The 9p13.3 amplicon in prostate cancer

Master's thesis

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Tiivistelmä:

Tutkimuksen tausta ja tavoitteet: Eturauhassyöpä on miesten yleisin syöpä Suomessa. Eturauhassyövän molekulaariset mekanismit ovat vielä epäselviä ja vain muutama todellinen kohdegeeni on löydetty. Tämän tutkimuksen tarkoituksena oli tunnistaa geeni(t), jonka monistunut kopioluku nostaa sen ilmentymistasoa, sekä määrittää 9p13.3 monistuman yleisyys ja kliinis-patologisten muuttujien assosiaatiot kliinisissä eturauhassyöpänäytteissä.

Tutkimusmenetelmät: Tutkimuksessa käytettiin 7 eturauhassyöpäsolulinjaa ja 19 eturauhassyöpävierassiirrännäistä. Suhteelliset lähetti-RNA -tasot määritettiin kvantitatiivisella käänteiskopiointipolymeraasiketjureaktiolla ja kopiolukumuutokset fluoresenssi *in situ* hybridisaatio (FISH) -tekniikalla. Lisäksi 143 prostatektomianäytettä ja 70 hormoniresistenttiä kasvainta analysoitiin FISH-menetelmällä.

Tutkimustulokset: Vierassiirrännäisen LuCaP35 avulla 9p13.3 monistuma rajattiin BAC-kloonien RP11-156G14 ja RP11-115O15 väliin. *PIGO* geenillä esiintyi merkittävää ja *UNC13B* geenillä melkein merkittävää assosiaatiota kopioluvun ja ilmentymistason välillä. Osa geeneistä osoitti kuvaajissa hyvää korrelaatiota, heikoista *p*-arvoista huolimatta. Prostatektomianäytteistä 10 % sisälsi korkean ja 32 % matalan kopioluvun monistuman, kun taas hormonirefraktorisista näytteistä 14 % sisälsi korkean ja 44 % matalan kopioluvun monistuman. Prostatektomia leikatuilla potilailla 9p13.3 monistumalla oli merkittävää assosiaatiota korkeampiin PSA tasoihin. Lisäksi, eloonjäämisanalyysi osoitti assosiaatiota 9p13.3 monistuman ja prostatektomia leikattujen potilaiden huonon progressiovapaan eloonjäämisen välillä.

Johtopäätöset: Lupaavimmat kohdegeeniehdokkaat 9p13.3-monistumalle ovat *PIGO* ja *UNC13B*, mutta myöskään *CD72*, *VCP*, *GALT*, *DCTN3*, *C9orf25*, *UBAP2*, *RUSC2* ja *UBE2R2* ei voida pois sulkea. Kliinisissä eturauhassyöpänäytteissä 9p13.3-monistuma oli hieman yleisempi hormonirefraktorisissa kuin hoitamattomissa eturauhassyövissä. Molemmissa havaittiin korkean kopioluvun monistumia, osoittaen että 9p13.3 monistuma on suhteellisen aikainen tapahtuma taudin etenemisessä, mutta saattaa liittyä myös aggressiivisiin eturauhassyöpä tapauksiin.

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Abstract

Background and Aims: Prostate cancer is the most common cancer in Finnish men. The molecular mechanisms of prostate cancer are poorly understood, and only a few real target genes have been identified. The aim of this research was to identify gene(s) whose amplified copy number increases its expression level, and to resolve the 9p13.3 amplification frequency and the associations of clinicopathological variables in clinical prostate cancer specimens.

Methods: Relative mRNA expression levels were measured by quantitative reverse transcriptase polymerase chain reaction (q-RT-PCR) and the copy number alterations by fluorescence *in situ* hybridisation from 7 prostate cancer cell lines and 19 prostate cancer xenografts. In addition 134 prostatectomy samples and 70 hormone-refractory (HR) tumours were analysed by FISH.

Results: In xenograft LuCaP35, the 9p13.3 amplification was found to be defined by BAC-clones RP11-156G14 and RP11-115O15. *PIGO* showed significant association between expression level and copy number. Gene *UNC13B* was classified as not quite significant. In addition, some other genes showed non-significant tendency between the gene copy number and expression. Of the prostatectomy specimens, 10% contained high-level amplification, and 32% low-level amplification. Of the HR tumours, 14% contained high-level amplification, and 44% low-level amplification. Amplification was associated with higher PSA-levels in prostatectomy-treated patients. In addition, survival analysis revealed a trend of association between the 9p13.3 amplification and poor progression-free survival of prostatectomy-treated patients.

Conclusion: The most promising candidate target genes for gain at 9p13.3 are *PIGO* and *UNC13B*, but also *CD72*, *VCP*, *GALT*, *DCTN3*, *C9orf25*, *UBAP2*, *RUSC2* and *UBE2R2* cannot be excluded. In clinical prostate samples, the 9p13.3 amplification was slightly more common in HR tumours than in untreated prostatectomy samples. In both prostatectomy and HR tumours high-level amplifications were observed suggesting that this genetic aberration is relatively early event but maybe associated with aggressive behaviour of the disease.

CONTENTS

ABBREVIATIONS

aCGH	array CGH
ANXA7	annexin 7
AR	androgen receptor
BAC	bacterial artificial chromosome
BCL2	B-cell CLL/lymphoma 2
BSA	bovine serum albumin
C9orf25	chromosome 9 open reading frame 25
CD44	CD44 molecule (Indian blood group)
CD72	CD72 molecule
CD82	CD82 molecule
CDH1	E-cadherin
cDNA	complementary DNA
cCGH	chromosomal CGH
CGH	comparative genomic hybridization
DAPI	4.6-diamidine 2-phenylindole
DAG	diacylglycerol
DCC	deleted in colorectal carcinoma
DCTN3	dynactin 3 (p22)
DHT	dihydrotestosterone
DNA	deoxyribonucleic acid
EGF	epidermal growth factor
EIF3S3	eukaryotic translation initiation factor 3, subunit H
ERBB2	v-erb-b2 erythroblastic leukemia viral oncogene homolog 2, neuro/glioblastoma derived oncogene homolog
ERG	v-ets erythroblastosis virus E26 oncogene homolog
ETV1	ets variant gene 1
ETV4	ets variant gene 4 (E1A enhancer binding protein, E1AF)
FISH	fluorescence in situ hybridisation
FITC	fluorescein-12-dUTP
GALT	galactose-1-phosphate uridylyltransferase
GPI	glycosylphosphatidylinositol
GSTP1	glutathione S-transferase π
GTP	guanosine-5’-triphosphate
HG	high-grade
ISH	in situ hybridisation
LOH	loss of heterozygosity
mRNA	messenger-RNA
MYC	v-myc myelocytomatosis viral oncogene homolog
NKX3.1	NK3 homeobox 1
PCR	polymerase chain reaction
PIA	proliferative inflammatory atrophy

PIGO	phosphatidylinositol glycan anchor biosynthesis, class O
PIN	prostatic intraepithelial neoplasia
PIP3	phosphatidylinositol (3, 4, 5)-trisphosphate
PKB	protein kinase B
PTEN	phosphatase and tensin homolog
RB1	retinoblastoma 1
RNA	ribonucleic acid
RT	room temperature
RUSC2	RUN and SH3 domain containing 2
TBP	TATA-box binding protein
TMA	tissue microarray
TMPRSS2	transmembrane protease, serine 2
TP53	tumour protein p53
TR	texas red-5-dUTP
UBAP1	ubiquitin associated protein 1
UBAP2	ubiquitin associated protein 2
UBE2R2	ubiquitin-conjugating enzyme E2R 2
UGS	urogenital sinus
UNC13B	unc-13 homolog B
VCP	valosin-containing protein
ZFHX3	zinc finger homeobox 3

1. INTRODUCTION

Prostate cancer is the most common cancer in men, both in Finland and other Western countries. It is an extremely heterogeneous cancer. Both slow and rapidly growing tumours occur, and the nature of the disease can vary from asymptomatic to aggressive and metastatic cases. Losses of genetic material are more common than gains or amplifications, and many of the chromosomal losses happen in the early-stages of the disease, while gains and amplifications, together with losses, are regarded mostly as late-stage events. The molecular mechanisms of prostate cancer are poorly understood (Dong, 2006).

Approximately 40% of men over the age of 50 have slow-growing and well-differentiated prostate cancer and the frequency increases with age. However, most of the cases are indolent and do no harm to the individuals affected, only 11% become clinically obvious, and 3% kill the patients (Dong, 2006). Histology and Gleason scoring are effective methods for the diagnosis of prostate cancer and prediction of outcomes. Problems are associated especially with patients with the intermediate grade of cancer (Gleason 6 or 7), because it is impossible with certainty to distinguish clinically indolent localised tumours from life-threatening disease with high metastatic potential (Saric *et al*., 1999; Dong, 2006).

The first treatments done with localised and organ-confined prostate cancer (existing only in the prostatic capsule) are radiotherapy or radical prostatectomy, aiming to remove or destroy the cancerous cells. These therapies do not cure all, and in about 20% of patients the cancer recurs, which is caused by micrometastatic dissemination. The only treatment for patients with metastatic disease is hormonal or androgen-ablative therapy (Latil & Lidereau, 1998). Prostate tumours are initially androgen dependent, but recurrent androgen-independent prostate cancers (AIPC) eventually

develop, when the androgen ablation therapy fails (Feldman & Feldman, 2001). For hormone-refractory prostate cancers, there is no effective treatment (Porkka, 2003).

Saramäki *et al*. (2006) performed array comparative genomic hybridisation (aCGH) to detect copy number alterations in 5 prostate cancer cell lines and 13 prostate cancer xenografts. They confirmed the previously identified common gains and losses, managed to narrow-down these minimal commonly altered regions and identified a new frequent gain at 9p13-q21. From this novel amplicon they identified two independent regions, 9p13.3 (39%) and 9p13.1-q21.11 (33%). The gain at 9p13-q21 has also been reported in other studies (Nupponen *et al*., 1998; Paris *et al*., 2003).

In this research the 9p13.3 amplification was examined by quantitative RT-PCR and/or FISH in 7 prostate cancer cell lines, in 19 prostate cancer xenografts and in 204 clinical prostate cancer specimens. The aim was to identify gene(s) whose amplified copy number increases its expression level, and to resolve the frequency at which this amplification occurs in clinical prostate cancers. Some of the identified genes could be potential target genes for this amplification, and might function as a prognostic marker for prostate cancer. Corresponding studies of this chromosomal region have not been published.

2. REVIEW OF THE LITERATURE

2.1. Prostate gland

Male sex accessory tissues are the prostate, seminal vesicles and bulbourethral glands, which all require testes for their development, growth, and secretory functions (Partin & Coffey, 1998, Campbell's urology). In the fetus, the caudal terminus of the hindgut, called the cloaca, is divided by the urorectal septum into the endodermal urogenital sinus (UGS) ventrally, and the rectum and anal canal dorsally. In males the UGS develops into the prostate, prostatic urethra and bulbourethral glands, and in females it gives rise to the lower vagina and urethra. In both sexes it also develops into the bladder (Cunha *et al*., 2004). The growth of benign and malignant tumours in accessory tissues is mostly limited to the prostate gland, the reason for this is unknown (Partin & Coffey, 1998, Campbell's urology).

The main function of the prostate is to store and secrete seminal fluid, which transports sperm. To function properly, the prostate needs male hormones. Prostate surrounds the upper part of the urethra, in front of the rectum, just below the urinary bladder and it is nearly the same size and shape as a walnut (Young & Heath, 2000).

The prostate develops during the third fetal month under the influence of e.g. DHT (dihydrotestosterone), produced from the fetal testosterone (Partin & Coffey, 1998, Campbell's urology). Because of the fetal testicular androgens, epithelial buds arise from the wall of the UGS and invade the surrounding mesenchyme. The androgens' effects are mediated through androgen receptors (AR) in context of mesenchymal-epithelial interactions. First the prostatic buds and ducts are solid, but in the neonatal period they canalise, when also luminal and basal epithelial cells differentiate. At the same time the mesenchyme differentiates into smooth muscle cells and fibroblasts

(Cunha *et al*., 2004). The prostate forms acini and collecting ducts in a treelike manner, which branches from the urethra (Partin & Coffey, 1998, Campbell's urology). During prostate development there is a close interaction between the stromal and epithelial tissue components. In the end of prostatic organogenesis the mature gland is composed of highly differentiated secretory epithelial cells and contractile smooth muscle cells. In the adult prostate, the homeostatic epithelial-stromal interactions are needed to maintain the highly differentiated growth-quiescent functional state of both epithelium and stroma (Cunha *et al*., 2004).

The prostate gland can be divided into four zones: peripheral zone, central zone, anterior fibro-muscular zone, and preprostatic region (including the periurethal ducts and transition zone, which surrounds the proximal prostatic urethra), (Young & Heath, 2000). The fibroelastic capsule, which is rich in smooth muscle, encloses the posterior and lateral surfaces of the prostate gland, while the apical and anterior parts are surrounded by the anterior fibro-muscular stroma (Young & Heath, 2000; Junqueira & Carneiro, 2003). The anterior fibro-muscular stroma consists of connective tissue and both smooth and skeletal muscle, and lacks entirely the glandular elements. The peripheral zone is the largest part of the prostate gland (approximately 70% of the gland). It consists of secretory tissues, which branch out from either side of the distal urethra. This is the region where most of the carcinomas arise. The central zone surrounds the ejaculatory ducts and it consists of approximately 20% of the glands volume. The preprostatic tissue is the smallest of the four zones and also the most complex one. It consists of both glandular and non-glandular regions. The transition zone is a small group of ducts, which comprise about 5% of the mass of the normal glandular prostate (Partin & Coffey, 1998, Campbell's urology).

The prostatic epithelial cells are composed of three major types: secretory epithelial cells, basal cells, and neuroendocrine cells. The most common tall columnar secretory epithelial cells are terminally differentiated and are distinguished by their morphology. They are connected to each other by cell adhesion molecules and to a basement membrane through integrin receptors. These cells produce the secretions into the acini that drain into the ducts that connect to the urethra. The basal cells are much smaller in

size, and comprise less than 10% of secretory cells. They always rest on the basement membrane. These basal cells are less differentiated than secretory cells, and nowadays it is believed that these undifferentiated basal cells function as stem cells for secretory cells. There are three types of endocrine cells in prostate gland. One has both serotonin and thyroid-stimulating hormone, and two others have calcitonin and somatostatin. These cells reside among the secretory cells and are found in the epithelium of the acini, in ducts of all parts of the gland, and in the urothelium of the prostatic urethral mucosa (Partin & Coffey, 1998, Campbell's urology).

2.2. Prostate cancer

Prostate cancer is the most common cancer in men, both in Finland and other Western countries. In 2005, over 5300 new cases were diagnosed in Finland (Finnish Cancer Registry, 20.3.2007), and the number is growing. The four well-known risk factors for getting this disease are family history, age, race, and country of origin. Many alterations in various genes have been found to be involved in the development of prostate cancer, but only a few real target genes have been identified. Chromosomal aberrations (deletions, gains, amplifications, and translocations) are thought to be one mechanism for the development and progression of a cancer (Saramäki, 2006). Environmental and genetic factors are known to be involved in the development and progression of prostate cancer.

Prostate cancer is an extremely heterogeneous cancer, its growth patterns can vary from slow growing tumours to very rapidly growing metastatic lesions. The multifactorial occurrence of this disease is demonstrated by the fact that both hereditary and sporadic cases occur in families, complicating, for example, linkage analyses (Nwosu *et al.*, 2001). Approximately 40% of men over age 50 have slow-growing and well-differentiated prostate cancer, and the number increases with age. The problem with patients with prostate cancer is the uncertainty about the aggressiveness of the disease.

Histology and Gleason scoring (Gleason score tells the microscopic appearance of prostate cancer) are used for the diagnosis of prostate cancer and prediction of outcomes, but the problem appears especially with patients with the intermediate grade of cancer (Gleason 6 or 7), which can be either aggressive or indolent (Dong, 2006). It is impossible to distinguish clinically indolent localised tumours from life-threatening disease with high metastatic potential (Saric *et al*., 1999).

In prostate cancer the essential mutations for hereditary cancer are mainly at the sequence level (e.g. point mutations, microdeletions, and –insertions), while for sporadic cancer the relevant alterations lie in both sequence and copy number level (Dong, 2006). Tumorigenesis is thought to be a multi-step process where point mutations and other alterations accumulate in the genome, and the cells with the most aggressive phenotype become dominant through clonal selection and expansion. It is believed that these genetic changes give the cancer cells a growth advantage over the normal cells, eventually leading to a metastatic phenotype.

The most common region in prostate gland where the adenocarcinomas develop is the peripheral zone. First, small groups of cancer cells remain restricted within normal prostate glands, a state called prostatic intraepithelial neoplasia (PIN). In normal prostate, the proliferation happens in the basal cell compartment, whereas in PIN lesions it mainly occurs on the luminal surface, but also in other sites. The incidence and extent of low- and high-grade PIN seems to increase with the patient age. PINs mostly develop in the peripheral zone of prostate, and they have many of the phenotypical, biochemical, and genetical changes of cancer, but no invasion of the basement membranes of the acini. Based on that, it is believed that they represent the premalignant stage of prostate carcinoma (Bostwick & Qian, 2004). However, it is not the only precursor of prostate cancer, because many early prostate carcinomas do not contain PIN lesions. It has been also suggested that precursor for PIN could be a proliferative inflammatory atrophy (PIA), which shares similar chromosomal abnormalities as PIN and prostate cancer (Marzo *et al*., 1999; Palapattu *et al*., 2004; Saric *et al*., 1999).

