but with 1.2 M KCl). Protein concentration was measured using the simplified Bradford method (BioRad Laboratories, Hercules, CA).

20 µg of proteins were separated on 8% (for RB, pRB) and 10% (for cyclin D1) sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and blotted to a polyvinylidene difluoride membranes (PVDF, Immobilon-P, Millipore Corp., Billerica, MA) using semi-dry transfer technique. The membranes were blocked in 4% BSA 2h in RT and then incubated with primary antibodies either against cyclin D1 (clone SP4, 1:100; Dako, Glostrup, Denmark), Rb (C-15: sc-50, 1:500; Santa Cruz Biotechnology, Inc., Dallas, TX), or phospho-Rb (Ser795, 1:1000, Cell Signaling, Danvers, MA). As a loading control, primary antibodies against fibrillarin (C13C3, 1:4000; Cell Signaling) or actin (pan AB-5 clone ACTN05, 1:400; Lab Vision Corp., Fremont, CA) were used. The blots were incubated with the primary antibodies overnight at +4°C. The primary antibodies were detected by secondary antibodies. Anti-rabbit-HRP (1:3000, DAKO) was used for phospho-RB, RB, cyclin D1, and fibrillarin detection, and anti-mouse-HRP (1:3000, DAKO) for Actin detection. The secondary antibodies were incubated for 1h at RT. Finally, the proteins were visualized by autoradiography.

4.5.2 Immunohistochemistry

The expression of cyclin D1 in clinical samples was performed by immunohistochemical analysis. Antibodies against cyclin D1 (dilution 1:100, clone SP4; Dako) and Ki-67 (dilution 1:500, MM1; Leica Biosystems Newcastle Ltd., Newcastle upon Tyne, UK) were used with a Power Vision+ Poly- HRP IHC kit (ImmunoVision Technologies Co.,

Hillsborough, CA) according to the manufacturer's instructions. Briefly, 4 μm tissue sections were deparaffinized and, for pretreatment, autoclaved in salt solution (5 mmol/L Tris-HCl / 1mmol/L EDTA; pH 9) at 98°C for 15 minutes. The primary antibody was diluted in preblocking solution and incubated overnight at +4°C. Lab Vision Autostainer 480S (Thermo Fisher Scientific, Waltham, MA, USA) was used to visualize the bound primary antibody. Tissue sections were counterstained with Mayer's hematoxylin (Histolab AB, Gothenburg, Sweden) for 2 minutes and mounted with Neo-Mount (Merck KGaA, Darmstadt, Germany). Every staining batch had a negative control, in which the primary antibody was omitted, and a positive control (formalin-fixed paraffin-embedded liver tissue).

The slides were scanned with an Aperio ScanScope XT scanner (Leica Microsystems GmbH, Wetzlar, Germany). The virtual microscope by Using JPEG2000 Interactive Protocol (Tuominen & Isola, 2010) and the ImmunoRatio web application (Tuominen et al., 2010) were used to score the cyclin D1-staining intensity (0–1 = negative and weak, 2 = moderate, and 3 = strong) within cancerous areas. For scoring the Ki-67 staining, the images were analyzed in ImageJ. Briefly, the immunostained and counterstained nuclei were discriminated from the background and segmented, and the relative percentages of the nuclei were calculated. The scoring was performed in a blinded fashion.

4.5.3 Quantitative reverse transcription PCR

Quantitative reverse transcription polymerase chain reaction (qRT-PCR) was used to study the expression of *CCND1* and miR-193b in transiently transfected 22rv1 cells. The CFX96 qRT-PCR detection system (Bio-Rad Laboratories Inc., Hercules, CA) was used for both experiments. In addition, the relative expressions were calculated using the $2^{-\Delta\Delta Cq}$ method.

For *CCND1* expression, first-strand complementary DNA synthesis was performed from 1 μg of total RNA with random hexamer primers using AMV reverse transcriptase (Finnzymes Inc., Espoo, Finland) according to the manufacturer's instructions. The Maxima SYBR Green qPCR Master Mix (Fermentas Inc., Burlington, ON, Canada) was used to determine the expression of *CCND1* and the expression was normalized to the β -Actin (*ACTB*) reference gene. The primer sequences used for the *CCND1* and β -Actin q-RT-PCR were:

CyclinD1for 5'-CCCTCGGTGGGTCCTACTTCAA-3'

CyclinD1rev 5'-TGGCATTGAGAGGAAGT-3'

Bactinf4 (for) 5'- TGGGACGACATGGAGAAAAT-3'

Bactinr4 (rev) 5'-AGAGGCGTACAGGGATAGCA-3'.

For miR-193b expression, TaqMan MicroRNA Reverse Transcription Kit and microRNA Assay for miR-193b (Applied Biosystems, Foster City, CA) were used according to the manufacturers' recommendations. Briefly, 50 ng of total RNA from each sample was reverse transcribed with miR-193b specific primer together with RNU6B specific primer using MultiScribe™ reverse transcriptase (Invitrogen). miRNA expression was normalized to RNU6B expression.

4.5.4 Microarray

miRNA expression profiling was performed on PC cell lines and xenograft samples using Agilent Technologies' (Santa Clara, CA) miRNA microarray system. Cell line RNA was hybridized to version 1 array (containing 470 human and 64 viral miRNAs) and LuCaP xenograft RNA to version 2 array (containing 723 human and 76 viral miRNAs). mRNA expression profiling from cell lines was done using Whole Human Genome Kit (4 × 44k) Chip (Agilent Technologies, Santa Clara, CA). mRNA expression profiling in LuCaP xenografts was performed on Affymetrix (Santa Clara, CA) HG U133 Plus 2.0 microarray. All arrays were done according to the manufacturer's protocols.

