

EEVALIISA HOPEASAARI THE ROLE OF A PUTATIVE TUMOR SUPPRESSOR MIGRATION AND INVASION INHIBITORY PROTEIN IN PROSTATE CANCER

Master of Science thesis

Examiners: Professor Minna Kellomäki, Professor Matti Nykter Examiners and topic approved by the Faculty Council of the Faculty of Natural Sciences on 8th June 2011

ABSTRACT

EEVALIISA HOPEASAARI: The role of a putative tumor suppressor migration and invasion inhibitory protein in prostate cancer

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Prostate cancer is the most common cancer in Western countries. As the disease proceeds to castration resistant phase, it often leads to death due to lack of effective treatment methods and lack of knowledge of molecular mechanisms leading to it. Migration and invasion inhibitory protein (MIIP) has been shown to have tumor suppressing effects in other cancers. The effect of it in prostate cancer has not been previously studied. The aims of this study were to discover the effect of *MIIP* on growth and migration of prostate cancer cells and to detect possible mutations and a single nucleotide polymorphism (SNP) rs2295283 previously shown to affect breast cancer risk from the gene.

DU145 and LAPC-4 prostate cancer cell lines were used. DU145 cells were stably transfected with a plasmid containing MIIP and an empty control plasmid. LAPC-4 cells were transfected with small interfering RNAs (siRNAs) targeting *MIIP* and a control siRNA. Proliferation of the cells was studied with image analysis and alamarBlue methods. Migration was studied with scratch assay. Mutations and SNPs were studied by sequencing.

Clear results from the effects of silencing *MIIP* in LAPC-4 cells were not achieved. In the proliferation assays, image analysis and alamarBlue methods gave contradictory result and due to difficulties in image analysis, results from alamarBlue should be considered more reliable. AlamarBlue assay showed the metabolic activity of DU145 cells overexpressing *MIIP* to be significantly lower than in cells transfected with control vector. However, DU145 cells overexpressing *MIIP* migrated significantly faster that cells transfected with control vector. Mutations in the gene were not detected and SNP rs2295283 was not found to be associated with prostate cancer risk.

It seems unlikely that MIIP would have a significant effect in most prostate cancer cases. However, it is possible that the role of MIIP is more notable in some of prostate cancer subtypes. More experiments are needed to fully uncover the effect of MIIP in prostate cancer cells.

TIIVISTELMÄ

EEVALIISA HOPEASAARI: Mahdollisen kasvua rajoittavan migraatio- ja

invaasioinhibiittoriproteiinin (MIIP) vaikutus eturauhassyövässä

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Eturauhassyöpä on miesten yleisin syöpä länsimaissa. Taudin edetessä hormoneista riippumattomaan vaiheeseen se johtaa usein kuolemaan, sillä tarpeeksi tehokkaita hoitokeinoja ei ole. Eturauhassyövän syntyyn ja etenemiseen liittyvät molekyylitason mekanismit ovat huonosti tunnettuja, mikä rajoittaa uusien hoitokeinojen kehittämistä. Migraatio- ja invaasioinhibiittoriproteiinilla (MIIP) on havaittu syöpää ehkäiseviä vaikutuksia muissa syövissä. Sen vaikutusta eturauhassyövässä ei ole aiemmin tutkittu. Tutkimuksen tavoitteena oli selvittää *MIIP:n* vaikutusta eturauhassyöpäsolujen kasvuun ja migraatioon sekä tunnistaa mahdollisia mutaatioita tai kohonneeseen rintasyöpäriskiin liitettyä yhden nukleotidin polymorfismia (SNP) rs2295283 geenissä.

Tutkimuksissa käytettiin DU145- ja LAPC-4-eturauhassyöpäsolulinjoja. DU145-solut transfektoitiin pysyvästi *MIIP*:n sisältävällä plasmidilla sekä kontrolliplasmidilla. LAPC-4-solut transfektoitiin *MIIP*:in kohdistuvilla pienillä häiritsevillä RNA:illa (siRNA) ja kontrolli-siRNA:lla. Solukantojen kasvua tutkittiin kuva-analyysin ja alamarBlue-menetelmän avulla. Migraatiota tutkittiin naarmukokeella. Mutaatioita ja SNP:a tutkittiin sekvensoimalla.

MIIP-geenin hiljentämisen vaikutuksista LAPC-4-soluissa ei saatu selkeitä tuloksia. Solukantojen kasvua arvioivissa kokeissa kuva-analyysin ja alamarBlue-menetelmän avulla saatiin ristiriitaisia tuloksia. Kuva-analyysiin liittyvien ongelmien takia, alamarBlue-menetelmän tuloksia tulee pitää luotettavampina. AlamarBlue-kokeissa huomattiin, että MIIP-geeniä yli-ilmentävien DU145-solukantojen metabolinen aktiivisuus oli huomattavasti matalampi kuin kontrollien. MIIP-geeniä yli-ilmentävien DU145-solukantojen migraatio oli kuitenkin odotusten vastaisesti huomattavasti nopeampaa kuin kontrollien. Mutaatioita geenissä ei havaittu eikä SNP rs2295283:n huomattu liittyvän kohonneeseen eturauhassyöpäriskiin.

Vaikuttaa epätodennäköiseltä, että MIIP:lla olisi yleisesti merkittävää vaikutusta eturauhassyövän synnyssä tai etenemisessä. On kuitenkin mahdollista, että MIIP:n rooli yksittäisissä eturauhassyövän alatyypeissä voi olla suurempi ja jatkotutkimuksia tarvitaan tämän selvittämiseksi.

PREFACE

The laboratory part of this thesis was done in Molecular Biology of Prostate Cancer group (MBPCG) led by Professor Tapio Visakorpi at Institute of Biosciences and Medical Technology (BioMediTech) (Previous: Institute of Biomedical Technology - IBT) at University of Tampere in collaboration with Computational Systems Biology (CSB) research group at Department of Signal Processing at Tampere University of Technology. The thesis was a part of FiDipro project "Systems Biology of Cancer: Mapping, Methods and Modeling for the Cancer Genome" funded by TEKES.

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Eevaliisa Hopeasaari

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1. INTRODUCTION

Prostate cancer (PC) is the most frequently diagnosed cancer in men and the second most common cause of cancer related deaths in Western countries (American Cancer Society 2011; European Cancer Observatory 2011). Most PCs grow quite slowly and remain confined to the prostate (Abate-Shen and Shen 2000). Organ-confined PCs are often relatively successfully treated with prostatectomy or radiation to remove or destroy the cancerous tissue (Heidenreich et al. 2012). Nevertheless, if the cancer is not detected early enough, or in more aggressive forms of PC, it can advance to invasive and metastatic stages, which usually lead to death (Abate-Shen and Shen 2000). Treatment of these more advanced stages of PC is more difficult. Because most PCs are androgen-dependent in the beginning, the standard treatment for advanced disease is androgen deprivation (Tammela 2012). However, as PC progresses during hormonal treatment, it turns from androgen-dependent to castration-resistant PC (CRPC) and androgen deprivation stops working (Best et al. 2005). Newer treatment methods have been developed, but they often offer only few more months in the life expectancy (Higano et al. 2009; Mita et al 2009; Tran et al. 2009; de Bono et al. 2010; Kantoff et al 2010; de Bono et al. 2011). PC is a very heterogeneous and complex disease and molecular mechanisms behind initiation and progression of it remain largely unclear (Barbieri and Tomlings 2014). Thus, better biological understanding is needed in order to be able to develop more efficient treatment methods.

Migration and invasion inhibitory protein (MIIP) has been seen to function as a possible tumor suppressor in gliomas. MIIP binds and inhibits insulin-like growth factor binding protein-2 (IGFBP-2) (Song et al. 2003), which is overexpressed in several cancers including prostate cancer (Kanety et al. 1993). The MIIP gene is also located on a chromosome region known to be deleted in various cancers including prostate cancer (Wang et al. 2011). The effect of it in prostate cancer has not been previously studied.

The aim of this thesis was to study the influence of MIIP on growth and migration of prostate cancer cells by overexpressing and silencing the gene in prostate cancer cells. Possible mutations and certain previously found single nucleotide polymorphisms (SNPs) were also studied by sequencing.