Localised, or organ-confined, prostate cancer is curable by either radiotherapy or radical prostatectomy (Porkka, 2003; Feldman & Feldman, 2001). These are usually the first treatments done, aiming to remove or destroy the cancerous cells still existing in the prostate capsule (Feldman & Feldman, 2001). These therapies do not cure all, and in about 20 % of patients the cancer recurs, caused by the micrometastatic dissemination. The predominant treatment for patients with metastatic disease is hormonal or androgen-ablative therapy (Latil & Lidereau, 1998). Prostate tumours are initially androgen dependent, and androgen ablation causes regression of these tumours (Feldman & Feldman, 2001). 70-80% of patients respond to the hormone therapy at first, but most of the tumours progress to androgen-independent growth. Only 10-20% of patients with hormone-refractory (HR) cancer are living 5 years after emergence of HR-cells (Latil & Lidereau, 1998). For hormone-refractory prostate cancers, there is no effective treatment (Porkka, 2003).

2.2.1. Chromosomal alterations in prostate cancer

In prostate cancers, gene copy number alterations are much more common than changes in the sequences (Dong, 2006). To identify common tumour suppressor genes and oncogenes, it is important to find genomic regions that are often deleted or gained in neoplastic cells. In primary tumours, losses of chromosomal material are found about five times more commonly than gains of DNA sequences. In hormone-refractory tumours both deletions and gains are observed (Visakorpi, 2003). These aberrant chromosomal occurrences implicate that recessive tumour suppressor genes are important in prostate cancer initiation, and the activation of oncogenes is involved, with the inactivated tumour suppressor genes, in late-stage prostate cancer (Porkka, 2003).

Losses in chromosomes 5q, 6q, 8p, 13q, 16q, and 18q, and gains in chromosomes 7p/q, 8q, and 16p, are the most frequent alterations in early stage clinical prostate cancer. These are found in 10-50% of untreated primary prostate cancers and to some extent in

pre-malignant lesions (for example high grade (HG)-PIN). Like other cancers, prostate cancer also acquires new genetic alterations when progressing to more malignant phenotype. In addition to the earlier alterations, losses in 10q, 15q, 17q, 19p/q and 22q, and gains in 1q, 3q and Xq are frequent in locally recurrent hormone-refractory prostate cancers (Saramäki, 2006).

Sun *et al*. (2007) did a combined analysis of published CGH studies (41 papers), where a total of 872 independent prostate tumours, including 51 cell lines and xenografts were analysed. They found eight deleted and five gained regions, which were observed in more than 10% of the prostate tumours. The most common deletion was 8p, which occurs in about a third of all tumours and about half of advanced tumours. The most commonly gained region was 8q, which occurs in about a quarter of all tumours and about half of all advanced tumours. According to the combined study, the most frequently deleted regions detected were: 8p23.1-8p21.2 (34%), 13q14.13-13p22.1 (28%), 6q14.1-6q21 (22%), 16q13-16q24.3 (18%), 18q12.2-18q23 (13%), 5q13.3-5q21.3 (13%), 2q21.2-2q22.3 (12%), and 10q23.1-10q25.3 (12%). The most commonly gained regions observed were: 8q21.3-8q24.3 (22%), 7q11.21-7q32.3 (12%), Xq11.1-Xq23 (11%), 17q24.1-17q25.3 (12%), and 3q23-3q26.33 (10%). They also noticed that the frequencies of these commonly altered regions were two- to threefold higher in advanced tumours than in early-stage tumours. In addition to these commonly altered regions they noticed chromosomal aberrations present only in advanced tumours. Six additional regions were deleted and seven additional regions were gained in more than 10% of cases. The deletions were: 15q15.1-15q23 (22%), 4q13.1-4qter (22%), 22q11.1-22qter (15%), 1pter-1p21.1 (15%), 9q21.13-9qter (14%), and 12p (12%). The gains were: 11p15.3-11p13 (14%), 1q21.3-1q42.3 (16%), 2p22.3-2p12 (12%), 9q22.11-9q33.3 (12%), 3p21.33-3p14.1 (11%), 4q21.1-4q31.3 (11%), and 6p21.33-6p11.1 (13%).

It is not found a single chromosomal alteration in prostate cancer, which would account for all or even most of the disease incidents. Many of the chromosomal alterations consist of several distinct regions (deletions or gains) indicating that there might be multiple target genes in these chromosomes (Isaacs & Kainu, 2001).

2.2.1.1. Losses of genetic material

The most commonly deleted regions in prostate cancer are 5q (13%), 6q (22%), 8p (34%), 10q (12%), 13q (28%), 16q (18%), and 18q (13%), (percents in early-stage prostate tumours, Sun *et al*., 2007). Other frequently occurring losses include regions 2q, 15q, 4q, 22q, 1p, 9q and 12p.

One of the most common chromosomal losses in prostate cancer is the 8p region, where at least two minimally deleted regions have been identified, 8p21 and 8p22 (Porkka, 2003). Loss of heterozygosity (LOH) at 8p21 appears to be an early event in tumorigenesis, and it is already seen in HG-PIN lesions (Savinainen, 2006; Porkka & Visakorpi, 2004). This loss has been detected approximately in 40% of prostate carcinoma cases and in up to 80% of metastatic and hormone-refractory tumour samples (Porkka, 2003). Candidate target genes for this loss are *NKX3.1* (NK3 homeobox 1, see section 2.2.2.1) at 8p21, and genes *TUSC3* (tumour suppressor candidate 3), *LZTS1* (leucine zipper, putative tumour suppressor 1), *PDGFRL* (platelet-derived growth factor receptor-like) and *MSR1* (macrophage scavenger receptor 1), all four located at 8p22 (Porkka, 2003; Savinainen, 2006).

The second most frequently deleted chromosomal region is 13q. It is lost in 32% of primary and in 56% of recurrent prostate cancers (Visakorpi *et al*., 1995). At least three independent deleted regions, 13q14, 13q21-22, and 13q33, have been detected (Porkka, 2003). The loss of 13q is observed in PIN lesions, but it is also associated with the clinical aggressiveness of the disease (Porkka, 2003; Savinainen, 2006). The most promising target gene for this loss has been the retinoblastoma gene (*RB1*) at 13q14, but mutations of *RB1* are infrequent in prostate cancers. (The *RB1* encodes a nuclear phosphoprotein, and functions in the negative regulation of cell proliferation.) Homozygous deletion is observed in the *RB1* promoter region in prostatic carcinomas (Latil & Lidereau, 1998), but it is generally thought that some other tumour suppressor gene has a key role at this deletion. Other candidate target genes for 13q loss are the *BRCA2* (breast cancer 2, early onset) at 13q12, *EDNRB* (endothelin receptor type B) at 13q21, *DBM* (dopamine beta-hydroxylase (dopamine beta-mono-oxygenase)) at 13q14,

DLEU1 and *DLEU2* (deleted in lymphocytic leukemia, 1 and 2) at 13q14 (Porkka, 2003; Savinainen, 2006).

Loss of 10q is frequently observed in prostate cancer and it usually occurs at the late-stages of disease progression (Savinainen, 2006; Porkka, 2003). In LOH studies this deletion has been detected in 30 to 60% of prostate tumour samples. The proposed target genes for this chromosomal loss are *PTEN* (phosphatase and tensin homolog, see section 2.2.2.1) at 10q23, *MXI1* (MAX interactor 1) at 10q25 (Porkka, 2003), and *ANXA7* (annexin 7) at 10q21 (Dong, 2006).

Loss of 16q is seen in both primary and metastatic tumours and it is associated with poor prognosis (Savinainen, 2006). This loss is detected in 19% of primary and in 56% of recurrent prostate cancers (Visakorpi *et al*., 1995). At least four distinct regions have been found, 16q21.1 16q22.1-22.3, 16q23.2-24.1, and 16q24.3-qter (Saramäki, 2006). Candidate target genes for 16q loss include *CDH1* (E-cadherin, see section 2.2.2.1) at 16q22, *CDH13* (H-cadherin) at 16q24.2, *FOXF1* (forkhead box F1) at 16q24, and *HSD17B2* (hydroxysteroid (17-beta) dehydrogenase) at 16q23.3 (Savinainen, 2006; Saramäki, 2006).

18q deletion is quite common in prostate cancer, and it is usually seen in an advanced stage of the disease. This loss has been detected in 19% of hormone-refractory prostate carcinomas (Nupponen *et al*., 1998). Possible target genes for this chromosomal loss are *SMAD2* (SMAD family member 2), and *SMAD4* (SMAD family member 4) both at 18q21.1, and *DCC* (deleted in colorectal carcinoma) at 18q21.3 (Porkka, 2003).

Loss of the chromosome arm 6q, with the minimal region at 6q15-q22, is observed more commonly in metastatic and recurrent (44%, Visakorpi *et al*., 1995) prostate cancers than in primary prostate tumours (22%, Visakorpi *et al*., 1995). Possible target gene for this loss is *FYN* (FYN oncogene related to SRC) at 6q21 (Sørensen *et al*., 2007).

2.2.1.2. Gains and amplifications of genetic material

Gains and amplifications of chromosomal regions are infrequent in primary prostate cancer. In hormone-refractory and metastatic tumours three chromosomal regions, 7p/q, 8q and Xq, have most commonly been gained or amplified. The other chromosomal regions seen to be gained or amplified in prostate cancer include 1q, 2p, 3q, 4q, 6p, 9q, 11p, 16p and 17q.

Gain at 8q is one of the most common chromosomal alterations in hormone-refractory and metastatic prostate carcinomas (almost 90% of advanced tumours, but only 5% of primary tumours have this gain) (Savinainen, 2006), and it has been shown to be associated with an aggressive phenotype of the disease (Porkka, 2003). At least four independent regions have been identified, 8q13.3-q21.11, 8q22.3, 8q24.13-q24.23, and 8q24.3 (Saramäki, 2006). *MYC* (v-myc myelocytomatosis viral oncogene homolog, see section 2.2.2.2), at 8q24, has been suggested as one of the target genes of this gain (Porkka, 2003). Other candidate target genes for this gain are *EIF3S3* (eukaryotic translation initiation factor 3, subunit H, see section 2.2.2.2) at 8q24.11, *TPD52* (tumour protein D52) at 8q21, *TCEB1* (transcription elongation factor B (SIII), polypeptide 1) at 8q21.11, *RAD21* (RAD21 homolog) at 8q24, *PSCA* (prostate stem cell antigen) at 8q24.2, and *KIAA0196* at 8q24.13 (Savinainen, 2006).

Gain of chromosome arm 7 has been detected in 56% of recurrent prostate cancers (Visakorpi *et al*., 1995). Three separate regions for gain at chromosome 7 have been found, 7p15-p21, 7q21, and 7q31. Candidate target genes for this chromosomal gain are *TES* (testis derived transcript), *CAV1* (caveolin 1), *CAV2* (caveolin 2), *MET* (met proto-oncogene), *CAPZA2* (capping protein (actin filament) muscle Z-line, alpha 2), and *WNT2* (wingless-type MMTV integration site family member 2), all located at 7q31 (Savinainen, 2006), and *MCM7* (minichromosome maintenance complex component 7) at 7q21.3 (Saramäki, 2006). Gain of chromosome 7 is seen frequently also in other solid tumours, such as bladder, brain, colon, and kidney. In prostate cancer this gain is associated with higher Gleason score and with advanced pathological tumour stage (Takahashi *et al*., 1994).

Amplification of Xq has been observed in about 30% of hormone-refractory tumours, and the target gene for this amplification is the androgen receptor (*AR*, see section 2.2.2.2) at Xq11-q12. This chromosomal alteration has not been detected in primary tumours. The amplification of the *AR* seems to occur during the hormone therapy, and may be one mechanism for the failure of the androgen deprivation treatments (Savinainen, 2006; Porkka, 2003).

2.2.1.3. Translocations

Gene fusion between the prostate-specific and androgen-regulated, 5'-untranslated region of *TMPRSS2* (transmembrane protease, serine 2, at 21q22) with the ETS transcription factor family members has been observed in clinical prostate cancers (Hermans *et al*., 2006; Tomlins *et al*., 2005). ETS transcription factors involved in this fusion are either *ERG* (v-es erythroblastosis virus E26 oncogene homolog, at 21q22), *ETV1* (ets variant gene 1, at 7p21), or *ETV4* (ets variant gene 4, at 17q21), which all are known oncogenes involved in translocations in Ewing's sarcoma and myeloid leukemias (Hermans *et al*., 2006; Saramäki, 2006). The translocation of these ETS transcription factors, with *TMPRSS2*, renders them also androgen-inducible and it is suggested that it is the mechanism for over-expression of the ETS genes in the majority of prostate cancers (Perner *et al*., 2006; Tomlins *et al*., 2005). This gene fusion is a common molecular event in prostate cancer (Mehra *et al*., 2007), and the reported frequencies of which the fusions are found in prostate cancers varies between 36 and 78% (Tomlins *et al*., 2007).

2.2.1.4. Epigenetic changes

Epigenetics is the study of chromatin and DNA modifications, such as chromatin acetylation, methylation, ubiquination, and phosphorylation, and DNA methylation.

These modifications do not change the DNA sequence, but can change the gene expression patterns. Because epigenetic features can be inherited from one generation to the next, this phenomenon gives the cells a stable inheritance of properties without changing the DNA sequence or content. Epigenetic modifications are responsible for the regulation of individual genes (parental imprinting), whole chromosomes (X-chromosome inactivation), cells (cell differentiation, maintenance of stem cells), and tissues (homeostatic epithelial-mesenchymal interactions) (Schulz & Hatina, 2006).

Epigenetic changes are observed in almost all cancer types and these modifications usually interact with genetic changes, such as point mutations or chromosomal aberrations, giving the cancer its phenotype (Schulz & Hatina, 2006). Although, the molecular mechanisms underlying the development and progression of prostate cancer remain poorly understood, it is known that also epigenetic changes influence malignant transformation of the cells and progression of this disease. In prostate cancer, epigenetic processes, such as DNA hypo- and hypermethylation and altered histone acetylation, have been observed (Li *et al*., 2005). Also changes in many histone-modifying enzymes and other chromatin proteins have been detected. It is suggested that DNA hypermethylation affects at least 30 individual genes, while hypomethylation influences repetitive sequences, like retroposons and certain individual genes. In early carcinogenesis, various genes are seen to be hypermethylated in a coordinated manner, whereas hypomethylation and further hypermethylation events arise with progression of prostate cancer (Schulz & Hatina, 2006).

Hypermethylation of the *GSTP1* (glutathione S-transferase π, at 11q13) promoter is one of the most common epigenetic alterations in prostate cancer, and it causes absence of the *GSTP1* expression. *GSTP1* promoter region is extensively methylated in 90% of prostate tumours. This hypermethylation has been detected also in about 70% of HG-PINs, and it seems to be a very early event in prostate cancer initiation (Visakorpi, 2003). *GSTP1* encodes for the phase II detoxification enzyme glutathione S-transferase π. The encoded enzyme has an important role in the cellular pathway to prevent damages from a variety of carcinogens. Inactivation causes the accumulation of

damaged DNA, making the prostate tissue more susceptible to tumour initiation and progression (Isaacs & Kainu, 2001).

Despite of the fact that both epigenetic and genetic alterations change the cell's gene expression affecting the cell's functions, epigenetic changes are in principle reversible. This allows the cells, and thus cancer, more versatile phenotypic possibilities, than genetic alterations do.

2.2.2. Tumour suppressor genes and oncogenes

Genes which are related to cancer are usually categorised into two groups, tumour suppressor genes and oncogenes. During development and progression of cancer, tumour suppressor genes lose their function, while oncogenes gain their function. Through successive mutations and alterations cells go through transformation, lose their contact inhibition and obtain the invasive capacity. When acquiring more mutations, the tumour progresses, with the help of clonal expansion. It is also generally thought that multiple genetic changes are needed for oncogenesis, and both tumour suppressor genes and oncogenes are needed for that (Latil & Lidereau, 1998). Because deletions of chromosomal regions are more common than gains or amplifications, it is believed that inactivation of tumour suppressor genes is more important in the early development of prostate cancer. In advanced prostate cancers, gains and amplifications become also more central, suggesting that oncogenes have a role in the late-stage progression of this disease.

2.2.2.1. Tumour suppressor genes

Tumour suppressor genes are genes whose function is to restrain cell growth, cell cycle and migration, and induce apoptosis. They control the cell's vital functions (e.g. proliferation, adhesion, apoptosis, and growth), and prevent cells from becoming malignant. When the tumour suppressor gene is inactivated, it makes the cells susceptible for transformation to malignant phenotype. First it was thought that tumour suppressor genes need to be altered at both alleles ("two-hit" theory) to be inactivated, but now it seems that loss of only one allele (haploinsufficiency) is sufficient for cancer formation (Porkka, 2003). Tumour suppressor genes can be divided into two classes, gatekeepers and caretakers. Gatekeepers are able to arrest the cell growth or induce apoptosis and their inactivation results in neoplastic growth, while caretakers prevent or repair genomic damages and their inactivation leads to an increased mutation rate (Savinainen, 2006). It seems that most of the common tumour suppressor genes (for example *RB1*) known to be crucial in other cancers, are not significant in prostate cancer (Porkka, 2003).

NKX3.1 (NK3 homeobox 1, at 8p21) encodes a homeodomain-containing transcription factor. It is expressed mostly in a prostate-specific and androgen-regulated manner, and absence of its expression is frequent in prostate carcinoma and PIN. *NKX3.1* has a role in the initiation of prostate development and in the maintenance of the differentiated state of prostatic epithelial cells. It is expressed in embryos and adults in the male urogenital system. Loss of one *NKX3.1* allele occurs in the majority of primary prostate cancers, and decreases the protein expression levels. Haploinsufficiency is enough to partly inactivate this gene, and happens as an early event in the initiation of prostate cancer (Ju *et al*., 2006). Inactivating mutations of the *NKX3.1* have not been observed in prostate cancer. It is also suggested that the expression of *NKX3.1* might be regulated in a post-transcriptional way (Saramäki, 2006).