4.6 Functional analyses

4.6.1 Cell proliferation assay

Different prostate cancer cell lines were treated with varying dosages of cyclin D1 target CDK4/6 inhibitor PD0332991 (Selleck Chemicals, Houston, TX) to study how the CDK4/6 blockage affects cell growth. Cells were seeded in 24-well plates in quadruplicate for each inhibitor concentration. The following day, the cells were imaged (day 0), and then treated with 0 nmol/L, 100 nmol/L, 500 nmol/L, or 2000 nmol/L inhibitor. The growth medium was replaced every other day with a fresh medium containing the inhibitor. The cells were imaged daily with an Olympus IX71 microscope with the OASIS automation control system and Surveyor imaging software version 5.5.5.26 (Objective Imaging Ltd.,

Cambridge, UK). To determine the cell growth, ImageJ software (NIH, Bethesda, MD) and an in-house macro were used to measure the cell surface area.

4.6.2 Luciferase reporter assay

Luciferase reporter assay was used to study whether miR-193b binds to the 3'UTR - region of the *CCND1* gene in prostate cancer cells. The pSGG-3UTR plasmid (SwitchGear Genomics, Menlo Park, CA) containing a luciferase gene fused with the 3'UTR of *CCND1* and an internal transfection efficiency control plasmid containing Renilla luciferase were utilized in this experiment. 22Rv1 cells were cotransfected with the 3'UTR plasmid, control plasmid, and pre-miR-193b or pre-miR-scramble using Lipofectamine™ 2000 transfection reagent (Invitrogen) according to the manufacturer's instructions. The Dual-Glo Luciferase Assay System (Promega, Madison, WI) was used according to the manufacturer's protocol to determine the firefly and Renilla luciferase activities 24 h after transfection. The measurement was done with Luminoscan Ascent luminometer (Thermo Fisher Scientific). The firefly luciferase light emission values were normalized against Renilla luciferase light emission values. The luciferase assay was performed in quadruplicate and repeated four times.

4.6.3 Flow cytometric analysis

Propidium iodide staining and flow cytometric analysis was used to determine the number of cells in S- and G2/M-phases of the cell cycle. First, 22Rv1 cells were harvested by trypsinization, washed with phosphate-buffered saline (PBS), and fixed in cold 70% EtOH. Next, ethanol was removed by aspiration, and the cells were washed and rehydrated with PBS. Finally, the cells were stained using propidium iodide (Sigma-Aldrich, St. Louis, MO), and the cell cycle profiles were analyzed with an Accuri™ C6 Flow cytometer and Accuri™ C6 Plus software (BD Biosciences, San Jose, CA).

4.7 Statistical analyses

Unpaired two-tailed Student's *t* test was used to calculate the significance between the experimental and control conditions in luciferase reporter assay and cell cycle analysis. In addition, the unpaired *t* test was also used to calculate the difference in miR-193b expression between differentially staged prostatectomy tumors. The P-value in the cell

proliferation assay was calculated using two-tailed paired *t* test. Fisher's exact test was used to assess the significant difference in miR-193b methylation in clinical samples. Fisher's exact, Chi-square, Mann–Whitney *U*, and unpaired *t* tests were used to analyse the association between clinicopathologic variables. Spearman's correlation was used to study the correlation of miR-193b and *CCND1* expression in cell lines and xenograft samples in microarray analysis.

P-values < 0.05 (*), < 0.01 (**), and < 0.001 (***) were considered statistically significant. All the statistical analyses and graphs used were performed using GraphPad Prism version 5.02 (GraphPad Software Inc, La Jolla, CA, USA). Error bars represent standard error of mean (SEM) in each graph.

5. RESULTS

5.1 *MIR193B* is hypermethylated in prostate cancer

A previous study by Rauhala et al., (2010) suggested that hsa-miR-193b-3p (aka miR-93b) is an epigenetically silenced putative tumor suppressor in prostate cancer. Their study analyzed the methylation levels of *MIR193B* by bisulfite sequencing in five untreated and four CRPC clinical specimens and different PC cell lines. Their data suggested that *MIR193B* is hypermethylated in 22Rv1 and moderately methylated in VCaP cells but is not methylated in LAPC-4, LNCaP, DU145, PC-3, EP156T, or PrEC cells. Furthermore, the methylation of miR-193b was increased in clinical samples. Because of the rather small group of clinical samples in the previous study, the aim here was to confirm if *MIR193B* hypermethylation in prostate cancer using a larger cohort of clinical specimens.

We have previously performed Methylated DNA immunoprecipitation sequencing (MeDIP-seq) on a set of clinical samples (unpublished data) and found that the most differentially methylated genomic region in proximity to *MIR193B* is 16:14396975–14397475 (GRCh37), which is located 349 bp upstream of the miR-193b gene. Here, MBD-qPCR method was utilized to measure the methylation of *MIR193B* in that genomic region in BPH ($n = 10$), PC ($n = 26$), and CRPC ($n = 9$) samples. In the analysis, samples were classified as methylated if they showed more than 20% methylation. According to MBD-qPCR data, *MIR193B* was significantly more methylated in cancer than in BPH ($p < 0.0001$, Figure 6).

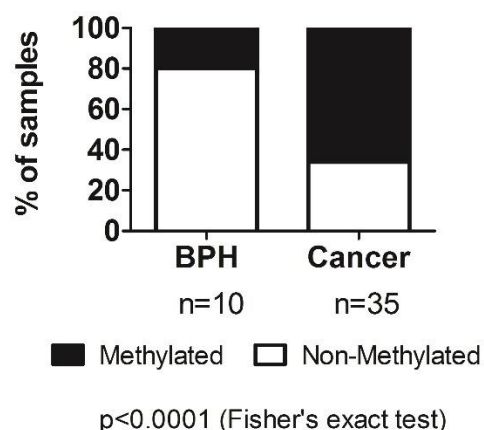


Figure 6. *MIR193B* is methylated in cancer samples. miR-193b methylation in clinical BPH and cancer samples was measured by MBD-qPCR. Fisher's exact test was used to calculate the p-value ($p < 0.0001$). Figure retrieved from Kaukoniemi et al., (2015).

5.2 Expression of miR-193b and *CCND1* in prostate cancer

To confirm the reduced expression of miR-193b in clinical prostate cancer samples detected by Rauhala et al., (2010), qRT-PCR was performed on a larger set ($n = 78$) of prostatectomy samples. The sample set included tumors ranging from pathological stage pT2 to pT3. The results indicated that the miR-193b expression was lower ($p < 0.05$) in stage pT3 tumors compared to stage pT2 tumors (Figure 7). However, there was no association between miR-193b expression and the Gleason score or progression-free survival (data not shown).