2. NORMAL ANATOMY AND FUNCTION OF THE PROSTATE GLAND

The prostate is a sex gland of the male reproductive system. It is approximately walnut sized and shaped and is located below the bladder, where it surrounds a part of the urethra. Location of the prostate gland in the male reproductive system can be seen in Figure 1. The prostate parenchyma can be divided into four distinct zones; peripheral zone, central zone, transitional zone and periurethral zone. The peripheral zone contains most (70%) of the main prostatic glands and it is the zone most prone to inflammation and prostatic carcinoma. A smaller part (25%) of the glandular tissue is located to the central zone. The transition zone contains the mucosal glands and cells of this zone are susceptible to extensive division, causing condition known as benign prostatic hyperplasia (BPH). The periurethral zone comprises mucosal and submucosal glands. Mucosal glands secrete directly into the urethra, whereas submucosal and main prostatic glands have ducts that open into the prostatic sinuses. (Ross and Pawlina 2006.)

The prostatic epithelial cells secrete several components of the semen such as prostatic acid phosphatase (ACPP), fibrinolysin, citric acid and a serine protease kallikrein-related peptidase 3 (KLK3) also known as prostate-specific antigen (PSA) (Ross and Pawlina 2006). The prostate gland also releases sugars, sulphate and a vitamin E-derivative to the seamen. (Boron and Boulpaep 2009). The seminal plasma, which is the remainder of the semen excluding sperm cells, functions to protect the sperm cells in different ways. (Ross and Pawlina 2006; Boron and Boulpaep 2009)

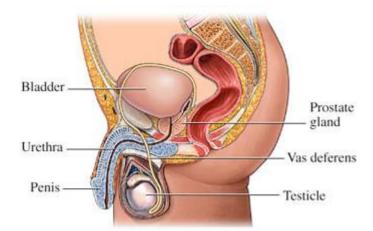


Figure 1. Location of the prostate gland in the male reproductive system (Prostate Health Tips 2011)

2.1 Testosterones

Male steroid hormones androgens are needed for the embryonic development and adult function of the prostate but also the growth of prostate cancer (PC) is generally dependent on androgens (Miyamoto et al. 2008). Most of the androgens are produced as testosterone (T) by Leydig cells in the testes (Taplin and Ho 2001). Adrenal cortex also secretes adrenal androgens, such as androstenedione, dehydroepiandosterone (DHEA) and its sulphate. Adrenal androgens are not as potent as T, but they still contribute to androgenic effects in the body and may be further metabolized to T. Androgen production in the Leydig cells is regulated primarily through hypothalamic-pituitarygonadal axis. Gonadotropin-releasing hormone (GnRH) is secreted by the hypothalamus in a pulsatile manner. In the anterior pituitary GnRH stimulates the release of luteinizing hormone (LH) and follicle-stimulating hormone (FSH). LH in turn stimulates the production of androgens in Leydig cells. Secretion of GnRH and LH are inhibited through negative feedback control. (Grossmann et al. 2001.) Adrenal androgen production is controlled through adrenocorticotropic hormone (ACTH). In the circulation, T is bound to serum proteins, mostly sex hormone-binding globulin (SHBG), albumin and corticosteroid-binding globulin. (Taplin and Ho 2001.) After entering the cells in the end organs, such as prostate, T is usually converted to a more potent dihydrotestosterone (DHT) by 5-α reductase (Grossmann et al. 2001; Taplin and Ho 2001). Intracellular DHT is metabolized rapidly to 3α,17β-androstenediol which can be converted back to DHT or to 3β,17β-androstenediol and further to water soluble, inactive triol steroids (Taplin and Ho 2001).

The action of androgens is mediated by a receptor molecule, a ligand-dependent nuclear transcription factor, androgen receptor (AR). In an inactive form AR is bound to heat-shock chaperone proteins, but when androgens bind to AR, conformation of AR changes, it dissociates from heat-shock proteins, forms dimers and the complex translocates rapidly to the nucleus. There the complex binds to AR response elements (AREs) in DNA and affects expression of hundreds of target genes, such as *KLK3* and *ACPP*. (Helenius et al. 2008.) Co-regulatory proteins are also recruited to the site and they too can affect gene expression of AR target genes (Taplin et al. 2004). Both T and DHT are capable of binding to AR, but the affinity of DHT is about two to tenfold compared to T making DHT the primary androgen bound by AR (Grossmann et al. 2001).

3. CANCER

Most cancers are believed to be caused by interactions between genes and the environment. There is evidence suggesting that tumorigenesis is a multistep process, each step involving genetic alterations that drive normal cells to transform into malignant cancer cells (Hanahan and Weinberg 2000). Cancer initiates from a single cell, which starts to divide uncontrollably after being damaged. Even more DNA-alterations accumulate to daughter cells, leading to the cancer tissue gaining growth advantage over other cells in the surrounding environment. (Joensuu et al. 1999.)

The best known and foundation setting alterations are mutations producing oncogenes and mutations inactivating tumor suppressor genes. Through these mutations, activated oncogenes gain the function to drive cells towards cancer cells and tumor suppressor genes lose their function to prevent cancer. (Hanahan and Weinberg 2000.) In normal cells, oncogenes are in their inactivated forms and are called proto-oncogenes. They can be activated as a consequence of DNA alteration such as mutation, chromosomal translocation or gene deletion or amplification. (Joensuu et al. 1999.) Also epigenetic factors and changes in gene expression can cause oncogene activation (Vogelstein et al. 2013). Alteration of only one allele can cause the oncogenic effect of a certain gene, but in order to cancer to develop, several genetic changes are needed. The function of most oncogenes is related to controlling cell growth. (Joensuu et al. 1999.)

In tumor suppressor genes, DNA-alteration leads to impairment of their normal function. Usually both alleles of the gene need to be altered in order for the cancer promoting effect to be generated. (Joensuu et al. 1999.) However, cancer can also initiate from a gene alteration in only one of the alleles, like with *PTEN*, as described later. The most common alteration causing inactivation of tumor suppressor genes is deletion. Inactivation can also be caused by DNA-viruses or DNA methylation. Products of these genes usually inhibit cell division. (Joensuu et al. 1999.)

Cancer cells have deficiencies in regulating cell proliferation and homeostasis. Even though there are hundreds of different cancer types and subtypes, it is suggested that the malignant growth in all of them is caused by a small amount of alterations in cell physiology. Assuming that these changes are in common with most cancers is justified by the fact that the same kind of molecular mechanisms regulate cellular processes in all mammalian cells. (Hanahan and Weinberg 2000.) These hallmarks of cancer include maintaining proliferative signalling, avoiding growth suppressors, hindering cell death, enabling replicative immortality, inducing angiogenesis and activating tissue invasion and metastasis (Hanahan and Weinberg 2011) (Figure 2).

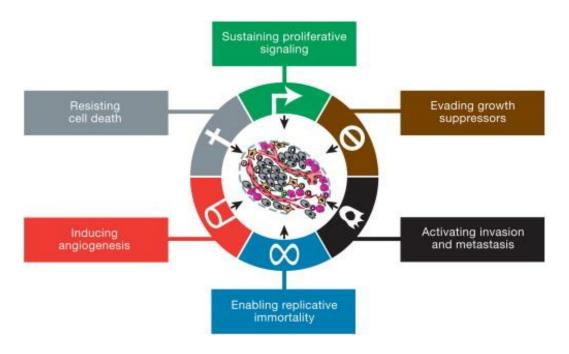


Figure 2: Characteristics of cancer presented by Hanahan and Weinberg (2011).

Genome instability and inflammation facilitate these changes. Recent progress in cancer research has also introduced two potential emerging hallmarks – reprogramming of energy metabolism and evading immune destruction (Figure 3). Tumor microenvironment also plays an important role in cancer development and progression. (Hanahan and Weinberg 2011.)