CDH1 (E-cadherin, at 16q22.1), encodes the epithelial cell adhesion molecule E-cadherin and it is assumed to function as an invasion-suppressor gene. Inactivation of this gene is thought to increase proliferation, invasion, and metastasis. In high-grade

primary prostate tumours reduced E-cadherin expression is observed, but the loss of expression is more frequent in advanced prostate cancers. The reduced expression is also associated with poor prognosis and lower survival (Latil & Lidereau, 1998; Saramäki, 2006). Mutations in this gene have been observed in gastric, breast, colorectal, thyroid, and ovarian cancers. Somatic mutations in *CDH1* have not been detected in prostate cancer (Porkka, 2003; Saramäki, 2006).

TP53 (tumour protein p53, at 17p13.1), encodes a DNA-binding protein, which has an essential role in the cell cycle regulation, and it is thought to inhibit growth and invasion of the cells. Mutations in *TP53* are known to occur in many human cancers. LOH at the *TP53* locus has been observed in only a few localised prostate tumours. *TP53* alterations are thought to be late events in prostate cancer and these alterations seem to be associated with the progression of prostate tumours, loss of differentiation, and also with poor outcome (Latil & Lidereau, 1998). It seems that LOH and point mutations of *TP53* do not occur tightly together to show the action of this tumour suppressor gene. Observed mutations in *TP53* are also extremely heterogeneous, within the same gland, and within different tumours (Isaacs & Kainu, 2001).

CD82 (CD82 molecule) tumour suppressor gene, at 11p11.2, belongs to the family of membrane glycoproteins and it is thought to function in cell-cell interactions and cell migration. Its expression is reduced in human cancer cell lines (Latil & Lidereau, 1998). In animal models, *CD82* expression is decreased in metastases and its expression suppresses metastases. Down-regulation of this protein is most probably caused by post-translational events (Isaacs & Kainu, 2001). Expression of *CD82* and *TP53* are known to correlate, and the loss of expression of these two genes is associated with poor survival of prostate cancer patients.

PTEN (phosphatase and tensin homologue 1), at 10q23.3, is inactivated by a combination of hemi- and homozygous deletion, point mutation, and promoter methylation. This gene encodes a protein that preferentially phosphorylates phosphoinositide substrates. It functions as a tumour suppressor by negatively regulating AKT/protein kinase B (PKB) signalling pathway. Inactivation of *PTEN*

causes the accumulation of its substrate PIP3 (phosphatidylinositol (3, 4, 5)-trisphosphate), and this constitutively present PIP3 activates the AKT/PKB pathway. Activation of this pathway causes decreased apoptosis and increased cell survival (Isaacs & Kainu, 2001). In prostate cancer, the rate of biallelic inactivation of *PTEN* by mutation or homozygous deletion is remarkably lower than the rate of LOH at chromosomal region 10q23.3 (Kwabi-abbo *et al.*, 2001). One explanation for this could be the haploinsufficiency of *PTEN*, and in mice it is observed that haploinsufficiency of *PTEN* promotes prostate cancer progression (Kwabi-abbo *et al.*, 2001; Saramäki, 2006). It is also seen that gene *LIPJ* (lipase, family member J), at 10q23.31, is frequently co-deleted with *PTEN*, and also genes *MINPP1* (multiple inositol polyphosphate histidine phosphatase, 1) at 10q23, *PAPSS2* (3'-phosphoadenosine 5'-phosphosulfate synthase 2) at 10q23-q24, and *ATAD1* (ATPase family, AAA domain containing 1) at 10q23.2 are commonly lost (Saramäki, 2006). These findings suggest that there can be also other target genes for this chromosomal loss.

There are also other candidate tumour suppressor genes in prostate cancer. *DCC* (deleted in colorectal carcinoma, at 18q21.3) tumour suppressor gene is often altered in colorectal cancer, and in prostate cancer LOH on chromosome arm 18q is observed, particularly in the *DCC* region (Latil & Lidereau, 1998). *CD44* (CD44 molecule, at 11p13), encodes a cell-surface glycoprotein, which functions in specific cell-cell and cell-extracellular matrix interactions, cell adhesion, and migration. Decreased expression and hypermethylation of the promoter region of *CD44* have been observed in prostate cancer, and it is thought that both of these are associated with the progression and metastasis of this disease (Porkka, 2003). *ANXA7* (annexin A7, at 10q21.1-q21.2), encodes a Ca^{2+}-activated GTPase (guanosine-5'-triphosphatase). Decreased expression and LOH of *ANXA7* have been observed in prostate cancer. In prostate cancer cell lines its expression has been shown to reduce cell proliferation and colony formation (Dong, 2006). *ZFHX3* (zinc finger homeobox 3, at 16q22.3-q23.1) has been shown to reduce cell proliferation rate, up-regulate *CDKN1A* (cyclin-dependent kinase inhibitor 1A) tumour suppressor gene, and down-regulate *AFP* (alpha-fetoprotein) oncoprotein (Latil & Lidereau, 1998; Dong, 2006). *ZFHX3* undergoes frequent somatic mutations in sporadic prostate cancer, and also

transcriptional down-regulation has been observed (Dong, 2006). Other candidate tumour suppressor genes in prostate cancer are *HIC-1* (hypermethylated in cancer 1, at 17p13.3), *CTNNA1* (catenin, alpha 1, at 5q31), *RAP2A* (member of RAS oncogene family, at 13q34), *CDKN2* (cyclin-dependent kinase inhibitor 2A, at 9p21), *NME1* and *NME2* (encodes A and B subunits of a human nucleoside diphosphate kinase, NDK, both at 17q21.3), and *GSTP1* (glutathione S-transferase pi, at 11q13) (Latil & Lidereau, 1998).

2.2.2.2. Oncogenes

Proto-oncogenes are cells' normal genes (e.g. regulate the cell proliferation and growth), which can be converted to cancer promoting oncogenes. The conversion can occur for example by gene amplification, translocation or point mutations, leading to changes in gene's function or over-expression of the gene product, and transforming the cell to more malignant phenotype (Porkka, 2003). Oncogenes act in a dominant manner and for them it is enough that only one allele is altered to have the malignant phenotypic consequences.

MYC (v-myc myelocytomatosis viral oncogene homolog) proto-oncogene, at 8q24.21, which is one of the minimally amplified regions at 8q, is commonly amplified in solid tumours (breast cancer, small-cell lung carcinoma, cervical carcinoma) and rearranged in haematological malignancies (Burkitt's lymphoma, T cell acute lymphocytic leukaemia). The gene encodes a transcription factor that is involved in the control of normal cell growth, proliferation, differentiation, and apoptosis. High rates of *MYC* expression have been observed in prostatic adenocarcinomas, and amplifications and rearrangements have been detected in prostate cancer cell lines (Latil & Lidereau, 1998). 29 % of tumours showing increased copy number of *MYC* show high-level amplification of that gene (Nupponen *et al*, 1998).

EIF3S3 (eukaryotic translation initiation factor 3, at 8q24.11) encodes a protein subunit of the eukaryotic translation initiator factor 3 (eIF3), which has a central role in the translation initiation pathway. In the absence of other initiation factors, eIF3 binds to the 40S ribosomal subunit and keeps it from interacting with the 60S ribosomal subunit (Savinainen, 2004). *EIF3S3* has been shown to be amplified in 9% of early-stage prostate tumours and in 33% of hormone-refractory, locally recurrent tumours, and in 50% of hormone-refractory, metastatic lesions (Saramäki *et al*., 2001). Its high-level amplification is associated with androgen independence, advanced stage, and poor differentiation (Savinainen, 2006).

ERBB2 (v-erb-b2 erythoblastic leukemia viral oncogene homolog 2, neuro/glioblastoma derived oncogene homolog) proto-oncogene, at 17q21.1, encodes a protein, which belongs to the epidermal growth factor (EGF) receptor family of receptor tyrosine kinases. The over-expression of *ERBB2* follows either through gene amplification, or transcriptional, or post-transcriptional deregulation (Latil & Lidereau, 1998). This gene is capable of activating the androgen receptor signalling pathway at low levels of androgens, and it is proposed that *ERBB2* could be involved in the development of androgen-independent prostate cancer (Porkka, 2003). It is suggested that over-expression of *ERBB2* can activate the androgen receptor pathway, providing the cells a pathway to circumvent the androgen deprivation therapy (Isaacs & Kainu, 2001). Despite its function in the androgen receptor signaling pathway, there has been contradictory results of *ERBB2* over-expression in clinical prostate tumours. Some studies have detected *ERBB2* over-expression in prostate cancer, while other studies have not (Savinainen, 2006).

BCL2 (B-cell CLL/lymphoma 2, at 18q21.3) encodes an outer mitochondrial membrane protein that prevents the apoptosis of some cell types. It is an anti-apoptotic oncogene, inhibiting apoptosis of prostate cancer cells, and especially cells which are under androgen deprivation (Isaacs & Kainu, 2001). Over-expression of *BCL2* has been observed in many malignancies (prostate, breast, lung, colorectal, and pancreas), and it has been proposed that it is associated with chemo- and radio-resistance (Savinainen, 2006). The over-expression of *BCL2* is detected commonly in recurrent prostate

tumours, but it seems not to be caused by the amplification of this gene (Isaacs & Kainu, 2001). Some studies have detected increased copy numbers, but not high-level amplifications (Savinainen, 2006).

AR (androgen receptor, at Xq11.2-q12) is a transcription factor that mediates the action of androgens, needed for the normal development of the prostate and the differentiation of secretory epithelial cells (Visakorpi, 2003; Saramäki, 2006). It belongs to the steroid and thyroid hormone receptor gene superfamily, and it has been identified as a target gene for the Xq amplification. It is expressed in both normal and malignant prostate. Amplification of this gene has been detected in about 30% of hormone-refractory cancers, but not in untreated primary tumours (Visakorpi, 2003). Amplification causes the increased *AR* expression, but this explains only about 30% of hormone-refractory prostate cancers (Saramäki, 2006). In the remaining cases the reason for over-expression remains unknown, polymorphisms and activating mutations act only in a small subset of cases. The over-expression of *AR* after the androgen deprivation, caused by the gene amplification or by some other mechanism, is a good way for prostate cancer cells to overcome the decreased levels of circulating androgens (Isaacs & Kainu, 2001).

2.3. Methods

2.3.1. Quantitative reverse transcriptase polymerase chain reaction

Reverse transcription polymerase chain reaction (RT-PCR) is a technique for amplifying a specific piece of a RNA molecule. The RNA molecules are first reverse transcribed into complementary DNA (cDNA) form and then amplified with the usual

PCR method. In this way it is possible to determine the abundance of specific RNA molecules within a cell or tissue, illustrating the gene expression.

Quantitative real time PCR is used to amplify and quantify a specific part of a certain DNA molecule. This procedure is as the general PCR, but the DNA is quantified after each round of amplification. Quantification of the PCR products can be done either with an intercalating fluorescence dyes (SYBR Green I, Roche Diagnostics), or target-specific probes.

SYBR Green I is a fluorescence dye that intercalates into double-stranded DNA. The intensity of the fluorescence varies depending on the amount of double-stranded DNA present after each round of PCR. Amplified PCR products can be recognised by the melting temperature profiles. To determine these, the temperature in steadily increased while at the same time the sample's fluorescence is monitored. The temperature at which DNA strands separate depends on the sequence, length, and CG content, giving each amplified DNA product its characteristic melting curve (The lightCycler System, A Proven Standard for Real-Time PCR, Roche Applied Science).

Three quantitative methods can be used: the absolute standard curve, the relative standard curve, and the comparative cycle time (Bookout & Mangelsdorf, 2003). The absolute standard curve assumes that controls, whose absolute RNA concentrations are known, are used (Applied Biosystems, 1997). At relative standard curve the sample expression values are compared to calibrator's expression values. The comparative cycle time uses cycle times, rather than interpolated template quantities, for the calculations, and it does not require the use of the template dilution series (Bookout & Mangelsdorf, 2003).

The obtained gene expression values have to be normalised by comparing them to reference gene expression values. This normalisation enables that the samples can be compared to each other, and that the variation between samples' expression levels are actually due to biological differences in the cells, rather than methodological artefacts. House-keeping genes (e.g. TATA box binding protein (TBP)) are usually thought of as

a good reference gene, and it is assumed that these genes are expressed equally in every cell and tissue.

2.3.2. Fluorescence in situ hybridisation

In situ hybridisation (ISH) enables the quantitative or localised demonstration of specific DNA or RNA sequences on chromosomes, in single cells, or in individual cells in tissue sections. It is based on annealing of the labelled and denatured complementary DNA or RNA strand to the denatured target sequence. First ISH was done using isotopically labelled probes, which were detected by autoradiography, but nowadays non-isotopic ISH is more commonly used. Probes, which can be used for non-isotopic ISH are repetitive-element probes (includes chromosome specific), whole-chromosome paint probes, locus-specific probes, nucleic acid sequence-based single-copy probes, and species-specific total genomic DNA probes (Knoll & Lichter, Genetic Protocols of Human Genetics, 2005).

Fluorescence *in situ* hybridisation (FISH) has been used to detect the changes in the number of chromosomes in autosomes and sex chromosomes of interphase amniocytes and tumour cells. It is a good technique for observing cryptic rearrangements and small deletions; mapping of genes and sequences; sequential ordering of close sequences in interphase cells; examining of chromosome structure and organisation, gene amplification and replication. Probes are most frequently labelled by nick translation, but other methods also exist. Biotin-labelled and digoxigen-labelled deoxynucleotides are most common, but also directly fluorescent-conjugated nucleotides are used. Biotin- and digoxigen-labelled probes are incubated with a fluorochrome conjugated antibody or avidin, and the probes are detected by fluorescent or enzymatic methods, and viewed by transmission, reflection, or epifluorescence microscopy (Knoll & Lichter, Genetic Protocols of Human Genetics, 2005).

3. AIMS OF THE RESEARCH

This research is based on Saramäki *et al*. (2006) study in screening the copy number and expression alterations in prostate cancer by microarray analysis. They used aCGH, and confirmed the previously identified common gains and losses. Due to the higher resolution of aCGH, they managed to narrow-down the minimal commonly altered regions, and identified a new gain at 9p13-q21. From this novel amplification they separated two independent regions, 9p13.3 and 9p13.1-q21.11.

The purpose of this research was to examine the amplification of chromosomal region 9p13.3 in prostate cancer (Figure 1). The aims of this research were:

1) To determine the boundaries of the 9p13.3 amplicon.

2) To define the frequencies and levels of 9p13.3 amplification in 7 prostate cancer cell lines and 19 prostate cancer xenografts.

3) To identify one or a few genes, whose amplified copy number affects increasingly to its expression level.

4) To examine whether the 9p13.3 chromosomal region is also amplified in clinical prostate tumours.

5) To determine the associations of clinicopathological variables with the 9p13.3 amplification.

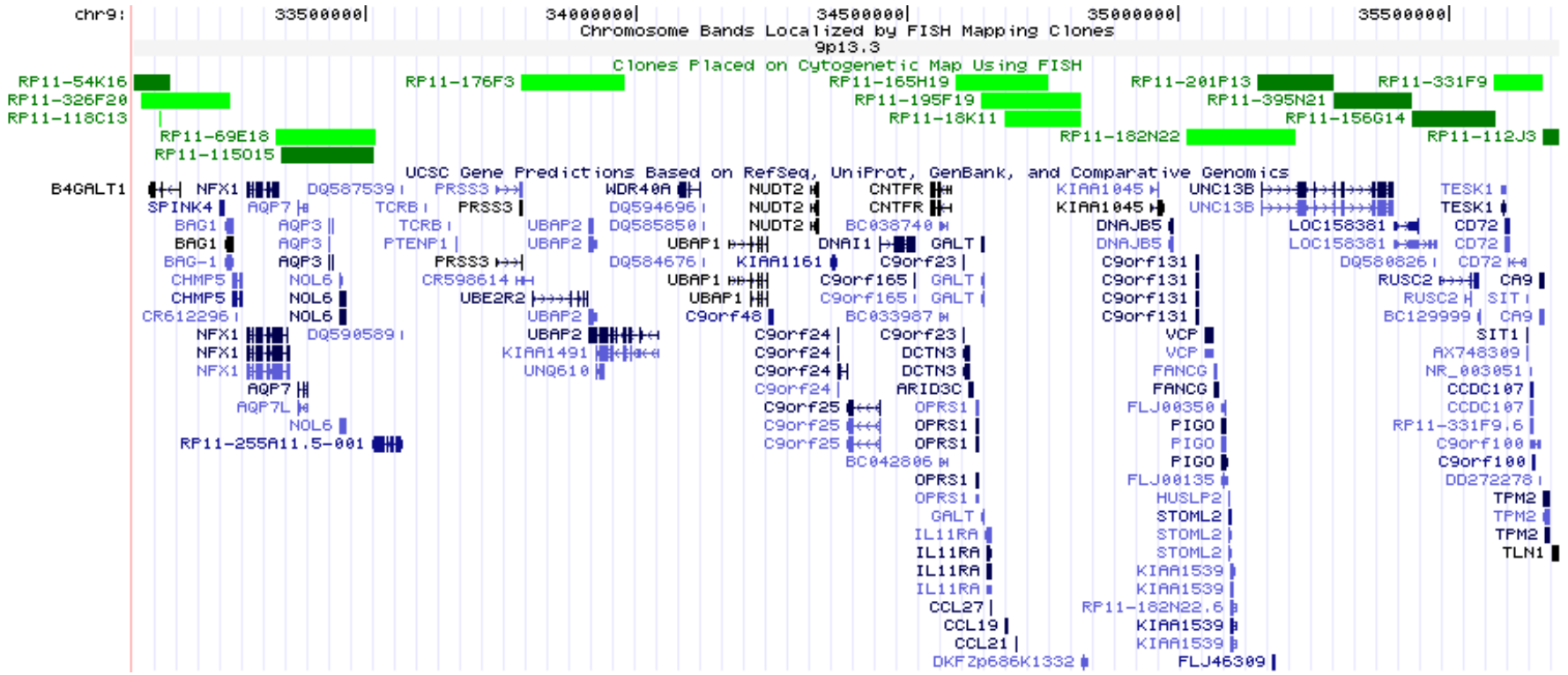

Figure 1. Chromosomal region 9p13.3. Base position, chromosome band, BAC (bacterial artificial chromosome) clones, and UCSC gene predictions are seen in picture, respectively. Picture is from UCSC Genome Browser website, http://genome.ucsc.edu/.