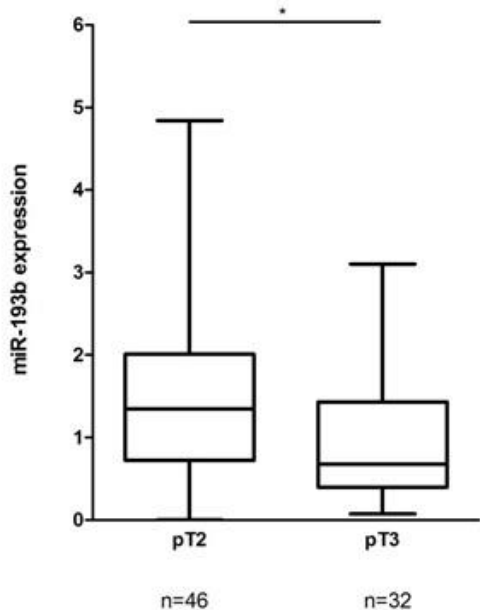


Figure 7. The expression level of miR-193b in clinical samples. miR-193b expression was analyzed from pathological stage 2 and 3 prostatectomy samples using qRT-PCR. The data was assessed with an unpaired two-tailed *t* test. *, $p < 0.05$. Figure retrieved from Kaukonen et al., 2015.

When this study was conducted, the target gene of miR-193 in prostate cancer had not yet been identified. Previous studies had suggested that *CCND1* could be a target of miR-193b in hepatocellular carcinoma, pancreatic carcinoma, and melanoma (J. Chen et al., 2010; Ikeda et al., 2012; C. Xu et al., 2010). Therefore, the hypothesis here was that *CCND1* might also be a target gene of miR-193b in prostate cancer. To investigate this possibility, the expression patterns of miR-193b and *CCND1* were first studied across various prostate cancer xenograft models, cell lines, and clinical samples.

First, the expression pattern of *CCND1* and miR-193b was studied in prostate cancer cell lines and xenografts using mRNA and miRNA microarray. The analyses showed that *CCND1* and miR-193b expressions are inversely correlated in PC cell lines (Figure 8A, $r = -0,7714$) and xenografts (Figure 8B, $r = -0,2522$). Especially in 22Rv1 and VCaP cells, the endogenous miR-193b expression was low whereas the *CCND1* expression was high. Instead, in LNCaP cells, the expression pattern was the opposite. The inverse expression pattern of miR-193b and *CCND1* was true also for seven LuCaP xenografts (23.6; 35; 70; 73; 77; 78 and 96) (Figure 8B).

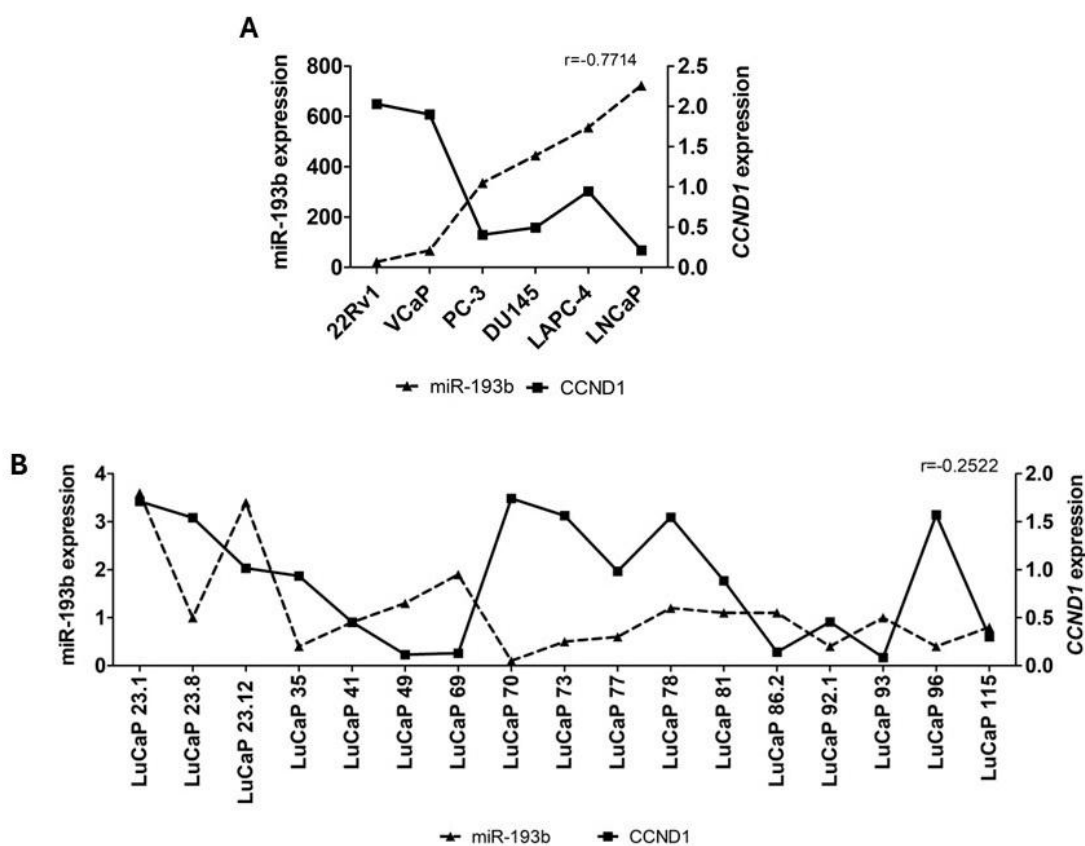


Figure 8. miR-193b and *CCND1* have inverse expression patterns in prostate cancer cells lines and xenografts. *CCND1* and miR-193b expressions were studied by miRNA and mRNA microarrays from (A) prostate cancer cell lines and (B) from xenografts. Spearman correlation coefficients are shown. Figure modified from Kaukonen et al., (2015).