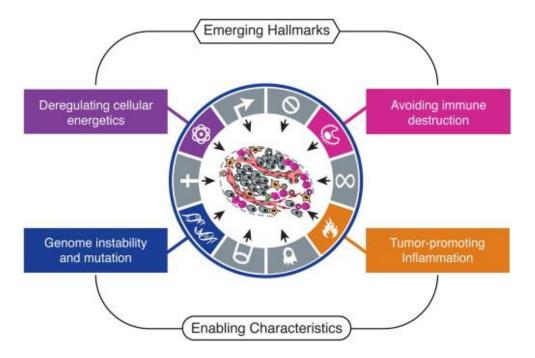


Figure 3: Emerging characteristics and facilitating features by Hanahan and Weinberg (2011).

4. PROSTATE CANCER

In western countries, PC is the most frequently diagnosed cancer in men and the second most common cause of cancer related deaths (American Cancer Society 2011; European Cancer Observatory 2011). PC is strongly correlated with aging. Although morphological changes associated with initiation of cancer are quite common and can be found also in younger men, PC is usually not clinically detected until the age of 60 or 70. (Abate-Shen and Shen 2000.)

In addition to being a component of the seminal fluid, KLK3 more commonly known as PSA, can also be found in the serum of normal males in much lower concentrations and the amount of it in blood is often used to define the condition of the prostate. PSA measurements from the blood have been routinely used in detection of PC since 1980s. (Diamandis 1998.) However, in addition to prostate cancer, also benign prostatic hyperplasia (BPH) and infections can result in elevated PSA levels, so PSA is not a PC-specific marker (Pin et al. 2013). It has also been discovered that high- or intermediate-grade PC can be present in men with low PSA levels (Thompson et al. 2004). Thus, better markers are needed for more efficient diagnosis.

Prostate cancer lesions are often heterogeneous and multifocal. Histological inspections of PC tissue often show benign glands, preneoplastic (PIN) foci and neoplastic foci next to each other. Gleason scoring is the predominant prognostic tool used by pathologists. (Abate-Shen and Shen 2000.) The Gleason score is a sum of the most prevalent pattern grade and the second most prevalent pattern grade. Grades range from 1 to 5, so the sum can be from 2 to 10. A higher Gleason score means more advanced cancer and a bleaker prognosis for the patient. In practice, Gleason scores from 2 to 5 are very rarely assigned due to poor reproducibility, lack of good correlation with prostatectomy grade, sampling issues, and potentially misleading clinical implications. (Shah 2009.) The basics of the Gleason system can be seen in Figure 4.

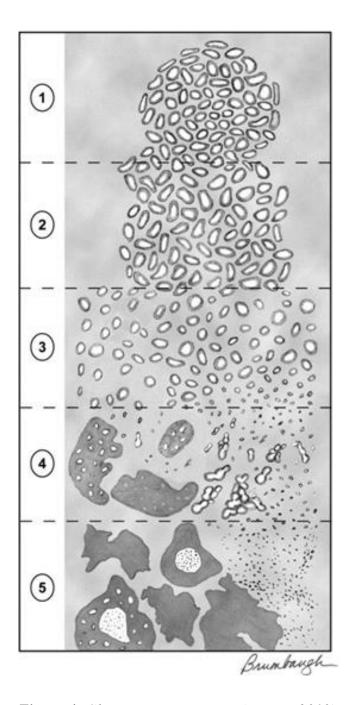


Figure 4: Gleason scoring system (Epstein 2010).

Gleason pattern 1 resembles normal prostate tissue the most and as the Gleason pattern becomes larger, the more abnormal characteristics it indicates (Ross and Pawlina 2006).

Most PCs are quite indolent, grow relatively slow and remain confined to the prostate. Actually, most men diagnosed with PC will eventually die of other causes. Nevertheless, if the cancer is not detected early enough, or in more aggressive forms of PC, it can advance to invasive and metastatic stages, which usually lead to death. (Abate-Shen and Shen 2000.) As PC progresses, it turns from androgen dependent to castration-resistant PC (CRPC) during hormonal treatment. The exact mechanisms leading to androgen-independence remain unclear. (Best et al. 2005.)

4.1 Genetic changes in prostate cancer

Previously it was thought that hereditary factors had quite small role in PCs and that those cases were often affiliated with early disease onset (Abate-Shen and Shen 2000). However, studies have given reason to believe that the effect of heritability in PC can be significantly higher than previously thought (up to 42 percent) (Lichtenstein et al. 2000). In hereditary cancer, an alteration predisposing to cancer is already in a gamete and is thereby passed on to all the cells of the body. The gene aberration is in one allele and does not yet intrude the function of the cells. Cancer initiates when also the other allele in a certain cell gets inactivated. For this reason, hereditary cancer occurs often earlier in life than other cancers. The mechanisms leading to malignancy are still often similar and involve same genes with both types. (Joensuu et al. 1999.)

In prostate cancer, genetic deletions have been found to occur most often in chromosomes 5q, 6q, 8p, 10q, 13q, 16q, 17p and 18q. Some of the target genes in these regions are known, such as *NK3 homeobox 1* (*NKX3-1*) (8p) and *phosphatase and tensin homolog* (*PTEN*) (10q), but many are still unknown. (Saramäki and Visakorpi 2007.) Gain-of-function alterations are less known in prostate cancer and only few have been identified (Taylor et al. 2010). Gains are most common in chromosomes 7p/q, 8q, 9p and Xq and the known target genes include among others *androgen receptor* (*AR*) (Xq) and *v-myc avian myelocytomatosis viral oncogene homolog* (*MYC*) (8q) (Saramäki and Visakorpi 2007). Chromosomal aberrations at Xq (*AR*) (in CRPC) and 21q (*TMPRSS2:ERG* fusion) are also common (Taylor et al. 2010).

NKX3-1 belongs to the NK subfamily of homeobox genes that are associated in cell differentiation and organogenesis in many species. NKX3-1 functions as a transcription factor and it has been shown to be very important in normal prostate development and function. Expression of *NKX3-1* is androgen-dependent and primarily restricted to the prostate gland. (Bieberich et al. 1996; He et al. 1997.) The gene is located at 8p21, a region commonly deleted in prostate cancer and *NKX3-1* expression has been shown to often be lost during PC progression (Bethel et al. 2006).

PTEN, located at 10q23, is one of the most frequently mutated tumor suppressors in human cancer. In addition to mutations, PTEN can also be inactivated by loss of heterozygosity (loss of one parental copy in a chromosomal region) resulting in only one copy of the gene. PTEN has been found frequently deleted or mutated in prostate cancer cell lines and especially late stage prostate cancers. (Li et al. 1997; Cairns et al. 1997.) PTEN is also important for embryonic development. PTEN has been shown to modulate cell growth, survival, migration and adhesion through its lipid phosphatase and protein tyrosine phosphatase activities. PTEN directly dephosphorylates two key tyrosine-phosphorylated proteins and thus inhibits interactions between integrins and extracellular matrix and integrin-triggered signalling pathways. PTEN also dephosphorylates a key signal transduction lipid that leads to maintaining cell sensitivity to apoptosis. (Tamura et al. 1999; Yamanda and Araki 2001.)

C-myc proteins are transcription factors that regulate genes involved in cell division, differentiation and apoptosis. Overexpression of *MYC* accelerates cell division and prevents differentiation. (Pelengaris et al 2002.) *MYC* is overexpressed in especially late stages of PC (Qian et al. 2002). However, the role of *MYC* overexpression in prostate cancer is not entirely clear.

The AR is a key molecule in the initiation and growth of prostate cancer and in its responsiveness to hormonal therapy. The AR is located at Xq11-12, composes of 8 exons and the full length protein contains four functional domains: an N-terminal regulatory domain, a DNA-binding domain, a hinge-region and a ligand binding domain. The N-terminus contains transcriptional activation function (TAF-1) site and polyglutamine (CAG) and polyglycine (GCC) repeats. Two zinc fingers in the DNA-binding domain are needed for ARE recognition. The nuclear localization signal is in the hinge region. The ligand binding domain has a second transcription activation function (TAF-2) site and the activity to bind ligands. (Taplin and Balk 2004.) During prostate carcinogenesis, AR signaling pathway is suggested to be converted from paracrine signaling, where stromal cells regulate the growth and development of prostatic epithelial cells, to autocrine signaling, where AR directly stimulates the growth of cancer cells (Gao et al. 2001).

