TARGETING THE GENES OF 9p13.3 AMPLICON IN PROSTATE CANCER

Teemu Grönlund
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TIIVISTELMÄ

Tutkimuksen tausta ja tavoitteet: Eturauhassyöpä on maailmanlaajuisesti yleisin miesten syöpäsairaus, jonka molekulaarisia mekanismeja ei tunneta täydellisesti. Eturauhassyövässä genomin kopiolukumuutokset ovat yleisiä, ja niiden kartoittaminen on auttanut tunnistamaan syövälle tunnusomaisia kohdegeenejä. Kromosomialueella 9p13.3 sijaitsee usein esiintyvä monistuma, jonka on havaittu olevan yhteydessä aggressiiviseen eturauhassyöpään, korkeampaan PSA-arvoon sekä lyhyempään progressiovapaaseen elinaikaan prostatektomiapotilailla. Alueella saattaa sijaita yksi tai useampia tuntemattomia onkogeeneja. NOL6, C9orf23, FANCG ja STOML2 ovat 9p13.3-monistuman mahdollisia kohdegeenejä ja tavoitteena oli selvittää, kuuluvatko NFX1- ja KIF24-geenit myös tähän ryhmään. Lisäksi tutkittiin, miten potentiaalisten kohdegeenien hiljentäminen vaikuttaa syöpäsolujen proliferaatioon ja migraatioon.

Tutkimusmenetelmät: NFX1- ja KIF24-geenien ilmentymistä tutkittiin kvantitatiivisella käänteiskopiointipolymeraasiketjureaktiolla eri 9p13.3-kopioluvun omaavissa syöpäsolulinjoissa ja ksenografteissa. Lupaavat kohdegeenit hiljennettiin transfektoimalla eri syöpäsolulinjoja kohdegeeneille suunnitelluilla siRNA:illa. Syöpäsolujen proliferaatiota ja migraatiota tutkittiin AlamarBlue-menetelmällä, mikroskopoimalla ja digitaalisella kuva-analyysillä.

Tutkimustulokset: KIF24-geenin ilmentyminen oli lisääntynyt 9p13.3-monistuman yhteydessä ja se valittiin mahdolliseksi alueen kohdegeeniksi. Muut potentiaaliset kohdegeenit olivat C9orf23, FANCG, NOL6 ja STOML2. C9orf23- ja NOL6-geenien hiljentämisellä oli negatiivinen vaikutus syöpäsolujen proliferaatioon ja migraatioon. FANCG- ja STOML2-geenien hiljentämisellä oli puolestaan positiivinen vaikutus syöpäsolujen proliferaatioon.

Johtopäätökset: C9orf23 ja NOL6 ovat 9p13.3-monistuma-alueen mahdollisia kohdegeenejä. Lisätutkimuksissa tulisi kartoittaa tarkemmin näiden geenien toimintaa ja merkitystä eturauhassyövässä. FANCG ja STOML2 ovat mahdollisia tuumorisupressorigeenejä. Niiden toimintaa ja merkitystä syövässä tulisi tutkia jatkossa tarkemmin. 9p13.3-monistuma-alueen kohdegeenin rajaaminen ja identifiointi voi tulevaisuudessa tarjota uuden merkkigeenin eturauhassyövän prognostiikkaan ja diagnostiikkaan.

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ABSTRACT

Background and aims: Prostate cancer is the most common cancer among men worldwide, and the molecular mechanisms involved in the disease still remain poorly understood. Prostate cancer is commonly associated with genomic copy number alterations that have helped to identify cancer associated target genes. 9p13.3 chromosomal region is recurrently amplified in prostate cancer and associated with the aggressive form of the disease, higher PSA-level, and poor progression-free survival in prostatectomy treated patients. 9p13.3 region may harbor one or more novel oncogenes. NOL6, C9orf23, FANCG, and STOML2 are possible 9p13.3 amplicon target genes that exhibit elevated gene expression in prostate cancer. Our aim was to investigate whether NFX1 and KIF24 genes are also part of this group. The possible effects of target gene silencing on cancer cell proliferation and migration were also studied.

Methods: NFX1 and KIF24 gene expression was studied with RT-qPCR in cancer cell lines xenografts with different copy number status. Putative target genes were silenced by transfecting cancer cell lines with target gene specific siRNAs. Cancer cell proliferation was assessed by AlamarBlue assay and by microscopy and digital image analysis. The effects on cell migration were studied with wound healing assay and digital image analysis.

Results: KIF24 exhibited elevated gene expression correlating with increased copy number of 9p13.3 and was therefore selected as putative target gene of the amplicon. The other genes outlined for our study were C9orf23, FANCG, NOL6, and STOML2. Silencing C9orf23 and NOL6 had a negative effect on cancer cell proliferation and migration. On the other hand, FANCG and STOML2 gene silencing had positive effect on cancer cell proliferation.

Conclusion: C9orf23 and NOL6 remain as putative target genes of 9p13.3 amplicon. Further investigations of these genes should be carried out in order to conclude their function and contribution to prostate cancer. FANCG and STOML2 genes remain as possible tumor suppressors, and their contribution to cancer needs further clarification. Confirmation of the 9p13.3 amplicon target gene may provide new prognostic or diagnostic marker for prostate cancer in the future.

CONTENTS

ABREVIATIONS

22Rv1	22Rv1 human prostate carcinoma epithelial cell line
aCGH	Array CGH
Ago	Argonaute
AKT	V-akt murine thymoma viral oncogene homolog
Alu	(Arthrobacter luteus) restriction endonuclease
APC	Adenomatous polyposis coli
AR	Androgene receptor
ATCC	American Type Culture Collection
BrCa	Breast cancer
BPH	Benign prostatic hyperplasia
BPE	Bening prostatic epithelium
BT-474	BT-474 ductal carsinoma cell line
C9orf23	Chromosome 9 open reading frame 23
cCGH	Chromosomal CGH
CD44	CD44 molecule (Indian blood group)
cDNA	Complementary DNA
CGH	Comparative genomic hybridization
CHD1	Chromodomain helicase DNA binding protein 1
CpG	Cytosine Guanine linear dinucleotide
CRPC	Castration resistant prostate cancer
CSCs	Cancer stem cells
Ct	Threshold cycle
DHT	5-alpha dihydrotestosterone
dNTP	Deoxynucleotide triphosphate
DROSHA	Ribonuclease type III
dsRNA	Double-stranded RNA

ELAC2	ElaC ribonuclease Z 2
ELK4	ETS-domain protein (SRF accessory protein 1)
ERG	Ets related gene
EMT	Epithelial to mesenchymal transition
ETS	E26 transformation-specific
ETV1/4/5	Ets variant 1/4/5
exo-siRNA	Exogenous small interfering RNA
EZH2	Enhancer of zeste 2 polycomb repressive complex 2 subunit
FANCG	Fanconi anemia, complementation group G
FISH	Fluorescence in situ hybridization
FLI1	Friend leukemia integration 1
FOXA1	Forkhead box A1
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
gDNA	Genomic DNA
GSTP1	Glutathione-S-transferase P1
H3K27Me2/3	Histone H3 di-/trimethyl Lysine 27
H3K18Ac	Histone H3 acetyl Lysine 18
HPC	Hereditary prostate cancer
Hox	Homeobox
KDM6A	Lysine (K)-specific demethylase 6A gene
KIF24	Kinesin family member 24
LINE1	Long interspersed nuclear element 1
LuCaP	Lucas Foundation Cancer of the Prostate, prostate cancer xenograft
MBPCG	Molecular Biology of Prostate Cancer Group
miRNA	MicroRNA
miR-141/ 375	MicroRNA 141/ 375
MLL2 / 3	Mixed-lineage leukemia 2/ 3
mRNA	Messenger RNA

MSR1	Macrophage scavenger receptor 1
MYC	v-myc myelocytomatosis viral oncogene homolog
NCOA2	Nuclear receptor coactivator 2
ncRNA	Non-coding RNA
NDRG1	N-myc downstream regulated 1
NFX1	Nuclear transcription factor, X-box binding 1
NGS	Next-generation sequencing
NKX3.1	NK3 homeobox 1
NO	Nitric oxide
NOL6	Nucleolar protein 6 (RNA associated)
PC-3	PC-3 human prostate cancer cell line
PI3K	Phosphoinositide 3-kinase
PIA	Proliferative inflammatory atrophy
PIN	Prostatic intraepithelial neoplasia
pre-miRNA	Precursor miRNA
pri-miRNA	Stem-loop dsRNA, precursor miRNA
PSA	Prostate specific antigen
PTEN	Phosphatase and tensin homolog
PTGS2	Prostaglandin-endoperoxide synthase 2
PTGS	Post-transcriptional gene silencing
RAF	Rapidly accelerated fibrosarcoma
RARb2	Retinoic acid receptor b2
RASSF1	Ras association (RalGDS/AF-6) domain family member 1
RB1	Retinoblastoma 1
RISC	RNA-induced silencing complex
RNAi	RNA interference
RNASEL	Ribonuclease L
RPP25L	Ribonuclease P/MRP 25kDa subunit-like

RT-qPCR	Real-time quantitative polymerase chain reaction
RUSC2	RUN and SH3 domain containing 2
Shh	Sonic hedgehog
shRNA	Short hairpin RNA
siRNA	Short interfering RNA
SLC45A3	Solute carrier family 45, member 3
SOX9	SRY (sex determining region Y)-box 9
SPINK1	Serine peptidase inhibitor, Kazal type 1
SPOP	Speckle-type POZ protein
STOML2	Stomatin (EPB72)-like 2
SQ	Starting quantity
TBP	TATA-box binding protein
TGS	Transcriptional gene silencing
TLR	Toll-like receptor
TMPRSS2	Transmembrane protease, serine 2
TP53	Tumor protein 53
TRBP	TAR-RNA-binding protein
UGS	Urogenital sinus
UTR	Untranslated region

1 INTRODUCTION

Prostate cancer is the most common cancer among men living in western countries and one of the most common reasons for cancer-related death among men worldwide (Center et al. 2012). Prostate cancer is an extremely heterogeneous cancer that presents a particular challenge regarding diagnosis and treatment of the disease (Boyd et al. 2012). Most of the cases appear as indolent and non-life-threatening, but some of the tumors exhibit malignant or highly metastasizing, aggressive form. The majority of patients under treatment respond initially to androgen-deprivation therapy, but in later stages the tumor becomes hormone-refractory, ultimately leading to castration resistant prostate cancer (CRPC) with poor prognosis.

At the moment there is a great demand of more accurate clinical classification and prognosis of prostate cancer. Better prognostic tools and new therapeutic targets could be found by identifying biomarkers and genetic alterations that are associated with different subtypes of prostate cancer (Shen & Abate-Shen 2010). It would be highly beneficial to be able to distinguish the molecular characteristics between indolent and aggressive types of prostate cancer at an early stage. This would enhance risk assessment of the disease, enable accurate diagnosis, and reveal new therapeutic targets.

There are multiple molecular events leading to pathogenesis and progression of prostate cancer that eventually establish the individual set of genetic alterations. Clinical heterogeneity arises from complex network of genome rearrangements, somatic mutations, and epigenetic alterations enhanced by genomic instability in a somewhat narrative fashion (Wyatt et al. 2013). Chromosomal copy number alterations are frequently observed in prostate cancer and occur commonly at chromosomal regions that harbor cancer-associated genes. Specific deletions and gains of particular genes are frequently found in prostate cancer, affecting ultimately to the level of transcribed gene product. Copy number alteration often silence or enhance their target gene expression, eventually contributing to carcinogenesis as an inactivated tumor suppressor gene or an activated oncogene.

A recurrent gain in chromosome 9p13.3 has been identified recently in prostate cancer (Saramäki 2006, Taylor et al. 2010). According to the previous studies, high-level 9p13.3 amplification is associated with aggressive behavior of prostate cancer (Leinonen 2007). In addition, 9p13.3 amplification has been found to be associated with higher PSA-value and poor progression-free survival in prostatectomy-treated patients. Multiple potential target genes reside at the 9p13.3 region, exhibiting elevated gene expression that correlates with increased copy number status.

The occurrence of 9p13.3 amplicon has previously been studied in various cancer cell lines and patient samples previously. The expression levels of multiple potential target genes of the region have been identified in previous studies, while no data has been obtained regarding to some of the genes. The most promising genes have been under functional studies, but so far no target gene has been identified for this amplification. This leaves open the possibility that the region may still harbor unknown genes associated to prostate cancer. Identification of the target gene of 9p13.3 amplification might provide a new prognostic marker or a novel oncogene for prostate cancer. It may also help to understand the molecular mechanisms underlying prostate cancer progression and offer novel therapeutic targets.

2 REVIEW OF THE LITERATURE

2.1 Structure and function of the prostate

The prostate is an exocrine gland that normally resembles the appearance and size of a walnut. It surrounds the urethra inside the pelvis and is located in front of the rectum, just under the bladder (Figure 1). The prostate acts to restrict urine flow and is a part of a man's reproductive system, secreting alkaline prostatic fluid (Ross & Wojciech 2010). Three male accessory sex glands are the seminal vesicles, the prostate gland, and the bulbourethral glands (Figure 1). They all rely on male hormones in their development, growth, maintenance and function throughout the individual´s lifetime. The prostate works in cooperation with the seminal vesicles, which produce seminal fluid, and the seminiferous tubules of the testes producing spermatozoa. Together they nurture, protect, and facilitate sperm transport for reproduction.

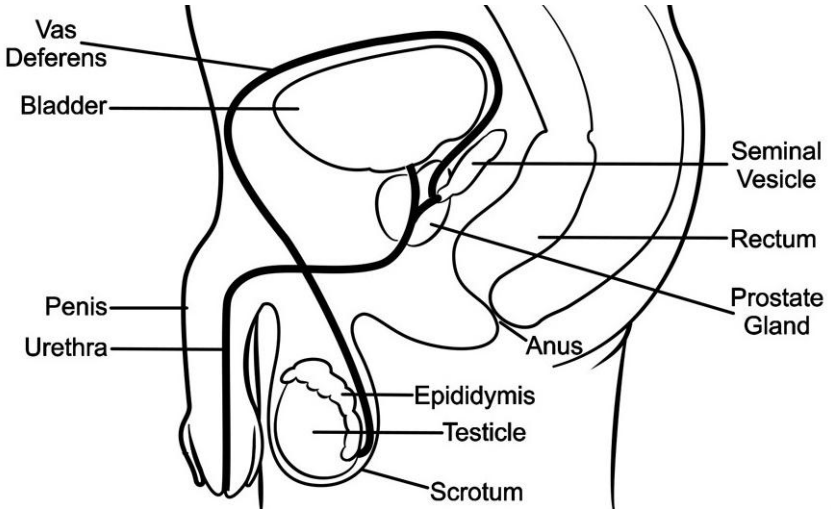


Figure 1. Male reproductive system. Image modified from http://teachers.teachingsexualhealth.ca/wp-content/uploads/MReproductive-System-1-labelled.pdf

The prostate gland is surrounded by a dense capsule formed by muscle and connective tissue fibers (Ross & Wojciech 2010). Glandular tissue of the prostate gland consists of 30 to 50 tubular-alveolar glands that open into the urethra. They are organized in three concentric layers: an inner mucosal layer, an intermediate submucosal layer, and a peripheral layer containing the main prostatic glands. The glands of the mucosal layer secrete directly into the urethra, while the other two layers have ducts that open into the prostatic sinuses located on either side of the urethral crest, on the posterior wall of the urethra.

McNeal's description from 1968 of zonal anatomy has replaced the previous lobe classification and is now accepted as the standard reference for understanding the normal and abnormal structure of the human prostate (McNeal 1968, Timms 2008). The adult prostate is classified into distinct glandular regions with different architecture. These include anterior fibromuscular zone, periurethral zone with mucosal glands, large peripheral zone, central zone surrounding the ejaculatory ducts, and small transitional zones lateral to the prostatic urethra (Figure 2). The outermost peripheral zone holding the most volume retains the majority of prostate carcinomas and is the most susceptible to inflammation (Ross & Wojciech 2010). The transition zone, on the other hand, gives rise to benign prostatic hyperplasia (BPH) which is a common nonmalignant condition found in older men. It allows prostate grow too large and squeeze the urethra disturbing the normal flow of urine. The central zone is resistant to both carcinoma and inflammation.