To study how the overexpression of miR-193b affects the RNA and protein expression of the *CCND1* gene, 22Rv1 cell line with the low endogenous miR-193b expression were used. 22Rv1 cells were transiently transfected with pre-miR-19b and pre-miR-scramble. As shown in Figure 9A, miR-193b overexpression reduced the expression of *CCND1*. Additionally, a clear reduction in the cyclin D1 protein level, encoded by *CCND1* gene, was detected by Western blot method in 22Rv1 cells with miR-193b overexpression (Figure 9B). Furthermore, the phosphorylation level of retinoblastoma protein RB, a downstream target of cyclin D1, was reduced in nuclear protein fraction of 22Rv1 cells overexpressing miR-193b (Figure 9C). In addition, also VCaP cells, another cell line with low endogenous miR-193b expression, were transfected similarly to 22Rv1 cells. Equal to 22Rv1 cells, the cyclin D1 protein level was diminished also in VCaP cells overexpressing miR-193b (Figure 9D).

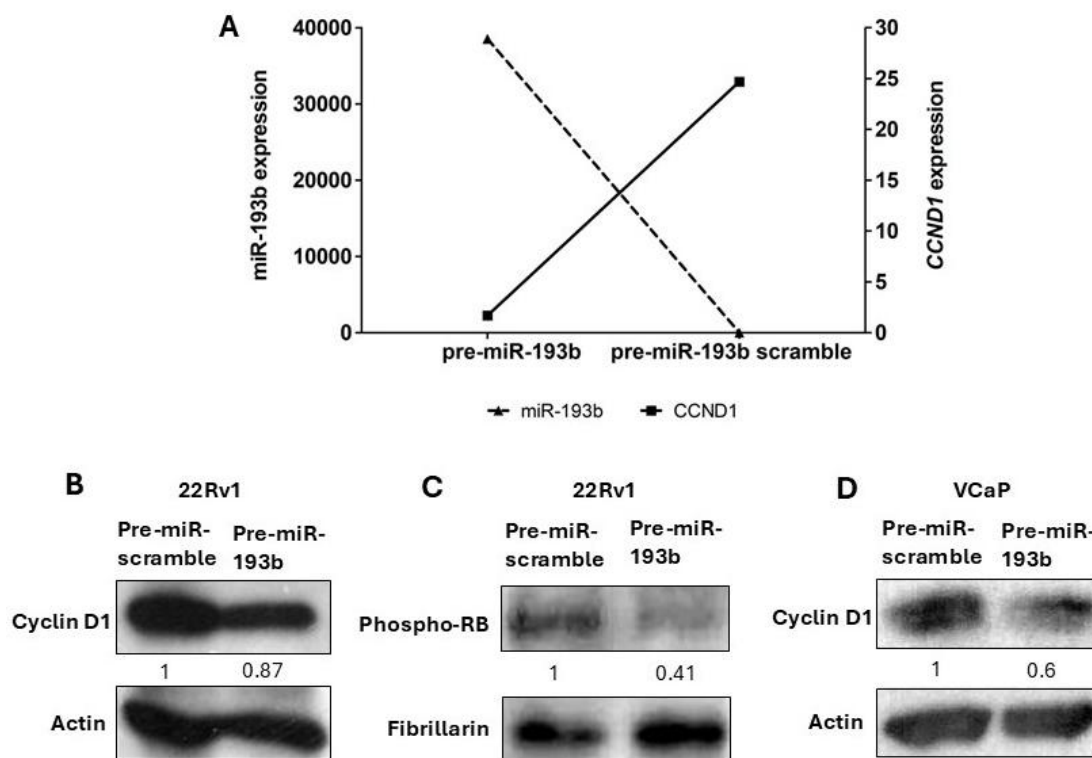


Figure 9. The overexpression of miR-193b reduces the expression of *CCND1*, the level of cyclin D1 protein, and the phosphorylation level of RB protein. (A) *CCND1* and miR-193b expressions by qRT-PCR in 22Rv1 cells transfected with pre-miR-193b and pre-miR-scramble. (B) Cyclin D1 protein expression in total proteins and (C) phosphorylation level of RB protein in nuclear fraction of 22Rv1 transfected with pre-miR-193b and pre-miR-scramble. (D) Cyclin D1 protein expression in total proteins of VCaP cells transfected with pre-miR-193b and pre-miR-scramble. Actin and fibrillarlin were used as loading controls. Protein expressions were determined using Western blot method. Figure modified from Kaukonen et al., (2015).

Cyclin D1 expression patterns were also determined in clinical samples using immunohistochemical analysis on tissue TMAs from PC ($n = 198$), and CRPC ($n = 69$) samples. Based on the results, cyclin D1 was expressed at a higher level in CRPC samples than in PC samples ($p = 0.0237$; Table 4). CRPC cells with high levels of cyclin D1 also had increased proliferation marker Ki-67 values but the association was not statistically significant. In prostatectomy samples, the cyclin D1 staining intensity was not associated with Gleason score, pT stage, or diagnostic PSA levels. However, there was a strong positive association between cyclin D1 and Ki-67 ($p < 0.0001$) in the prostatectomy cohort.

Table 4. Association between cyclin D1 expression and clinicopathological variables. Table retrieved from Kaukonen et al., 2015.