AR has been found present in different stages and grades of prostate cancer from primary to metastatic and CRPCs (Ruizeveld de Winter et al. 1991). It was previously believed that CRPCs were independent of AR signalling for growth and survival (Ryan et al. 2010). However, it has been shown that tissue levels of testosterone and dihydrotestosterone remain relatively high in recurrent prostate cancer tissues despite of androgen deprivation therapy and enable AR signalling to continue (Titus et al. 2005; Ryan et al. 2010). Sufficient T and DHT tissue levels in recurrent prostate cancer have been hypothesized to possibly result from intracrine metabolism of circulating adrenal androgens or synthesis from plasma membrane cholesterol (Titus et al. 2005).

There are different mechanisms for cancer cells to utilize low levels of androgens, which are present in patients during castration. Potential mechanisms include amplification of the AR gene or increased protein expression by other ways. The AR gene has been reported to be amplified in 20-30 % of CRPCs. Untreated primary tumors, however, do not seem to harbor AR amplifications. Samples taken from the same patients before and after hormonal therapy also show no amplifications prior to therapy and AR amplifications after treatments and recurrence. (Visakorpi et al. 1995; Koivisto et al. 1997.) Amplification does not fully explain the high expression levels in CRPC tumors and other mechanisms leading to overexpression of AR are involved (Linja et al. 2001). These include for example tumor suppressor protein retinoblastoma (RB) loss, which may induce cell cycle activating transcription factor E2F1-mediated deregulation of AR locus leading higher AR expression. This in turn drives progression to CRPC. (Sharma et al. 2010.)

AR point mutations are found in some local and locally advanced tumors, but more widely in advanced androgen-independent cancers, especially after treatment with

antiandrogens. (Taplin et al. 1995; Tilley et al. 1996.) Most *AR* gene missense mutations in the ligand-binding domain in prostate cancer have clustered in three regions, codons 670-678, codons 701-730 and codons 874-910. Codons 670-678 are located near the hinge region and mutations in that region can affect AR interactions with co-regulators. Codons 701-730 contain the "signature sequence", a highly conserved loop common for many steroid hormone receptors. Codons 874-910 flank AF-2, which is the primary binding site for co-activator proteins needed for AR activity. Mutations in these areas affect the ligand-binding pocket and allow binding of ligands other than testosterone or DHT, such as other steroid hormones and pharmaceutical antiandrogens. (Buchanan et al. 2001; Gelmann 2002.) The most commonly found and first identified point mutation of *AR* is the T877A mutation changing threonine at position 877 to alanine. It was first described in LNCaP cell line. (Veldscholte et al. 1990.) The T877A mutation changes the stereochemistry of the binding pocket and facilitates binding of progesterone and other ligands (Sack et al. 2001).

The influence of AR cofactors has been studied in prostate cancer progression. Levels of some cofactors, such as transcriptional intermediary factor 2 (TIF2) and steroid receptor coactivator 1 (SCR1) have been found to increase with increases in AR expression in androgen independent prostate cancers (Gregory et al. 2001). It has been hypothesized that overexpression of AR cofactors could make AR more responsive to low levels of androgen or broaden ligand specificity like mutations of AR do, but the confirmation of the role of AR cofactors in prostate cancer progression awaits further studies (Taplin and Balk 2004).

Alternative splicing can also be one way to alter the function of AR. Three novel AR splice variants lacking the ligand binding domain have been found in CRPC. One of the isoforms, AR3 (designated by the researchers) appears to be constitutively active and the activity is not regulated by androgens or antiandrogens. It has seen to be upregulated during prostate cancer progression and the expression levels correlate with the risk of tumor recurrence after radical prostatectomy. It also seems to regulate some genes not regulated by the wild-type AR. (Guo et al. 2009.) The amount of a constitutively active splice variant of AR has also been shown to rapidly increase right after androgen deprivation. The levels of the splice variant are not high enough to restore the high-level AR activity in relapsed tumors, but may allow tumors to preserve basal AR activity needed for survival until more powerful mechanisms arise to activate AR. (Yu et al. 2014.)

Gene fusions involving *E-twenty-six* family (*ETS*) genes and androgen regulated prostate-specific *transmembrane protease*, *serine* 2 (*TMPRSS2*) gene occurs in approximately 40-50 % of prostate cancers (Tomlins et al. 2005; Huang and Waknitz 2009). Fusion of *TMPRSS2* and *ETS*-family transcriptional activation factor *ERG* is the most frequent rearrangement. *TMPRSS2* is constitutively expressed in prostate and the expression is controlled by androgens. (Paoloni-Giacobino et al. 1997; Lin et al. 1999; Tu et al. 2007). Several *TMPRSS-ERG* fusions have been identified and most of them involve only the 5' untranslated region of *TMPRSS2*. These fusions thus cause

overexpression of the ERG or ETV protein induced by the *TMPRSS2* promoter. (Tu et al. 2007.) The expression of ERG proteins also becomes androgen regulated as a consequence of the fusion. In prostate cancer, ERG activates WNT signaling and this leads to loss of cell adhesion. ERG also modulates prostate cancer prostaglandin signaling and affects tumor metastasis. ERG is not expressed in normal prostate tissue. (Wu et al. 2013.)

5. MIGRATION AND INVASION INHIBITORY PROTEIN

The migration and invasion inhibitory protein (MIIP), also known as invasion inhibitory protein 45 (IIp45), was first discovered in a yeast two-hybrid screen in an attempt to identify binding partners of insulin-like growth factor binding protein 2 (IGFBP-2) frequently overexpressed in aggressive gliomas (Song et al. 2003). MIIP was found to bind and inhibit IGFBP-2 and other mechanisms of action related to cell migration and invasion have been discovered since.

MIIP gene is located on chromosome 1p36.22, it has 10 exons and it comprises 12.6 kb of genomic DNA (Wang et al. 2011). The transcript has 1588 base pairs. The MIIP protein consists of 388 amino acids with an estimated molecular mass of 43 kDa. In protein analysis MIIP was found to be highly hydrophilic and have three segments of low compositional complexity (SEG) domains and an arginine–glycine–aspartic acid (RGD) motif. (Song et al. 2003.)

5.1 MIIP and cancer

There are several factors pointing to the possibility of MIIP having a role in genesis and progression of cancer. First, MIIP inhibiting cancer cell migration and invasion promoting IGFBP-2 (Song et al. 2003). Second, the location of *MIIP* gene being on chromosome 1p36, a chromosome region known to be deleted in various cancers including prostate cancer, lung cancer, glioblastoma and breast cancer (Gibbs et al. 1999; Yanada et al. 2005; Ichimura et al. 2008; Climent et al. 2010; Heinrich et al. 2012). Many tumor-suppressor genes have been found to be located in commonly deleted chromosome areas. Such genes include *TP53* on chromosome 17p, *PTEN* on chromosome 10 and *Rb* on chromosome 13. (Donehower el al 1992; Li et al. 1997; Burkhart and Sage 2008.)

In addition to being deleted, tumor-suppressor genes are often found mutated. Although clear mutations in the *MIIP* gene have not been found in tissues or cell lines sequenced so far (Song et al. 2005, Wang et al. 2010), it does not rule out the possibility of *MIIP* being a tumor-suppressor. Tumor-suppressors can also function by other mechanisms, such as *CHD5* and *PTEN* (Cairns et al. 1998; Fujita et al. 2008). In these cases cancer development is caused by loss of one copy of the gene without any mutations.

Several single-nucleotide polymorphisms (SNPs) have also been found in different regions of the MIIP gene and can be found from the database of single nucleotide

polymorphisms, dbSNP (The National Center for Biotechnology Information 2013). SNPs in tumor-suppressor genes have been seen to affect cancer risk (Imyanitov 2009). SNPs of *MIIP* were investigated in a case-control study with breast cancer patients and healthy women and SNP rs2295283 was found to affect breast cancer risk. SNP rs2295283 has two variants; adenosine (A) and guanine (G) and they result in two different amino acids, lysine (Lys, K) or glutamic acid (Glu, E) at codon 167. Presence of G allele was found to be a protective factor for breast cancer in both AG (one A allele and one G allele) and GG (both G alleles) genotypes when compared with the AA genotype. Glu amino acid at codon 167 was associated with lower risk of breast cancer, but also to smaller and lower-grade tumors. (Song et al. 2010.)