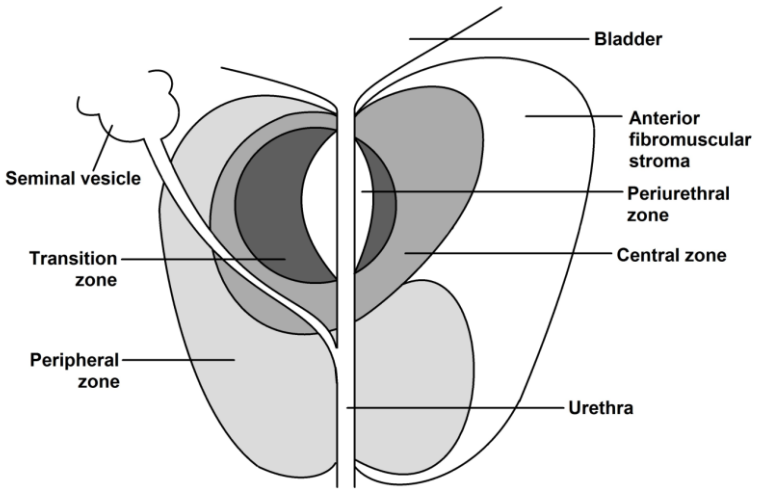


Figure 2. Schematic representation of the zones of the prostate gland. Image modified from Ross & Wojciech 2010.

Two major cellular compartments of the prostatic epithelium are composed of epithelial cells and stromal cells. The main types of epithelial cells are basal epithelial cells, intermediate cells that share phenotypic features between basal and luminal cells, neuroendocrine cells that secrete bioactive peptides, and luminal secretory epithelial cells that are responsible for epithelial barrier integrity and production of prostatic secretion (De Marzo et al. 1998). The epithelial cells in prostate gland produce several enzymes, particularly prostate-specific antigen (PSA), prostatic acid phosphatase (PAP), fibrinolysin, human kallikrein-2 (HK2), and citric acid. Stem cells reside in the basal epithelial compartment and give rise to all of the other epithelial cell types, as well as neuroendocrine cells.

The stromal compartment architecture serves as structural support, where testosterone is converted to more potent 5-alpha dihydrotestosterone (DHT) by 5α-reductase (Berman et al. 2012). It is mainly consisted of connective tissue, smooth muscle cells, and fibroblasts, but also includes immune cells, endothelial cells, and nerve cells. Nitric oxide (NO) affects smooth muscle contraction in the prostate and is mainly formed by nerve cells, endothelial cells, or macrophages.

Androgens are considered as male sex hormones. The most abundant androgen is testosterone, which is synthesized in testis. Other androgen species remain relatively minor and are synthesized by the adrenal gland (Ross & Wojciech 2010). These include androstenedione

and dehydroepiandrosterone (DHEA) that can be converted into testosterone. In the normal prostate, androgens have different effects on different cell types, and they act through paracrine signaling (Isaacs & Isaacs 2004).

Androgen binds to androgen receptor (AR) which is a nuclear hormone receptor. The binding of DHT to AR at the prostatic epithelium cells results in a conformational change of the receptor, dimerization, and translocation to the nucleus of epithelial and stromal cells (Ross & Wojciech 2010). Phosphorylated dimers of AR complex bind to a specific sequence of DNA known as a hormone-response element residing in the promoter regions of target genes. Androgen receptor interacts with other proteins in the nucleus and regulates transcription of multiple genes. DHT stimulates growth of normal prostatic epithelium, proliferation and growth of BPH and androgen-dependent prostate cancer. Therefore, androgen ablation is usually the initial therapeutic approach for the treatment of advanced prostate cancer patients.

Stromal-epithelial interactions in the prostate epithelium are highly essential for information transfer and regulation within the prostate (Berman et al. 2012). Testosterone and growth factors interact on and between stromal and epithelial cell compartments. The production of growth factors is either stimulated or inhibited by androgens and function is transmitted in cells either autocrine or paracrine fashion. In the stromal cells, AR activates secretion of growth and survival factors that diffuse across the basement membrane on basal cells and secretory luminal cells (Isaacs & Isaacs 2004). This activates signaling pathways for cell proliferation and epithelial cell survival controlling the normal growth and maintenance of prostate epithelium. In the nuclei of the epithelial secretory luminal cells, AR stimulates the transcription of a series of genes and production of prostatic secretion, while the growth of these cells is suppressed by inhibition of growth and survival factors inducing proliferation. Basal epithelial cells do not express AR.

2.1.1 Morphogenesis

The stepwise formation of the prostate originates from early embryogenesis. A cloaca appears as a part of the regional differentiation of the primitive endoderm. In placental mammals it gives rice to urogenital sinus (UGS) and digestive outlets (Berman et al. 2012). In both sexes the UGS develops into urinary bladder and urethra. The UGS differentiates and becomes sex-

ually dimorphic in response to androgens which begin to be excreted by Leydig cells in male gonads (Staack, Donjacour et al. 2003, Meeks et al. 2011).

After 10 weeks of gestation, molecular events mark the initiation of prostatic development from the UGS (Kurzrock et al. 1999). The epithelial buds of the UGS epithelium proliferate in response to testosterone stimulation. During ductal budding multiple epithelial outgrowths invade the mesenchyma and form ducts that elongate and branch out from the urethra, terminating into gland development (Shen et al. 2008, Tomuleasa et al. 2010). In the course of development, the UGS epithelium differentiates into distinct basal and luminal layers, while the mesenchymal cells differentiate into stromal elements (Wang et al. 2001). The prostate grows as a single organ and glandular regions become histologically distinct after sexual maturity.

Development of the prostate is driven by the intact AR pathway that requires stromal-epithelial interaction, sufficient androgen exposure and conversion of testosterone into more potent DHT (Andersson et al. 1991, Mahendroo & Russell 1999, Berman et al. 2012). Together androgens and AR signaling take care of the prostate homeostasis in adulthood. During development they act on the mesenchyme to indirectly induce prostate epithelial outgrowth. Their role is vital for controlling the timing of the developmental events, but not their location (Berman et al. 2012). The exact developmental events of the prostate are still relatively poorly understood.

Disruption of some of the key molecular events taking part in prostate development has been found to be associated with prostate carcinogenesis. For instance, paralogous homeobox (Hox) genes have been shown to be re-expressed in several different types of cancers (Podlasek et al. 1997 & 1999, Javed & Langley 2014). In normal prostate development these genes are involved in branching morphogenesis, where prostate epithelial buds form at precise locations. The earliest indicator of prostate development at the molecular level is the epithelial expression of NK homeobox transcription family member NK3 homeobox 1(Nkx3.1) gene that works as a tumor suppressor in mature prostate and is commonly defected in prostate cancer (Bieberich et al. 1996, Bhatia-Gaur et al. 1999, Abate-Shen et al. 2008). Nkx 3.1 transcription factor influences the degree of branching in the mature prostate while other important molecules in emerging prostate buds are SRY (sex determining region Y)-box 9 (Sox9) and sonic hedgehog (Shh) (Podlasek et al. 1999, Lupien et al. 2008, Schaeffer et al. 2008,

Thomsen et al. 2008). Sox9 and Shh genes have been shown to be associated with prostate cancer, as well as other cancers (Cai et al. 2013, Gonnissen et al. 2013).

2.2 Prostate cancer

Prostate cancer is the second most common malignancy and the sixth leading cause of cancer-related death among men worldwide (Ferlay et al. 2013). It is the most common cancer in Finnish men, with over 4600 new cases annually, and the second leading cause of cancer related death (Finnish Cancer Registry 2014). The global population growth and ageing are expected to increase the worldwide number of new prostate cancer cases to 1.7 million with 499 000 new deaths by the year 2030 (Ferlay et al. 2010). Even though the number of prostate cancer cases is increasing the mortality ratio has remained relatively low due to early-stage diagnosis and improved treatment options (Center et al. 2012).

In the development of a multidisciplinary disease such as cancer, the environmental, physiological, and molecular factors constitute a strong interaction with ageing. Almost all (97%) prostate cancer cases occur in men 50 years and older (American Cancer Society 2013). More than half (60%) occur in men 65 years and older. Familial prostate cancer refers to a clustering of the disease within families. Positive family history of prostate cancer in a first-degree relative multiplies the disease risk approximately twofold (Potter & Partin 2000). 13-25% of men with prostate cancer have a familial form of the disease that tends to develop about a decade earlier than the more common type of prostate cancer (Bratt 2002, Alberti 2010).

PSA is a kallikrein-related serine protease that is produced in normal prostate secretions. When normal prostate architecture is disrupted PSA is released into the blood (Lilja et al. 2008). If elevated blood PSA levels are detected, the patient usually undergoes biopsy to assess the potential presence of prostate cancer, based on histopathological grading system called the Gleason score. The great increase in prostate cancer cases is partly due to extensive PSA screening and digital rectal examination in western countries. In the other hand some parts of Asia and Africa obtain the lowest incidence rates due to low rates of testing. In prostate cancer, advanced age, geographic region, ethnicity, and family history remain the most important risk factors (Platz et al. 2006).

Even though prostate cancer initiation may take place at a relatively early age it may result in latent form of cancer. Multifocal and highly heterogeneous tumor may exhibit various neoplastic alterations that may develop to a latent form of prostate cancer instead of a clinically detectable one (Shen & Abate-Shen 2010). Many of diagnosed prostate cancer cases have proved to be latent or indolent forms of the disease. More effective prostate cancer diagnostics and treatment options would require improved molecular markers and other supporting approaches to assist histological assessment of prostate cancer. This would help to distinguish cancers that potentially develop to the form of more aggressive ones. Molecular markers can be discovered through better understanding of the molecular basis of cancer initiation.

Intact AR signaling maintains homeostasis of both epithelial and stromal tissues in the normal prostate (Shen & Abate-Shen 2010). The paracrine mechanism of AR action is replaced by autocrine mechanism during transformation to prostate cancer. In this mode of action the cancer cells become less dependent on stromal cell factors, while androgens act through AR directly stimulating the production of growth and survival factors. Therefore, prostate cancer is highly dependent on androgens (Huggins et al. 1941).

Most prostate cancer patients are diagnosed with organ-confined disease that remains potentially curable (Chiam et al. 2014). The primary treatments for this type of a localized prostate cancer include the removal of the prostate gland by surgery and/or radiation therapy together with active surveillance. More than 95% of prostate cancers are classified pathologically as adenocarcinoma that arises from prostatic epithelial cells, which express AR (Figure 3) (Bostwick 1989, Shen & Abate Shen 2010). Prostate cancer can acquire diverse histological patterns and clinical heterogeneity, complicating disease diagnoses. This also reflects the highly variable response of patients to standard therapies. It has been estimated that approximately 30% of patients relapse after the initial treatment and require further treatment.

DHT stimulates growth of normal prostatic epithelium, but also the proliferation and growth of BPH as well androgen-dependent prostate cancer (Ross & Wojciech 2010). Therefore, androgen deprivation is usually the preferred initial therapeutic approach for the treatment of advanced prostate cancer patients with progressive or metastatic disease. This is achieved by targeting androgen production and its mediator, the AR, with anti-androgens that block the functional action of AR (Scher et al. 2004). Under androgen deprivation, stromal cells pro-

duce paracrine proapoptotic signals that act on neighboring epithelial cells, promoting regression of normal prostate.

After an initial period of tumor regression, prostate cancers become unresponsive to androgen deprivation and eventually progress to castration-resistant prostate cancer (CRPC) (Figure 3) (Chiam et al. 2014). In this state tumors exhibit sensitization to low levels of androgens, commonly through elevated AR expression and androgen-independent activation of AR signaling by various molecular mechanisms. These mechanisms include AR gene amplification through chromosomal copy number alteration, the overexpression of AR or AR cofactors, gain-of-function mutation of AR, or possible ligand-independent AR activation by up-regulation of other signaling pathways (Buchanan et al. 2001, Heemers & Tindall 2007, Locke et al. 2008, Zhu & Kyprianou 2008, Steinkamp et al. 2009, Shen & Abate-Shen 2010). Endogenous biosynthesis of androgens by tumor cells may also occur and constitutively active mRNA splice variants of AR have also been detected (Bergerat & Ceraline 2009).

The latency period before CRPC development is highly variable, and only a fraction of tumors will transform into lethal form, again highlighting the clinical heterogeneity of the disease (Chiam et al. 2014). Rising serum PSA indicates the inappropriately restored AR activity in CRPC that can be detected by active surveillance of the patients (Figure 3) (Ryan et al. 2006). Chemotherapy has been shown to have limited benefits in improving survival, and at the moment there are no effective or curative treatments available for treating advanced CRPC.

The progression of advanced hormone-refractory prostate cancer is followed by metastasis formation, but metastases may also occur at early stage of cancer development (Figure 3) (Heinlein & Chang, 2004). The molecular biology of prostate cancer metastasis is poorly understood. Generally metastasis proceeds through a complex series of molecular events that include angiogenesis at the site of the original tumor, local invasion and migration within the primary site through extracellular matrix and surrounding stromal cells, intravasation into the blood stream, survival within the blood circulation, and finally extravasation of the tumor cells to the target organ (Leber & Efferth 2009, Hudson et al. 2013). Metastasis formation is preceded by colonization and proliferation of these cells within the secondary site. In prostate cancer this takes place most commonly in the bone tissue, causing osteolysis and abnormal

bone formation (Bubendorf et al. 2000, Keller et al. 2001). It has been estimated that more than 80% of men who die from prostate cancer develop bone metastases.

2.2.1 Pathogenesis

The prostate cancer initiation has been characterized to take place, when changes in the proliferation of normal epithelium occur (Bostwick & Cheng 2012). This hypothesis suggests that chemicals, physical factors, or bacteria may induce inflammation and oxidative stress that results in cellular injury and abnormal cellular regeneration. The regenerative proliferation of cells exposes them to mutations that may lead to cancer initiation, promotion, and progression.

The multistep prostate cancer progression arises from the glandular epithelium and most often in the peripheral zone of the prostate (Shen & Abate Shen 2010). Prostatic intraepithelial neoplasia (PIN) is currently accepted as the precursor of prostate cancer (Figure 3). PIN arises from epithelial cells of prostatic ducts and acini with distinguished cytological abnormalities, indicating impairment of cell proliferation, differentiation, and regulatory control with advancing stages of prostatic carcinogenesis. (Shen & Abate Shen 2010, Bostwick & Cheng 2012). Progressive abnormalities with characteristic phenotypic and genomic changes in PIN appear as an intermediate form of bening prostatic epithelium (BPE) and cancer.

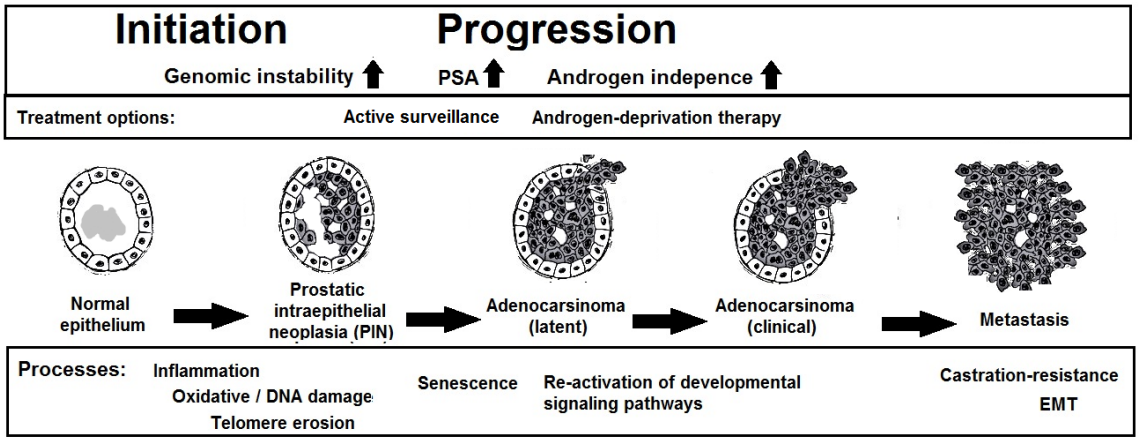


Figure 3. Progression of prostate cancer. Different stages of progression are linked to specific molecular processes. Image modified from Shen & Abate-Shen 2010.

The precise molecular mechanism of prostate cancer initiation and progression is still poorly understood and under debate. It has been suggested that the initiation of clinical prostate can-

cer would follow a different pathogenic program than latent form of the cancer (Shen & Abate-Shen 2010). The other hypothesis proposes that certain critical events lead to the activation of latent prostate cancer to clinical disease, while the latent form can be maintained by the suppression of these activating events. This is supported by the observation of primary tumors exhibiting multiple and separate independent tumor focis that express clinical heterogeneity in the form of histology and genetic alterations (Aihara et al. 1994, Bostwick et al. 1998, Macintosh et al. 1998, Mehra et al. 2007, Clark et al. 2008, Boyd et al. 2012). Similar heterogeneity is shown also in different cells within the same tumor foci.