Variable	Cyclin D1 expression		P
	Negative (0/1)	Positive (2-3)	
Prostatectomy specimens, n (%)	74 (37)	124 (63)	
Locally recurrent CRPCs, n (%) ^a	20 (29)	49 (71)	0.0237
Prostatectomy specimens			
Gleason score, n (%) ^a			
<7	24 (33)	46 (37)	
7	38 (52)	60 (48)	
>7	11 (15)	19 (15)	0.8337
pT Stage, n (%) ^b			
pT2	54 (75)	88 (72)	
pT3	18 (25)	35 (28)	0.6216
PSA ng/mL (mean ± SD) ^c	20.0 ± 31.5	14.3 ± 11.3	0.9786
Age (mean ± SD) ^d	62.6 ± 5.2	63.2 ± 4.9	0.4167
Ki-67 (mean ± SD)	7.1 ± 7.0	13.6 ± 14.5	< 0.0001
Locally recurrent CRPCs, n (%) ^c			
Ki-67 (mean ± SD)	13.2 ± 9.3	20.7 ± 15.8	0.0950

^a χ^2 test
^b Fisher's exact test
^c Mann-Whitney U-test
^d Unpaired t test

5.3 miR-193b targets *CCND1* in prostate cancer

Following the *CCND1* and miR-19b expression analyses, the next aim was to confirm that miR-193b binds to the 3'UTR -region of the *CCND1* gene in prostate cancer cells. For this, a luciferase reporter assay was performed. Again, 22Rv1 cells with low endogenous expression of miR-193b were transfected with a pSGG-luciferase plasmid containing *CCND1* 3'UTR at the 3' end of the firefly luciferase gene together with either pre-miR-193b or pre-miR-scramble and control plasmid containing Renilla luciferase. Indeed, luciferase activity inhibition was observed in cells transfected together with the pSGG-plasmid and pre- miR-193b ($p < 0.05$) but not with pre-miR-scramble (Figure 10A), confirming that miR- 193b targets the 3'UTR of *CCND1*.

Next, a rescue experiment was performed to validate that miR-193b targets *CCND1*. In this experiment, the phosphorylation of RB was used as a measure of cyclin D1 activity.

In 22Rv1 cells the overexpression of miR-193b reduced the phosphorylation of RB protein. Such a reduction in phospho-RB level was not detected in 22Rv1 cells transfected with pCMV-CCND1 plasmid lacking the 3'UTR of *CCND1* (Figure 10B). Furthermore, cell cycle analysis from miR-193b overexpressing 22Rv1 cells showed a reduction in the proportion of cells in S (SPF) and G2/M phase fractions (Figure 10C, *p*-value <0.05). However, no significant difference was observed in 22Rv1 cells co-transfected with pre-miR-193b and *CCND1* lacking 3'UTR (Figure 10C).

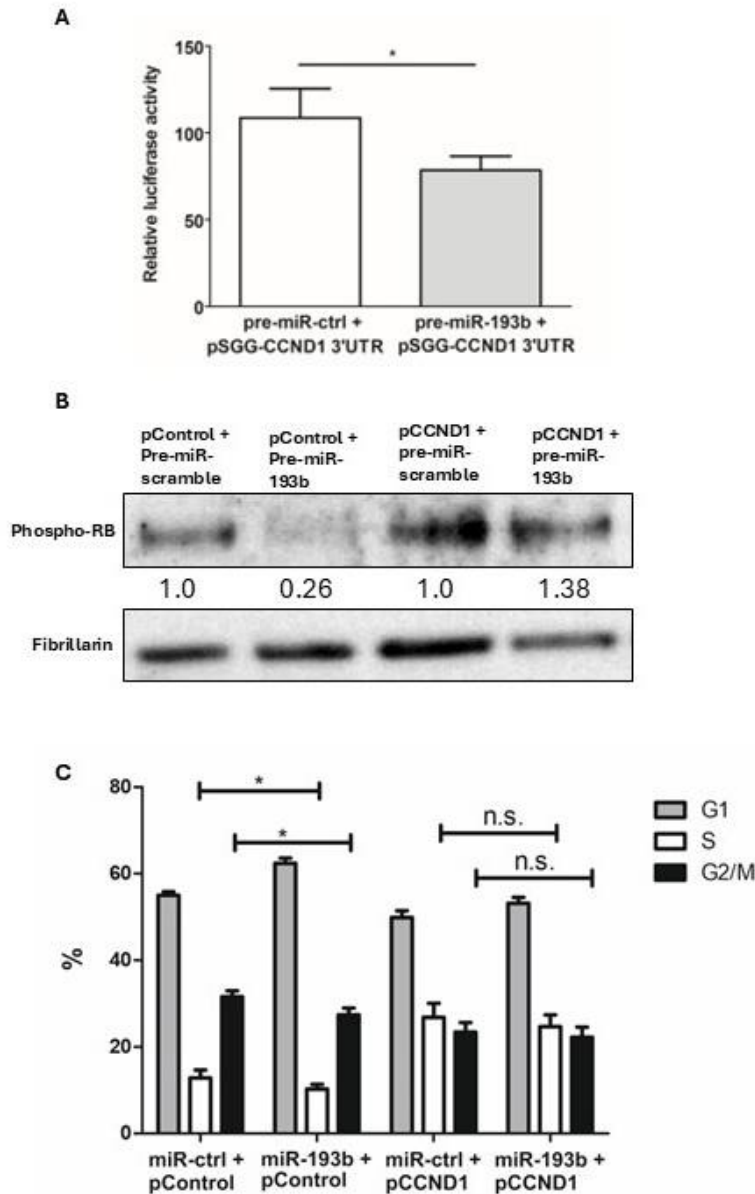


Figure 10. miR-193b targets *CCND1* 3'UTR. (A) In luciferase reporter assay 22Rv1 cells were cotransfected with pSGG-plasmid containing *CCND1* 3'UTR, Renilla luciferase plasmid, and pre-miR-193b or pre-miR-scramble. Renilla luciferase activity was used for normalization. The means of four experiments \pm SEM are shown. p -value was calculated using an unpaired t test. *, p -value < 0.05 . (B) A rescue experiment was performed using Western blot method for 22Rv1 cells transfected together with either control plasmid or pCMV-*CCND1* plasmid lacking *CCND1* 3'UTR and pre-miR-scramble or pre-miR-193b. Fibrillarin antibody was used as a loading control for nuclear proteins. (C) Cell cycle analysis to 22Rv1 cells transfected with pre-miR-scramble or pre-miR-193b together with either control plasmid or with p*CCND1*-plasmid containing *CCND1* gene without its 3'UTR. The means of four experiments \pm SEM are shown. p -value was calculated using an unpaired t test. *, p -value < 0.05 . Figure modified from Kaukonen et al., (2015).