Expression of *MIIP* has been shown to inhibit glioma cell invasion both *in vitro* and *in vivo*. Correspondingly, in glioma tissues including highly invasive glioblastoma multiforme, expression of *MIIP* has been seen to be reduced. (Song et al. 2003.) In addition, *MIIP* is alternatively spliced in gliomas. Alternative splicing produces an unstable isoform of the protein and the frequency of it correlates with glioma grade. (Song et al. 2005.)

5.2 Mechanisms of action of MIIP

5.2.1 Insulin-like growth factor binding protein 2

MIIP has been found to bind and inhibit IGFBP-2 (Song et al. 2003). As part of the insulin-like growth factor (IGF) network, insulin-like growth factor binding proteins (IGFBPs) are important in regulating cell proliferation, differentiation, migration, survival and metabolism (Duan and Xu 2005). IGFBP-2 has also been found to be overexpressed in several cancers including glioblastoma multiforme, prostate, ovarian, breast and colon cancers (Kanety et al. 1993; Fuller et al. 1999; Renehan et al. 2000; Busund et al 2005; Wang et al 2006).

In addition to its effects on IGFs, IGFBP-2 has also been shown to specifically bind to the α5β1 integrin on the cell surface with its RGD-motif and promote de-adhesion of tumor cells (Schütt et al 2004). Integrins affect signalling through integrin-linked kinase (ILK), which binds to β1 and β3 integrins. ILK stimulates invasion and migration and induces tumor angiogenesis. IGFBP-2 has been shown to be connected to the integrin/ILK/NF-κB network. IGFBP-2 was seen to activate integrin β1 and its downstream pathways, require ILK for cell migration and to activate NF-κB, a transcriptional factor, which activates the transcription of many cancer-promoting genes. Blocking any constituent of the integrin/ILK/NF-κB network also hindered progression of gliomas driven by IGFBP-2 *in vivo*. (Holmes et al. 2012.) IGFBP-2 has also been discovered to contribute to glioma progression by promoting tumor cell invasion through increased *matrix metalloproteinase-2 (MMP-2)* transcription (Wang et al. 2003).

In addition, it has been shown that IGFBP-2 has an inhibitory effect on non-malignant prostate cells, but for DU145 and LAPC-4 prostate cancer cells the effect is stimulatory in a mitogen-activated protein (MAP) kinase and androgen-modulated process. This suggests that there could be a molecular switch conversing IGFBP-2 from growth inhibitor to tumor promoting molecule. (Moore et al 2003.)

In an invasion study with BioCoat Matrigel invasion chamber, LN-229 cells were transfected with MIIP, IGFBP-2 or MIIP+IGFBP-2. The invasiveness of MIIP-expressing cells decreased by approximately 50 %, whereas that of IGFBP-2-expressing cells increased approximately 250 %, when compared with the invasiveness of cells transfected with control vector. Invasion was also attenuated in cells transfected with both MIIP and IGFBP-2, compared with cells expressing only IGFBP-2. (Song et al 2003.)

MIIP was found to bind to the same thyroglobulin-RGD region of IGFBP-2 that is also utilized in IGFBP-2 binding to integrin $\alpha 5$ (Song et al. 2003). Therefore binding of MIIP to IGFBP-2 could inhibit IGFBP-2 from binding to integrin and interfere with the downstream pathways (Wang et al. 2011).

5.2.2 Histone deacetylase 6

By using MIIP as a bait molecule in a yeast two-hybrid assay, Wu et al. found MIIP to bind to histone deacetylase 6 (HDAC6) (Wu et al. 2010). HDAC6 is a cytoplasmic enzyme that deacetylates α -tubulin, heat-shock protein 90 (Hsp90) and cortactin, and forms complexes with other partner proteins. Through these actions HDAC6 regulates many important biological processes such as cell migration. (Valenzuela-Fernandez et al. 2008.) HDAC6 possesses two functional deacetylase domains, which are both needed for intact enzymatic activity (Zhang et al. 2006) and a zinc finger motif that could be involved in regulating ubiquitination (Seigneurin-Berny et al. 2001). Reversible acetylation of α -tubulin, an important component of the cytoskeleton, regulates microtubule stability and function and thus affects cell motility (Hubbert et al. 2002).

MIIP binds to both catalytic domains of HDAC6. Overexpression of *MIIP* and knockdown of *HDAC6* was seen to cause increased levels of α -tubulin acetylation, whereas transfection with *MIIP* small interfering RNA (siRNA) decreased α -tubulin acetylation. This strongly suggests that MIIP inhibits deacetylase activity of HDAC6. MIIP was also found to reduce the protein stability of HDAC6. Through these mechanisms MIIP increases acetylation of α -tubulin and decreased cell motility. (Wu et al. 2010.)

Cortactin is another protein that has an important role in regulating cell motility. Its interaction with F-actin promotes polymerization and branching of the actin assembly. The acetylation status of cortactin influences its F-actin binding activity. (Zhang et al.

2007.) MIIP could inhibit cell migration also by decreasing HDAC6 mediated deacetylation of cortactin (Wu et al. 2010).

Hsp90 is a molecular chaperone that interacts with a large number of client proteins and regulates signal transduction pathways. Acetylation of Hsp90 is one of the regulators of Hsp90 activity. (Li et al. 2012.) When Hsp90 is inhibited, tyrosine phosphorylation of focal-adhesion kinase (FAK) and assembly of focal adhesions are reduced and further FAK-dependent actions such as actin cytoskeleton rearrangement, cell migration and cell invasion are inhibited (Rousseau et al. 2000; Masson-Gadais et al. 2003; Koga et al. 2007). Thus, inhibition of cell migration by MIIP may also function through deacetylation of Hsp90 by HDAC6 (Wu et al. 2010).

5.2.3 Mitosis

In normal conditions, cell cycle is tightly controlled by numerous regulatory elements and molecules. Cells go through the phases of cell cycle G1, S, G2 and finally M in a highly orchestrated manner and disruption of this balance may lead to different diseases.

MIIP may also have an important role in regulation of mitosis. In flow cytometry analysis, *MIIP* expression was shown to markedly delay G₂/M transition in the cell cycle in LN229 cells. MIIP was discovered to bind to cell division cycle protein 20 (Cdc20), a molecule that activates anaphase-promoting complex/cyclosome (APC/C) ubiquitin-ligase E3. (Ji et al. 2010.) APC/C allows ubiquitination and degradation of securing and cyclin B1 which in turn contributes to mitotic exit (Yu 2007). A significant decrease in an APC/C substrate cyclin B1 ubiquitination was observed in the presence of MIIP, implying that the interaction of MIIP and Cdc20 causes reduction in APC/C ubiquitin-ligase activity. Cyclin B1 protein levels were also seen to positively correlate with levels of MIIP in several cancer cell lines, indicating that MIIP modulates cyclin B1 stability. *MIIP* expression in LN229 glioma cells was connected to increased chromosomal abnormalities and mitotic catastrophe. (Ji et al. 2010.)

MIIP might regulate mitosis also through its previously described effect on HDAC6 mediated α -tubulin acetylation (Wang et al. 2011). Microtubules consisting of the $\alpha\beta$ -tubulin heterodimers form the mitotic spindle during cell division and facilitate the separation of replicated sister chromatids. Microtubules also function in aiding the intracellular organelle and vesicle transportation. Acetylation of α -tubulin has a role in regulating these functions. (Westermann and Weber 2003.)