Mainly two models have been proposed to explain tumor heterogeneity and functional differences among the cancer cells within a single patient. The more stochastic clonal evolution model suggests that some individual cancer cells acquire regenerative proliferation and high malignant potential (Shackleton et al. 2009, Tang 2012, Visvader & Lindeman 2012). This is obtained through genetic and epigenetic alterations, driven by genomic instability. Some of these cells acquire selective advantage, resulting in a dominant clone and the following progeny with similar tumor-initiating capacities, ultimately responsible for cancer progression (Nowell 1976, Shackleton et al. 2009, Baylin & Jones 2011).

In comparison to clonal evolution, cancer stem cell model suggests that cancers cells receding in tumors are organized hierarchically (Shackleton et al. 2009, Tang 2012, Visvader & Lindeman 2012). This model suggests that only a subset of the cancer cells is capable of tumor-initiating and -maintaining activity. These cancer stem cells (CSCs) resemble normal stem cells with many phenotypic and functional properties and differentiate into non-tumorigenic progeny. CSCs have been proposed to be responsible for tumor initiation, progression, metastasis and treatment resistance. The two distinctive models may not necessarily be mutually exclusive as cancer stem cells may evolve by clonal evolution.

Some of the most recent studies have given more support for the monoclonal origin of early prostate cancer (Boyd et al. 2012, Lindberg 2013a). According to this theory the multifocal tumor derive from a single cell with individual neoplastic alterations. This prostate cancer precursor clone is speculated to give rise to separate PIN foci that may progress to multifocal invasive prostate cancer. It has been suggested that advanced prostate cancer would also express a monoclonal origin (Mehra et al. 2008; Liu et al. 2009). Even though advanced metas-

tatic prostate cancer shows phenotypic heterogeneity the metastases seem to be clonally related (Shah et al. 2004). Based on these observations metastatic aggressive form of prostate cancer has been suggested to progress through clonal evolution (Shen & Abate-Shen 2010). Therapeutic interventions may affect this evolution by the elimination of specific cells with altered malignant potential. Therefore, it is not yet known whether clinical tumors express the naturally occurring evolution of cancer progression or just the final outcome of cancer intervention (Shen & Abate-Shen 2010).

2.3 Prostate cancer genetics

New technologies and extensive analysis of prostate cancer genomes has vastly increased our understanding about prostate cancer genetics in recent years. Next-generation sequencing (NGS) has changed our view about genetics and genome biology, while revolutionizing cancer genomics research. High-resolution analysis and global view of the cancer genomes have confirmed their truly complex nature (Barbieri et al. 2013). At the same time, the huge amount of data obtained with modern technology has set new challenges and limitations to comprehend new findings.

Prostate cancer is characterized by multiple genomic alterations, including somatically acquired point mutations, chromosomal rearrangements, and epigenetic alterations (Shen & Abate-Shen 2010, Baca et al. 2013). Cancer progression from localized tumor to metastasizing and aggressive form is driven by the accelerated accumulation of these genetic alterations, promoted by genomic instability (Figure 3) (Cheng et al. 2012). Genetic heterogeneity of prostate cancer is displayed by clinically heterogeneous and multifocal tumors.

Chromosomal and epigenetic alterations are relatively common in prostate cancer, while somatic point mutation altering protein coding sequences occur at low rate compared to other cancers (Chan et al. 2008, Taylor et al. 2010, Chiam et al. 2014). The most important somatic alterations in prostate cancer have been identified as chromosomal copy number alterations or gene fusions, inactivating recessive tumor suppressors at an early stage of disease development and activating oncogenes at later stages (Shen & Abate-Shen 2010, Barbieri et al. 2012). Altered expression of genes associated with ageing, inflammation, oxidative stress, DNA

damage, and cellular senescence are commonly detected in prostate carcinogenesis (Figure 3) (Shen & Abate-Shen 2010, Baca et al. 2013).

Oncogenic aberrations may accumulate gradually or in a single catastrophic event. In both cases simultaneous deletion of tumor suppressors and creation of oncogenic rearrangements are required (Stephens et al. 2011, Kim et al. 2013, Wyatt et al 2013). Large scale rearrangements may be more difficult for the cell to survive and may require concurrent oncogenic alterations to become fixed in a tumor.

The interaction of environmental factors and multiple low-penetrance susceptibility genes are important for prostate cancer development (Boyd, Mayo & Lu 2012). Positive family history accounts about 13-25% of prostate cancer cases (Alberti 2010). Only few high-penetrance susceptibility genes are characterized for prostate cancer carcinogenesis so far. Dominantly inherited germ line mutations occur commonly in ribonuclease L (RNASEL), elaC ribonuclease Z 2 (ELAC2) and macrophage scavenger receptor 1(MSR1) genes in hereditary prostate cancer (HPC), causing 5-10% of all prostate cancer cases (Brat 2002, Alberti 2010). Somatic mutations in these genes lead to an early onset of the disease in 30-40% of all prostate cancer cases.

The clonal evolution theory of prostate cancer highlights the evolutionary processes in the background of cancer genomes. In clonal evolution the cancer arises from the step-wise accumulation of a large number of somatic mutations in the same cell in a variable time frame (Greaves & Maley 2012). During cancer evolution, repetitive rounds of clonal expansion, genetic diversification, and clonal selection take place in adaptive tissue environment. Clonal evolution involves the interplay of selectively advantageous driver mutations, and selectively neutral passenger mutations. At the same time there may be lesions in the background that increase the rate of other genetic changes. Changes in the tissue microenvironment on the other hand may change the fitness effects of acquired genetic alterations.

Recent discoveries in prostate cancer genetics have suggested a series of recurrent lesions taking part in prostate carsinogenesis in a narrative fashion, resembling the clonal evolution of prostate cancer progression (Baca et al. 2013, Barbieri et al. 2013). According to this theory, ERG rearrangement, NKX3.1 deletion, and mutations in SPOP and FOXA1 genes have been

denoted as early events of prostate cancer. This is followed by lesions in CHD1 and TP53 genes that ultimately lead to PTEN tumor suppressor inactivation among other late event lesions. These alterations are discussed more detailed in the following sections.

2.3.1 Oncogenes and tumor suppressor genes

Multiple genetic alterations take part in prostate carsinogenesis, usually requiring the inactivation of tumor suppressor genes and the activation of oncogenes. Proto-oncogene is a normal cellular gene that encodes a protein usually involved in regulation of essential cellular pathways, such as cell growth or differentiation, while tumor-suppressor genes generally encode proteins that inhibit cell proliferation (Lodish et al. 2000). Tumor supressors are commonly involved in cell cycle progression, cell proliferation, DNA repair, cell cycle arrest, or apoptosis. Loss of function mutations of one or more of these genes contributes to the initiation and development of many cancers. A proto-oncogene can be mutated into a cancer-promoting oncogene, either by changing the protein-coding sequence or by altering its expression. Activation of a proto-oncogene into an oncogene generally involves a gain-of-function mutation, which takes place at more advanced stages of cancer.

The inactivation of a tumor suppressor can occur through point mutation, chromosomal deletion or epigenetic gene silencing. Inheritance of a single mutant allele of many tumor-suppressor genes greatly increases the risk for developing certain types of cancer, but usually homozygous tumor-suppressor lesions are required for the ultimate cancer development. Oncogene activation usually occurs through a point mutation, gene amplification, or chromosomal translocation that brings a growth-regulatory gene under the control of a different promoter, causing altered expression of the gene.

2.3.2 Chromosomal alterations in prostate cancer

Characterization of aberrant chromosomal alterations in prostate cancer has led to the identification of common tumor suppressors and oncogenes involved in disease progression (Boyd, Maoy & Lu 2012). Some of the recurrent alterations have been discovered to be associated with certain stages of disease progression (Baca et al. 2013, Barbieri et al. 2013). Common loss of function mutations inactivating tumor suppressors seem to occur in early development of the disease, while gain of function mutations activating oncogenes are detected more in

later stages. Several chromosomal regions are aberrant at high frequency in prostate cancer, but at the same time chromosomal alterations appear as variable combinations (Wyatt et al. 2013). This is shown as individual tumor heterogeneity that is enhanced by simultaneous tumor specific alterations.

Chromosomal single-copy losses and a few additional copy gains are more commonly observed in prostate cancer, compared to homozygous loss and more extensive amplifications of specific DNA sequences (Boyd, Maoy & Lu 2012). According to current view, there are in total of 90 chromosomal regions that are recurrently altered in prostate cancer, categorized in 73 gains (low-level amplifications) and 17 deletions (Beroukhim et al. 2007, Kim et al. 2013). These include the most common chromosomal gain at 8q and losses at 3p, 8p, 10q, 13q and 17p (Paris et al. 2004, Kim et al. 2007, Lapointe et al. 2007, Saramäki & Visakorpi 2007, Liu et al. 2008, Demichelis et al. 2009, Taylor et al. 2010).

Chromosomal alterations in chromosome 8 remain frequent and highly essential for prostate cancer initiation and tumorigenesis (Abate-Shen et al. 2008). 8p is the most common deletion in prostate cancer, affecting about a third of all tumors and half of advanced tumors (Sun et al. 2007). NKX3.1 homeobox gene represents a haploinsufficient tumor suppressor gene that is located within a 150 Mb minimal deletion region of chromosome 8p21.2 (Emmert-Buck et al. 1995, Vocke et al. 1996, Haggman et al. 1997, Swalwell et al. 2002, Bethel et al. 2006). NKX3.1 loss of heterozygosity is apparent in 85% of high-grade PIN lesions and prostate adenocarcinomas, representing an early event in tumorigenesis. The remaining allele remains unmutated, but may undergo epigenetic down-regulation during prostate cancer progression (Vocke et al. 1996, Voeller et al. 1997, Ornstein et al. 2001, Bethel et al. 2006, Asatiani et al. 2005).

8q amplification often involves amplification of the entire arm of chromosome 8, leading to the possibility of multiple potential oncogenes in the region. The amplification of chromosomal region 8q24 has been demonstrated in a variety of advanced prostate tumors harboring v-myc myelocytomatosis viral oncogene homolog (MYC) (Jenkins et al. 1997; Sato et al. 1999). MYC oncogene overexpression has been detected in a variety of carcinomas and PIN lesions, while denoted to take part in cancer initiation as well (Gurel et al. 2008). Long-range DNA regulatory elements may also undergo dysregulation in carsinogenesis, therefore alter-

ing regulation of MYC gene expression (Jia et al. 2009; Sotelo et al. 2010). Androgen receptor coactivator NCOA2 is commonly amplified in primary and metastatic disease at 8q13 (Taylor et al. 2010).

A deletion of a chromosomal region 10q is observed in more than 10% of prostate cancer tumors (Sun et al. 2007). An allelic loss of 10q23 remains as an early event in prostate carcinogenesis and results in a copy number loss of a common tumor suppressor gene phosphatase and tensin homolog (PTEN) at 10q23.1 (Wang et al. 1998; Whang et al. 1998; McMenamin et al. 1999; Dong et al. 2007). The reduction or loss of PTEN gene expression is correlated with progression to aggressive CRPC, but may be retained in the indolent form of the disease (Gao et al. 2004, Abate-Shen et al. 2008, Shen & Abate-Shen 2010).

Some of the chromosomal alterations mainly occur in the later stages of cancer progression. These include frequent deletion of the chromosome 13q, which harbors known tumor suppressor genes such as retinoblastoma 1(RB1) at 13q14.2, and also the deletion of the chromosome 17p, which leads to copy number loss of TP53 tumor suppressor gene at 17p31.1 (Taylor et al. 2010, Berger et al. 2011, Grasso et al. 2012). The copy number alteration of TP53 remains more frequent than somatic mutation of TP53 in prostate cancer (Taylor et al. 2010). These common deletions are observed rarely in primary cancers, but occur at higher frequencies in metastatic and/or hormone refractory lesions. Similarly, the gain of AR gene copies at the commonly gained region Xq12 may lead to overexpression of AR and prostate tumor progression towards more androgen independent disease (Visakorpi et al. 1995). Xq12 amplification occurs almost exclusively in the hormone refractory state of prostate cancer.

2.3.2.1 9p13.3 amplification

The chromosome 9p13.3 gain is located at the pericentromic region of chromosome 9 and recurrently detected in prostate cancer. 9p13-21 gain was originally detected in DU145 cell line and prostate cancer metastasis sample using chromosomal comparative genomic hybridization (cCGH) method (Nupponen et al. 1998, Paris et al. 2003). The minimal region of amplification was later mapped to 9p13.3 by using array comparative genomic hybridization (aCGH) method (Saramäki et al. 2006). So far 9p13.3 gain has been detected from multiple

prostatectomy samples and cell lines, exhibiting variability in the size of the amplified region (Saramäki et al. 2006, Leinonen 2007, Taylor et al. 2010).

High level amplifications seem to be relatively rare in prostate cancer, but even low level copy number alterations can have an effect on gene expression (Saramäki 2006, Leinonen 2007). 9p13-21 gain has been shown to have a frequency of 39% in 13 prostate cancer xenografts and 5 prostate cancer cell lines by aCGH (Figure 3) (Saramäki et al. 2006). 9p13.3 amplification has been detected in three breast cancer cell lines, while low-level amplification has been denoted in three other breast cancer cell lines (unpublished). High-level amplification has been detected in 10% and a low-level amplification in 32% of prostatectomy treated patients (Leinonen 2007). 14% of hormone refractory tumors have been shown to have high-level amplification, while 44% contained low-level amplification. 9p13.3 amplification has been reported to be associated with a higher PSA-level and poor progression-free survival in prostatectomy treated patients. The amplification frequency seems to increase with disease progression to hormone-refractory stage and high-level 9p13.3 amplification is associated with aggressive behavior of prostate cancer.

There are over 40 protein coding genes located at the 9p13.3 chromosomal region with no known tumorigenic function (Leinonen 2007). Some of these genes exhibit association between their copy numbers status and expression levels, therefore remaining as promising oncogenic target genes of the amplicon. Corresponding studies of this chromosomal region have not been published. Despite broad analysis and functional studies, the target gene(s) of the amplicon have not yet been defined (Leinonen 2007, Taylor et al. 2010, Leppälä 2013). Since 9p13.3 gain has also been found in several breast cancer cell lines, it is possible that the amplification target gene(s) may be involved in breast cancer tumorigenesis in addition to prostate cancer.

2.3.3 Gene fusions

Genome rearrangements and DNA breakpoints occur preferentially in transcriptionally active regions and are frequently observed in cancer (Mani et al. 2009, Lin et al. 2009, Nambiar & Raghavan 2011). In addition to chromosomal copy number changes, amplifications, and deletions there are several other types of aberrations strongly promoted by genomic instability.

Translocations between non-homologous chromosomes, inversions, and insertions also take part in recurrent aberrations in cancer genomics.

Oncogenic translocations or deletions usually involve abnormal fusion of two genes. Also, parts of gene coding sequences from separate genes or regulatory DNA element may take part in these fusions. This results in deregulated gene expression, altered levels of expression, or the expression of chimeric proteins with new or altered activity. Recently, it has been suggested that poly-gene fusion transcripts containing genetic material from more than two genes could occur in prostate cancer through drastic shattering and reassembly of chromosomes in a phenomenon called chromothripsis (Wu et al. 2012). The fusion gene profile of an individual tumor can provide valuable biomarkers for diagnose and could be considered as a glimpse into gene expression history, and therefore disease etiology (Wyatt et al. 2013).

Erythroblast transformation-specific (ETS) transcription factor gene translocations are common in prostate cancer, resulting in oncogenic fusion gene transcripts (Clark & Cooper 2009, Rubin et al. 2011). ETS transcription factors can alter the expression of proteins involved in a range of pathways, including stem cell development, cell senescence, proliferation, migration, apoptosis, and tumorigenesis. The rearrangement occurs as an early event, but cannot induce cancer formation on its own. The rearrangement continues to be expressed in metastatic and castration-resistant prostate cancer.

Androgen-regulated TMPRSS2–ERG fusion gene is the most common fusion, found in approximately 50-70% of prostate cancers (Clark & Cooper 2009, Rubin et al. 2011). The androgen regulated transcriptional promoter of transmembrane protease, serine 2 gene (TMPRSS2, 21q22.3) becomes fused to ERG (21q22.2) gene encoding an ETS transcription factor (Tomlins et al. 2005, Clark & Cooper 2009, Rubin et al. 2011). The formation of TMPRSS2–ERG fusion gene results in high level expression of the ERG gene in the presence of androgens and truncated ERG transcription factor proteins. Altered gene expressions of ETS transcription factors provide a mechanism for cancer transformation. This leads to cancer promoting events such as β-estradiol signaling, higher levels of histone deacetylase 1 (HDAC1) expression and altered ion channel gene expression.