4. MATERIALS AND METHODS

4.1. Prostate cancer cell lines and xenografts

The prostate cancer cell lines LNCaP, DU145, PC-3, 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, The Netherlands). The cell lines were grown in recommended media. Interphase and metaphase spreads were prepared following standard laboratory protocols. Genomic DNA was extracted and purified according to common laboratory protocols and total RNA was extracted with the TRIZol®-reagent (Invitrogen Life Technologies, Carlsbad, CA, USA), following the manufacturer's protocols (Saramäki, 2006).

Materials for the 19 prostate cancer xenografts (LuCaPs 23.1, 23.8, 23.12, 35, 41, 49, 58, 69, 70, 73, 77, 78, 81, 86.2, 92.1, 93, 96, 105, and 115) were kindly provided by Dr. Robert Vessella (department of Urology, University of Washington, Seattle, WA, USA). Genomic DNA and total RNA of the xenografts were extracted from snap frozen pieces of tissue. DNA extraction was done with the DNAzol reagent according to the manufacturer's protocol (Molecular Research Center, Inc. Cincinnati, OH, USA) and RNA was isolated with the Tri-Pure reagent and protocol (Roche Diagnostics, Mannheim, Germany). 5µm sections were cut from snap frozen tissue blocks for FISH analyses (Saramäki, 2006).

4.2. Clinical prostate tumour specimens

Formalin-fixed and paraffin-embedded samples of locally recurrent hormone-refractory prostate cancers and prostatectomy prostate tumours were obtained from Tampere University Hospital. Tissue micro-arrays (TMA) were created of the cancer samples according to published guidelines (Kononen *et al*., 1998). The material was used with consent of the patients and with the approvals of the ethical committee of the Tampere University Hospital and the National Authority for Medicolegal Affairs (TEO), applied for previously (Saramäki, 2006).

4.3. Quantitative RT-PCR

Genes' mRNA expression levels were studied with quantitative reverse transcription polymerase chain reaction (q-RT-PCR). The primers were designed for different exons to avoid the amplification of contaminating genomic DNA. Primer sequences are listed in Table 1. The standard curve was prepared from a mixture of LNCaP/Universal Total Human RNA (1/3, BD Biosciences Clontech, Palo Alto, CA, USA), and dilutions were made to correspond to nominal concentrations of 1500, 300, 60, 12, 2.4, 0.48, and 0.096µg/µl. All the expression values were normalised to the expression values of the housekeeping gene *TBP*. Sterile water was used as the negative control.

Quantitative RT-PCR reactions were done with LightCycler 2.0 (Roche Diagnostics, Mannheim, Germany), in 20µl capillaries with LightCycler FastStart DNA Master SYBR Green I Kit. In every q-RT-PCR reaction, there were 2µl of sample, 0.5µl of each primer (reverse and forward primer, which were diluted in 20 µM concentration), 2.4 µl of $MgCl_2$ solution (so that the final $MgCl_2$ concentration in capillaries were 4mM), 2 µl of Master mix (which includes polymerase, buffer, nucleotides, SYBR

Table 1. *Primer sequences used in q-RT-PCR*

Gene	Primer sequences 5'-3'	Gene	Primer sequences 5'-3'
PRSS3	AGCGAACAGTGGGTGGTATC TGTCCAGAGTGTCCCTGTTG	***CCL21***	CCCAAGGCAGTGATGGAGGG TTGGAGCCCTTTCCTTTCTT
UBE2R2	ACTGCCTTCTGAAAGGTGGA CTCCATCCTTTTCTGCTTCG	***KIAA1045***	TGGGCTACATCCAAGGAGAC CGGTAACGCAGAAAGTCCTC
UBAP2	TGGTCGAAGTCAGCAGACAC GCTGAGAGAGAGGGCTGCTA	***DNAJB5***	TTCAGCTGGAGCCCTTAAAA CATAGGCCTCTGCAATCTCC
WDR40A	CCAAGATCCCCATTCTGAAA TGAGCCAGACACTGCCATAG	***C9orf131***	GGCTAAGGGAAATGGAATGG[1] GGCAGAGAACAAAGGAAGGA[2] TCCAGAGATGCCTCGTCTTC[3]
UBAP1	CTTGGACAGTGGCACAGAGA ACGAGTAGCCCATGTTGACC	***VCP***	ATCCGTGAATCCATCGAGAG GACTCTGCTGAAGGGTCTGG
KIF24	CCAGCTGGGTCACAAAATCT GCCGATCTCAAGGTGCTTAG	***FANCG***	GGACTTCCTCCTCAGTGTGC GGACGGATCCAGCTCAAATA
NUDT2	GAAGATGCCTCATTCCCAAA CTATGCCTGCTTCCTCTTGG	***PIGO***	AGCTGGGCCTCAAGTACCTC GCTATGCCCAGGAGAAGTCC
C9orf24	CCAGGATGGAGACAGCAGTT GGTGCTCGTAGGGAGGTACA	***STOML2***	GGCCAAGGACTCCAACACTA ACATCTCTGCTGCTCCCACT
C9orf25	AAGAATGGCAGCATGGGTAG CTTCAGCAGCTGGATGTTCA	***KIAA1539***	AAGCCACACCCTTCTGGAG AAGGGGTTCAGATCCACGA
DNAI1	ATCCACCCCATCATCATTGT AGCCCCTCAGGTCTTGATTT	***UNC13B***	ACGCTATGCCCTGTCTCTGT TCTGCCACTTGAGGTCATTG
C9orf165	CAGGCTCCCAAGCCTTAGTC GACAGTGGAGCTGCCTCAAT	***LOC158381***	GATTGGTGCTATGGCTTGGT CATGTTCCTCCATTGCACAC
CNTFR	CATGGAGTGACTGGAGCGTA CGGGCTCAGATCAAGAGACT	***RUSC2***	AGTCATACCATGCGCTTCAA ACACACGGTATGAGCTGCAC
C9orf23	ATCTTGGCTCCGGATCGT CGCATCTCAAGGGTATCAGG	***TESK1***	TGCTCCTGGCCATCCACT CAACAGAGCCCTGATTGTGT
DCTN3	AGCAGCAGGACCAGTGTGTG ACAAAGGCAGGTTGGCATAG	***CD72***	GGCCTCAGCTCTAACAAGGA AATCTGGAAACCTGAAAGCTG
ARID3C	TCAACGGGGTGGTCTACACT CAGGGCAAGATGCTGGAAG	***SIT1***	TACAGACAGGACGGCTGTCT CTCAGAGTCCAGCACCACCT
OPRS1	GCAGTGGGTGTTCGTGAAT CTCCACCATCCATGTGTTTG	***CCDC107***	CGGCTTTTGTGCTGTACAAGT GGTTGTTCAGGTGCTGCTCT
GALT	CGTCTGGTCCTAACCAGTGA GCTTCTTCATGATGGAGGCTA	***C9orf100***	TCACACCTGTAATCCCAGCA CCCACAATCCAGTGAGGAGT
IL11RA	CCCACTTCCTGCTCAAGTTC CCTTTGGTATGGTCCCAGTG	***CA9***	TAAGCAGCTCCACACCCTCT TCTCATCTGCACAAGGAACG
CCL27	GGCTTTCGTGCTTCACCTG ATTTGGCTATTGGGGGCTTC	***TPM2***	CCCTCCAGAAGAAGCTGAAG CTCCTCCTCAACCAGCTGAA
CCL19	CCTGCTGGTTCTCTGGACTT GTACCCAGGGATGGGTTTCT		

For each gene: upper sequence is forward primer, and bottom sequence reverse primer. [1]Forward sequence for isoforms A and B; [2]forward sequence for isoforms C and D; [3]reverse sequence.

Green I dye, and $MgCl_2$), and water was added to fill the final volume to 20µl. Table 2 summarises the quantitative RT-PCR reaction conditions.

Table 2. *The quantitative RT-PCR reaction conditions.*

Program		**Temperature/°C**	**Time**
Denaturation		95	10 min
40-50 cycles:			
	Denaturation	95	2 sec
	Annealing	55-60[1]	10 sec
	Elongation	72	6-15 sec[2]
Melting:			
	Denaturation	95	1 sec
	Annealing	65-70[3]	15 sec
	Denaturation	To 95[4]	0.1°C/sec[4]
Cooling		40	1 min

[1]Annealing temperature was dependent on the size and CG content of the primers; [2]Elongation time was dependent on the size of the product. With LightCycler it is thought that approximately 25 bp are synthesised in a second; [3]The annealing temperature was 10°C higher than annealing temperature in cycles; [4]Temperature was increased from annealing temperature to 95°C at the rate of 0.1°C/second.

Before running q-RT-PCR reactions with all the sample material, screening for genes located at 9p13.3 was done (9p13.3 region includes over 40 known or predicted protein coding genes). In screening the same reaction conditions were used (Table 2). Normalised expression values from LuCap35 were divided with normalised LNCaP values. When this quotient was two or more the gene passed the screening, and q-RT-PCR was performed in all samples. The expression levels were analysed from 7 prostate cancer cell lines and from 19 prostate cancer xenografts.

4.4. Fluorescence in situ hybridisation (FISH)

Locus-specific BAC (bacterial artificial chromosome) probes were labelled with digoxigenin-dUTP (Roche) by nick translation. Centromere 9 was used as a reference. In prostate cancer cell line and xenograft slides pHuR98 (centromere 9) was labelled with fluorescein-12-dUTP (FITC) or texas red-5-dUTP (TR) (both PerkinElmer Life Science), and in tissue microarrays (TMAs) a commercial labelled centromere 9 probe (CEP 9 SpectrumGreen, Abbott Molecular Inc.) was used. The probes used in FISH studies are listed in Table 3.

Table 3. *FISH probes*

Gene(s)/locus	Clone	Accession number(s)	label
BAG1	RP11-326F20	AQ538767, AQ548728	digoxigenin
NFX1, AQP7, NOL6	RP11-115O15	AQ344588, AQ344591	digoxigenin
PRSS3, UBE2R2, UBAP2	RP11-176F3	AL139113	digoxigenin
DCTN3, GALT, IL11RA	RP11-165H19	AQ415409, AQ382511	digoxigenin
VCP, PIGO, STOML2	RP11-182N22	AQ415988, AL353795	digoxigenin
UNC13B	RP11-201P13	AL354669	digoxigenin
RUSC2	RP11-156G14	AL133476	digoxigenin
TPM2, TLN1	RP11-112J3	AQ313109, AL133410	digoxigenin
cen9	pHuR98		FITC/TR
cen9/9p11-q11	Alpha satellite DNA		spectrum green

To ensure that the probes recognised only a single target and to estimate the hybridisation efficiencies of the probes, hybridisations to normal lymphocyte metaphase preparations were performed (data not shown).

The interphase and metaphase slides of the cell lines, and Carnoy-fixed (3:1 methanol: acetic acid glacial; 50%, 75%, 2x100%, 10 minutes in each) frozen sections of the xenografts were denatured in denaturation solution (appendix 1) for 2 minutes (72-75°C), after which they were dehydrated in an ascending ethanol series (70%, 85%, and 100%, 2 min in each) and air dried. Hybridisation mixture was prepared: 0.5-1µl Cot-1

DNA (1 ng/µl, Roche), 0.5-1 µl TR or FITC labelled centromeric probe, 1.5µl digoxigenin-labelled locus-specific probe, and 7µl Master mix (MM1.0, appendix 1). Probes were denatured in the hybridisation mixture for 5 minutes at 70-75°C, applied to the slides and hybridised in a humid chamber for 1 to 3 days at 37°C.

After hybridisation the cell line slides were washed in 0.4xSSC/0.3% NP-40 at 72°C for 2 minutes, in 2xSSC/0.1% NP-40 for 1 minute at RT, and in 4xSSC for 5 minutes at RT. Xenograft slides were washed in 50% formamide/2xSSC at 45°C for 2x5 minutes, in 4xSSC/0.05% Tween for 2x5 minutes at RT, and in 4xSSC for 5 minutes at RT. For both cell line and xenograft slides pre-blocking with 4xSSC/1%BSA (bovine serum albumin) for 5 minutes, and staining in 2µl/ml anti-digoxigenin-rhodamine or -FITC for 20 minutes. After this, the slides were washed in PN (3x10 minutes, appendix 1), rinsed in water, and allowed to dry for 15 to 20 minutes. The slides were counterstained with 0.1 or 0.05 µM DAPI (4.6-diamidine 2-phenylindole) in anti-fade solution (Vectashield, Vector Laboratories, Burlingame, CA, USA).

From TMAs, the paraffine was removed in 100% xylene by incubating for 2x15 minutes at RT, and the xylene was removed in 100% methanol for 2x5 minutes at RT, after which the slides were air dried. Deparaffinised slides were autoclaved in pH-9 salt solution (appendix 1) for 2 minutes at 121°C, cooled at RT for 15 minutes, and dipped in water. Slides were then incubated in 3.25 mg/ml pepsin (Sigma-Aldrich Co) in 0.9% NaCl (pH 1.5, 40ml) for 20 minutes at 37°C. Slides were rinsed in water to remove the excess pepsin and washed in 2xSSC (2 minutes), dehydrated in ethanol series (70%, 85%, and 100%, 2 minutes in each), and air dried. Hybridisation mixture (0.5 µl Cot-1 DNA, 1 µl commercial centromere 9 probe, 1.5µl digoxigenin-labelled locus-specific probe, and 7µl Master mix (MM 1.0, appendix 1) were applied to the slides and co-denatured on an 80°C water bath for 7 minutes. Slides were hybridised in a humid chamber for 2-3 days at 37°C. Post-hybridisation washes were same as for xenograft slides and staining same as for both xenograft and cell line slides.

The probe and centromeric signals from non-overlapping nuclei were scored with an Olympus BX50 epifluorescence microscope (Tokyo, Japan). High-level amplification

(amplification) was defined as greater than two-fold higher copy number of probe than reference probe (centromere 9), or five or more copies of probe, or a tight cluster of probe signals. Low-level amplification (gain) was defined as three or four copies of probe.

4.5. Statistical analyses

The association between copy number and expression level of individual genes was tested with Kruskal-Wallis test. The outliers were detected from quantitative RT-PCR data with Grubb's test.

The association of 9p13.3 amplification and tumour type (prostatectomy or HR) was tested with χ^2 test. From untreated prostatectomy specimens the association of Gleason score with 9p13.3 amplification was tested with χ^2 test. Fisher's exact test was used to test the association between amplification and tumour progression, and the clinical tumour stage. Mann-Whitney test was used to test the association between amplification and PSA (prostate specific antigen), Ki-67, age at diagnosis, and tumour percent. Kaplan-Meier analysis was used to test the progression-free survival of patients treated by prostatectomy.

5. RESULTS

5.1. FISH data from prostate cancer cell lines and xenografts

Fluorescence in situ hybridisation was done on 19 prostate cancer xenografts and on 7 prostate cancer cell lines to detect the copy number changes in different parts of 9p13.3 region. High-level amplification was defined as greater than two-fold higher copy number of probe than reference probe (centromere 9), or five or more copies of probe, or tight clusters of probe signals. Low-level amplification was defined as three or four copies of probe. Normal copy number status was defined as two copies of both probe and centromere.

Of 19 prostate cancer xenografts, 3 samples, LuCaPs 35 (Figure 2), 41 (Figure 3), and 58 showed high-level amplification. Low-level amplification was observed in 10 xenografts, LuCaPs 69, 70, 77, 81, 86.2, 92.1, 93, 96, 105, and 115, and normal copy number status was seen in 6 samples, LuCaPs 23.1, 23.8, 23.12, 49, 73, and 78. Table 5 summarises the FISH results obtained from prostate cancer xenografts. From 7 prostate cancer cell lines, 6 samples, DU145, LAPC-4, LNCaP, 22Rv1, VCaP, and DuCaP, showed low-level amplification and the normal copy number staus was detected only in the cell line PC-3 (data not shown). The distributions of high-level amplification, low-level amplification and normal copy number status in prostate cancer xenografts and cell lines are summarised in Table 4.

Table 4. *Copy number changes in prostate cancer xenografts and cell lines.*

Sample	Normal	Low-level amplification	High-level amplification	Total
Xenografts	6 (31.6%)	10 (52.6%)	3 (15.8%)	19 (100%)
Cell lines	1 (14.3%)	6 (85.7%)	0 (0%)	7 (100%)

Results are shown as No (%).