Following the rescue analyses, the effect of cyclin D1 on the proliferation of different PC cell lines was evaluated. Since cyclin D1 complexes with and activates CDK4/6 to control

the progression of the cell cycle from G1 to S phase, CDK4/6 inhibitor PD0332991 was used to treat different PC cell lines with varying concentrations (0, 100, 500, and 2000 nmol/L) of the inhibitor. 22Rv1 and VCaP cells with low level expression of miR-193b and high-level expression of *CCND1*, showed significant growth suppression at a 500 nmol/L concentration (Figures 11 A-B, $p < 0.05$). The inhibitor had only little effect on the growth of DU145, LNCaP, LAPC-4, and PC-3 (Figure 11C-11F).

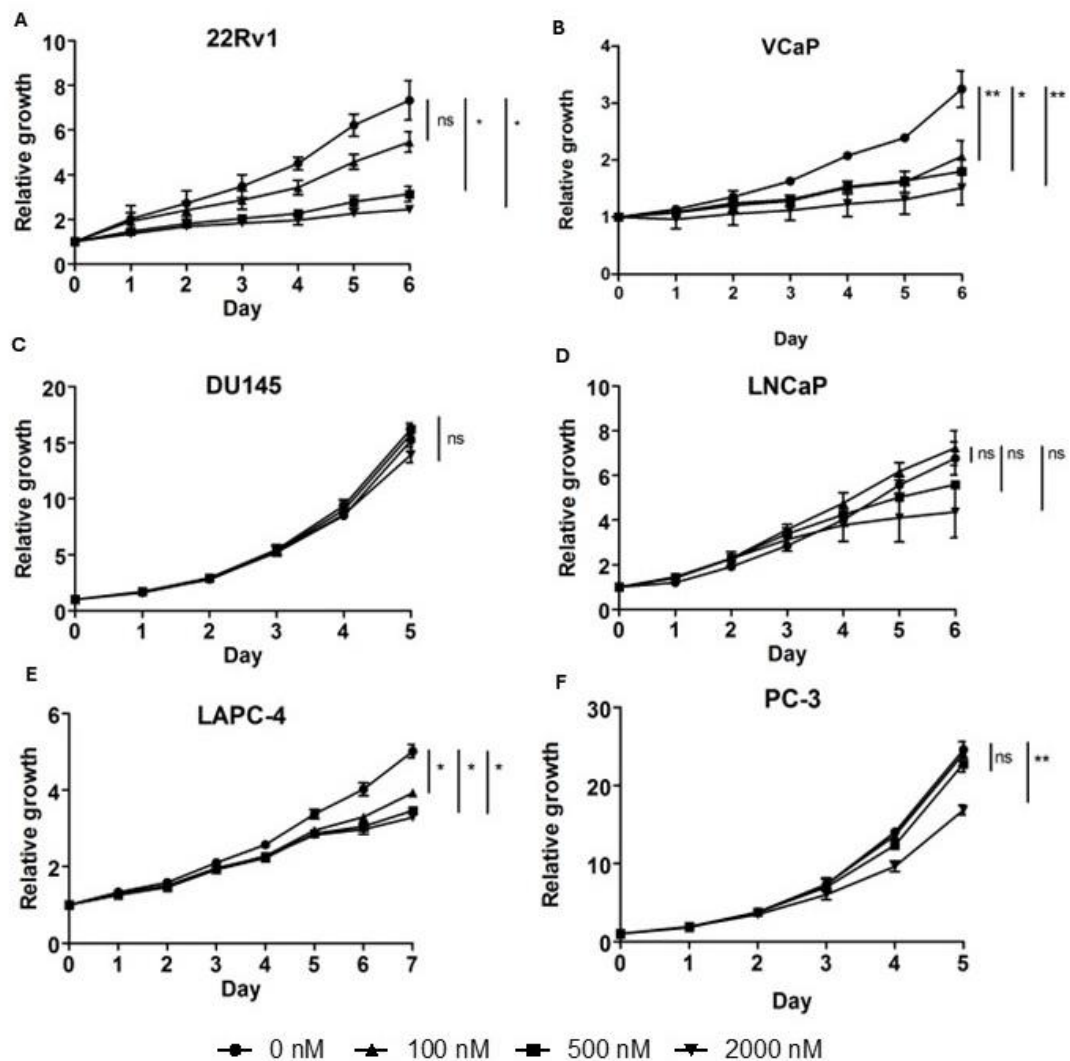


Figure 11. The effect of CDK 4/6 inhibitor PD0332991 on the growth of PC cell lines. (A) 22Rv1, (B) VCaP, (C) DU145, (D) LNCaP, (E) LAPC-4 and (F) PC-3 cells were treated with 0, 100, 500, and 2000 nmol/L concentrations of the inhibitor, and growth was followed for 5 to 6 days. Each concentration was done in quadruplicates and each experiment was repeated three times. The average from experiments \pm SEM is shown. P -values were calculated using paired t test, * $p < 0.05$, ** $p < 0.01$. Figure modified from Kaukonieni et al., (2015).

6. DISCUSSION

All cancers, including prostate cancer, accumulate various genetic and epigenetic aberrations affecting multiple genes that regulate signaling pathways, cell cycle, chromatin structure, etc. before transformation to malignant disease. Furthermore, the disease can continue to evolve, acquiring additional changes that drive metastasis and, potentially, lead to an incurable state. MicroRNAs are one part of the epigenetic system that regulates chromatin structure and gene expression, and their dysregulation plays a pivotal role in cancer pathogenesis, including in prostate cancer. Moreover, miRNAs themselves are subject to epigenetic regulation in cancers.

Rauhala et al. (2010) proposed that *MIR193B* is hypermethylated in prostate cancer. Their analysis, based on a limited clinical sample set of 4 benign prostatic hyperplasia (BPH), 5 prostate cancer (PC), and 4 castration-resistant prostate cancer (CRPC) tissues, examined the methylation status of *MIR193B*. However, these findings required further validation. Methylation analysis using MBD-qPCR on a larger cohort (10 BPH and 35 cancer samples) confirmed that the *MIR193B* promoter exhibited significantly higher methylation levels in PC tissues compared to BPH. Similarly, miR-193b expression was notably reduced in more advanced pT3 prostatectomy tumors compared to pT2 tumors. Subsequent studies have corroborated the hypermethylation of *MIR193B* in prostate cancer. Torres-Ferreira et al. (2017) showed similar promoter hypermethylation of *MIR193B* in prostatectomy samples and more interestingly, in urine sediments. Further confirmation of *MIR193B* promoter hypermethylation and its reduced expression was provided by Mazzu et al. (2019) based on microarray data from 330 prostatectomy samples.