5.2.4 Other

In an attempt to find genes associated to expression of MIIP, a cDNA microarray expression profiling was made and several adhesion- and motility-associated genes were seen to be downregulated in cells expressing MIIP. Among them were integrin $\alpha L/\beta 8$, cell division cycle protein 42 (Cdc42), transcription factor nuclear factor kappa B

 $(NF\kappa B)$ and its target genes intracellular adhesion molecule 1 (ICAM-1), interleukin 10 (IL-10) and E-selectin (SELE). (Song et al. 2003.) However, it remains unclear how and how much MIIP affects these gene expressions.

6. PROSTATE CANCER TREATMENT

6.1 Clinically localized prostate cancer

Treatment methods for clinically localized prostate cancer currently include active surveillance (AS), radical prostatectomy, external-beam radiation therapy, and interstitial radiation therapy (brachytherapy) (Heidenreich et al. 2012). Active surveillance is an option for men with low-risk prostate cancer. It builds upon the assumption that in low-risk disease, the time between diagnosis and clinical progression is usually long, so men with diagnosed low-risk prostate cancer will not necessarily have any clinical symptoms of the condition during their lifetime. Men are not treated with anything, but are closely followed by serial PSA assessments, repeat biopsies, and possible other tests to identify early signs of progression. At the first signs of higher-risk disease, treatment is initiated. (Klotz 2010; Klotz 2012.)

The gold standard in the surgical management of localized prostate cancer is radical prostatectomy (Mullins et al. 2012). The goal of radical prostatectomy is to remove all of the carcinoma tissue. The operation can be done as an open surgery or laparoscopically either manually or using robot-assisted techniques. All options have been found to be safe presenting similar overall complication rates. (Coelho et al. 2010.) Pelvic lymph nodes can be removed in the same surgery (El-Galley et al. 2002). Disease-specific mortality, overall mortality, and the risks of metastasis and local progression have been proven to be reduced with radical prostatectomy. Though the reduction in the risk of death after 10 years is small, the reductions in the risks of metastasis and local tumor progression are significant. (Bill-Axelson et al. 2005). However, radical prostatectomy can cause side-effects such as erectile dysfunction and urinary leakage, which have an impairing effect on quality of life after surgery (Steineck et al 2002).

If surgical intervention is not possible or not wanted, another option is radiation therapy. Radiation therapy can also be given as adjuvant treatment after prostatectomy when the risk of recurrence is high. Three-dimensional conformal radiotherapy (3D-CRT) is widely used but newer techniques like intensity-modulated radiotherapy (IMRT) and image-guided radiotherapy (IGRT) are also becoming more common. (Heidenreich et al. 2012.)

It has been stated that the dose of external radiotherapy should be at least 74 grays (Gy) because biochemical disease-free survival is significantly higher when compared with a dose <72 Gy (Hayden et al. 2010). Studies have shown that higher, up to 78 Gy doses result in a significant improvement in freedom from failure for patients with intermediate to high risk tumors. However, escalated doses also increase the incidence

of rectal side effects, so techniques preventing radiation to the surrounding tissues are preferable. (Pollack et al. 2002.) In addition to the prostate gland, seminal vehicles are often also treated. Seminal vehicles can be given the same dose as the prostate gland or they can be left outside the treatment area after 45-50 Gy. (Prostate cancer: Current Care Guidelines 2007.) Pelvic lymph nodes can also be radiated at a dose of 45-50 Gy when there is a high risk for lymph node metastasis (Roach et al 2006).

IMRT allows delivery of higher doses of radiation with very low toxicity and lesser side-effect (Vora et al. 2007). IMRT is delivered using a multileaf collimator (MLC) to shape the particle beams (Luxton et al. 2004).

In IGRT the exact position of the prostate is being located with the help of imaging methods. Different methods such as rectal balloon catheters, fiducial markers (e.g. 1 mm diameter gold seeds), three-dimensional ultrasound and computer tomographic imaging can be used. When the precise location is known, smaller margins can be used and radiation to normal tissues can be reduced. (Button and Staffurth 2010.)

Increasing the radiation dose alone is not enough for high-risk PC and adjuvant androgen deprivation therapy (ADT) is used for 3 years. For high-risk PC, a short course of neoadjuvant hormonal ablation can also be applied for 2 months before as well as during radiotherapy. (Heidenreich et al. 2012.) The principle of ADT in more detail is explained in the following chapter.

For low-risk PC, low dose rate (LDR) prostate brachytherapy can also be used (Heidenreich et al. 2012). In LDR brachytherapy radioactive seeds are delivered into the prostate using transrectal ultrasound imaging guidance. Radioisotopes can be either Iodine-125 (I-125) or Palladium-103 (Pd-103). (Machtens et al. 2006.) Finnish centers use I-125 (Prostate cancer: Current Care Guidelines 2007). The half-life of I-125 is 60 days and it has an energy of 0.028 keV. 100-120 Gy is generally delivered in 2 Gy fractions. (Machtens et al. 2006.) In Finland, a dose of 140 Gy is used (Prostate cancer: Current Care Guidelines 2007). Good long-term results have been achieved with LDR brachytherapy (Taira et al. 2011).

Cryosurgical ablation has been considered as a treatment method, but while there are no reliable long-term results, it is not in the official treatment recommendations (Heidenreich et al. 2012). In cryosurgical ablation, liquid nitrogen is used to freeze the prostate or areas of it using ultrasound guidance (Babaian et al. 2008).

Several studies have shown that patients with localized disease do not benefit from added antiandrogen bicalutamide. However, adding bicalutamide to standard care results in considerable clinical benefits in patients with locally advanced prostate cancer. (McLeod et al. 2006; See et al. 2006.)

6.2 Advanced and castration resistant prostate cancer

6.2.1 Traditional treatment methods

Endocrine treatment of prostate cancer aims to deprive the cancer cells of androgens. This can be executed by eliminating the testosterone production of the testes or by blocking the androgen receptors (AR) with antiandrogens (Tammela 2012). The first method of permanent testosterone synthesis prevention was bilateral orchiectomy (surgical removal of the testes) and reversible castration was first achieved with estrogen treatment (Huggins and Hodges 1941). Charles B. Huggings received a Nobel Prize price for his discoveries concerning hormonal treatment of prostatic cancer in 1966.

Orchiectomy is still considered the primary option for ADT in cases related with high spinal cord compression risk, brain metastasis or severe pain (Prostate cancer: Current Care Guidelines 2007). Medically castration can be achieved with gonadotropin-releasing hormone (GnRH) (often used as a synonym of luteinizing hormone-releasing hormone (LHRH)) agonists and antagonists of which LHRH agonists represent the standard care (Tammela 2012; Heidenreich et al. 2013). Castration with oestrogen agonists has widely been abandoned due to unwanted side effects (Prostate cancer: Current Care Guidelines 2007; Tammela 2012).

Continuous stimulation of the pituitary with high concentrations of GnRH agonist causes receptor desensitization and inhibition of LH release, which further blocks testosterone synthesis by the testes. GnRH agonists available include for example goserelin, leuprorelin, buserelin and tritorelin. During first 1-2 weeks of GnRH agonist therapy, there is an increase in LH production and plasma testosterone levels, referred often as the flare effect. Therefore, co-treatment with antiandrogen is used for 2-3 weeks from the initiation of GnRH agonist treatment. (Tammela 2012.) GnRH antagonists on the other hand rapidly block the release of both LH and FSH resulting in more rapid and crucial decrease in serum testosterone level without the flare effect (Tammela 2012). GnRH antagonists have proven useful in patients with locally advanced or metastatic disease, but the benefits in other situations remain to be verified (Heidenreich et al 2013). Intermittent androgen deprivation (IAD) in which androgen blockade and treatment cessation alternate to enable hormonal recovery between cycles has been tested to improve tolerability and quality of life. Meta-analysis of 13 trials comprising 6419 patients showed that overall survival, time to progression and quality of life were similar in IAD and continuous androgen deprivation. However, patients treated with IAD had lower incidence of hot flushes and higher sexual activity scores. (Botrel et al 2014.)

In maximum androgen blockade (MAB), the action of testosterone is inhibited by preventing the production by the testes using castration, and by blocking AR by antiandrogens to inhibit the effects of adrenal and locally produced androgens (Tammela 2012). However, MAB provides only small survival advantage compared

with GnRH agonist monotherapy and is associated with significant impairment of quality of life (Heidenreich et al. 2013).