Table 5. *FISH data from prostate cancer xenografts.*

LuCaPs	**23.1**	**23.8**	**23.12**	**35**	**41**	**49**	**58**	**69**	**70**	**73**	**77**	**78**	**81**	**86.2**	**92.1**	**93**	**96**	**105**	**115**
RP11-326F20[1]	2	2	2	2-3	8-10	2	4	1	3	2	3	2	1	3	2	3	3	6	2
RP11-115O15	-	-	-	*8-10*	*10-13*	-	*5-7*	*3-4*	-	-	-	-	*3*	-	*3-4*	-	-	*3-4*	-
RP11-176F3	-	*3*	-	*12-15*	*10-15*	*2*	-	*3*	*3-4*	-	-	-	-	*4*	-	-	-	-	-
RP11-165H19[2]	*2*	*2*	*2*	*10-13*	*7-10*	*2*	*5-7*	*3*	*3-4*	*2*	*3*	*2*	*3*	*4*	*3*	*3*	*3-4*	*3-5*	*3*
RP11-182N22	-	-	-	*10-12*	*8-10*	-	-	*3*	-	-	-	-	*3*	-	*3-4*	-	-	-	-
RP11-201P13	-	-	-	*10-12*	*8-10*	-	-	*3*	-	-	-	-	-	-	-	-	-	-	-
RP11-156G14	-	-	-	*6-10*	*8-10*	-	*5-7*	*3*	-	-	-	-	-	-	-	-	-	-	-
RP11-112J3	-	-	2	3	6-8	-	5	3	3-4	-	-	-	-	-	-	-	-	3-4	-
pHuR98	3	2	2	3-4	2-3	2	4	3	3-4	2	3	2	2	4	4	3	3-4	3-5	3

The minimal commonly gained region is grey-shaded and in cursive. [1]Mäki et al., 2006; [2]Saramäki et al., 2006.

With the help of xenograft LuCaP35, the 9p13.3 amplification was limited between the BAC-clones RP11-326F20 and RP11-112J3. In LuCaP35 BAC-clones RP11-326F20 and RP11-112J3 showed only 3 probe copies, while in BAC-clones between these two, more than 5 (up to 15) probe copies were detected (Figure 2). The centromere 9 showed 3 to 4 copies in LuCaP35.

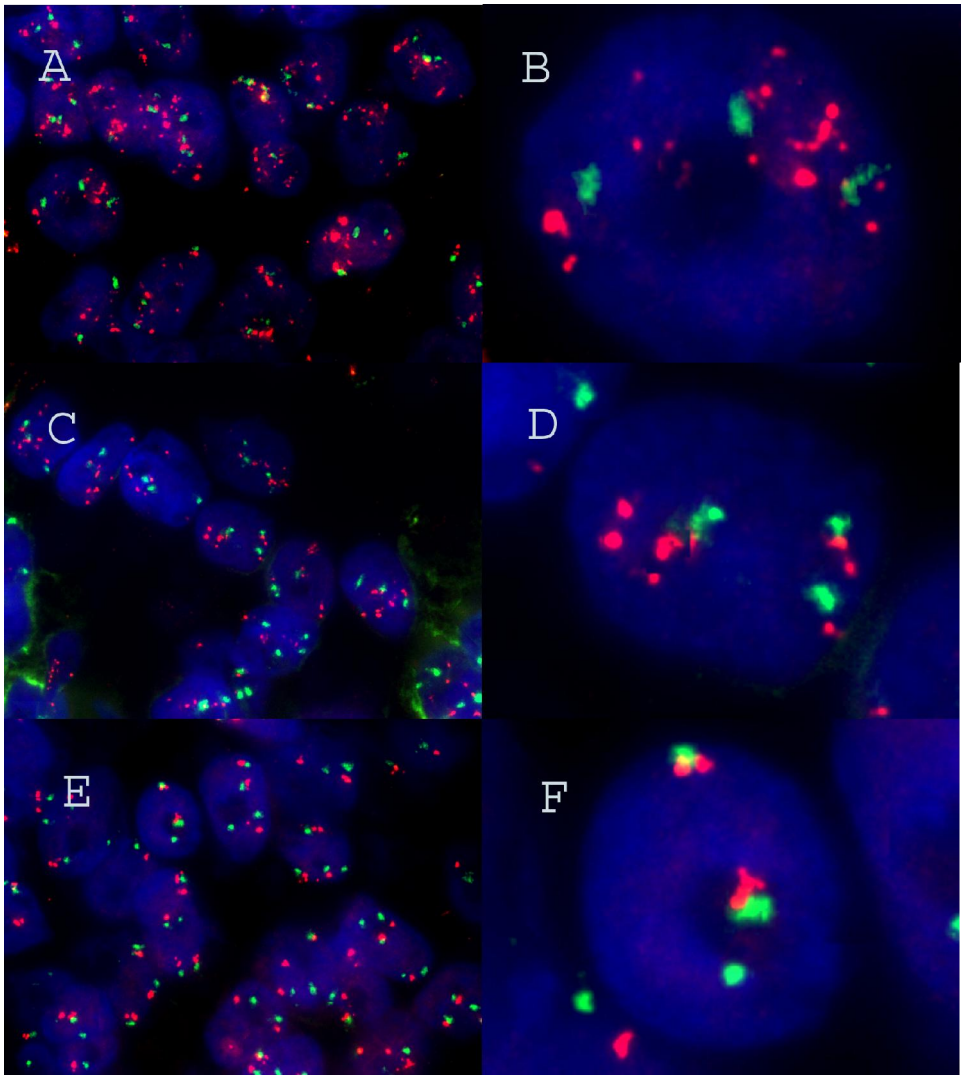

Figure 2. FISH for prostate cancer xenograft LuCaP35. Probe signals are seen in red and centromere 9 signals in green. A, B: BAC-clone RP11-115O15 used as a probe, and 8-10 probe signals are detected. C, D: BAC-clone RP11- 156G14 used as a probe, and 6-10 probe signals are detected. E, F: BAC-clone RP11-326F20 used as a probe, and only 3 probe signals are detected, showing border of the 9p13.3 amplicon.

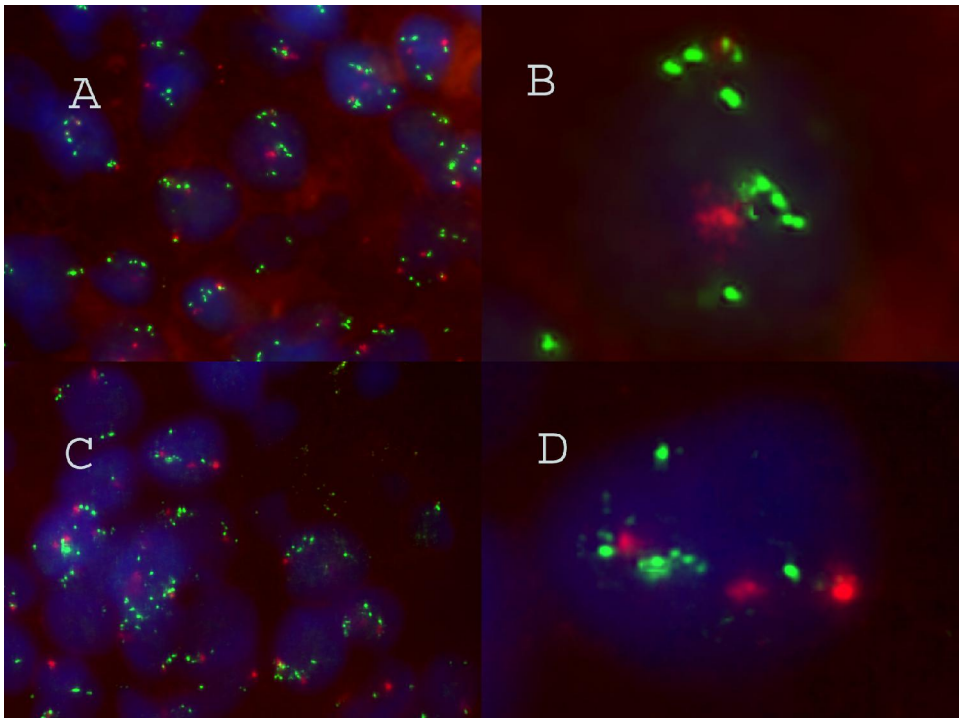

Figure 3. FISH for prostate cancer xenograft LuCaP41. The probe signals are seen in green and centromere 9 signals in red. A, B: BAC-clone RP11-201P13 used as a probe, and 8-10 probe signals are detected. C, D: BAC-clone RP11- 115O15 used as a probe, and 10-13 probe signals are detected.

5.2. Screening the chromosomal region 9p13.3

The chromosomal region 9p13.3 includes over 40 known or predicted protein coding genes. To reduce the amount of genes to run with quantitative RT-PCR with all samples (19 prostate cancer xenografts and 7 prostate cancer cell lines), screening was performed. Prostate cancer xenograft LuCaP35 and prostate cancer cell line LNCaP were used, because they contain high-level amplification and low-level amplification at 9p13.3 region, respectively. The expression was normalised to TBP. These normalised LuCaP35 expression values were divided with normalised LNCaP expression values. When this quotient was two or more, and also the product melting curve and agarose gel

showed good specificity of the primers and reaction conditions, the gene passed the screening. Table 6 summarises the list of genes, which passed the screening.

Genes *UBE2R2*, *WDR40A*, *DNAI1*, and *DCTN3* were previously studied by Saramäki *et al*. (2006). The quantitative RT-PCRs of these genes were conducted in the same way as for the other genes.

Table 6. *Genes, which passed the screening.*

Gene symbol	Full name/description	Q[1]
PRSS3	Protease, serine, 3 (mesotrypsin)	8.41
UBE2R2	Ubiquitin-conjugating enzyme E2R 2	2.14
UBAP2	Ubiquitin associated protein 2	3.80
WDR40A	WD repeat domain 40A	1.83
UBAP1	Ubiquitin associated protein 1	5.16
C9orf25	Chromosome 9 open reading frame 25	6.50
DNAI1	Dynein, axonemal, intermediate chain 1	5.59
C9orf165	Chromosome 9 open reading frame 165	15.19
DCTN3	Dynactin 3 (p22)	4.47
GALT	Galactose-1-phosphate uridylyltransferase	3.49
IL11RA	Interleukin 11 receptor, alpha	4.24
CCL19	Chemokine (C-C motif) ligand 19	17.73
KIAA1045	KIAA1045	3,46
DNAJB5	DnaJ (Hsp40) homolog, subfamily B, member 5	4.05
VCP	Valosin-containing protein	2.33
PIGO	Phosphatidylinositol glycan anchor biosynthesis, class O	2.18
UNC13B	Unc-13 homolog B (C. elegans)	4.73
RUSC2	RUN and SH3 domain containing 2	4.41
TESK1	Testis-specific kinase 1	6.76
CD72	CD72 molecule	85.15
SIT1	Signaling threshold regulating transmembrane adaptor 1	3.90
CCDC107	Coiled-coil domain containing 107	4.03

[1]Quotient (normalised LuCaP35 expression values divided with normalised LNCaP expression values).

5.3. Quantitative RT-PCR

The relative mRNA expression of genes *PRSS3*, *UBE2R2*, *UBAP2*, *WDR40A*, *UBAP1*, *C9orf25*, *DNAI1*, *C9orf165*, *DCTN3*, *GALT*, *IL11RA*, *CCL19*, *DNAJB5*, *VCP*, *PIGO*, *UNC13B*, *RUSC2*, *TESK1*, *CD72*, *SIT1*, *CCDC107*, and *KIAA1045* (Listed in table 6) were measured with quantitative real-time RT-PCR in 19 prostate cancer xenografts and in 7 prostate cancer cell lines. The expression values were normalised to the expression value of TBP. The genes' normalised expression values and their copy numbers are shown in appendix 2. In addition to melting curve analysis 1% agarose gel was run to check the specificity of the primers and ensure that only the correct size product(s) was amplified (data not shown). Grubb's test was used to detect the outliers and they are shown in appendix 2. The outliers for each gene were searched separately from normal and low-level amplification copy number groups.

5.4. The combined data from Q-RT-PCR and FISH results

Kruskal-Wallis test was used to examine the association between gene expression values and gene copy number status. Of the 22 genes studied, only one, *PIGO* (*p*-value 0.0468) (Figure 4), showed significant association between expression level and copy number. *UNC13B* (*p*-value 0.0826) (Figure 4), was classified as not quite significant. Rest of the genes showed no significant correlation. Despite of the poor *p*-values, some genes (Figure 4), such as *VCP*, *GALT*, *UBAP2*, *DCTN3* and *C9orf25*, showed nice correlation in graphs. Table 7 summarises the results obtained by comparing gene expression levels with copy number status.

Figure 4. (The graphs below) The graphs show the relative mRNA expression level versus gene copy number status. Genes *PIGO*, *UNC13B*, *VCP*, *GALT*, *UBAP2*, *C9orf25*, *DCTN3*, *RUSC2*, *CD72*, and *UBE2R2* showed the most promising correlation between gene expression levels and copy number state.

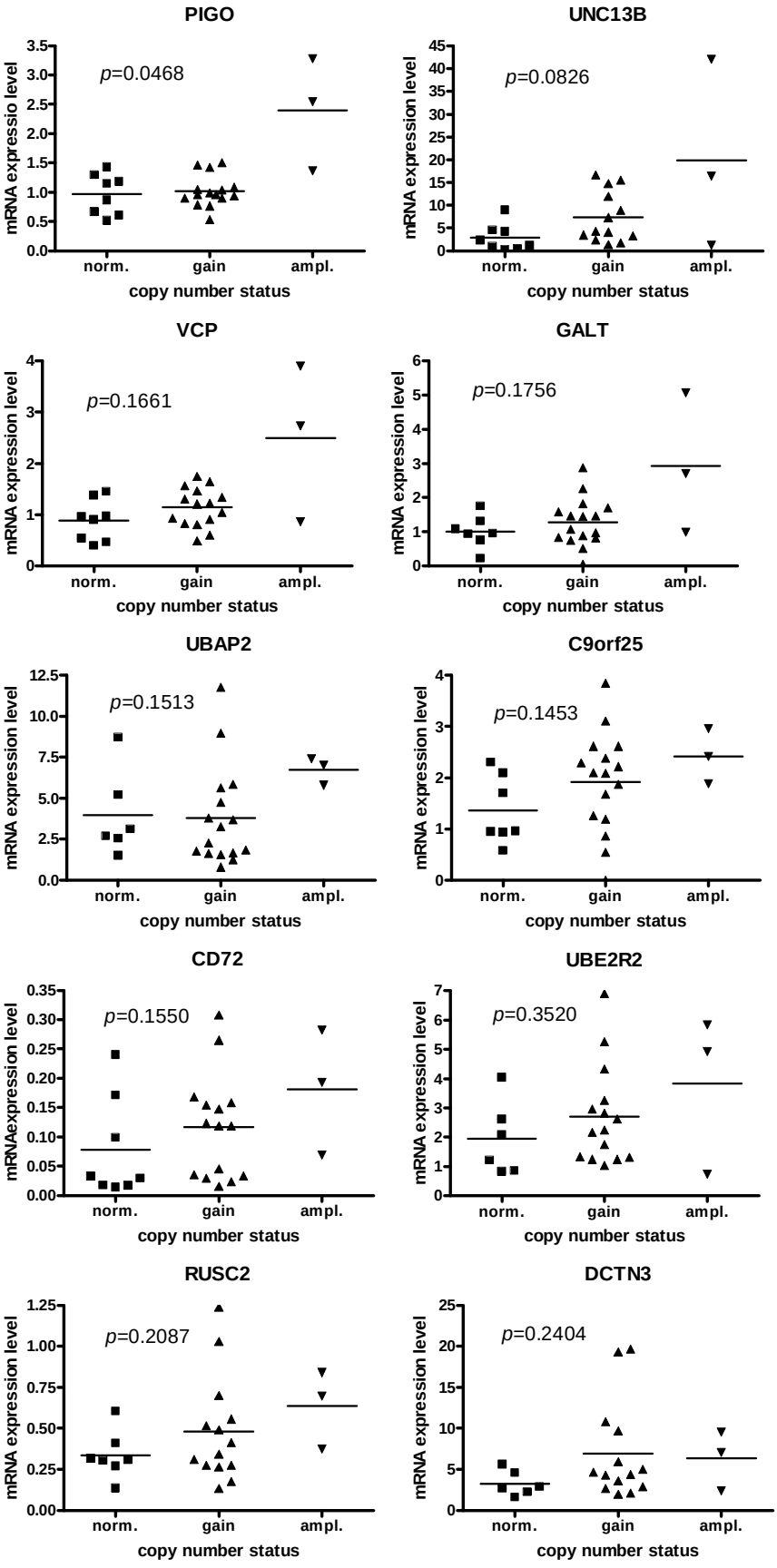
PIGO
p=0.0468
mRNA expressio level
copy number status
norm.
gain
ampl.
UNC13B
p=0.0826
mRNA expression level
VCP
p=0.1661
GALT
p=0.1756
UBAP2
p=0.1513
C9orf25
p=0.1453
CD72
p=0.1550
mRNAexpression level
UBE2R2
p=0.3520
RUSC2
p=0.2087
DCTN3
p=0.2404

Table 7. *Gene expression correlation with copy number status.*

Start/bp	Stop/bp	Gene symbol	Gene full name	GeneID	*p*-value[1]
33785559	33789228	***PRSS3***	Protease, serine, 3 (mesotrypsin)	5646	0.3404
33807182	33910401	***UBE2R2***	Ubiquitin-conjugating enzyme E2R 2	54926	0.3520
33911691	34038947	***UBAP2***	Ubiquitin associated protein 2	55833	0.1513
34076386	34116736	***WDR40A***	WD repeat domain 40A	25853	0.3835
34169011	34242521	***UBAP1***	Ubiquitin associated protein 1	51271	0.1475
34388182	34448568	***C9orf25***	Chromosome 9 open reading frame 25	203259	0.1453
34448811	34510982	***DNAI1***	Dynein, axonemal, intermediate chain 1	27019	0.4102
34511040	34513037	***C9orf165***	Chromosome 9 open reading frame 165	375704	0.9619
34603548	34610496	***DCTN3***	Dynactin 3 (p22)	11258	0.2404
34636635	34640574	***GALT***	Galactose-1-phosphate uridylyltransferase	2592	0.1756
34624719	34657384	***IL11RA***	Interleukin 11 receptor, alpha	3590	0.2741
34679567	34681274	***CCL19***	Chemokine (C-C motif) ligand 19	6363	0.9007
34925837	34972541	***KIAA1045***	KIAA1045	23349	0.9825
34979785	34988428	***DNAJB5***	DnaJ (Hsp40) homolog, subfamily B, member 5	25822	0.7141
35046560	35062564	***VCP***	Valosin-containing protein	7415	0.1661
35078688	**35086579**	***PIGO***	**Phosphatidylinositol glycan anchor biosynthesis, class O**	**84720**	**0.0468**
35152059	**35395332**	***UNC13B***	**Unc-13 homolog B (C. elegans)**	**10497**	**0.0826**
35480124	35551889	***RUSC2***	RUN and SH3 domain containing 2	9853	0.2087
35595281	35600038	***TESK1***	Testis-specific kinase 1	7016	0.9892
35599976	35608408	***CD72***	CD72 molecule	971	0.1550
35639295	35640947	***SIT1***	Signaling threshold regulating transmembrane adaptor 1	27240	0.7304
35648311	35651184	***CCDC107***	Coiled-coil domain containing 107	203260	0.4115

[1]The outliers were left out of the calculations.