To evaluate the impact of miRNA methylation or expression in a given tissue, it is essential to identify the target gene(s) through which the miRNA exerts its regulatory function. At the time this study was conducted, no validated target genes for miR-193b in prostate cancer had been published. MicroRNA target genes can be predicted by using miRNA target prediction algorithms which can be based on characteristics of miRNA sequence, miRNA-miRNA target interaction, and Machine Learning-driven statistical deduction (Liu & Wang, 2019). Rauhala et al. (2010) attempted to identify miR-193b targets using prediction programs such as Miranda and TargetScan. However, no consensus targets suitable for experimental validation were identified, as the top 20

predicted targets varied across the programs. However, miR-193b was suggested to target *CCND1* in hepatocellular carcinoma, pancreatic carcinoma, and melanoma (Chen et al., 2010; Ikeda et al., 2012; Xu et al., 2010). Cyclin D1 (encoded by *CCND1*) plays a key role in cell cycle regulation by directly targeting and phosphorylating the RB protein, thereby promoting the G1/S phase transition (Montalto & De Amicis, 2020). As Rauhala et al. (2010) had demonstrated that the overexpression of miR-193b inhibited the proliferation and reduced the number of cells in the S-phase of the cell cycle, it was natural to investigate whether *CCND1* could be a target of miR-193b also in prostate cancer.

First, the expression levels of both genes were examined in prostate cancer cell lines and xenografts. The results revealed an inverse correlation between miR-193b and *CCND1* expression in these models, suggesting a regulatory relationship. Furthermore, in 22Rv1 cells with low endogenous miR-193b expression and high endogenous *CCND1* expression, transient miR-193b overexpression reduced the expression levels of *CCND1*/Cyclin D1, RB, and phospho-RB further elucidating the connection between miR-193b and *CCND1*/Cyclin D1.

The relationship between miR-193b and *CCND1* was further investigated using several approaches. First, a luciferase assay was used to show that miR-193b targets *CCND1* through binding to its 3'UTR in 22Rv1 prostate cancer cells. This finding aligns with previous and subsequent studies showing that miR-193b binds to the 3'UTR of *CCND1* to downregulate its expression in various cancers, including e.g. malignant melanoma (Chen et al., 2010), hepatocellular cancer (C. Xu et al., 2010), cervical cancer (Huang et al. 2021), and meningiomas (Kober et al. 2023).

Luciferase assay result was validated through a rescue experiment. In 22Rv1 cells with high endogenous cyclin D1 expression, the forced overexpression of miR-193b decreased significantly the phosphorylation of RB and the number of cells in the S and G2/M phases of the cell cycle compared to the control. The function of *CCND1* was rescued when 22Rv1 cells were transfected with miR-193 and plasmid containing *CCND1* without its 3'UTR. In these cells, miR-193b was able to target only the endogenous *CCND1* but not the overexpressed one, and therefore the phosphorylation of RB and the number of cells in the S and G2/M phases of the cell cycle did not decrease significantly. In the rescue Western blot (Figure 10B), the quantitation of the 22Rv1 cells transfected with miR-193b and p*CCND1* plasmid (*CCND1* without its 3'UTR) is slightly higher compared to control cells (miR-scramble + control plasmid). The higher

quantitation can be caused for example by uneven expression of *CCND1* transgene in the cells. Another possibility could be technical issues, such as errors in sample loading, antibody incubation, autoradiography, etc. during Western blot method. Anyhow, the effect of miR-193b through cyclin D1 on cell cycle progression and RB phosphorylation is clear.

In addition, when the prostate cancer cell lines were treated with CDK4/6 inhibitor PD0332991 (Palbociclib), the cell lines' response to the treatment was influenced by miR-193b expression and methylation status, and cyclin D1 levels. Rauhala et al. (2010) had previously studied that *MIR193B* is hypermethylated in 22Rv1 and VCaP cell lines. These cells have low miR-193b expression and high *CCND1*/cyclin D1 expression. Therefore, when cells were treated with PD0332991, the proliferation of these cells was significantly reduced as the catalytic activity of CDK4/6 in the cyclin D1-CDK4/6 complex was inhibited and cells were not able to continue cell cycle from G1 to S phase. The inhibitor was effective in VCaP cells in every concentration whereas in 22Rv1 cells the 100nM treatment did not cause significant growth reduction although the effect was still visible.

In contrast to 22Rv1 and VCaP, *MIR193B* is not methylated in PC-3, DU145, LNCaP, and LAPC-4 cell lines. Thus, in these cells, the regulation of cell cycle progression is controlled by other proteins than cyclin D1-CDK4/6 complex, and therefore the inhibitor PD0332991 did not affect the proliferation of these cells. In DU145 and LNCaP cells, the inhibitor did not affect cell growth at any concentration whereas in PC-3 cells only the 2000 nM concentration reduced cell proliferation. In LAPC-4 cells all inhibitor concentrations slightly decreased the proliferation of the cells.

Previously, Fry et al. (2004) assessed the function of PD0332991 in several cell lines representing multiple cancers and found that the effective concentration of PD0332991 to obtain 50 % proliferation reduction varies from 40 nM to over 3000 nM depending on the cell line. In PC-3 cells the only significantly effective inhibitor concentration was 2000 nM which is rather high and the significance is apparent only on the last day of imaging. Similarly in LAPC-4, the inhibitor treatment was significant only after several days of treatment. In 22Rv1 and VCaP cells the effect was apparent already on the first days of treatment. It is possible that 100 nM concentration of PD0332991 is not enough to suppress CDK4/6 function in 22Rv1 cells and that this cell line requires higher concentration despite low endogenous expression of cyclin D1.