There are steroidal and non-steroidal antiandrogens and they can be used as monotherapy or in combination with castration. Antiandrogens competitively inhibit the binding of DHT and testosterone to AR. Steroidal antiandrogens (cyproterone acetate) also binds to progesterone receptors in the pituitary inhibiting the release of LH and production of testosterone by the testicles. However, steroidal antiandrogens have been seen to cause more adverse effects than GnRH agonists and non-steroidal antiandrogens such as bicalutamide, flutamide and nilutamide. Currently, bicalutamide is the best tolerated antiandrogen. Bicalutamide offers benefits in quality of life, so bicalutamide has been considered as an option to castration for younger, sexually active men with locally advanced prostate cancer. (Tammela 2012.) However, clinical benefits are minor or nonexistent and therefore bicalutamide monotherapy is not recommended as a standard care (Heidenreich 2013).

All types of endocrine treatment have adverse events which influence quality of life in different ways. The proper use and timing of endocrine therapy still remain subjects of debate. (Tammela 2012).

In case of recurrence, local recurrences are often treated by salvage radiation therapy with 64-66 Gy. High-intensity focused ultrasound (HIFU) or cryotherapy have also been proposed as an alternative because of equal efficacy but less morbidity. (Heidenreich et al. 2013.) With metastatic CRPC, life-expectancy is short, only about one and a half years, and treatment often aims in alleviating the symptoms and improving quality of life (Prostate cancer: Current Care Guidelines 2007).

Chemotherapy with docetaxel can be used for metastatic CRPC. Docetaxel is a semi-synthetic taxane which functions by disrupting the function of cellular microtubules and thus preventing cell division (Shelley et al 2006). It can offer pain relief and improve quality of life compared to other treatments and also extend life expectancy (Tannock et al 2004). New and improved agents have also been developed and they are becoming more widely used.

6.2.2 Newer treatment methods

Although orchiectomy and chemical castration effectively block androgen synthesis of the testes, androgen synthesis still continues in adrenals and tumor cells resulting in adequate levels of androgens in the prostate to stimulate PC cell growth (Titus et al. 2005). Abiraterone is a selective cytochrome P450 c17 (CYP17) inhibitor. CYP17 is a crucial enzyme in androgen biosynthesis despite production location, so abiraterone blocks androgen synthesis of adrenals and tumor cells in addition to that of testes. (de Bono et al. 2011.) Abiraterone also significantly increases adrenocorticotropic hormone (ACTH) levels and plasma levels of 11-deoxycorticosterone and corticosterone, leading to unwanted effects such as hypokalemia, hypertension and edema. These are suppressed by co-administration of a synthetic glucocorticoid. (Attard et al. 2008.) Abiraterone has also been found to reduce AR protein expression and R1881-induced

AR transactivation in prostate cancer cell lines, which may influence the anti-tumor effects (Solifer et al 2012). In patients with metastatic CRPC previously treated with chemotherapy, overall survival was seen to be longer with abiraterone compared to placebo (14.8 months vs. 10.9 months) (de Bono et al 2011).

More effective AR antagonists have been developed to overcome issues related to first generation antagonists. First generation AR antagonists such as bicalutamide and flutamide have lower affinity to AR than DHT and they demonstrate partial antagonism, which leads to weak results especially when AR is highly overexpressed (Chen et al. 2004). The AR affinity of the second generation AR antagonist enzalutamide is 8-fold higher than that of bicalutamide and no agonism is shown. Enzalutamide inhibits AR translocation into nucleus and impairs its DNA binding and co-activator recruiting, but also induces apoptosis in VCaP cells which have AR gene amplification. (Tran et al. 2009.) Enzalutamide was seen to extend the survival of men with metastatic CRPC previously treated with chemotherapy. In the ezalutamide group, the median overall survival was 18.4 whereas in the placebo group it was 13.6 months. (Scher et al 2012.)

Cabazitaxel belongs to taxanes like docetaxel and acts with the same mechanism by binding to tubulin and inhibiting microtubule depolymerization and consequently arresting mitosis. Cabazitaxel demonstrates greater antitumor activity and can be used also in docetaxel-resistant cancers. (Mita et al 2009; de Bono et al. 2010.) Cabazitaxel has a lower affinity for P-glycoprotein, the drug efflux pump responsible for multidrug resistance, which may affect the better antitumor properties (Mita et al. 2009). Cabazitaxel was found to improve overall survival in patients with metastatic castration-resistant prostate cancer whose disease has progressed during or after docetaxel treatment. The median survival was 15.1 months in the cabazitaxel group and 12.7 months in the mitoxantrone (control) group. Median progression-free survival was 1.4 months higher in the cabazitaxel group than in the mitoxantrone group. (de Bono et al. 2010.)

Several gene therapy approaches have been proposed as possible treatment methods for prostate cancer. The anatomy and biology of the prostate gland allow prostate cancer to be an extremely powerful candidate for gene therapy. The prostate gland is not an essential organ so its complete removal or ablation is possible. In addition, it can be easily accessed for implantation of therapeutic agents or taking tissue samples. A number of genes, including *KLK3*, *h-kallikrein-2*, *prostate specific membrane antigen*, *probasin* and *relaxin H2*, are also specifically expressed in prostate tissue under tight control of tissue specific promoters. The *osteocalcin* gene encoding a major noncollagenous bone protein is also overexpressed in prostate cancer. These facts can be used in targeting the expression of therapeutic genes selectively to prostate cancer. (Harrington et al. 2001.)

In gene therapy, a functional gene can be introduced to the target tissue to restore absent or deficient protein production, to modulate the immune system or to destroy cells in a controlled manner to slow down the disease (Kootstra and Verma 2003). Although the principle of gene therapy is relatively simple, one major problem has been

efficient delivery of the gene to the target tissue (Verma and Weitzman 2005). Viral and non-viral vectors can be used for gene delivery purposes. Viral vectors are more popular in clinical trials at the moment and of those adenoviral vectors are most widely used (Gene Therapy Clinical Trials Worldwide 2011). Other viral vectors used for gene delivery include retroviral vectors, poxvirus vectors, adeno-associated virus vectors and herpes simplex virus vectors (Harrington et al. 2001; El-Aneed 2004). Common non-viral vectors include naked DNA injections, cationic polymers, cationic peptides, cationic lipids and cells (Harrington et al. 2001; El-Aneed 2004; MacRae et al. 2006; Kitchen et al. 2011).

Gene therapy for cancer can be divided into three main categories: immunotherapy, oncolytic virotherapy and gene transfer. The purpose of immunotherapy is to strengthen the immune system to attack and destroy the cancer cells. Cancer vaccines are an example of immunotherapy. (Cross and Burmester 2006.) In oncolytic virotherapy oncolytic virus (OV) vectors are used in destroying the tumor. Oncolytic viruses are able or can be modified to replicate specifically in cancer cells and cause their death as a consequence of the lytic viral cycle. Due to targeting to cancer cells, normal cells of the body remain intact. (Mullen and Tanabe 2002.) In gene transfer, a foreign gene is introduced into the cancer cells or the surrounding tissues. Genes with several different functions can be used, including genes causing cell death when expressed (suicide genes), antiangiogenesis genes, genes that restore the function of a deleted or mutated gene (often a tumor suppressor gene), genes that negate the effect of a tumor promoting gene (oncogene) or genes that enhance immune responses against tumor tissues. (Harrington et al. 2001; Cross and Burmester 2006.)

Sipuleucel-T is one example of a successful gene therapy approach in prostate cancer. It is a cancer vaccine containing autologous peripheral-blood mononuclear cells (PBMCs); including antigen-presenting cells (APCs), that have been activated *ex vivo* with a recombinant fusion protein (PA2024). In PA2024, a prostate antigen, prostatic acid phosphatase (ACPP) is fused to granulocyte—macrophage colony-stimulating factor (GM-CSF). (Kantoff et al. 2010.) GM-CSF activates the APCs and ACPP works as the target antigen and navigating epitope. Back in the body, sipuleucel-T induces an immune response to ACPP, which is one of the major proteins secreted by prostate. ACPP serum levels are also significantly elevated in many metastatic prostate cancers. (Provenge: EPAR — Public assessment report 2013.) Sipuleucel-T was found to reduce the risk of death significantly compared to placebo in men with advanced prostate cancer (Higano et al. 2009; Kantoff et al 2010).