The 5´-untranslated region of TMPRSS2 has been identified to fuse with different ETS transcription factor family members among ERG such as ETV1, ETV4, ETV 5, ELK4, or FLI1 (Rickman et al. 2009, Paulo et al. 2010). By contrast, ETV1, ETV4, and ETV5 have multiple 5'-fusion partners that are mostly androgen activated genes such as SLC45A3 and NDRG1 (Clark & Cooper 2009, Rubin et al. 2011). The non ETS-fusions include RAF kinase family fusions associated with advanced prostate cancer and SPINK1-positive cases characterized as a distinctive subtype of prostate cancer.

2.3.4 Epigenetics

Epigenetic modifications are heritable and reversible biochemical changes of the chromatin structure. Epigenetic alterations are frequent in prostate cancer initiation and progression, occurring at a much higher frequency than somatic mutations (Chan et al 2008, Chiam et al. 2014). These alterations include changes in DNA methylation, histone modifications, and microRNAs (miRNAs). Their role in prostate cancer tumorigenesis is still poorly understood, and the mechanisms inducing them remain largely unknown. Their occurrence in premalignant stages of prostate cancer may provide biomarkers for diagnosis, prognosis, and treatment response for the disease.

CpG site is an area in DNA sequence where cytosine (C) lies next to guanine (G) and p indicates that C and G are connected by a phosphodiester bond. Cytosines in CpG dinucleotides can be methylated to form 5-methylcytosine. Methylation of DNA occurs at any CpG site. The epigenetic therapeutic and biomarker research of prostate cancers has been largely focused on gene-specific hypermethylation (Baylin & Herman 2000, Miyamoto & Ushijima 2005). This phenomenon is characterized by the increased DNA methylation of CpG rich areas in the promoter regions of specific genes. Commonly this leads to the inactivation or transcriptional repression of these genes. Gene-specific hypermethylation in cancer has been associated with inactivation of genes involved in DNA repair, cell-cycle regulation, apoptosis, and tumor-suppression.

There is a large number of characterized hypermethylated genes involved in prostate cancer. The most established epigenetic biomarker for prostate cancer is the hypermethylation of glutathione-S-transferase P1(GSTP1) gene (Henrique & Jeronimo 2004). This gene encodes an

enzyme required for detoxification and protection of DNA from oxidants and electrophilic metabolites. GSTP1 exhibits hypermethylation in all stages of prostate cancer and is commonly assessed with other hypermethylated genes such as adenomatous polyposis coli (APC) and retinoic acid receptor b2 (RARb2) (Jeronimo et al. 2004, Chiam et al. 2014). APC is a tumor suppressor gene that is involved in several cellular processes such as Wnt signaling pathway, cell migration and adhesion. RARb2 is a hormone receptor that regulates cellular processes such as cell growth and differentiation.

Ras association (RalGDS/AF-6) domain family member 1(RASSF1) is a tumor suppressor gene involved in cell cycle and apoptosis, while it is hypermethylated in early stages of prostate cancer assessed with GSTP1 (Chiam et al. 2014). Another two genes discovered to be hypermethylated in all stages of prostate cancer are prostaglandin-endoperoxide synthase 2 (PTGS2) and CD44 (Cho et al. 2007, Bastian et al. 2007). PTGS2 is a pro-inflammatory enzyme required for prostaglandin biosynthesis, while CD44is a cell-surface glycoprotein involved in cell-cell interaction, cell migration and adhesion.

Global hypomethylation arises in later stages of prostate cancer development than CpG island hypermethylation and it is shown to be associated with advanced, metastatic stage of the disease (Yegnasubramanian et al. 2008). In global hypomethylation the genomic DNA methylation level has decreased. This event is frequently observed in cancer since it leads to the activation of proto-oncogenes and chromosomal instability (Hake et al. 2004). The promoter regions of proto-ocogenes may become hypomethylated increasing the expression of the promoter regulated genes. On the other hand, repetitive DNA sequences such as retrotransposon elements are normally methylated in normal tissues. It has been shown that during prostate cancer progression long Interspersed Element–1 (LINE-1) retrotransposons and Alu repeatative DNA elements become hypomethylated (Cho et al. 2007).

H3K27Me2 and H3K18Ac histone modifications are commonly associated with active gene expression whereby H3K27Me3 is associated with repressed gene expression. In prostate cancer the global levels of these modifications may become aberrant (Seligson et al. 2005, Bianco-Miotto et al. 2010). Specific alterations in histone modifications provide an epigenetic signature that may lead in aberrant gene expression promoting prostate cancer progression.

Mutations and chromosomal alterations targeting genes involved in histone modifications have been identified in prostate cancer. Most commonly these alterations are related to the post-transcriptional modification of histone variant H3, which is highly essential in transcriptional control and regulation of chromatin states. Enhancer of zeste 2 polycomb repressive complex 2 subunit (EZH2) is a histone-lysine N-methyltransferase that methylates H3K27 histones. EZH2 is commonly hypomethylated and overexpressed during prostate cancer progression (Varambally et al. 2002). Mixed-lineage leukemia 2 gene (MLL2) histone methyltransferase that methylates the Lys-4 position of histone H3 is mutated in 8.6% of prostate cancers (Grasso et al. 2012).

The chromodomain helicase DNA binding protein 1 gene (CHD1) is located at 5q21chromosomal locus that is recurrently deleted in 10–25% of primary and metastatic tumors of prostate cancer (Taylor et al. 2010, Barbieri et al. 2012, Grasso et al. 2012). Mutations and rearrangements of CHD1 gene have also been observed. The CHD1 gene product acts genome-wide in chromatin remodeling and transcriptional control. Deletion of CHD1 results in increase of genomic rearrangements (Liu et al. 2012, Baca et al. 2013).

MicroRNAs (miRNAs) are small non-coding RNAs that regulate gene expression of numerous cellular functions such as development and differentiation. miRNAs can bind to complete or partial complementary target mRNAs, usually at the 3´-untranslated region (Chiam et al. 2014. Most commonly miRNAs induce gene silencing by mRNA degradation or translational repression. miRNAs can also act through epigenetic regulation of genes, while being under epigenetic regulation themselves (Lujambio & Esteller 2009, Sato et al. 2011). They can induce gene silencing by targeting a specific gene region for DNA methylation and histone modifications or regulate epigenetic enzymes, inducing global epigenetic regulation. The strong interactions between miRNAs and other epigenetic mechanisms display a complex regulatory network of chromosomal remodelling and regulation of gene expression, which is still poorly understood.

Several miRNAs have been identified to be altered in prostate cancer. Some of the most promising biomarkers at the moment are miR-141 and miR-375 that have shown to be overexpressed in prostate cancer metastasis (Brase et al. 2011, Bryant et al. 2012). These miRNAs have multiple target genes, where miR-141 is a member of miR-200 family that most com-

monly regulates epithelial to mesenchymal transition (EMT). Their expression level may be useful to identify patients with high-risk of prostate cancer.

2.3.5 Somatic mutations

The phosphatase and tensin homolog (PTEN) gene is one of the most commonly mutated and inactivated tumor suppressor gene in prostate cancer and carsinogenesis, located on chromosome 10q23 (Cairns et al. 1997, Barbieri et al. 2012b, Barbieri et al. 2013). Recurrent deletions at the PTEN locus are mostly heterozygous, occurring in 40% of primary prostate cancers, while inactivating mutations are apparent in 5-10% and more common in advanced disease. PTEN inhibits phosphoinositide-3-kinase /protein kinase B (PI3K/Akt) pathway affecting the main regulation of cell proliferation, survival, and invasion. In addition, PI3K/Akt pathway is activated by aberrant signaling pathway components in approximately 25–70% of prostate cancers (Barbieri et al. 2013). Activating lesions in PI3K/Akt pathway components and inactivation of PTEN alter the normal cell regulatory system and have central significance in the pathogenesis of prostate cancer.

Tumor protein p53 (TP53) is a highly essential transcription factor and the most commonly mutated tumor suppressor gene in cancer (Barbieri et al. 2013). TP53 activates genes involved in cell cycle arrest, DNA repair, and apoptosis.TP53 deletions occur in 25–40% and point mutations in 5–40% of prostate cancer incidences (Taylor et al. 2010, Barbieri et al. 2012b, Grasso et al. 2012, Lindberg et al. 2013b).

The v-myc myelocytomatosis viral oncogene homolog (MYC) gene encodes a transcription factor protein c-Myc. MYC is one of the most common oncogenes in cancer located at chromosome 8q24. MYC is involved in the regulation of cell growth, apoptosis, and metabolism (Dang 1999, Dang 2013). Oncogenic MYC transcription factor alters global gene expression of these essential pathways through transcriptional regulation and strongly promotes tumorigenesis. MYC gene amplifications are common in prostate cancer, but it also may undergo oncogenesis through rearrangements, translocations, over expression, or mutations (Taylor et al. 2010, Barbieri et al. 2012b, Grasso et al. 2012, Beltran et al. 2013).

The key players of androgen signaling pathway undergo recurrent lesions in prostate cancer (Barbieri et al. 2013). These include alterations of AR itself, the factors altering AR activity

and transcription, as well as downstream target factors of AR (Taylor et al. 2010, Barbieri et al. 2012b, Grasso et al. 2012, Lindberg et al. 2013b). AR signaling interacts also with other oncogenic pathways and alterations in this network remain in the centre of prostate cancer development and progression.

The increased activity of AR remains highly essential for metastatic CRPC (Visakorpi et al. 1995, Koivisto et al. 1997, Linja & Visakorpi 2004). AR is a ligand-dependent nuclear transcription factor that that often transforms in to an oncogene during prostate cancer development. AR alterations include gene amplification, point mutations, and alternative splicing of transcripts (Hu et al. 2012). These alterations lead to constitutively active AR variants and androgen signaling pathway as a resistance mechanism for androgen deprivation therapies in prostate cancer (Barbieri et al. 2013). AR amplification are recurrent in 40%, while AR mutations in 10% of metastatic prostate cancers (Taylor et al. 2010).

The forkhead-box family of transcription factors has been recently implicated in prostate cancer pathogenesis. This protein family is involved in cell growth and differentiation. Some of these genes are possible tumor suppressor genes located in recurrently deleted chromosomal regions in prostate cancer (Taylor et al. 2010, Barbieri et al. 2012b). On the other hand, recurrent point mutations of the forkhead box A1 (FOXA1) modulating AR expression have been found in both primary tumors and metastatic lesions of prostate cancer (Barbieri et al. 2012a, Grasso et al. 2012).

Recently detected point mutations in the speckle-type POZ protein (SPOP) gene are the most common in primary prostate cancer (Barbieri et al. 2012a, Lindberg et al. 2013a, Lindberg et al. 2013b). SPOP encodes for the substrate-recognition component of a Cullin3-based E3-ubiquitin ligase. The enzyme substrate binding is altered in prostate cancer due to the missense mutations in the substrate binding-cleft.

Genes involved in histone modifications undergo recurrent mutations in prostate cancer. Lysine (K)-specific demethylase 6A gene (KDM6A), mixed-lineage leukemia 2 (MLL2) gene, and MLL3 gene participate in altering H3 histone variant methylation status (Barbieri et al. 2012b, Grasso et al. 2012, Lindberg et al. 2013b). Also CHD1 gene rearrangements and point mutations have been identified. The proteins encoded by these genes all act to alter methyla-

tion of the histone variant H3 and affect indirectly to the regulation of chromatin states and transcriptional control.

2.4 Methods

2.4.1 Real-time quantitative polymerase chain reaction

Real-time quantitative polymerase chain reaction (RT-qPCR) is a technique used to amplify, detect, and simultaneously quantify the number of copies of a targeted piece of DNA molecule (Gibson et al. 1996). The accumulation of amplified product can be detected and measured in real-time by including the reaction with a fluorescent molecule that reports an increase in the amount of DNA with a proportional increase in fluorescent signal (Higuchi et al 1992, 1993). With RT-qPCR it is possible to determine accurately the starting DNA or complementary DNA (cDNA) template copy number of a given sample (Bustin 2000).

RT-qPCR can be applied for quantifying chromosomal DNA gene copy number alterations or detecting mutations and genetic polymorphisms (Van Guilder et al. 2008). Most importantly, RT-qPCR is widely applied to quantify and study the expression levels of particular genes from isolated total RNA of different cell types or tissues (Stahlberg et al. 2004). Since the mRNA molecules are transcribed from the gene of interest their abundance resembles the level of expression of the gene of interest. Before RT-qPCR amplification the total RNA is extracted, reverse transcribed, and amplified into cDNA in a reverse transcription polymerase chain reaction (RT-PCR). This enables to study the initial abundance of specific mRNA molecules of interest in a given sample.

Quantification of the RT-qPCR products can be done either with an intercalating fluorescent dyes or sequence specific DNA-probes (Higuchi et al 1992, 1993). SYBR Green I is a non-specific DNA-binding fluorescent dye that intercalate with any double-stranded DNA (Bio-Rad Laboratories, Inc. 2006). It is an asymmetrical cyanine dye that has low level fluorescence when it is free in solution, but its fluorescence increases up to 1,000-fold when it binds double stranded DNA. The method is highly efficient because, only two primers are needed for PCR reaction to amplify target area to double stranded DNA molecule and no probe design will be necessary. Specialized thermal cyclers with fluorescence detection modules are used to measure the fluorescence that reflects the amount of amplified product in each PCR

cycle. Therefore, the overall fluorescent signal from a reaction is proportional to the amount of double stranded DNA present, and will increase as the target is amplified.

The primers for RT-qPCR are designed as for standard PCR. The gene-specific primers and probes are usually designed as complementary to a cDNA region covering an exon-exon junction (Ye et al. 2012). This prevents the primer or the probe from binding to genomic DNA (gDNA) and thereby reduces the amount of false positive results (Proudnikov et al. 2003). Before amplifying the gene of interest, primers and probes are annealed with the template. The annealing temperature in the PCR reaction is proportional of the nucleic acid content and sequence of each primer pair that can be optimized prior to study.

Amplified RT-qPCR products can be identified from the melting temperature curve to separate different reaction products from nonspecific products (Nolan et al. 2006). This confirms the specificity of the amplification reaction. The melting curve is generated after the PCR amplification reaction by increasing the temperature in small increments and monitoring the fluorescent signal at each step. DNA melts when the double stranded DNA in the reaction denaturates and at the same time the fluorescence decreases. The melting temperature is characteristic to every amplified product and depends on their sequence, length, and guanine-cytosine (GC) content.

Two quantitative methods are primarily used for RT-qPCR data-analysis: absolute quantification and relative quantification. Absolute quantification gives the exact quantity of target DNA molecules in a given amount of sample. This is achieved by comparing the threshold cycle (Ct) values of the test samples to a standard curve, using serial dilutions of a known template (Bustin 2000). The standard curve of the gene of interest and the reference gene is used to calculate the target gene expression based on the obtained values from RT-qPCR assay.

In relative quantification the changes in gene expression in a given sample and under a specific treatment or state is analyzed relative to a reference sample such as an untreated control sample (Livak & Schmittgen 2001). This can be based on internal reference genes to determine fold-differences in expression of the target gene. Also, it is easier to carry out as it does not require a standard curve because the amount of the studied gene is compared to the

amount of a control reference gene. A reference gene is thought to have a constant expression level throughout the test samples while it´s expression is not affected by the experimental treatment under study (Kozera & Rapacz 2013). House-keeping genes are commonly used as reference genes because of their gene expression in every cell and tissue is assumed to be similar. For exambleTATA-box binding protein (TBP) and Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) are widely used as housekeeping genes in prostate cancer research.

When using a standard curve, it is essential that the sample and the standard have the same amplification efficiency (Dhanasekaran et al. 2010). The efficiency of the assay should be 90–105% and the standard curve should have a correlation coefficient (R2) of at least 0.98. The obtained Ct values of the replicate samples should be similar to confirm replicate consistency. In order to find biological differences, the cause of methodological artifacts has to be excluded when comparing the variation of expression levels between different samples (Leong et al. 2007).

For both absolute and relative quantification methods the obtained gene expression quantities from RT-q PCR must be normalized by comparing them to reference gene expression quantities. Normalization to the reference gene and comparison of the treated samples to their controls gives the relative change in gene expression. In absolute quantification, normalizers are used to adjust or standardize the obtained target quantity to the unit amount of sample (Bio-Rad Laboratories, Inc. 2006). In relative quantification, normalizers are used to ensure that the target quantities from equivalent amounts of samples are compared. The use of a reference gene is advantageous in many cases because the precise quantification of input template amount is not possible. This occurs for example in cases of cDNA-template from extracted total RNA or in cases where only a small amount of starting template is available (Bio-Rad Laboratories, Inc. 2006).