Finally, the cyclin D1 expression was studied also from clinical samples. IHC analysis of Cyclin D1 expression on tissue microarrays (TMAs) showed that Cyclin D1 is more highly expressed in CRPC than in hormone-naïve PC. In both PC and CRPC, the cyclin D1 staining was localized to the nucleus, with higher intensity observed in CRPC. In PC, there was a strong association between cyclin D1 and proliferation marker Ki-67, which is expressed during all phases of the cell cycle in actively proliferating cells but is absent in non-dividing cells (Gerdes et al., 1991; Guillaud et al., 1989). Instead, in CRPC the association was not significant although Ki-67 values were high in cells with high cyclin D1.

Although it is well known that cyclin D1 has two isoforms, cyclin D1a and D1b, caused by possible G870A polymorphism in the *CCND1* gene, the results in this study do not separate these isoforms. Primers for *CCND1* and antibodies for cyclin D1 (WB and IHC) identify both isoforms. Distinguishing these isoforms was deemed unnecessary, as the primary objective was to investigate whether miR-193b targets Cyclin D1 rather than explore the differential expression of the isoforms. Both isoforms share the 3'UTR region where miRNAs bind, meaning that alternative splicing would not have influenced the RNA and protein expression outcomes observed in the forced overexpression studies using miR-193b and miR-scramble in 22Rv1 cells.

Altogether, these results show the importance of miR-193b in regulating *CCND1* expression and cell cycle progression in prostate cancer. By targeting *CCND1*, miR-193b inhibits its activity to activate RB through phosphorylation. However, the regulatory network involving miRNAs is highly complex, as one mRNA can be targeted by multiple miRNAs, and conversely, one miRNA can target multiple mRNAs. This complexity is further intensified by the fact that miRNA genes, miRNA target genes, and miRNAs themselves are regulated at multiple levels, including genetic, epigenetic, transcriptional, translational, and post-translational mechanisms.

Given these layers of regulation and the central role of *CCND1* as a master regulator of the cell cycle, it is expected that miR-193b is not the only miRNA targeting *CCND1* in prostate cancer or other cancers. For example, miR15-a and miR-16-1 directly bind *CCND1* 3'UTR and diminish its expression in prostate cancer (Bonci et al., 2008). Unlike *MIR193B* which is hypermethylated in PC (Rauhala et al., 2010), *MIR15A* and *MIR16-1* are frequently deleted, thus conferring to their reduced regulatory function (Bonci et al., 2008). Although these miRNAs are dysregulated through different mechanisms, their disruption collectively leads to *CCND1* overexpression in prostate cancer. Beyond miR-

193b, *CCND1* is also targeted by various miRNAs in other cancers, such as miR-374b in breast cancer (Liu et al., 2021), miR-628 in colorectal cancer (Guo & Xue, 2020) and miR-342 in chronic myeloid leukemia (Wu et al., 2021).

MicroRNA-193b also targets other genes than *CCND1* in prostate cancer. Mazzu et al. (2019) identified a network of 41 target genes for miR-193b that are activated in aggressive prostate cancer. Among these, they validated *FOXM1* (forkhead box M1) and *RRM2* (ribonucleotide reductase regulatory subunit M2) as direct targets, showing that miR-193b downregulates the expression of these genes (Mazzu et al., 2019). Notably, although the study confirmed the promoter hypermethylation of *MIR193B*, it did not identify *CCND1* as a miR-193b target, possibly due to differences in target identification methods. While Rauhala et al. (2010) used target prediction programs, Mazzu et al. (2019) relied on a pre-established set of target genes identified from liposarcoma using microarray and SILAC analysis (Mazzu et al., 2017). They then searched for miR-193b targets within prostate cancer datasets based on this liposarcoma target gene set (Mazzu et al., 2019).

Additionally, *PLAU* (also known as uPA) has been suggested as a potential miR-193b target in prostate cancer, based on experiments conducted in PC-3 cells, which express miR-193b (Xie et al., 2013). However, the 22Rv1 and VCaP cells used in this study have low endogenous miR-193b expression compared to PC-3 cells and do not express uPA (Helenius et al., 2006; Prensner et al., 2011), suggesting that uPA is not primary target of miR-193b in these prostate cancer cells. This further highlights the cellular context dependency of miRNA-mRNA targeting.

Circulating microRNAs have been considered as potential biomarkers for cancer detection, prognostic, and follow-up. Interestingly, hypermethylation of the miR-193b promoter has been proposed as a potential diagnostic and prognostic biomarker for prostate cancer, detectable in both tissue and urine (Torres-Ferreira et al., 2017). However, before miRNA-based biomarkers can be integrated into clinical practice, further research is needed to generate a larger body of consistent data across different study groups to validate the biomarker potential of specific miRNAs, as reviewed by Condrat et al. (2020) and Gujrati et al., (2023).

7. CONCLUSIONS

The results presented in this thesis confirmed that the promoter of *MIR193B* is hypermethylated, leading to reduced miR-193b expression in clinical prostate cancer samples. The results also demonstrated that miR-193b directly targets *CCND1* in prostate cancer. Consequently, the overexpression of Cyclin D1 in prostate cancer is, at least in part, induced by the methylation-driven downregulation of miR-193b. However, due to the complexity of the regulatory networks and processes involved in cancer initiation and progression, no single gene, or even a pair of genes, is solely responsible for tumor development. Given that Cyclin D1 regulates the cell cycle, plays a role in AR signaling, and DSB repair, the aberrant hypermethylation of *MIR193B* in prostate cancer may disrupt all these crucial processes. From a theoretical clinical perspective, reduced expression of miR-193b could indicate increased sensitivity of prostate cancer cells to Cyclin D1 inhibition. Conversely, restoring miR-193b expression could potentially suppress prostate cancer cell proliferation.

Further research is needed to validate these findings and evaluate their clinical significance in larger cohorts using standardized methodologies. It would also be valuable to explore the potential of *MIR193B* hypermethylation as a biomarker and to map additional targets of miR-193b, which could reveal other cellular functions disrupted by its methylation beyond cell cycle regulation.

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