5.5. FISH data from clinical prostate tumour specimens

134 prostatectomy tumours and 70 locally recurrent hormone-refractory prostate cancers were analysed, using BAC-clone RP11-165H19 as a probe, and centromere 9 as a reference. Amplifications were classified as for xenografts and cell lines.

Of the 134 prostatectomy tumours, 13 (9.7%) showed high-level amplification (Figure 6), 43 (32.1%) low-level amplification, and 78 (58.2%) had the normal copy number status. Of the 70 hormone-refractory prostate cancers analysed, high-level amplification was seen in 10 cases (14.3%) (Figure 7), low-level amplification in 31 cases (44.3%), and normal copy number status in 29 (41.4%) cases. Examination of the associations of clinicopathological variables with the 9p13.3 amplification showed that increased PSA levels were significantly associated with 9p13.3 amplification. Table 8 summarises the results of analysed clinical prostate cancer specimens, and the associations of clinicopathological variables with the 9p13.3 amplicon.

Kaplan-Meier survival analysis showed that the survival was slightly poorer for patients with high-level amplification than patients with no amplification or low-level amplification (Figure 5). However, the difference was not statistically significant ($p=0.1533$).

Table 8. *Association of clinicopathological variables with the 9p13.3 amplification.*

Variables		Normal	Low-level amplification	High-level amplification	*p*-value
Prostatectomy specimens		78/134 (58.2%)	43/134 (32.1%)	13/134 (9.7%)	
Hormone-refractory tumours		29/70 (41.4%)	31/70 (44.3%)	10/70 (14.3%)	0.0739[1]
Prostatectomy specimens:					
Gleason score:					
	<7	45 (34.1%)		3 (2.3%)	
	=7	63 (47.7%)		6 (4.5%)	
	>7	11 (8.3%)		4 (3.0%)	0.0614[1,2]
pT-stage:					
	pT2	82 (61%)		7 (5%)	
	pT3	39 (29%)		6 (4%)	0.3598[3]
PSA (mean±SD)		***16.91 ± 23.64***		***23.84 ± 15.75***	***0.0251[4]***
Ki-67 (mean±SD)		7.66 ± 10.23		11.00 ± 13.91	0.6304[4]
Age (mean±SD)		63.2 ± 4.7		62.9 ± 6.8	0.9132[4]
Tumour-% (mean±SD)		26.65 ± 16.68		32.29 ± 23.23	0.5542[4]

Results are shown as No (%).Examination of the association between gene expression and copy number status revealed that only high-level amplification had notable influence on gene expression. Because of that, normal and low-level amplification groups were integrated when the associations of clinicopathological variables were examined. [1]Chi-square test; [2]Chi-square calculations are not valid, because required conditions were not met (amount of samples too low in a few groups); [3]Fisher's exact test; [4]Mann-Whitney test.

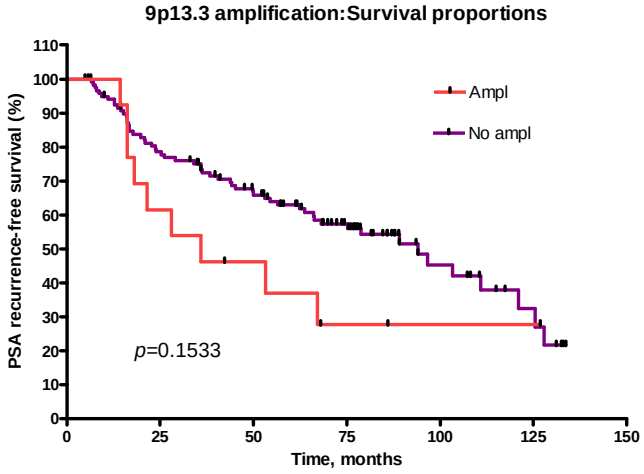

Figure 5. Kaplan-Meier survival analysis of progression-free survival of prostatectomy-treated patients. Examination of the association between gene expression and copy number status revealed that only high-level amplification had notable influence on gene expression. Because of that, normal and low level amplification groups were integrated, and handled as a no amplification group.

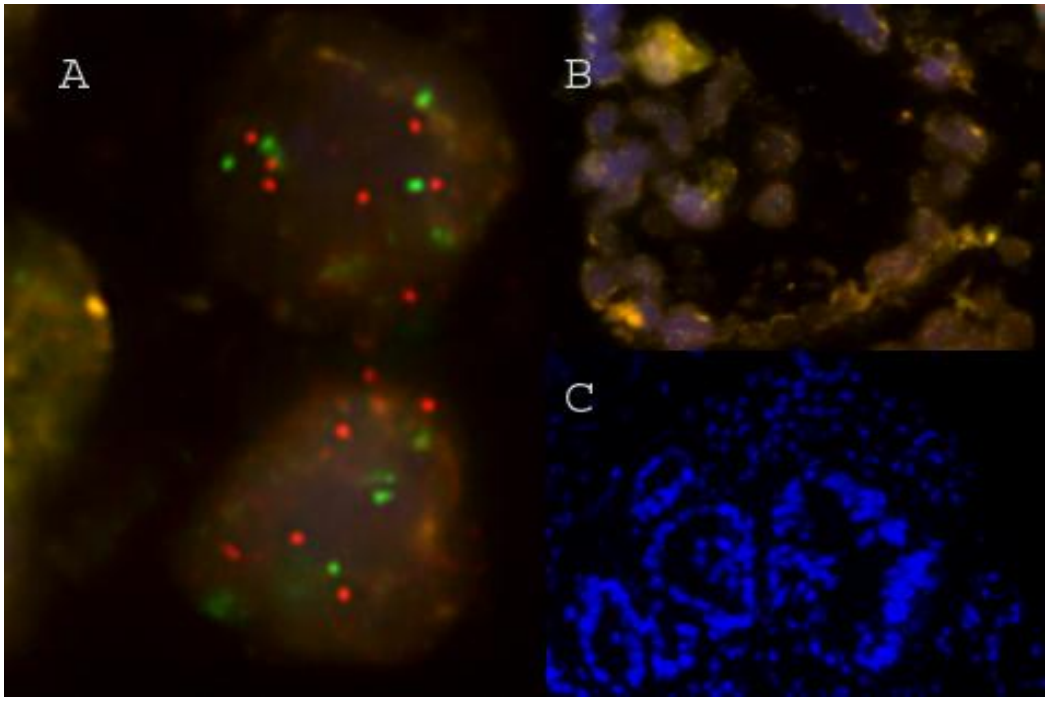

Figure 6. FISH for a prostatectomy sample. All three pictures are from the same spot. Closer view of cells with high-level amplification are seen in pictures A and B. Morphology of the sample is seen in picture C. BAC-clone RP11-165H19 was used as a probe, and centromere 9 as a reference. Probe signals are seen in red, centromere 9 signals in green.

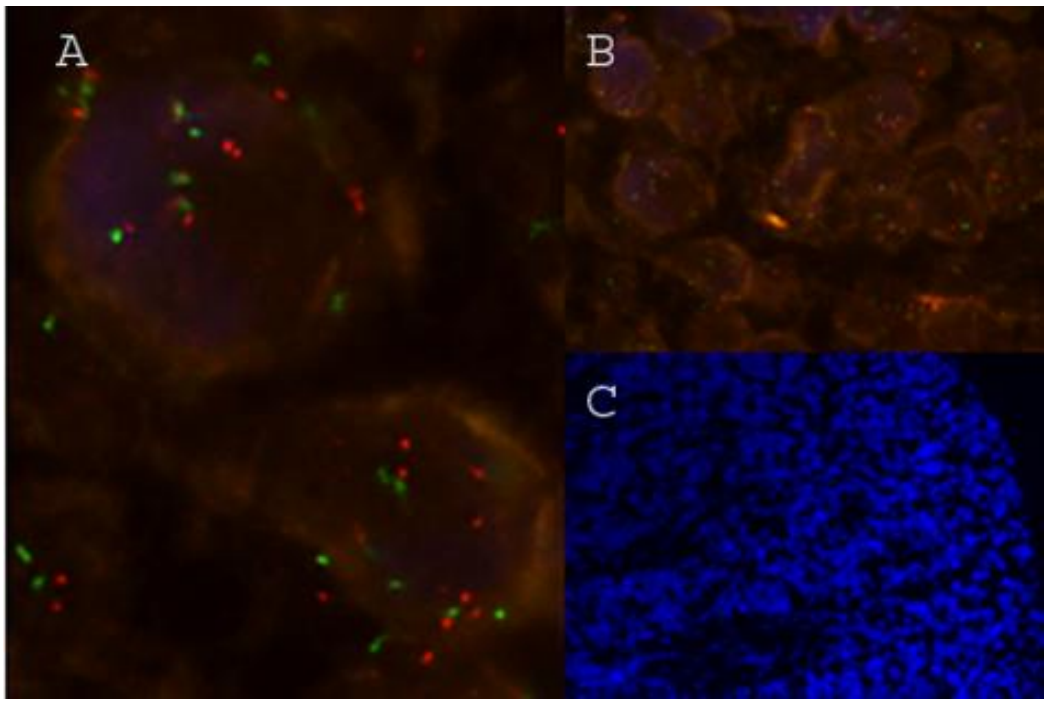

Figure 7. FISH for an HR tumour sample. All three pictures are from the same spot. Closer view of cells with high-level amplification are seen in pictures A and B. Morphology of the sample is seen in picture C. BAC-clone RP11-165H19 was used as a probe, and centromere 9 as a reference. Probe signals are seen in red, centromere 9 signals in green.

6. DISCUSSION

This research is based on Saramäki *et al*. (2006) study, where they performed aCGH to detect copy number alterations in 5 prostate cancer cell lines and 13 prostate cancer xenografts. They confirmed the previously identified common gains and losses (gains at 1q, 7, 8q, 16p, and 17q, and losses at 2q, 4p/q, 6q, 8p, 13q, 16q, 17p, and 18q). Because of the higher resolution of aCGH, they were able to narrow-down these minimal commonly altered regions and also to identify a new frequent gain at 9p13-q21. From this novel recurrent aberration they separated two independent regions, 9p13.3 (39%) and 9p13.1-q21.11 (33%). They observed that genes *UBE2R2*, *WDR40A* and *DCTN3* from 9p13.3 region showed significant association between increased copy number status and gene over-expression level. They also combined their expression and copy number data and came to the conclusion that even the low-level copy number alterations can have an effect on gene expression. This statement is especially interesting because high-level amplifications are rare in prostate cancer. According to their data the most common gains were at chromosomal arms 1q (44%), 7p (33%), 8q (67%), 9p/q (39%), 14q (39%), 16p (44%), and 17q(39%), and losses at 2q (44%), 4p/q (44%), 6q (44%), 8p (61%), 13q (66%), 16q (44%), 17p (39%), 18q (50%), and 22q (56%).

The gain at 9p13-q21 has been also reported by other studies. Nupponen *et al*. (1998) used chromosomal CGH (cCGH) to detect genetic alterations in prostate cancer cell lines PC-3, DU145, and LNCaP. From the cell line DU145 they found a gain at 9p13-q21. Paris *et al*. (2003) used aCGH and cCGH to study 20 formalin-fixed and paraffin-embedded prostate cancer samples. With aCGH they found gain at 9p12-p13 from one sample, and gain at 9p13-p21 from one sample. Also deletions to this region have been identified. von Knobloch *et al*. (2004) performed a microsatellite allelotyping of chromosomal regions, which are known to be involved in the development of prostate cancer. They analysed 100 paired normal and tumour DNA samples with markers for different chromosomes, and found out that the chromosomal arm 9p and the locus

9p21.3, near the 9p13.3 region, is crucial for prostate cancer progression. They observed that alterations at chromosomes 8p, 9p, 13q, and 17p were significantly associated with advanced tumour stage, and that the allelic imbalance (AI) at 5q and 9p were associated with regional lymph node metastasis.

The aim of this research was to examine the 9p13.3 amplification in 7 prostate cancer cell lines, 19 prostate cancer xenografts and in clinical prostate tumour specimens. The plan was to determine the boundaries of the 9p13.3 amplicon, define the frequencies and levels of 9p13.3 amplification in cell lines and xenografts, identify one or a few genes, whose amplified copy number is manifested as an elevated expression level, and examine whether this region is also amplified in clinical untreated primary prostate cancers and locally recurrent hormone-refractory prostate cancers. Equally comprehensive studies of this chromosomal region have not been published, and the intention was to search for potential target genes for this amplification.

6.1. Q-RT-PCR and FISH analyses for prostate cancer cell lines and xenografts

Proto-oncogenes are cell's normal genes, which code proteins that function, for example, in cell growth and differentiation. One way to transform proto-oncogene into oncogene is through gene amplification, which leads to over-expression of the gene product. Therefore oncogenes are usually assumed to lie in the gained chromosomal regions.

To study the correlation between gene over-expression and copy number, q-RT-PCR and FISH were used, respectively. Gene copy number status of the amplification was investigated by FISH. This method is an advantageous and quite easy method for detecting chromosomal alterations *in situ*, but on the other hand the resolution and reliability of the signals can sometimes be fairly poor. The treatments, required to allow

the probe to penetrate into the specimen and to minimize the background in sample, can be so severe that they can damage the morphology of the sample. In those cases the identification and visualisation of the signals from morphologically identifiable nuclei can be impossible. When these barriers are overcome, FISH is a good method for analysing chromosomal aberrations.

Gene over-expression was examined by q-RT-PCR, to detect potential target genes for this amplification. In q-RT-PCR, the measurement of relative mRNA level is based on the detection of the PCR-cycle in which the product amplification is in the exponential phase, and not in the amount of final product. This means that there is no rate limiting elements in the measurement. Quantitative RT-PCR is nowadays considered as a highly quantitative and reliable method. Problems can come along the sensitivity of the method and specificity of primers. However these issues are usually solved with good primer designing and adjusting the parameters of the PCR reaction. When all functions properly this method it is extremely easy, fast, and sensitive working method to use.

In this study, prostate cancer cell lines and xenografts were used, most of which are established from hormone-refractory metastatic prostate cancers. Both of these sample materials are epithelial origin. Xenografts represent the clinical prostate tumours more closely, and they contain chromosomal alterations, which are typical for clinical prostate cancer. The benefit of using these cell lines and xenografts in research studies is that they are not contaminated by normal cells, and they are quite informative of this disease. Despite these good aspects it should be kept in mind that they do not really represent the true frequencies in clinical prostate cancers. Cell lines can also contain artefacts, emerged from cell culturing. Also worth of notice is the fact that cell lines and xenografts represent quite different kind of sample material and comparing them with each other and with clinical samples should be done with caution.

FISH analyses revealed that of 19 xenograft samples, 3 showed high-level amplification, low-level amplification was seen in 10 cases, and 6 had the normal copy number status. Of the three samples with high-level amplification, LuCaPs 35 and 41 showed more amplified 9p13.3 region. With LuCaP35, 6 to 15 probe signals and 3 to 4

centromere 9 signals were detected, whereas with LuCaP41, 7 to 15 probe signals and 2 to 3 centromere 9 signals were detected. LuCaP58 showed 5 to 7 probe signals and 4 centromere 9 signals, which is classified as a high-level amplification, but cannot be considered to belong in the same group as LuCaPs 35 and 41. LuCaP35 is known to be originated from prostate cancer lymph node metastases, and it is hormone sensitive tumour sample expressing both AR and PSA. LuCaP41 is a hormone-refractory, primary tumour sample. LuCaP58 is derived from lymph node prostate cancer metastasis prior to hormonal therapy, and it is hormone sensitive sample expressing both AR and PSA. Low-level amplification was observed in LuCaPs 69, 70, 77, 81, 86.2, 92.1, 93, 96, 105, and 115. Normal copy number status was seen in LuCaPs 23.1, 23.8, 23.12, 49, 73, and 78, of which LuCaPs 23.1, 23.8, and 23.12 are derived from the same patient's different metastases.