2.4.2 Endogenous RNA interference

RNA interference (RNAi) is a relatively new concept in the field of biology. It is an efficient and specific gene silencing mechanism for the regulation of gene expression. The process can be triggered by various species of double-stranded RNA (dsRNA) molecules, targeting specific mRNA gene product based on their nucleic acid sequence. The process can lead to altered

mRNA splicing, inhibition of mRNA translation, or degradation of mRNA (Carthew & Sontheimer 2009).

Several different kinds of regulatory RNA species are transcribed endogenously during normal processes of the cell, where the mechanism of microRNA (miRNA) and short interfering RNA (siRNA) remain the best understood (Figure 4). RNAi pathway occurs during normal animal development and physiological function, while RNAi dysregulation has been linked to several pathologies (Burnett & Rossi 2012). This process also occurs in cellular defense mechanism against parasitic nucleotide sequences, viruses and transposons. RNAi has been one of the most rapidly expanding areas of biological research in recent years, and today it is providing us efficient tools for research and novel therapeutic approaches for disease treatments (Kurreck 2009, Burnet & Rossi 2012).

miRNAs are highly abundant in eukaryotes and may originate from the introns of coding genes, from large non-coding RNAs (ncRNA) or even from pseudogenes (Kaikkonen et al. 2011). Endogenous miRNAs are transcribed into large primary transcripts that fold into double stranded secondary structure with incomplete base pairing. This stem-loop precursor dsRNA (pri-miRNA) is cleaved in the nucleus by the ribonuclease (RNase) III Drosha to a pre-miRNA precursor and exported to the cytoplasm (Figure 4) (Lee et al. 2003). siRNA precursors are thought to be long, linear and perfectly base-paired dsRNAs, without stem-loop structure (Kaikkonen et al. 2011). siRNA precursors arise mostly from exogenous origin, such as transgenes or viruses, but endogenous precursors may reside for instance in introns, intergenic areas, or transposable elements (Carthew & Sontheimer 2009). Both naturally occurring miRNA and siRNA species in the cytoplasm are double stranded, consisting of sense and antisense strand.

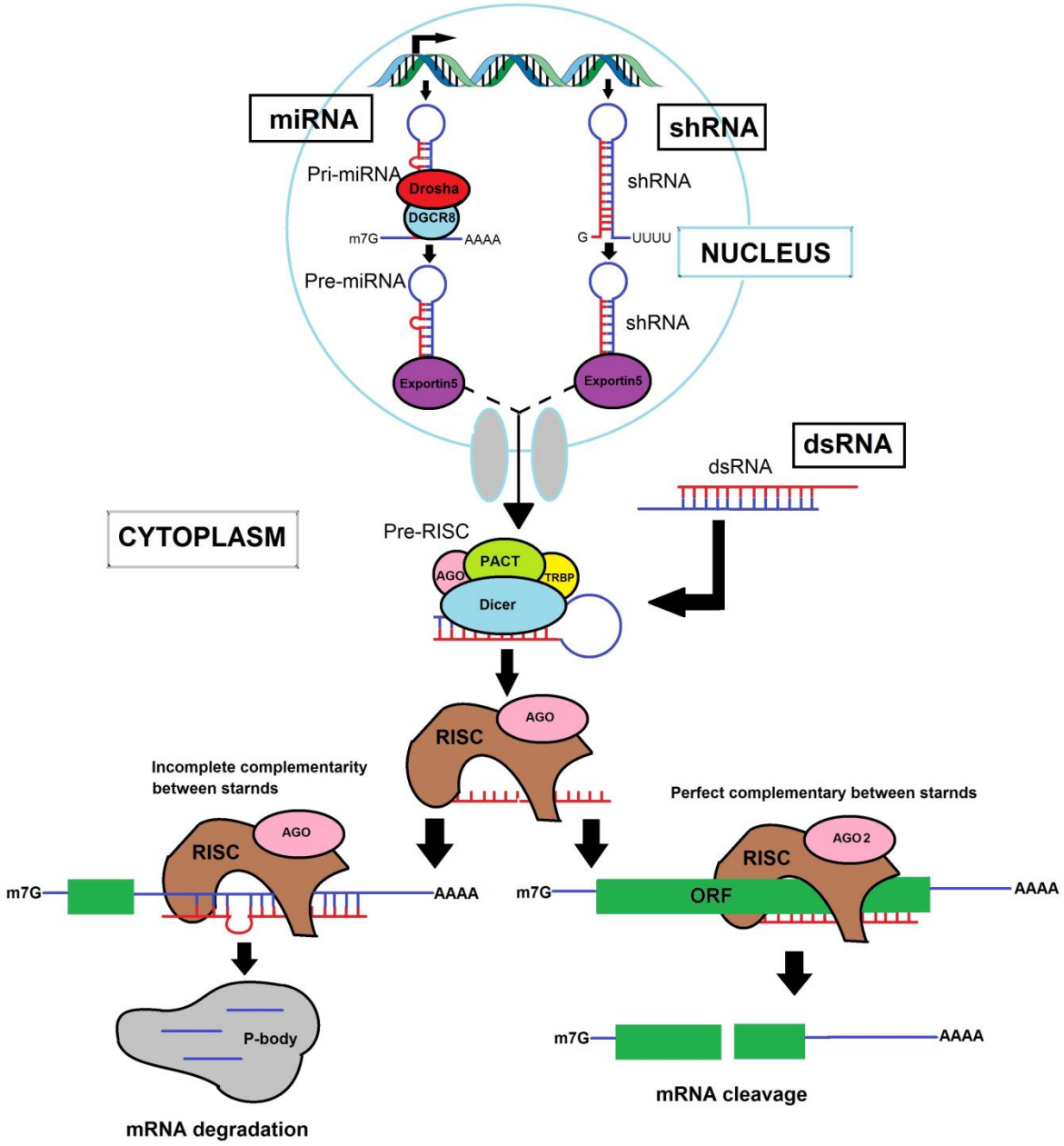


Figure 4. Mammalian RNAi pathway for miRNAs, shRNAs, and siRNAs. The endogenous RNAi pathway precursor RNAs are transcribed from DNA in the nucleus. Stem-loop precursors are transported to the cytoplasm. Linear and double-stranded RNA sequences are modified by the RNase Dicer in the cytoplasm. Exogenous RNA inhibitors bound to multimer-protein complex RISC and the antisense-strand (red) is guided to the target mRNA sequence (blue). The target sequence is recognized by base-pairing and sequence similarity, while RISC complex catalyses the final proteolytic processing. Image modified from Burnett & Rossi 2012.

Upon introduction to the cell cytoplasm, the long precursor dsRNA molecules form a complex with dsRNA-specific RNAse III Dicer (Figure 4) (Bernstein et al. 2001). Dicer cleaves the pre-miRNAs to their shorter, mature 21-23 base pair form with ~2 nucleotide single

stranded ends. Dicer also cleaves linear siRNA duplexes into mature siRNAs, 21-26 nucleotides in lenght with characteristic of 2-3 nucleotide overhangs at the 3'-OH ends. The cleaved products in the cytoplasm are then incorporated into the RNA-Induced Silencing Complex (RISC), which is composed of argonaute (AGO) protein family member, Dicer, and TAR-RNA-binding protein (TRBP) (Figure 4) (Han et al. 2006). The antisense strand of original dsRNA is complementary to the target mRNA sequence, and the sense strand mediates the binding to AGO in the catalytic site of the RISC (Burnett & Rossi 2012). The RNA duplex is separated and the RISC cleaves the sense strand guiding the antisense strand to the 3´ untranslated region (UTR) of the target mRNA sequence.

Gene silencing in RNAi pathway can occur through transcriptional gene silencing (TGS) or post-transcriptional gene silencing (PTGS), where PTGS remains the main focus of clinical applications (Castanotto & Rossi 2009, Burnett & Rossi 2012). The antisense strand of dsRNA may have imperfect complementarity to target mRNA, inducing translational repression, deadenylation, or mRNA cleavage. In the case of complete or near-complete complementarity of dsRNA to the target sequence, the mRNA will be degradated (Martinez et al. 2002).

2.4.2.1 Exogenous siRNA gene silencing

RNAi provides an approach for silencing specific target gene expression under study. Cells in tissue culture can be transfected with exogenous, synthetic RNA inhibitors, such as exogenous small interfering RNAs (exo-siRNAs) or short hairpin RNAs (shRNAs). Exogenously introduced RNA inhibitors can act as endogenously transcribed ones since they are processed and targeted by RISC complex (Rao et al. 2009b). Synthetic siRNA oligonucleotides are usually 19-21 nucleotide long double-stranded sequences with two nucleotide overhang in both 3'-OH ends (Jackson & Linsley 2010). The antisense strand is designed for perfect complementarity to the mRNA sequence of the target gene of interest to obtain efficient inhibition of protein translation and target mRNA degradation (Burnett & Rossi 2012).

Exogenous RNA inhibition exhibits limitations and may trigger various unwelcome off target effects in the model system. Off-target activity of siRNAs can lead to unanticipated phenotypes and complicate interpretation of the effects of target gene silencing (Jackson & Linsley

2010). Therefore these challenges need to be addressed in order to understand the ways of reducing them and selecting the suitable purpose of use.

Firstly, the exogenous transfection of RNA sequences can only provide transient target gene silencing. Cell proliferation and endogenous degradation of the exo-siRNA sequences result in siRNA dilution and eventually loss of RNAi effect (Rao et al, 2009a; Sandy et al, 2005). Secondly, synthetic siRNAs and/or their transfection vehicles may activate an inflammatory response through activation of Toll-like receptors (TLRs) (Jackson & Linsley 2010). Transfection approach should be carefully addressed to avoid cell toxicity and possible effects on the expression of other genes and proteins. Different approaches of chemical transfection are currently available for commercial siRNAs. For instance, former problems encountered with cationic lipids have been reduced by using alternative endocytosis based cell delivery, such as cationic polymers to complex with the negatively charged DNA.

The estimation of synthetic siRNA inhibition efficiency is challenging. All exogenous siRNAs exhibit sequence-dependent regulation of their target mRNA, but they may have also partial sequence complementarity to the 3′ UTRs of unintended transcripts (Jackson & Linsley 2010). The target gene exhibits specific GC-content, point-specific nucleotides, and specific motif sequences (Chan et al. 2009, Takasaki 2010). Exogenously implicated dsRNAs may also compete with the production and function of natural dsRNAs since they utilize the same endogenous factors of naturally occurring RNAi pathway. Therefore exogenous siRNAs may have effects on normal endogenous microRNA processing and function through saturation of the endogenous RNAi machinery (Jackson & Linsley 2010).

Commercial exogenous siRNAs are nowadays designed, using bioinformatic algorithms and synthesized with novel chemical modifications to enhance their specificity and exhibit higher overall potency with lower concentrations (Burnett & Rossi 2012). Positive control siRNA labeled with fluorescent dye are used to monitor transfection efficiency by observing the cellular uptake of fluorescently-labeled siRNA (Ambion, Inc. 2006, RNA Interference Research Guide). Non-targeting negative control siRNAs have no homology to any known mammalian gene and serve as a baseline for evaluation of experimental target knockdown. Minimal non-specific effects ensure that comparison of the gene-specific siRNA to the negative control gives a true picture of the effects of target-gene knockdown on gene expression and pheno-

type. Housekeeping gene expression levels between non-transfected and negative control siRNA transfected cultures can be also used to obtain information about transfection effects on cell viability.

3 AIMS OF THE RESEARCH

The aim of this Master's thesis study was to screen potential target genes of 9p13.3 amplicon in prostate cancer. Putative amplification target gene is expected to have a strong correlation between increased chromosomal copy number status and elevated mRNA expression (Leinonen 2007). Therefore it is highly important to determine the function of these genes and to clarify their role in prostate cancer. With the results from previous studies and data mining from public databases the Molecular Biology of Prostate Cancer Group (MBPCG) at the University of Tampere had selected the possible target genes of 9p.13.3 amplicon yet unstudied.

The potential target genes determined in this study were NOL6, C9orf23, FANCG and STOML2. These genes were known to have elevated mRNA expression correlating with the amplification (unpublished). Their function and role in prostate cancer were unknown. The functional effects of these genes on cancer cell proliferation and migration of 9p13.3 amplicon harboring cell lines were also unknown. In addition, the genes NFX1 and KIF24 are located at the 9p13.3 amplicon area, but no information about their gene expression had been obtained in previous studies. Based on previous studies and gene expression data obtained in this study, low level amplification (gain) 9p13.3 harboring prostate cancer cell line 22Rv1 and amplicon harboring breast cancer cell line BT-474 were selected for functionality studies. 9p13.3 amplicon deficient PC3 prostate cancer cell line was selected to be used as a control cell line.

The main aims:

1) Explore the extent of correlation between expression and 9p13.3 amplification status of NFX1 and KIF24 genes.

2) Perform successful gene silencing of potential target genes of 9p13.3 amplicon with siRNA transfection to understand their contribution to prostate cancer, and analyze the effects on target gene expression, cell proliferation, and cell migration.

3) Outline putative target genes for further investigations.

4 MATERIALS AND METHODS

4.1 Cancer cell lines and prostate cancer xenografts

The prostate cancer cell lines LNCaP, DU145, PC3, and 22Rv1 were obtained from American Type Culture Collection (ATCC, Rockville, MD, USA). The prostate cancer cell line LAPC-4 was kindly provided by Dr. Charles Sawyers (Jonsson Cancer Center, UCLA, Los Angeles, USA), and the prostate cancer cell lines VCaP and DuCaP were kindly provided by Dr. Jack Schalken (Radbound University Nijmegen Medical Centre, Nijmegen, Netherlands).

Materials for the 21 prostate cancer xenografts (LuCaPs 23.1, 23.8, 23.12, 35, 35A1, 41, 49, 58, 69, 70, 73, 77, 78, 81, 86.2, 92.1, 93, 96, 102, 105 and 115) were kindly provided by Dr. Robert Vessella (Department of Urology, University of Washington, Seattle, WA, USA). The 22 breast cancer cell lines (BT-474, CAMA-1, DU4475, EFM192A, HBL-100, HCC1419, HCC1954, MDA-MB-231, JIMT-1, MCF 10A, MCF-7, MDA-MB-194, MDA-MB-361, MDA-MB-415, MDA-MB-436, MDA-MB-453, MPE600, SK-BR-3, T-47D, UACC-812, UACC-893, ZR-75-30) were obtained from American Type Culture Collection (ATCC, Rockville, MD, USA).

The cell lines used for siRNA transfection to study gene expression, cell proliferation and migration were PC-3, 22Rv1, and BT-474 cell lines. The cells were cultured at 37 °C and 5% CO2. PC3 and 22Rv1cell cultures were subcultured every three to four days. BT-474 was subcultured every four to five days. Interphase and methaphase spreads were prepared following the standard laboratory protocols. The cell lines had been grown in recommended media. The used basal media was Ham's F12 with 10% fetal bovine serum and 2 mM L-glutamine for PC-3 cell line and RPMI 1640 with 10% fetal bovine serum and 2 mM L-glutamine for

22Rv1 and BT-474 cell lines. Basal media and fetal bovine serum were purchased from Lonza.

PC-3 cell line is initiated from a bone metastasis of a grade IV prostatic adenocarcinoma. It was selected as the control cell line because of normal 9p13.3 copy number status. 22Rv1 is an epithelial human prostate carcinoma cell line derived from a mice xenograft. This cell line had been shown to have low level 9p13.3 amplification and elevated gene expression of our selected, putative target genes. BT-474 represents solid, invasive ductal carcinoma of the breast and harbours 9p13.3 amplification.

4.2 RNA extraction

Total RNA from PCa and BrCa cell lines and LuCaP xenografts used for gene expression assays in this study was extracted with TRIZol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA), following the manufacturer´s protocol. Total RNA from prostate cancer cell lines LNCaP, PC3 and 22Rv1 were used for cDNA synthesis and later as standard templates for RT-qPCR assays. cDNA samples were used as templates in RT-qPCR assays for the gene expression screening of NFX1 & KIF24 genes. cDNA samples prepared from from each LuCaP xenograft total RNA were previously extracted by Outi Saramäki, PhD. cDNA samples prepared from each PCa and BrCa cell line total RNA were previously extracted by Leena Latonen, PhD.

In order to determine successful target gene silencing of transient siRNA transfection, the total RNA from PC-3, 22Rv1, and BT-474 were isolated for the RT-qPCR assays. For total RNA extraction, 200 000 cells were seeded on 6-well plate and transfected with 10nM target gene specific and negative control siRNAs. After 3 days of growth, the RNA was extracted with the TRIZol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA), following the manufacturer´s protocol.

4.3 cDNA synthesis and real-time quantitative polymerase chain reaction

The total RNA of the specimens were reverse transcribed to cDNA with Maxima Reverse Transcriptase (Thermo Fisher Scientific Inc., Waltham, MA, USA) by using 5’-d(NNNNNN)-3’ (N = G, A, T or C) Random Hexamer Primers (Thermo Fisher Scientific Inc., Waltham, MA, USA). Standard curves were created by using 5-fold dilution series and prepared from

pooled mixture of cDNA from 1µg of total RNA of three non-transfected prostate cancer cell lines: LNCaP, 22Rv1 and PC3. Dilution series consisted of 1:5, 1:25, 1:125, 1:625, and 1: 3125 dilutions.

RT-qPCRs were carried out in Bio-Rad CFX96™ Real-Time PCR Detection System thermal cycler in 96-well plates (Bio-Rad Laboratories Inc., Berkeley, CA, USA) with 20 µl reaction volume. 10µl of Maxima™ SYBR Green/ROX qPCR Master Mix (Fermentas Inc., Burligton, Ontario, Canada) was used for every reaction containing Maxima™ Hot Start Taq DNA Polymerase, SYBR® Green dye I, and dNTPs in an optimized PCR buffer. Every reaction was included with 0.1 µM of each reverse and forward primer. The template cDNA samples were diluted to 1:10 (PCa), 1:20 (BrCa) or 1:100 (LuCaP) and the sample volume were 1 µl for LuCaP template cDNA and 2µl for other cDNA templates. DEPC treated sterile water was added to fill the final volume of 20µl. The reaction conditions are summarized in Table 1.

Table 1. *Reaction coditions of real-time qPCR with SYBRGreen I fluorescent dye .*

Step	Program	Temperature /°C	Time
1	Denaturation	95	10 min
2	Denaturation	95	15 sec
3	Annealing	65	30 sec
4	Elongation	72	30 sec[2]
5	49 cycles: jump step 1 to 5		
6	Enhancement	50	30 sec
7	Melt curve & plate read	65-95	0,5°C/sec[1]
8	Cooling	4	Forever

Temperature was increased from annealing temperature of 65°C to 95°C at the rate of 0,5 °C/second.

Manufacturer's instructions were followed in every step of RT-qPCR, and reaction conditions were optimized for each primer pair. The primers for the genes of interest were designed online with NCBI Primer-BLAST program for amplifying regions of the mRNAs derived from different exons to avoid amplification of gDNA. The primers (Oligomer Oy, Helsinki, Finland) were tested with gradient-PCR before RT-qPCR assay to conclude their specificity and suitable annealing temperature. The primer sequences used are summarized in Table 2.

Table 2. *Primer sequences used in RT-qPCR .*

Gene	Primer Sequence 5´-3´
C9orf23	ATCTTGGCTCCGGATCGT CGCATCTCAAGGGTATCAGG
FANCG	GGGACCTGGCCTTGTTACTA CTGATCCCTCTTTCAGGGCT
KIF24	ACTGTTAGGTTGCGGGCTTT TTGGAAGAGACGTTTGCGGT
NFX1	TCTCCCGAGGCAAACAGAAC GCCGTCACACGAACCAATTC
NOL6	GGGCCTGGAGTTAGGCATTT TGTACAGTTCTGCCCTGCTG
STOML2	CCGAAACACCGTGGTACTGT TGCCCACAACAGTAACCTGG
TBP3	GGGGAGCTGTGATGTGAAGT GAGCCATTACGTCGTCTTCC

For each gene: upper sequence is forward primer and bottom sequence reverse primer.

Following RT-qPCR reaction, the specificity of the primers and the correct size of the amplified PCR products were analyzed by melting curve analyzis and 1% agarose gel electrophoresis. PCR results were analyzed using CFX Manager Software (Bio-Rad Laboratories Inc., Berkeley, CA, USA). All expression values were normalized to the expression values of the housekeeping gene TBP and diethylpyrocarbonate (DEPC) treated sterile water was used as a negative control. All samples were run in triplicates. Only the screening of NFX1 and KIF24 gene expression in multiple BrCa and LuCaP cell lines was done in single reactions due to the small cDNA template sample amounts available.

4.4 Transient siRNA transfection

siRNA transfections of PC3, 22Rv1, and BT-474 cell lines were performed using INTERFERin® in vitro siRNA transfection reagent (Polyplus-transfection Inc., NY, USA). For the growth curve assay, 20 000 cells of 22Rv1, 20 000 cells of BT-474, and 15 000 cells of PC3 cells were seeded on 24-well plate and incubated 1h before transfection.

Silencer® Select Pre-designed siRNAs were used for the gene specific silencing (Ambion® Life Technologies, Thermo Fisher Scientific Inc., Waltham, MA, USA). Equal mass amounts of different target gene specific siRNAs and non-specific negative control siRNAs were used to transfect cells. All functional siRNA experiments were performed in six parallel replicates and were repeated at least twice.

In this study all the potential target genes and their functions were characterized by the extent of siRNA mediated gene silencing. The results were quantified between negative control siRNA and gene specific siRNA transfection in parallel for each cell line. The efficacy of target gene specific silencing was quantified using RT-qPCR assay. The manufacturer announces >80% reduction in target gene expression.

Table 3. *Silencer® select siRNAs used for target gene specific silencing (Ambion®, Life technologies)*

Target gene symbol	siRNA ID #	Sence sequence (5´- 3´)
C9orf23	s44127	GCUACCUCCUGAUACCCUUtt
	s44128	AGUUCUGCAUUGCUAAAAUAtt
FANCG	s5020	CAGGUAAUCGAGACACUUAtt
	s5018	GAUCUGCUGUUACUUCUGAtt
KIF24	s51220	GUUCCACUCUUACUCUGAAtt
	s51222	GGGUCAAAGAACUAAAGAAtt
NOL6	s35199	GUUUAUGUCUCAGCUCAGAtt
	s35200	GGGCAGAACUGUACAAGGAtt
STOML2	s26969	AAACAACCAUGAGAUCAGAtt
	s26971	AAAUCGAUGGAGUCCUUUAtt

The sence sequence is identical to the mRNA sequence transcribed from the gene

4.5 Proliferation assay

Cells were quantified by using light microscopy right after transient siRNA transfection and also at one, three, and five day time points after transfection. Six parallel samples were used in each experiment. The growth area of cells was quantified by Olympus IX71 inverted light microscope (Olympus Corporation, Tokyo, Japan) imaging and Surveyor software (Objective Imaging Inc., Kansasville, WI, USA). Images were analyzed, and cell surface areas were determined by using software algorithm developed in-house to produce relative growth curves.

The growth area was calculated at each time point and normalized to the initial growth area between negative control siRNA and gene specific siRNA treatments.

Cell proliferation was also quantified by AlamarBlue assay (Invitrogen, Life Technologies, Thermo Fisher Scientific Inc., Waltham, MA, USA) five days after transfection. In this assay, increase or decrease in the metabolic activity of cell culture was measured by assaying the relative absorbances of the samples (Fields & Lancaster 1993). The overall metabolic activity of the cell culture generally correlates with the number of cells in the culture. Six parallel samples were used in each experiment. For AlamarBlue assay, 50 µl of AlamarBlue reagent was added to 0.5 ml of medium and 100 µl of medium was collected after 120 minutes adding the reagent on 96 well plates. Absorbances of collected samples were then measured spectrophotometrically at 570 nm using the 2104 EnVision® Multilabel Reader (PerkinElmer Inc., Waltham, MA, USA).

4.6 Cell migration assay

The scratch wound healing assay is widely used, economical method to measure cell migration *in vitro*. In this study 24-well plates and six parallel samples were used in each experiment. The ‘‘scratch’’ was created in a confluent cell monolayer at the bottom of each well with a 200 µl pipette tip. The cells on the edge of the newly created gap are expected to move toward the opening to close the ‘‘scratch’’ until new cell-cell contacts are established again.

With 22Rv1 cell line, 80 000 cells were seeded on each well, while 70 000 cells were plated when using PC3. The cells were transfected after plating with gene specific and negative control siRNAs. The images were captured at the beginning, right after making the scratch, and at regular intervals during cell migration. For 22Rv1 cell line the time points were 0h, 24h, and 48h. For PC3 cell line the time points were 0h, 8h, and 16h.

The images were analyzed to quantify cell migration. The wound area in each well was monitored and quantified by Olympus IX71 inverted light microscope imaging (Olympus Corporation, Tokyo, Japan) and Surveyor software (Objective Imaging Inc., Kansasville, WI, USA). The images of PC3 cell line were analyzed with ImageJ program (Rasband, W.S, 1997-2014, U. S. National Institutes of Health, Bethesda, Maryland, USA) and images of 22Rv1 cell line were analyzed with WHA 0.1 program developed in-house. The wound area was calculated at

each time point and normalized to the initial wound size, and compared between negative control siRNA and gene specific siRNA treatments.

4.7 Statistical analyses

All results obtained from qRT-PCR studies were normalized to TBP housekeeping gene. Data from gene silencing experiments, proliferation and wound healing assay analyses were compared to negative control siRNA transfected cells. Probability (p) values were calculated using Student's two-tailed heteroscedastic t-test with values obtained from the negative control siRNA transfected cells as the control data set. P-values < 0.05 were considered statistically significant.

5 RESULTS

5.1 Screening of NFX1 and KIF24 gene expression

Potential target genes for RT-qPCR assays were selected based on previous microarray and RT-qPCR studies made by MBPCG at the University of Tampere, with the assistance of data mining from public data bases (unpublished). Gene expression levels of putative target genes NFX1 and KIF24 were analysed from 7 PCa cell lines, 22 BrCa cell lines, and 21 LuCaP xenografts. Based on data obtained from this study the potential target genes were outlined to C9orf23, FANCG, KIF24, NOL6, and STOML2. The expression levels of these genes were quantified between negative control siRNA and target gene specific siRNA transfection in parallel for PC3, 22Rv1, and BT-474 cell lines.

In this study NFX1 did not show strong association between elevated mRNA expression and 9p13.3amplification (Figure 5). NFX1 had low expression level in 9p13.3 amplicon harboring cell line BT-474 (Figure 5 A). There was no statistically significant difference between the NFX1 mean expression level in the amplification harboring group compared to normal copy number status group in BrCa cell lines ($p > 0.05$, Figure 5 B). Similar observation was made in LuCaP xenografts (data not shown).The relative mean values of NFX1 expression in low level amplification PCa cell lines DU145 and LAPC-4 did not show increased expression compared to normal copy number cell line PC3 (Figure 5 C). In low level amplification cell

lines DuCaP, LNCaP, VCaP, and 22Rv1 the expression was elevated (Figure 5 C). In 22Rv1 cells the expression was 3.4 fold higher than in PC3 cell line (Figure 5 C).

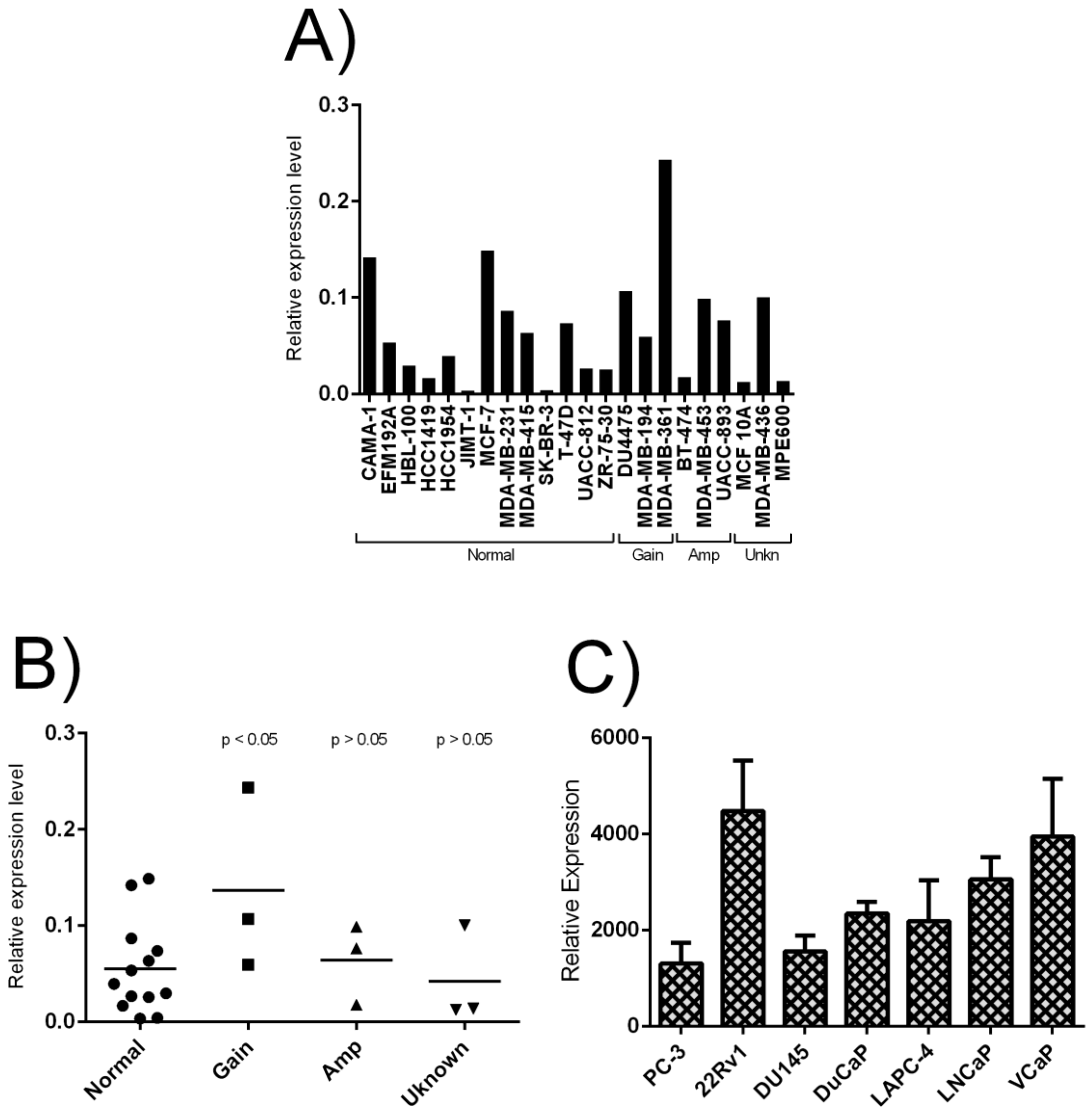


Figure 5. NFX1 relative mRNA expression in BrCa and PCa cell lines. A) NFX1 expression in BrCa cell lines: Normal copy number (Normal), low level 9p13.3 amplification (Gain), high level 9p13.3 amplification (Amp), and unknown 9p13.3 copy number status (Unkn). **B)** NFX1 mean expression levels between different copy number status groups of the cell lines shown in A. The bar in the scatter graph indicates the mean value of NFX1mRNA expression in each group. **C)** NFX1expression in low level 9p13.3 amplification PCa cell lines. Normal copy number status in PC3 control cell line. The data is expressed as the relative starting quantity (SQ) of the respective mRNAs normalized to the housekeeping gene TBP. The data is presented as individual samples in A and as the average of three individual samples ± the standard deviation in C.

KIF24 expression level was elevated in 9p13.3 amplicon harboring LuCaP 35 and low level amplification LuCaPs 35A1, 77, and 96 compared to normal copy number LuCaPs (Figure 6 A). KIF24 mean expression level in the amplification harboring group was higher compared

to non-amplified group in LuCaP xenografts ($p < 0.05$, Figure 6 B). Similar observation was detected in BrCa cell lines (data not shown). Furthermore, KIF24 was strongly over-expressed in low level amplification 22Rv1 prostate cancer cell line and amplicon harboring BT-474 breast cancer cell line (Figure 6 C). The relative mean expression value of KIF24 was 12.6 fold higher in 22Rv1 cell line and 6.3 fold higher in BT-474 cell line compared to PC3 cell line (Figure 6 C). KIF24 was selected for further siRNA mediated gene silencing experiments as a potential target gene of 9p13.3 amplicon.