Of the prostate cancer cell lines used in this study none, showed the high-level amplification. Low-level amplification was detected in DU145, LAPC-4, LNCaP, 22RV1, VCaP, and DuCaP. Saramäki *et al*. (2006) identified in their study by aCGH that in 22RV1 the gain is slightly distal to the minimal region detected, they also confirmed it by FISH. The cell line PC-3 showed normal copy number status.

The gain of 9p13.3 region was defined with the help of LuCaP35. In this sample the BAC-clones RP11-326F20 and RP11-112J3 showed only 3 probe signals, while the BAC-clones between them (RP11-115O15, RP11-176F3, RP11-165H19, RP11-182N22, RP11-201P13, and RP11-156G14) showed 6 to 15 probe signals. In all hybridisations three to four centromere 9 signals were seen. This minimal region was then taken into closer examination. Genes distal from BAC-clone RP11-112J3 and proximal to RP11-326F20 were screened, and with those that passed, the q-RT-PCR reaction was preformed in all the xenografts and cell lines.

To examine the effect of copy number on gene expression, samples were subdivided into three different groups depending on the copy number in that particular gene area investigated. The groups were normal, gain (low-level amplification), and amplification (high-level amplification). Kruskal-Wallis test was used to test the association between

copy number and gene expression. Both xenografts and cell lines were handled together, in order to have sufficiently large sample size. Of 22 genes only one, *PIGO*, showed significant association ($p<0.05$) between copy number and gene expression. *UNC13B* was classified as not quite significant ($0.1<p>0.05$), and the rest of the genes were classified as not significant ($p>0.1$). When looking only at these *p*-values the error happens in the fact that LuCaP58 is not actually as amplified as LuCaPs 35 and 41. Deviations also occur when cell lines and xenografts are processed together. Concentrating only to gene expression and copy number data, and overlooking the *p*-values there appeared some other genes (in addition to these two just mentioned), which showed nice correlation between copy number and expression level (Figure 4).

In this study mRNA expression levels were examined. It would be interesting to see whether these raised mRNA expression levels would also increase the protein levels. And if that is the case how much of the protein over-expression is caused by the gene amplification. Overall, it is important to examine the function of these genes in prostate cancer development, and to find the real target gene of the 9p13.3 amplicon. Following is a review of genes, which were the most promising when the correlation between gene expression level and copy number status was measured.

PIGO (phosphatidylinositol glycan anchor biosynthesis, class O) encodes a protein that takes part in glycosylphosphatidylinositol (GPI)-anchor biosynthesis, which anchors proteins to the cell surface. The encoded protein functions in the transfer of ethanolaminephosphate (EtNP) to the third mannose in GPI (Hong *et al*., 2000). Other members of the GPI –anchor biosynthesis pathway have been implicated to contribute in the development of other human cancers. The human GPI transmidase complex is known to contain five subunits: *PIGT* (phosphatidylinositol glycan anchor biosynthesis, class T, at 21q12-q13.12), *GPAA1* (glycosylphosphatidylinositol anchor attachment protein 1 homolog, at 8q24.3), *PIGK* (phosphatidylinositol glycan anchor biosynthesis, class K, at 1p31.1), *PIGS* (phosphatidylinositol glycan anchor biosynthesis, class S, at 17p13.2), and *PIGU* (phosphatidylinositol glycan anchor biosynthesis, class U, at 20q11.22). Guo *et al.* (2004) identified *PIGU* (phosphatidylinositol glycan anchor biosynthesis) as a promising oncogene in human bladder cancer. Wu *et al*. (2006)

identified *PIGT*, *GPAA1*, and *PIGU* as candidate oncogenes in breast cancer development. In addition, they found correlation between gene expression level and the copy number for each gene.

UNC13B is a cytosolic diacylglycerol (DAG)-binding protein. In response to DAG activation *UNC13B* translocates in the Golgi. UNC13B belongs to the UNC13/munc13 family of proteins, which are known to function in the regulation of cell secretion at the plasma membrane as part of postsynaptic transmission of neurotransmitters, insulin secretion, and secretion of lysosomes (Speight *et al*., 2005). Over-expression of UNC13 proteins can lead to increased synaptic transmission, whereas decreased expression almost completely eliminates the synaptic vesicle (SV) priming and fusion (McEwen *et al*., 2006). *UNC13B* is also known to be up-regulated and activated by hyperglycemia (NCBI, Entrez Gene database).

VCP (valosin-containing protein) encodes a protein that is implicated in many cellular events that are regulated during mitosis (e.g. homotypic membrane fusion, spindle pole body function, ubiquitin-dependent protein degradation). It is known to interact with e.g. clathrin, heat-shock protein Hsc70, and BRCA1 (the breast/ovarian cancer susceptibility gene product). High *VCP* expression levels have been detected in e.g. non-small-cell lung carcinomas (Yamamoto *et al*., 2004), gastric carcinomas (Yamamoto *et al*., 2003), pancreatic ductal adenocarcinomas (Yamamoto *et al*., 2004), colorectal carcinomas (Yamamoto *et al*., 2004), and prostate cancer (Tsujimoto et al., 2004). Noteworthy, all these results are from the same research study group, and other independent studies are waited to confirm these findings. It is also proposed that the VCP is one of the targets of Akt signalling in cell survival (e.g. Vandermoere *et al*., 2006).

CD72, B cell surface molecule and a type II transmembrane glycoprotein, has been shown to be tyrosine-phosphorylated and to recruit SH2-containing tyrosine phosphatese-1, when cross-linking of the Ag receptor of B cells (BCR). BCR activates the cytoplasmic kinase Syk and Src-family kinases, which then activates various signalling cascades, affecting cell proliferation, anergy, or apoptosis (Adachi *et al*.,

2000). This BCR signalling is regulated either positively or negatively. It has been observed that CD72 negatively regulates the BCR signalling and in that way promotes cell cycle arrest and cell death in primary mature B cells (Adachi *et al*., 2000; Li *et al*., 2006).

GALT (galactose-1-phosphate uridylyltransferase) encodes a protein, which catalyses the second step of the Leloir pathway of galactose metabolism. In humans the absence of this enzyme causes the classic galactosemia. The deficient galactose metabolism has been proposed as a risk factor for ovarian cancer and also for endometriosis, based on the knowledge that galactose causes ovarian failure and that ovarian cancer arises from premature ovarian failure (Cramer *et al*., 1996; Morland *et al*., 1998). The results obtained from studies examining the association between improper galactose metabolism and ovarian cancer have been controversial, where some studies have seen association, while others have not.

DCTN3 (dynactin 3 (p22)) encodes the smallest subunit of dynactin, and it exists only as a part of this complex. The macromolecule complex (consisting of 10 subunits) binds to both microtubules and cytoplasmic dynein, and has a role in many cellular functions, such as ER-to-Golgi transport, the centripetal movement of lysosomes and endosomes, spindle formation, cytokinesis, chromosome movement, nuclear positioning, and axonogenesis (NCBI, Entrez Gene database).

UBE2R2 (ubiquitin-conjugating enzyme E2R 2) encodes a protein, which is similar to the E2 ubiquitin conjugating enzyme CDC34. CDC34 catalyses the polyubiquitination reaction mediated by the SCF (Skp1.CUL1.F-box) protein E3 ubiquitin ligase (Gazdoiu *et al*., 2005; Semplici *et al*., 2002). It is suggested that CK2-dependent phosphorylation of UBE2R2 functions by regulating β-TrCP substrate recognition, enhancing β-catenin degradation (Semplici *et al*., 2002).

UBAP1 (ubiquitin associated protein 1) belongs to the UBA domain (ubiquitin associated domain) family, which includes proteins that have connections to ubiquitin and the ubiquitination pathway. Its exact function is unknown. In nasopharyngeal

carcinoma (NPC) LOH has been frequently detected in this chromosomal locus and *UBAP1* has been proposed as target gene. It is also suggested that decreased expression of UBAP1 protein could have a role in progression of NPC (Xiao *et al*., 2006).

UBAP2 (ubiquitin associated protein 2) encodes a protein that contains a UBA domain and also a region that is similar to a domain found in members of the atrophin-1 family. The function of this protein is unknown. *C9orf25*, chromosome 9 open reading frame 25, encodes a protein that has a putative prenyl group binding site (CAAX box) at its C-terminus. The function of this protein is unknown (NCBI, Entrez Gene database).

RUSC2 (RUN and SH3 domain containing 2) encodes a protein that may function as a link between the targeting of ER derived vesicles, triggered by the rab1 and a signalling pathway regulated by molecules containing the SH3 or poly-proline regions. Rab1 is a small GTPase (two isoforms (a and b), which encodes proteins localised on ER-Golgi membranes, regulating the exocytotic transport from the ER to the Golgi apparatus (Bayer *et al*., 2005).

6.2. FISH data from clinical prostate tumour specimens

The clinical samples were examined by FISH to acquire real 9p13.3 amplicon frequencies in prostate cancer. The disadvantage with clinical samples is that cancer cells are surrounded by normal cells, and it should be controlled that only cancer cells are examined. Problems came with samples' thickness, which complicated the penetration of the probes and with proteins, which disturbed probes from working properly. Removal of proteins demanded extremely hard treatments, sometimes damaging the morphology of the specimen, and making the identification of individual nuclei laborious. Regardless of these obstacles, clinical samples were quite informative about the 9p13.3 amplification frequencies occurring in prostate cancer.

From untreated prostatectomy tumours 9.7% showed high-level amplification, 32.1% low-level amplification, and 58.2% had normal copy number status. From hormone-refractory tumours 14.3% showed high-level amplification, 44.3% low-level amplification, and 41.4% had the normal copy number status. These results suggest that the 9p13.3 amplification was slightly more common in HR tumours than in untreated prostatectomy samples. In addition, in both prostatectomy and HR tumours high-level amplifications were observed, suggesting that this genetic aberration is relatively early event but maybe associated with aggressive behaviour of the disease (hormone-refractory prostate tumours represent the advanced state of the disease and it is assumed that they contain the highest amount of genetic alterations).

Examination of the associations of clinicopathological variables with the 9p13.3 amplification showed that increased PSA levels were significantly associated with 9p13.3 amplification (p=0.0251). To determine is this amplification associated with poor progression-free survival of prostatectomy-treated patients Kaplan-Meier survival analyses was done. Despite of the not significant p-value, 0.1533, it showed that there is a trend that the progression-free survival was slightly poorer for patients with high-level amplification than patients with no amplification or low-level amplification. Weak point of this analysis was that there were only few samples in the amplification group, and more samples should be examined to study the progression-free survival in more detail. In future it would be interesting to determine whether this amplification could work as a prognosis marker to be used in detecting new cancer cases, which are clinically significant and require aggressive treatment.

7. CONCLUSIONS

One of the main focuses of this study was to identify one or a few genes, whose amplified copy number state increases its mRNA expression levels. The most significant correlation between copy number and expression level was seen in *PIGO*, which encodes a protein that takes part in glycosylphosphatidylinositol (GPI)-anchor biosynthesis. Other genes showing correlation were *UNC13B*, *CD72*, *VCP*, *GALT*, *DCTN3*, *C9orf25*, *UBAP2*, *RUSC2*, and *UBE2R2*. Although this examination is not enough in identification of real target genes, the further investigation of the function of these genes in prostate cancer should be considered, and they should be thought of as putative target genes of 9p13.3 amplicon. If confirmed as a target gene, it may be used as a prognostic or diagnostic marker in prostate cancer.

The other important aim of this study was to examine the occurrence of 9p13.3 amplicon in clinical prostate cancers. The gain was slightly more common in locally recurrent hormone-refractory prostate cancers than in untreated prostatectomy tumours. The frequencies of high- and low-level amplifications were in HR cancers 58.6% and in prostatectomy tumours 39.9%. High-level amplifications were seen in both, HR (14.3%) and prostatectomy specimens (9.2%). These results suggest that this genetic aberration is relatively early event but maybe associated with aggressive behaviour of the disease. Survival analysis revealed a trend of association between the 9p13.3 amplification and poor progression-free survival of prostatectomy-treated patients. In addition, PSA levels were significantly increased in prostatectomy-treated patients with 9p13.3 amplification.

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9. APPENDICES

Appendix 1: Solutions used in FISH.

Denaturation solution:
700 ml formamide (Flug. 476718)
100 ml 20xSSC
pH adjusted to 7
water added to the volume 1000 ml

MM1.0:
1g dextran sulphate (MW 500 000)
5 ml formamide (high-grade)
1 ml 20xSSC
pH adjusted to 7
water added to the volume 7 ml

PN-buffer pH8:
0.1 M NaH_2PO_4
0.1M Na_2HPO_4
0.1% NP-40
A: 13.8 g NaH_2PO_4 and water added to 1000 ml
B: 89 g Na_2HPO_4 and water added to 5000 ml
pH of solution B adjusted to 8 with solution A.
To this buffer (pH 8) NP-40 added to 0.1% concentration.

pH-9 salt solution:
0,05M Tris-HCl
0,01M EDTA)

Appendix 2: mRNA expression levels and copy numbers in prostate cancer xenografts and cell lines. Tables are in alphabetical order according to genes.

Table 9. *C9orf25.*

Sample	mRNA expression	Copy number	Sample	mRNA expression	Copy number
LuCaP23.1	0.939	2	LuCaP92.1	2.088	3
LuCaP23.8	2.302	2	LuCaP93	1.195	3
LuCaP23.12	1.705	2	LuCaP96	2.218	3-4
LuCaP35	***2.413***	***10-13***	LuCaP105	3.849	3-5
LuCaP41	***2.965***	***7-10***	LuCaP115	2.380	3
LuCaP49	0.962	2			
LuCaP58	***1.879***	***5-7***			
LuCaP69	0.546	3	DU145	2.290	4
LuCaP70	1.873	3-4	DuCaP	2.094	4
LuCaP73	0.578	2	VCaP	2.616	4
LuCaP77	2.614	3	LNCaP	0.000	4
LuCaP78	2.094	2	22Rv1	0.865	3
LuCaP81	1.256	3	PC-3	0.948	2
LuCaP86.2	1.674	4	LAPC-4	3.104	3

Table 10. *C9orf165.*

Sample	mRNA expression	Copy number	Sample	mRNA expression	Copy number
LuCaP23.1	2.613	2	LuCaP92.1	8.885	3
LuCaP23.8	5.720	2	LuCaP93	11.524	3
LuCaP23.12	6.222	2	LuCaP96*	28.491	3-4
LuCaP35	***3.694***	***10-13***	LuCaP105	22.039	3-5
LuCaP41	***3.589***	***7-10***	LuCaP115	8.208	3
LuCaP49	2.797	2			
LuCaP58	***2.990***	***5-7***			
LuCaP69	4.258	3	DU145	0.00002	4
LuCaP70	6.797	3-4	DuCaP	0.02452	4
LuCaP73	0.867	2	VCaP	0.00827	4
LuCaP77	1.412	3	LNCaP	0.00005	4
LuCaP78	6.160	2	22Rv1	0.00930	3
LuCaP81	6.698	3	PC-3	0.23563	2
LuCaP86.2	1.908	4	LAPC-4	0.08129	3

*Outlier.

Table 11. *CCDC107.*

Sample	mRNA expression	Copy number	Sample	mRNA expression	Copy number
LuCaP23.1	0.556	2	LuCaP92.1	1.041	3
LuCaP23.8	1.451	2	LuCaP93	0.818	3
LuCaP23.12	0.972	2	LuCaP96	1.793	3-4
LuCaP35	1.217	3	LuCaP105	2.759	3-4
LuCaP41	***3.033***	***8-6***	LuCaP115	1.523	3
LuCaP49	0.664	2			
LuCaP58	***0.722***	***5***			
LuCaP69	1.156	3	DU145	0.257	4
LuCaP70	1.119	3-4	DuCaP	0.153	3-4
LuCaP73	0.411	2	VCaP	0.131	4
LuCaP77	0.719	3	LNCaP	0.211	4
LuCaP78	0.662	2	22Rv1*	2.565	2
LuCaP81	1.452	3	PC-3	0.269	2
LuCaP86.2	0.424	4	LAPC-4	0.196	3

*Outlier.

Table 12. *CCL19.*

Sample	mRNA expression	Copy number	Sample	mRNA expression	Copy number
LuCaP23.1	0.009	2	LuCaP92.1	0.044	3
LuCaP23.8*	0.140	2	LuCaP93	0.037	3
LuCaP23.12	0.034	2	LuCaP96	0.054	3-4
LuCaP35	***0.013***	***10-13***	LuCaP105	0.079	3-5
LuCaP41	***0.021***	***7-10***	LuCaP115	0.088	3
LuCaP49	0.040	2			
LuCaP58	***0.022***	***5-7***			
LuCaP69	0.029	3	DU145	0.009	4
LuCaP70	0.025	3-4	DuCaP	0.002	4
LuCaP73	0.011	2	VCaP	0.003	4
LuCaP77	0.017	3	LNCaP	0.004	4
LuCaP78	0.024	2	22Rv1	0.002	3
LuCaP81	0.038	3	PC-3	0.001	2
LuCaP86.2	0.016	4	LAPC-4	0.001	3

*Outlier.