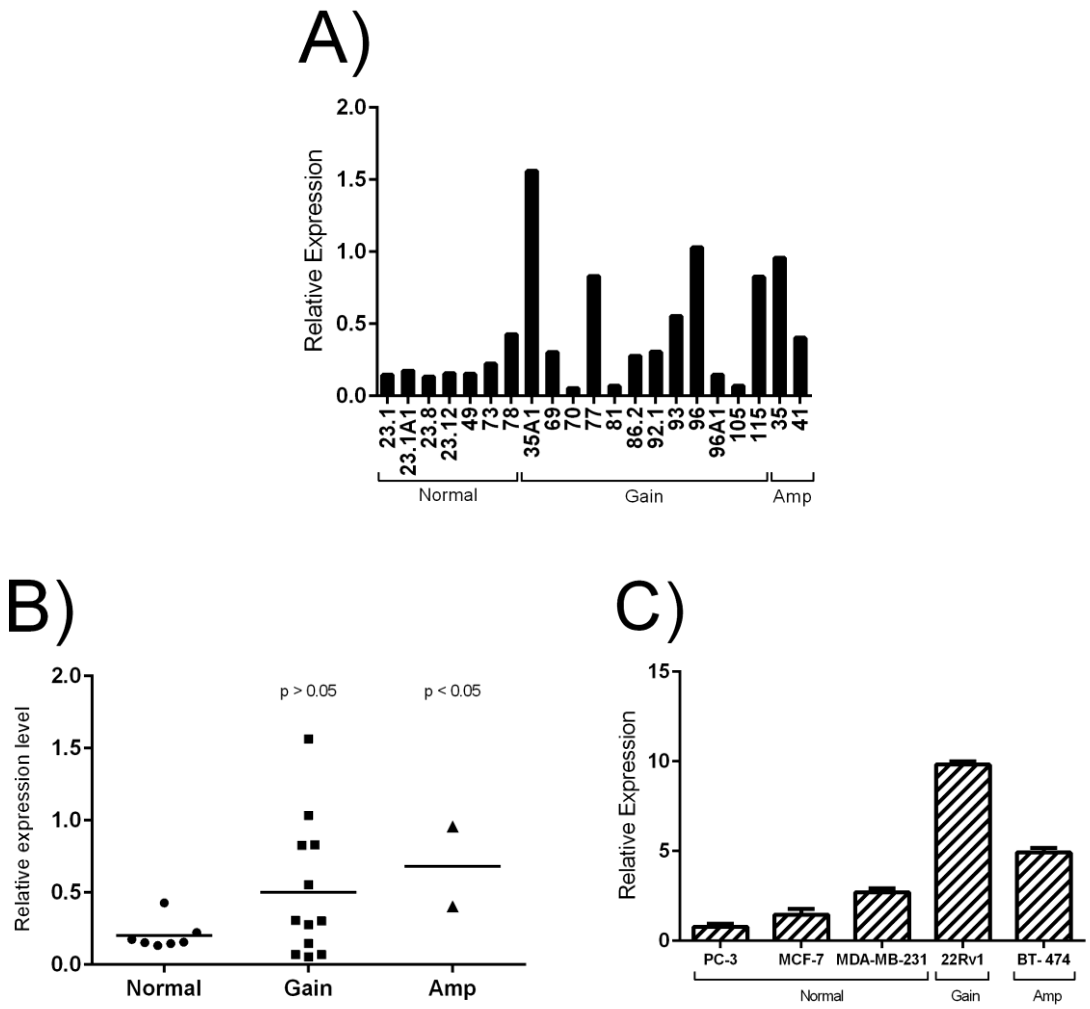


Figure 6. KIF24 relative target mRNA expression in LuCaP xenografts, PCa, and BrCa cell lines. **A)** KIF24expression in LuCaP cell lines: Normal copy number (Normal), low level 9p13.3 amplification (Gain) and high level 9p13.3 amplification (Amp). **B)** KIF24 mean expression levels between different copy number status groups of samples shown in A. The bar in the scatter graph indicates the mean value of expression in each group. **C)** KIF24 expression in PCa (PC3 & 22Rv1) and BrCa (BT-474, MCF-7, MDA-MB-231) cell lines. The data is expressed as the relative starting quantity (SQ) of the respective mRNAs normalized to the housekeeping gene TBP. The data is presented as individual samples in A and as the average of three individual samples ± the standard deviation in C.

5.2 Verification of siRNA mediated gene silencing by RT-qPCR

Successful silencing of potential 9p13.3 amplicon target genes in siRNA transfected prostate and breast cancer cell lines was verified by RT-qPCR (Figure 7). Both 22Rv1 and BT-474 cell lines were transfected with target gene-specific siRNAs (Table 3) and negative control siRNA with no sequence similarity to human gene sequences. After silencing of C9orf23, FANCG, KIF24, NOL6, and STOML2, cells expressed these mRNAs at lower levels compared with cells transfected with negative control siRNA. Reduction of KIF24 mRNA expression was relatively low in 22Rv1 and BT-474 cell lines (Figure 7 A & B). Therefore, successful gene silencing of KIF24 gene was confirmed using PC3 control cell line (Figure 7 C).

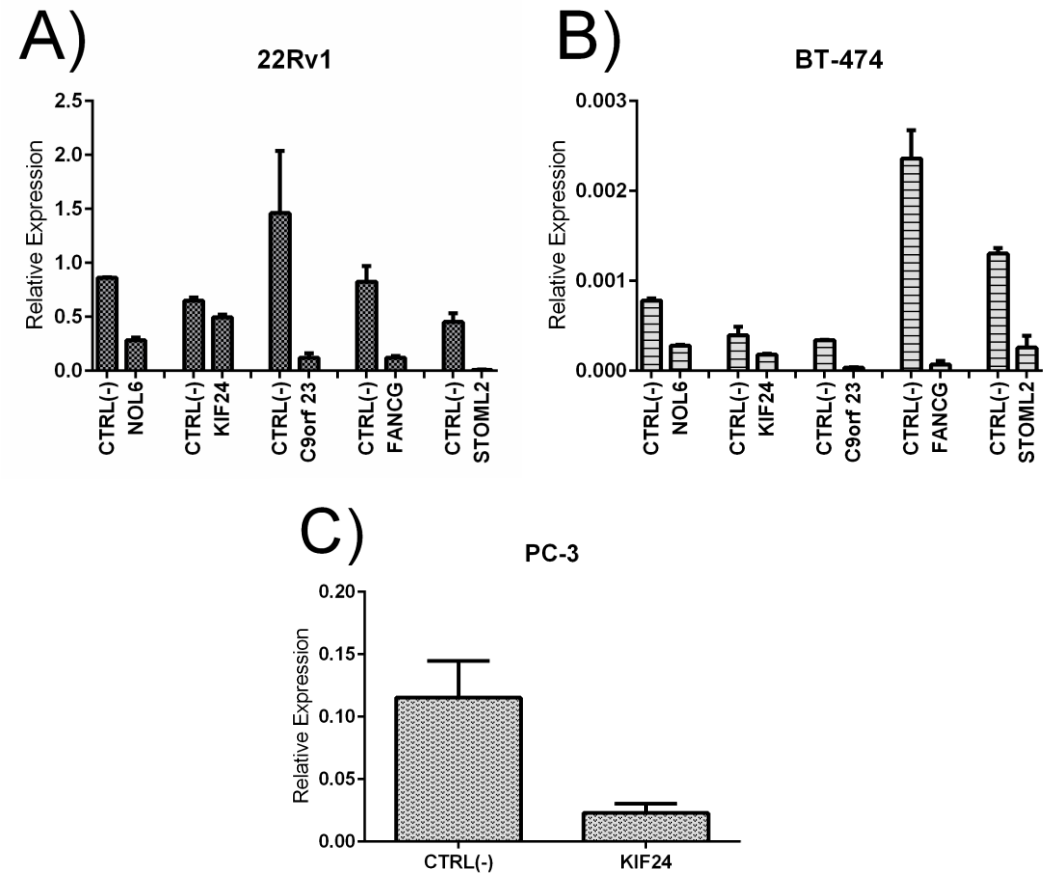


Figure 7. Relative mRNA expression levels of C9orf23, FANCG, KIF24, NOL6, and STOML2 genes 48h after 10nM target gene specific siRNA and negative control siRNA transfection of cancer cells. A) 22Rv1 **B)** BT-474 **C)** PC3. The data is expressed as the relative starting quantity (SQ) of the respective mRNAs normalized to housekeeping gene TBP. The data is presented as the average of three individual samples ± the standard deviation.

5.3 The effects of target gene silencing on cancer cell growth and proliferation

The effects of target gene specific silencing on cancer cell growth and proliferation were studied in PC3, 22Rv1, and BT-474 cell lines. Cancer cell lines were treated with siRNAs for C9orf23, FANCG, KIF24, NOL6, and STOML2 in parallel with negative control siRNA. The obtained relative growth curves are gathered in Figure 8. The p-values were defined at the 5^{th} day endpoint relative to control treatment. The growth endpoints at day 5 were also analyzed with AlamarBlue assay and are shown in Figure 9.

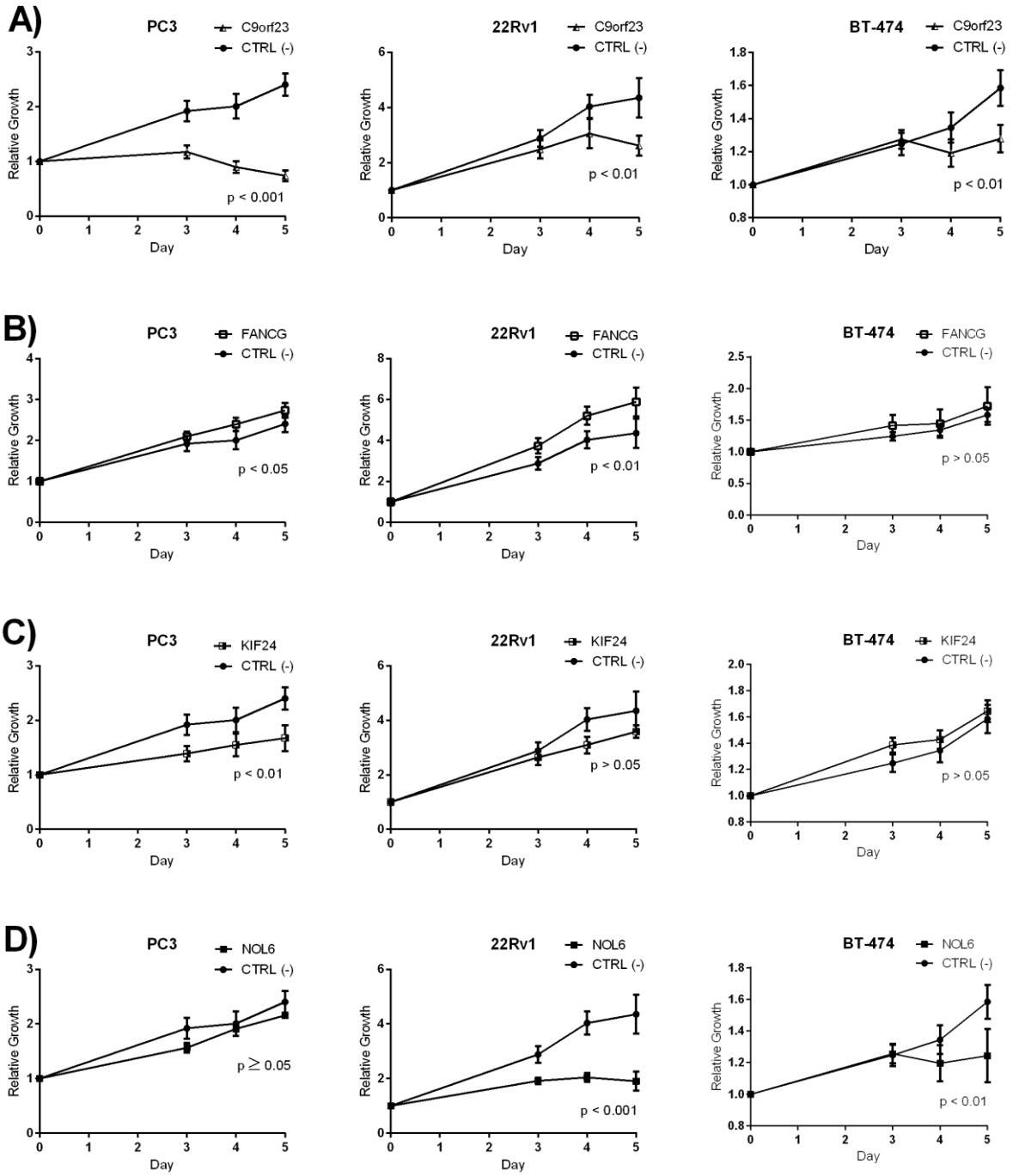
Silencing of C9orf23 had negative effect on growth in 22Rv1 cell line ($p < 0.01$) (Figure 8 A). In BT-474 cell line similar negative effect on growth was detected, but it was not statistically significant ($p > 0.05$). A strong negative effect on growth was detected also in PC3 control cell line ($p < 0.001$). By using AlamarBlue assay after five days of growth, C9orf23 silencing was shown to have statistically significant ($p < 0.05$) and negative effect on growth in each of the three cell lines (Figure 9).

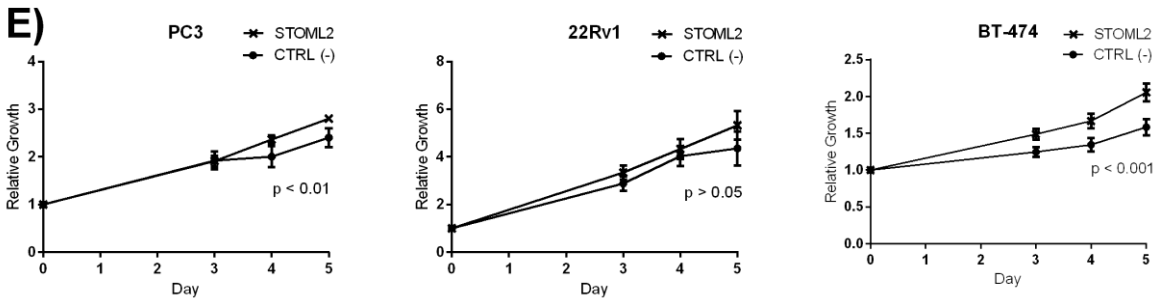
FANCG gene silencing led to increased proliferation and growth of 22Rv1cell line ($p < 0.01$) (Figure 8 B). In BT-474 cell line there was no statistically significant effect on growth. In PC3 control cell line FANCG silencing had positive effect on growth ($p < 0.05$) that was also confirmed in AlamarBlue assay ($p < 0.001$) (Figure 8 B, Figure 9 A). AlamarBlue assay did not show statistically significant difference in proliferation of 22Rv1 and BT-474 cell lines ($p > 0.05$) (Figure 9 B & C).

KIF24 gene silencing did not have statistically significant effect on cell growth and proliferation in 22Rv1 and BT-474 cell lines (Figure 8 C). In normal 9p13.3 copy-number PC3 cell line negative effect on growth was detected ($p < 0.01$). With AlamarBlue assay KIF24 was shown to have negative effect ($p < 0.01$) on growth in 22Rv1 cell line (Figure 9 B).

Silencing of NOL6 had negative effect on growth in 22Rv1 ($p < 0.01$) and BT-474 ($p < 0.05$) cell lines (Figure 8 D). These results were also confirmed with AlamarBlue assay (Figure 9 B & C). In PC3 cell line minor negative effect on growth was detected in growth curve assay ($p = 0.053$) (Figure 8 D). In AlamarBlue assay no statistically significant effect was observed in PC3 cell line.

Silencing of STOML2 had proliferative effect in PC3 and BT-474cancer cell lines (Figure 8 E). In 22Rv1 cell line similar positive effect on growth was also detected, but it was not statistically significant. In AlamarBlue assay the proliferative effect was detected in PC3 cell line ($p > 0.001$) but no statistically significant effect was observed in 22Rv1 or BT-474 cell lines (Figure 9).





Previous page, Figure 8. The relative growth of PC-3, 22Rv1, and BT-474 cell lines transfected with gene specific siRNAs. A) C9orf23 **B)** FANCG **C)** KIF24 **D)** NOL6 and **E)** STOML2. Cells were seeded on 24-plate wells and transiently transfected with 10nM gene specific siRNA in parallel with negative control siRNA.. Cell growth was analyzed at indicated timepoints. The p-values were defined at the 5th day endpoint relative to control treatment.