Table 13. *CD72.*

Sample	mRNA expression	Copy number	Sample	mRNA expression	Copy number
LuCaP23.1	0.017	2	LuCaP92.1	0.015	3
LuCaP23.8	0.018	2	LuCaP93	0.158	3
LuCaP23.12	0.033	2	LuCaP96	0.123	3-4
LuCaP35	***0.193***	***6-10***	LuCaP105	0.046	3-5
LuCaP41	***0.282***	***8-10***	LuCaP115	0.030	3
LuCaP49	0.030	2			
LuCaP58	***0.070***	***5-7***			
LuCaP69	0.119	3	DU145	0.309	4
LuCaP70	0.147	3-4	DuCaP	0.035	4
LuCaP73	0.014	2	VCaP	0.119	4
LuCaP77	0.024	3	LNCaP	0.155	4
LuCaP78	0.240	2	22Rv1	0.099	2
LuCaP81	0.168	3	PC-3	0.171	2
LuCaP86.2	0.265	4	LAPC-4	0.033	3

Table 14. *DCTN3.*

Sample	mRNA expression	Copy number	Sample	mRNA expression	Copy number
LuCaP23.1	2.235	2	LuCaP92.1	4.637	3
LuCaP23.8*	17.706	2	LuCaP93	4.282	3
LuCaP23.12	5.616	2	LuCaP96	9.689	3-4
LuCaP35	***9.552***	***10-13***	LuCaP105	19.312	3-5
LuCaP35V	***7.050***	***10-13***	LuCaP115	10.836	3
LuCaP49	2.878	2			
LuCaP58	***2.380***	***5-7***			
LuCaP69	5.928	3	DU145	2.122	4
LuCaP70	5.002	3-4	DuCaP	-	-
LuCaP73	2.679	2	VCaP	-	-
LuCaP77	4.371	3	LNCaP	2.688	4
LuCaP78	4.593	2	22Rv1	19.691	3
LuCaP81	3.595	3	PC-3	1.609	2
LuCaP86.2	1.962	4	LAPC-4	2.900	3

*Outlier.

Table 15. *DNAI1.*

Sample	mRNA expression	Copy number	Sample	mRNA expression	Copy number
LuCaP23.1	0.039	2	LuCaP92.1	0.302	3
LuCaP23.8*	0.865	2	LuCaP93	0.218	3
LuCaP23.12	0.185	2	LuCaP96	0.190	3-4
LuCaP35	***0.168***	***10-13***	LuCaP105*	2.890	3-5
LuCaP35V	***0.390***	***10-13***	LuCaP115	1.329	3
LuCaP49	0.034	2			
LuCaP58	***0.016***	***5-7***			
LuCaP69	0.075	3	DU145	0.061	4
LuCaP70	0.191	3-4	DuCaP	-	-
LuCaP73	0.168	2	VCaP	-	-
LuCaP77	0.070	3	LNCaP	0.021	4
LuCaP78	0.077	2	22Rv1	0.206	3
LuCaP81	0.043	3	PC-3	0.009	2
LuCaP86.2	0.036	4	LAPC-4	0.005	3

*Outliers.

Table 16. *DNAJB5.*

Sample	mRNA expression	Copy number	Sample	mRNA expression	Copy number
LuCaP23.1	0.134	2	LuCaP92.1	0.183	3-4
LuCaP23.8	0.357	2	LuCaP93	0.627	3
LuCaP23.12	0.295	2	LuCaP96	0.270	3-4
LuCaP35	***0.175***	***10-12***	LuCaP105	0.749	3-5
LuCaP41	***0.321***	***8-10***	LuCaP115	0.288	3
LuCaP49	0.182	2			
LuCaP58	***0.344***	***5-7***			
LuCaP69	0.162	3	DU145	0.060	4
LuCaP70	1.129	3-4	DuCaP	0.257	4
LuCaP73	0.330	2	VCaP	0.076	4
LuCaP77	0.370	3	LNCaP	0.932	4
LuCaP78	0.326	2	22Rv1	0.060	2
LuCaP81	0.574	3	PC-3	0.088	2
LuCaP86.2	0.172	4	LAPC-4	0.111	3

Table 17. *GALT.*

Sample	mRNA expression	Copy number	Sample	mRNA expression	Copy number
LuCaP23.1	0.954	2	LuCaP92.1	1.443	3
LuCaP23.8	1.753	2	LuCaP93	0.513	3
LuCaP23.12	1.081	2	LuCaP96	1.818	3-4
LuCaP35	***5.079***	***10-13***	LuCaP105	1.699	3-5
LuCaP41	***2.703***	***7-10***	LuCaP115	2.258	3
LuCaP49	0.932	2			
LuCaP58	***0.993***	***5-7***			
LuCaP69	1.457	3	DU145	0.831	4
LuCaP70	1.587	3-4	DuCaP	0.742	4
LuCaP73	0.757	2	VCaP	0.816	4
LuCaP77	2.875	3	LNCaP	0.882	4
LuCaP78	1.309	2	22Rv1	1.072	3
LuCaP81	1.458	3	PC-3	0.217	2
LuCaP86.2	0.972	4	LAPC-4	0.050	3

Table 18. *IL11RA.*

Sample	mRNA expression	Copy number	Sample	mRNA expression	Copy number
LuCaP23.1	10.882	2	LuCaP92.1	2.702	3
LuCaP23.8	6.362	2	LuCaP93	3.662	3
LuCaP23.12	5.153	2	LuCaP96	4.098	3-4
LuCaP35	***2.440***	***10-13***	LuCaP105	7.035	3-5
LuCaP41	***3.021***	***7-10***	LuCaP115	1.211	3
LuCaP49	2.546	2			
LuCaP58	***1.868***	***5-7***			
LuCaP69	2.491	3	DU145	0.249	4
LuCaP70	2.139	3-4	DuCaP	1.060	4
LuCaP73	0.954	2	VCaP	0.855	4
LuCaP77	1.536	3	LNCaP	0.598	4
LuCaP78	2.302	2	22Rv1	0.707	3
LuCaP81*	13.078	3	PC-3	0.654	2
LuCaP86.2	0.875	4	LAPC-4	0.641	3

*Outlier.

Table 19. *KIAA1045.*

Sample	mRNA expression	Copy number	Sample	mRNA expression	Copy number
LuCaP23.1	0.112	2	LuCaP92.1	0.047	3
LuCaP23.8	0.208	2	LuCaP93	0.047	3
LuCaP23.12	0.122	2	LuCaP96	0.076	3-4
LuCaP35	***0.387***	***10-12***	LuCaP105	0.245	3-5
LuCaP41	***0.078***	***8-10***	LuCaP115	0.222	3
LuCaP49	0.048	2			
LuCaP58	***0.095***	***5-7***			
LuCaP69	0.088	3	DU145	0.369	4
LuCaP70	0.391	3-4	DuCaP	0.661	4
LuCaP73	0.044	2	VCaP	0.651	4
LuCaP77	0.057	3	LNCaP	0.427	4
LuCaP78	0.435	2	22Rv1	0.240	2
LuCaP81	0.092	3-4	PC-3	0.293	2
LuCaP86.2	0.054	4	LAPC-4	0.332	3

Table 20. *PIGO.*

Sample	mRNA expression	Copy number	Sample	mRNA expression	Copy number
LuCaP23.1	1.435	2	LuCaP92.1	1.423	3-4
LuCaP23.8	0.608	2	LuCaP93	0.958	3
LuCaP23.12	1.187	2	LuCaP96	0.962	3-4
LuCaP35	***3.276***	***10-12***	LuCaP105	0.992	3-5
LuCaP41	***2.547***	***8-10***	LuCaP115	1.468	3
LuCaP49	0.665	2			
LuCaP58	***1.367***	***5-7***			
LuCaP69	1.050	3	DU145	0.898	4
LuCaP70	0.902	3-4	DuCaP	1.084	4
LuCaP73	1.298	2	VCaP	1.044	4
LuCaP77	1.504	3	LNCaP	0.767	4
LuCaP78	1.150	2	22Rv1	0.869	2
LuCaP81	0.938	3	PC-3	0.520	2
LuCaP86.2	0.786	4	LAPC-4	0.532	3

Table 21. *PRSS3.*

Sample	mRNA expression	Copy number	Sample	mRNA expression	Copy number
LuCaP23.1	0.588	2	LuCaP92.1	1.839	3-4
LuCaP23.8	1.418	3	LuCaP93	1.608	3
LuCaP23.12	1.707	2	LuCaP96	3.440	3-4
LuCaP35	***1.081***	***12-15***	LuCaP105	8.053	3-5
LuCaP41	***3.036***	***10-15***	LuCaP115	1.737	3
LuCaP49	0.816	2			
LuCaP58	***1.101***	***5-7***			
LuCaP69	1.321	3	DU145	0.505	2
LuCaP70	1.155	3-4	DuCaP*	12.349	4
LuCaP73	0.474	2	VCaP	0.018	4
LuCaP77	0.750	3	LNCaP	0.320	4
LuCaP78	0.892	2	22Rv1	0.370	3
LuCaP81	0.645	3	PC-3	0.063	2
LuCaP86.2	0.458	4	LAPC-4	0.300	3

*Outlier.

Table 22. *RUSC2.*

Sample	mRNA expression	Copy number	Sample	mRNA expression	Copy number
LuCaP23.1	0.270	2	LuCaP92.1	0.518	3-4
LuCaP23.8	0.411	2	LuCaP93	0.413	3
LuCaP23.12	0.305	2	LuCaP96	1.238	3-4
LuCaP35	***0.840***	***6-10***	LuCaP105	0.557	3-5
LuCaP41	***0.697***	***8-10***	LuCaP115	0.492	3
LuCaP49	0.605	2			
LuCaP58	***0.373***	***5-7***			
LuCaP69	0.341	3	DU145*	1.809	4
LuCaP70	0.277	3-4	DuCaP	0.176	4
LuCaP73	0.308	2	VCaP	0.312	4
LuCaP77	1.028	3	LNCaP	0.700	4
LuCaP78	0.318	2	22Rv1	0.136	2
LuCaP81	0.265	3	PC-3*	1.383	2
LuCaP86.2	0.276	4	LAPC-4	0.134	3

*Outliers.

Table 23. *SIT1.*

Sample	mRNA expression	Copy number	Sample	mRNA expression	Copy number
LuCaP23.1	0.629	2	LuCaP92.1	0.837	3-4
LuCaP23.8	0.789	2	LuCaP93	0.630	3
LuCaP23.12	0.612	2	LuCaP96	0.867	3-4
LuCaP35	0.660	3	LuCaP105	0.731	3-4
LuCaP41	***1.272***	***8-6***	LuCaP115	0.460	3
LuCaP49	0.381	2			
LuCaP58	***0.580***	***5***			
LuCaP69	0.434	3	DU145	0.296	4
LuCaP70	0.601	3-4	DuCaP	0.594	4
LuCaP73	0.619	2	VCaP	0.290	4
LuCaP77	0.600	3	LNCaP	0.386	4
LuCaP78	1.303	2	22Rv1	0.222	2
LuCaP81	0.766	3	PC-3	0.122	2
LuCaP86.2	0.676	4	LAPC-4	0.340	3

Table 24. *TESK1.*

Sample	mRNA expression	Copy number	Sample	mRNA expression	Copy number
LuCaP23.1	2.077	2	LuCaP92.1	3.075	3-4
LuCaP23.8	4.707	2	LuCaP93	2.563	3
LuCaP23.12	4.595	2	LuCaP96	5.148	3-4
LuCaP35	***1.877***	***6-10***	LuCaP105	6.527	3-5
LuCaP41	***4.315***	***8-10***	LuCaP115	5.720	3
LuCaP49	2.421	2			
LuCaP58	***1.811***	***5-7***			
LuCaP69	3.605	3	DU145	0.364	4
LuCaP70	2.724	3-4	DuCaP	0.650	4
LuCaP73	1.266	2	VCaP	0.879	4
LuCaP77	2.331	3	LNCaP	0.410	4
LuCaP78	2.663	2	22Rv1	0.490	2
LuCaP81	3.403	3	PC-3	0.857	2
LuCaP86.2	0.668	4	LAPC-4	0.704	3

Table 25. *UBAP1.*

Sample	mRNA expression	Copy number	Sample	mRNA expression	Copy number
LuCaP23.1	1.593	2	LuCaP92.1	1.476	3
LuCaP23.8	4.038	3	LuCaP93	3.017	3
LuCaP23.12	2.134	2	LuCaP96	1.828	3-4
LuCaP35	***2.214***	***12-15***	LuCaP105	4.671	3-5
LuCaP41	***2.239***	***10-15***	LuCaP115	1.804	3
LuCaP49	1.422	2			
LuCaP58	***1.592***	***5-7***			
LuCaP69	2.035	3	DU145	4.849	4
LuCaP70	2.325	3-4	DuCaP	1.832	4
LuCaP73	1.251	2	VCaP	1.984	4
LuCaP77	2.083	3	LNCaP	3.165	4
LuCaP78	2.151	2	22Rv1	2.104	3
LuCaP81	2.365	3	PC-3	1.155	2
LuCaP86.2	0.869	4	LAPC-4	1.915	3

Table 26. *UBAP2.*

Sample	mRNA expression	Copy number	Sample	mRNA expression	Copy number
LuCaP23.1	3.133	2	LuCaP92.1	11.771	3
LuCaP23.8	3.673	3	LuCaP93	3.248	3
LuCaP23.12	8.722	2	LuCaP96	5.856	3-4
LuCaP35	***5.795***	***12-15***	LuCaP105*	22.425	3-5
LuCaP41	***7.004***	***10-15***	LuCaP115	8.965	3
LuCaP49	2.555	2			
LuCaP58	***7.414***	***5-7***			
LuCaP69	4.741	3	DU145	2.275	2-4
LuCaP70	5.654	3-4	DuCaP	1.619	4
LuCaP73	2.708	2	VCaP	1.670	4
LuCaP77	3.803	3	LNCaP	1.564	4
LuCaP78	5.209	2	22Rv1	0.800	3
LuCaP81	1.781	3	PC-3	1.508	2
LuCaP86.2	1.833	4	LAPC-4	1.251	3

*Outlier.

Table 27. *UBE2R2.*

Sample	mRNA expression	Copy number	Sample	mRNA expression	Copy number
LuCaP23.1	2.089	2	LuCaP92.1	1.313	3
LuCaP23.8	6.914	3	LuCaP93	2.157	3
LuCaP23.12	4.048	2	LuCaP96	5.269	3-4
LuCaP35	***5.844***	***12-15***	LuCaP105	2.969	3-5
LuCaP35V	***4.919***	***12-15***	LuCaP115	1.237	3
LuCaP49	1.212	2			
LuCaP58	***0.732***	***5-7***			
LuCaP69	2.239	3	DU145	2.815	2-4
LuCaP70	1.237	3-4	DuCaP	-	-
LuCaP73	0.828	2	VCaP	-	-
LuCaP77	3.251	3	LNCaP	1.742	4
LuCaP78	2.609	2	22Rv1	4.317	3
LuCaP81	2.629	3	PC-3	0.854	2
LuCaP86.2	1.029	4	LAPC-4	1.332	3

Table 28. *UNC13B.*

Sample	mRNA expression	Copy number	Sample	mRNA expression	Copy number
LuCaP23.1	1.019	2	LuCaP92.1	7.294	3-4
LuCaP23.8	8.988	2	LuCaP93	4.239	3
LuCaP23.12	2.328	2	LuCaP96	15.527	3-4
LuCaP35	***16.375***	***10-12***	LuCaP105	14.760	3-5
LuCaP35V	***42.006***	***10-12***	LuCaP115	8.915	3
LuCaP49	1.191	2			
LuCaP58	***1.283***	***5-7***			
LuCaP69	4.116	3	DU145	2.378	4
LuCaP70	3.480	3-4	DuCaP	-	-
LuCaP73	4.547	2	VCaP	-	-
LuCaP77	16.711	3	LNCaP	1.751	4
LuCaP78	4.250	2	22Rv1	0.458	2
LuCaP81	3.264	3	PC-3	0.343	2
LuCaP86.2	11.948	4	LAPC-4	1.387	3

Table 29. *VCP.*

Sample	mRNA expression	Copy number	Sample	mRNA expression	Copy number
LuCaP23.1	1.378	2	LuCaP92.1	0.929	3
LuCaP23.8	0.399	2	LuCaP93	0.490	3
LuCaP23.12	0.962	2	LuCaP96	1.469	3-4
LuCaP35	***2.733***	***10-12***	LuCaP105	0.602	3-5
LuCaP41	***3.908***	***8-10***	LuCaP115	1.335	3
LuCaP49	0.542	2			
LuCaP58	***0.859***	***5-7***			
LuCaP69	1.039	3	DU145	1.229	4
LuCaP70	0.826	3-4	DuCaP	1.305	4
LuCaP73	0.905	2	VCaP	1.649	4
LuCaP77	1.751	3	LNCaP	1.569	4
LuCaP78	0.973	2	22Rv1	1.452	2
LuCaP81	1.207	3-4	PC-3	0.466	2
LuCaP86.2	0.904	4	LAPC-4	0.803	3

Table 30. *WDR40A.*

Sample	mRNA expression	Copy number	Sample	mRNA expression	Copy number
LuCaP23.1	2.188	2	LuCaP92.1	1.191	3
LuCaP23.8	0.420	3	LuCaP93	2.610	3
LuCaP23.12	1.649	2	LuCaP96	3.073	3-4
LuCaP35	***3.884***	***12-15***	LuCaP105	3.735	3-5
LuCaP35V	***2.017***	***12-15***	LuCaP115	1.671	3
LuCaP49	0.953	2			
LuCaP58	***0.734***	***5-7***			
LuCaP69	1.404	3	DU145	2.714	4
LuCaP70	1.006	3-4	DuCaP	-	-
LuCaP73	0.865	2	VCaP	-	-
LuCaP77	2.007	3	LNCaP	1.547	4
LuCaP78	1.107	2	22Rv1	1.332	3
LuCaP81	0.848	3	PC-3	0.645	2
LuCaP86.2	1.355	4	LAPC-4	1.346	3