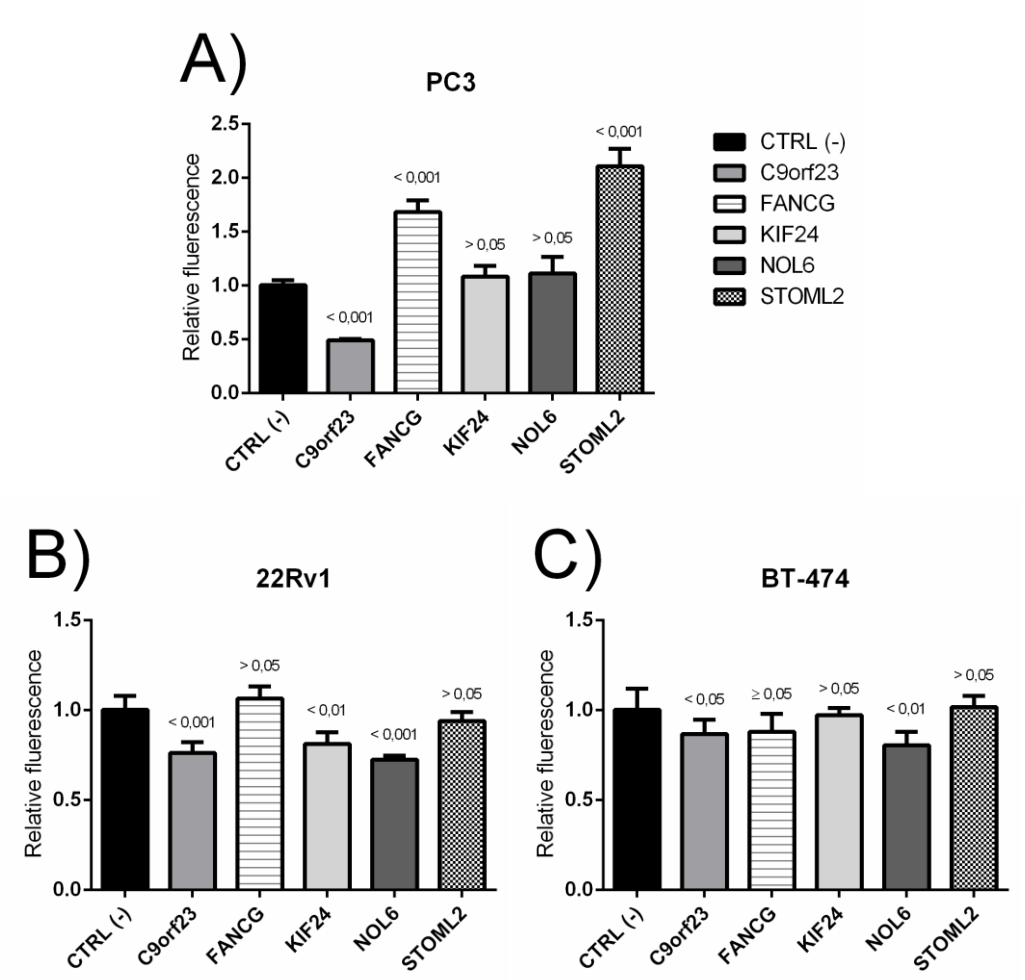


Figure 9. Relative growth of cancer cell lines after five days of growth quantified by AlamarBlue assay. A) PC3 **B)** 22Rv1 **C)** BT-474. Cells were seeded on 24-plate wells and transiently transfected with 10nM gene specific siRNA in parallel with negative control siRNA. The numbers on top of the bars represent p-values relative to control treatment.

5.4 The effects of target gene silencing on cell migration

The effects of target gene specific silencing on cell migration were studied in PC3 and 22Rv1 prostate cancer cell lines. Cells were treated with target gene specific siRNAs for C9orf23, FANCG, KIF24, NOL6, and STOML2 in parallel with negative control siRNA. The results are presented in Figure 10. Silencing of C9orf23 had positive effect on cell migration in 22Rv1 cell line ($p < 0.01$), but no statistically significant effect in PC3 cells was detected. Similarly, silencing of FANCG affected migration in a positive way in 22Rv1 cell line, while no such effect was detected in PC 3. KIF24 gene silencing seemed to have positive effect on migration in PC3 cell line, but it was not statistically significant ($p = 0.0599$). No statistically significant differences were detected in cancer cell migration after silencing NOL6 or STOML2.

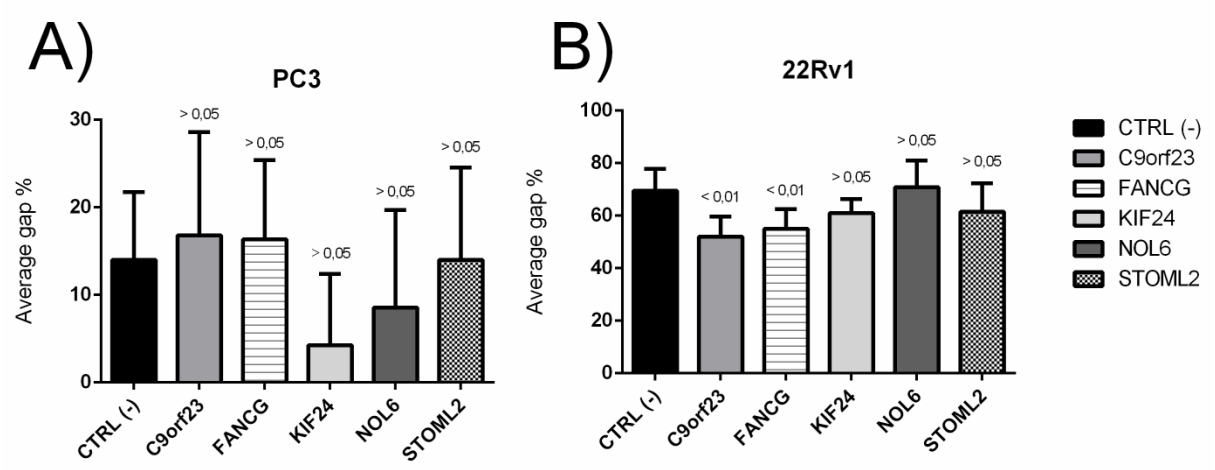


Figure 10. Relative cell migration of PC3 and 22Rv1 cell lines after 10nM target gene specific siRNA and negative control siRNA transfection of cancer cells. A) Average gap of PC3 cell line after 16 hours of making the scratch wound **B)** Average gap of 22Rv1 cell line after 24 hours of making the scratch wound. The numbers on top of the bars represent p-values relative to control treatment.

6 DISCUSSION

The 9p13.3 gain in prostate cancer was originally mapped by our research group and since then corresponding studies have been on a quest to outline the oncogenic target gene of this chromosomal region. Proto-oncogenes are normal cellular genes that encode a protein usually involved in regulation of essential cellular pathways, such as cell growth or differentiation (Lodish et al. 2000). A proto-oncogene can be altered into cancer-promoting oncogene by gene amplification that results in over-expression of the gene product. Therefore, amplified chromosomal regions are appealing targets of closer examination since they may carry novel oncogenes yet to be discovered.

There are multiple protein coding genes located at the 9p13.3 chromosomal region, but none of them have been shown to have tumorigenic function (Leinonen 2007). Some of these genes exhibit association between their copy numbers status and elevated gene expression levels, therefore remaining as promising oncogenic target genes of the amplicon. Despite broad analysis and functional studies, the target gene of the 9p13.3 amplicon has not yet been defined (Leinonen 2007, Taylor et al. 2010, Leppälä 2013). Identification of the target gene of 9p13.3 amplification might provide a new prognostic marker or a novel oncogene for prostate cancer. It may also help to understand the molecular mechanisms underlying prostate cancer progression and offer novel therapeutic targets.

Recurrent 9p13.3 amplification in prostate cancer has been reported to be associated with a higher PSA-level and poor progression-free survival in prostatectomy treated patients (Saramäki 2006, Leinonen 2007, Taylor et al. 2010). The amplification frequency seems to increase with disease progression to hormone-refractory stage, and high-level 9p13.3 amplification is associated with aggressive behavior of prostate cancer (Leinonen 2007). So far 9p13.3 gain has been detected from multiple prostatectomy samples and cell lines, exhibiting variability in the size of the amplified region (Saramäki et al. 2006, Leinonen 2007, Taylor et al. 2010).

In this study, we combined results from previous studies, data mining from public databases, and conducted further gene expression analyzes in order to outline the putative, yet unstudied

target genes of 9p.13.3 amplicon. The potential target genes selected for this study were C9orf23, FANCG, KIF24, NOL6, and STOML2. These genes were known to have elevated mRNA expression correlating with increased chromosomal copy number status (unpublished). We wanted to study the function of these genes and to clarify their role in prostate cancer. In the present study we focused on understanding the functional effects of these genes on cancer cell proliferation and migration in 9p13.3 amplicon harboring cell lines by the use of siRNA mediated target gene silencing. Based on the results obtained in this study we could outline putative target genes for further investigations.

These types of questions are extremely difficult to study using *in vivo* models or freshly isolated tumor cells. Established cell lines grown in defined conditions provide important and convenient model systems for these types of studies. In this study, cancer cell lines were used as a model system and sample material. Sample material was also extracted from prostate cancer xenografts. PCa cell lines and xenografts used in this study were epithelial origin and most of them were established from hormone-refractory and metastatic prostate cancer. This type of sample material is widely used in cancer studies since they are informative about the disease without being contaminated by normal cells. Xenografts represent the clinical prostate tumours more closely, but neither xenografts nor cancer cell lines represent the true clinical disease. This type of sample material also expresses some heterogeneity. For instance, cell lines may contain artifacts from cell culturing. Therefore considerable caution should be exercised when comparing this type of sample material with each other and with clinical material.

6.1 Studying the effects of gene silencing with siRNA transfection

No difficulties were detected in the transfection method or siRNA delivery, and the successful target gene silencing was confirmed with RT-qPCR in this study. Significant reduction in gene expression was detected 48 hours after siRNA transfection in every target gene. It would have been useful to obtain positive control siRNAs to visualize the siRNA delivery to target cells after transfection. Positive control siRNA specific for TBP or similar housekeepping gene could have been used to detect possible off-target effects of the transfection method in parallel with non-targeting negative control siRNA.

In this master´s thesis project limited time and resources did not allow the visualization of gene silencing as possible knock down at the protein level. On the other hand, there was no commercial antibodies specific for all of our target gene coded protein products that would have been suitable for western blot assays.

The cell migration assay performed in this study did not show clear evidence about the effects of target gene silencing on cancer cell migration. With BT-474 cell line, the wound healing assay was not successful due to the low level of cell proliferation and migration characteristic for this cell line. For the future studies the use of commercial migration and invasion assays could be used.

6.2 The results obtained from functional studies

Possible NFX1 and KIF24 over-expression was studied with RT-qPCR assays in order to assess their role as a putative target gene of 9p13.3 amplicon. We studied their expression in PCa and BrCa cell lines as well as in LuCaP xenografts. We used multiple samples with different 9p13.3 copy number status. Statistical difference between different copy number status groups and gene expression of NFX1 and KIF24 were also studied. The groups were divided based on normal, low-level amplification (gain), amplification, and unknown chromosomal copy number status of 9p13.3 region.

QRT-PCR is a standard method nowadays for studying gene expression. We wanted to ensure the sensitivity of the method and primer specificity by using gradient PCR for primer annealing temperature optimization. The primers were carefully pre-designed using software algorithm and data from previous studies. In this study it was relevant to obtain information about the relative expression levels of the genes of interest between each sample. Since the samples had different levels of 9p13.3 amplification there was variation in the absolute gene expression profiles of target genes between samples. In this study the relative gene expression of target genes between amplicon harboring samples and negative control sample was the most relevant.

Because of the limited sample cDNA available, were run BrCa and LuCaP samples in single reactions instead of triplicates. This may have caused an error in our gene expression assay since no replicates were used. On the other hand, the sample size was quit extensive: 7 pros-

tate cancer cell lines (PCa), 22 breast cancer cell lines (BrCa), and 21 prostate cancer xenografts (LuCaP). Also, detecting elevated expression of putative target gene in amplicon harboring cell lines was sufficient enough for the nature of this study. If there were no elevated expression in amplified cell line, or a lot of variation we would simply not consider the gene as putative target gene and outline it from the gene silencing experiment.

In this study, KIF24 exhibited elevated gene expression correlating with increased copy number of 9p13.3 and was therefore selected for further studies. The other genes outlined for our study were C9orf23, FANCG, NOL6, and STOML2 based on data mining and previous studies by the MBPCG group. These genes and their contribution in this study are reviewed in the following paragraphs.

Kinesin family member 24(KIF24) is a microtubule-dependent motor protein that mediates depolymerization of microtubules of centriolar origin (Venturelli et al. 2010). It belongs to kinesin protein family that takes part in intracellular vesicle transport and cell division. In this study, KIF24 demonstrated correlation between elevated mRNA level and 9p13.3 amplification and was therefore selected for further siRNA mediated gene silencing experiment as a potential target gene of 9p13.3 amplicon. In this experiment, KIF24 gene silencing did not seem to have effect on cancer cell growth and proliferation in cell lines used in this experiment. Allthough, in AlamarBlue assay a negative effect on 22Rv1 cell line growth was detected.

At the moment, little is known about nucleolar protein 6 (NOL6) gene encoding a nucleolar RNA-associated protein (Pruitt et al. 2005). Evidence in this study suggests antiproliferative effect for NOL6 gene silencing in 9p13.3 amplicon harboring 22Rv1 and BT-474 cancer cell lines. Therefore, NOL6 remains as a potential oncogene and putative target of the 9p13.3 amplicon. Further investigation should be carried out to conclude target gene function and its contribution to prostate cancer.

Chromosome 9 open reading frame 23 (C9orf23) gene is also known by the name of ribonuclease P/MRP 25kDa subunit-like (RPP25L) gene that encodes a protein that belongs to a family of evolutionarily related archaebacterial proteins with no known function (Pruitt et al. 2005). Results in this study suggest antiproliferative function for C9orf23 gene silencing in

PC3, 22Rv1, and BT-474 cancer cell lines. Based on these observations the possibility of C9orf23 as a potential target gene of 9p13.3 amplicon can´t be fully rejected, but further studies have to be made in order to conclude C9orf23 function in prostate cancer.

Fanconi anemia pathway is one of the central nodes in the network of genome stability and tumor suppressor pathways (Bogliolo et al. 2002). Fanconi anemia, complementation group G (FANCG), is one of the players in this pathway, and it has been previously denoted to have possible tumor suppressor function. This is supported by the results of this study where silencing of FANCG gene had proliferative effect on the growth of 22Rv1 and BT-474 cell lines.

Stomatin-like protein 2 (STOML2) has been reported to be up-regulated in several human cancers and its function as an independent, prognostic factor in gastric adenocarsinoma has been supported by recent studies (Li et al. 2014). Silencing of STOML2 gene had strong proliferative effect in each cancer cell line used in this experiment, suggesting possible tumor suppressor function for this gene.

Based on evidence obtained in this study the possible protein level knock down of at least C9orf23, NOL6, FANCG, and STOML2 should be confirmed in the future studies. In further investigations, it should be concluded whether the effect of C9orf23 and NOL6 gene silencing is truly anti-proliferative, or perhaps apoptotic, for cancer cells. It should also be characterized, which phase of cell cycle is possibly affected. In addition, the possible tumor suppressor function of FANCG and STOML2 should be also confirmed in further investigations. After all of these characterizations and functional studies, the final candidate target gene overexpression should be performed in prostate cancer cell line in order to investigate true oncogene function.

7 CONCLUSIONS

The aim of this study was to investigate putative 9p13.3 target genes C9orf23, FANCG, KIF24, NOL6, and STOML2 in order to explore their role in prostate cancer by the extent of siRNA mediated gene silencing. These putative target genes of the 9p13.3 chromosomal gain were identified by previous studies, data mining and further gene expression assays of this study. These genes have been shown to have elevated gene expression correlating with the increased copy number status of 9p13.3 amplicon. In this study, target gene silencing was successfully obtained with transient siRNA transfection and used to studying the functional effects in PC3, 22Rv1, and BT-474 cancer cell lines.

We propose C9orf23 and NOL6 as putative target genes of 9p13.3 amplicon. In this study, these genes exhibited cancer-relevant functions in cancer cell proliferation and migration. However, these need to be confirmed in additional studies. FANCG and STOML2 genes remain as possible tumor suppressors, and their role in cancer needs further clarification. Further investigations of the functions of putative target genes suggested by this study should be considered. Confirmation of the amplicon target gene may provide new prognostic or diagnostic marker for prostate cancer in the future.

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