7. CELL LINES AND XENOGRAFTS AS PROSTATE CANCER MODELS

One of the biggest obstacles to gaining more biological information about prostate cancer and developing drugs to fight it has been the paucity of adequate model systems. Prostate cancer is very heterogeneous morphologically and molecularly, it grows relatively slowly and is highly dependent on paracrine and endocrine signaling. Due to all this, it has been very challenging to grow human prostate cancer in the laboratory *in vitro* or *in vivo*. (Pienta et al. 2008.)

Xenografts are widely used models in cancer research and drug development. Prostate cancer xenografts can be established by implanting surgically removed human prostate cancer explants into immunodeficient mice. The tissue can be from primary tumors or from metastatic sites and also prostate cancer cell lines can be used. However, xenografts from primary tumors are extremely difficult to carry out. (Chauchereau 2011.) Xenografting is usually done to the three major graft sites; subcutaneous, under the renal capsule and orthotopic. Orthotopic xenograft model is achieved by implanting human prostate tumor cells into mouse prostate glands. Different graft sites have their advantages and disadvantages. (Wang et al.2005)

The subcutaneous site has been the most commonly used because it can be easily accessed and large amounts of tumor can be grafted there. However, vascularization is relatively poor and especially with primary tumors the survival rates are low. The subrenal capsule has proven relatively successful with different tissue types, especially with primary tissues including localized prostate cancer. The vascularization under the renal capsule is high, assuring sufficient amount of nutrients to the tumor prior to developing their own vasculature. Yet, the surgery is more difficult and less tissue can be transplanted under the renal capsule than subcutaneously. Different approaches such as maintaining host androgen levels by implanting testosterone and precision cutting of tissue slices have been tested to achieve better survival rates. (Wang et al. 2005; Zhao et al. 2010.) Orthotopic graft site has the advantage of having similar microenvironment as the original tumor site and orthotopic xenografts seem to also mimic the actual disease better (Chauchereau 2011). Survival rate of grafts is lower than with subrenal graft site, but the proportion of glands in every surviving graft seems to be higher. However, rodent prostate is not very easily accessible and the capacity to carry xenografts is limited. (Wang et al. 2005)

After implantation and tumor growth, it can be serially transplanted into other animals. Xenografts are able to retain various biological properties of the original human tumors they are derived from, but the major disadvantage of these models is the

need for continuous transplantation in animals and due to high transplantation failure rate, a large number of animals is needed, which is both expensive and laborious. (Chauchereau 2011.) Immunodeficient hosts are required for xenografting so that the host does not reject the transplanted tissue. Either athymic or severe combined immunodefiency (SCID) animals can be used. Athymic animals lack a thymus and are unable to produce T-cells, whereas SCID is a genetic disorder. However, the absence of an immune system is not a normal situation with prostate cancer patients and it should be considered when applying results achieved with this type of model to practice. (Wang et al. 2005.) Ethical issues may also cause concern.

The advantages of in vitro cell cultures are relatively low costs and high replicative capacity ensuring enough material for long-term use. They offer a simple approach to study cell behavior and molecular pathways involved in PC. However, the biological properties of the original cancer tissue are not well preserved in in vitro twodimensionally cultured cell lines. (Sampson et al 2013.) Immortalized cell lines can also harbor genetic alteration and mutations that are not typical for the original disease. The loss of AR and KLK3 is a common characteristic for several cell lines. (Peehl 2005). Most of the PC cell lines are derived from advanced or metastatic disease and same few most common cell lines are used for most experiments. This hinders the study of earlier stages of PC and lacks diversity normally seen in human prostate cancer. (Peehl 2005; Pienta et al. 2008.) Nevertheless, the usefulness of these models is proven by our continuously increasing knowledge of molecular mechanisms regulating PC development and progression. Development of three-dimensional co-culture systems has also given new extent to investigation of stromal-epithelial interactions, angiogenesis and metastasis. Several in vitro models have been created and each model system has its own characteristics. (Sampson et al 2013.) However, developing these cell lines is anything but simple and there are very few models available for PC despite the fact that PC is a common cancer.

LNCaP, PC3 and DU145 were the first human prostatic tumor epithelial cell lines established and they are still the most commonly used PC cell lines (Sampson et al 2013). LNCaP cells have been derived from lymph node metastasis of human prostate adenocarcinoma. They are androgen responsive and DHT regulates their proliferation. LNCaP cultures also produce acid phosphatase and KLK3. (Horoszewicz et al. 1983.) LNCaP cells express *AR* that carries the T877 mutation in the AR ligand binding domain changing threonine 877 with alanine which affects both binding specificity and the induction of gene expression (Taplin et al. 1995). LNCaP is widely used for studying AR signalling in prostate cancer. PC3 cells are established from bone metastasis and DU145 cells from brain metastasis and neither of these cell lines responds to androgens. (Sampson et al. 2013.)

In addition to these most commonly used cell lines established directly form tissues of PC patients, there are several cell lines derived from human PC tissue first heterotransplanted into immune-deficient mice. Such cell lines include VCaP and DuCaP that were established from bone and brain metastasis of the same patient with

CRPC by xenografting them first into SCID mice and harvesting them later for in vitro culture. Both cell lines are sensitive to androgens and express wild-type AR. In addition, they harbour the TMPRSS2:ERG fusion and high level AR gene amplification. (Saramäki et al. 2008; Sampson et al. 2013.) The LAPC cell lines were established with the same principle from eight different patients (Sampson et al. 2013). LAPC-4 expresses wild-type AR, KLK3 and HER-2/neu receptor tyrosine kinase and it has been used for example to compare drug efficacy with cell lines expressing mutated forms of AR (Cherian et al. 2012; Craft et al. 1999; Sampson et al. 2013). 22Rv1 is a cell line derived from a xenograft that was serially propagated in mice after castration-induced regression and relapse of the parental, androgen-dependent CWR22 xenograft (Sramkoski et al 1999). 22Rv1 cells secrete KLK3 and express AR, but in lower levels than LNCaPs. 22Rv1s also carry a H874Y mutation in AR, which results in change in codon 874 from CAT for histidine to TAT for tyrosine and leads to reduced specificity of AR. (Attardi et al. 2004.) In addition, these cells harbour two distinct forms of AR, a larger one with an extra zinc finger motif and a C-terminally truncated one (Sampson et al. 2013).

8. MATERIALS AND METHODS

8.1 Cell lines and xenografts

DU145, LNCaP, PC3 and 22Rv1 cell lines used in the research were obtained from American Type Cell Collection (Manassas, VA, USA). VCaP and DuCaP cell lines were kindly provided by Dr. Jack Schalken (Radboud University Nijmegen Medical Center, Nijmegen, the Netherlands) and LAPC-4 cell line by Dr. Charles Sawyers (University of California, Los Angeles, CA, USA). All cell lines were stored in liquid nitrogen and cultured under recommended conditions. 19 previously established LuCaP-xenografts (Royai et al. 1996) were available for the analyses by collaboration with Professor Vessella (University of Washington, Seattle, WA, USA). All samples were snap frozen in liquid nitrogen and stored at -80°C.

8.2 Plasmids and siRNAs

pCMV6-AC-HA expression vector inserted with *MIIP* coding region was kindly donated by Wei Zhang (The University of Texas MD Anderson Cancer Center, Houston, USA) and verified by PCR and sequencing. PCR for plasmids containing *MIIP* was done using Dynazyme II DNA polymerase (Thermo Scientific, Thermo Fisher Scientific, Inc., Waltham, USA) and specially designed primers (sequences can be found from Table 4). Reaction volume of 25 µl was used. 20 nanograms of plasmid DNA was used in each reaction and manufacturer's protocol was followed with the exceptions of not adding any MgCl₂ to the reaction mix and using slightly modified denaturation times and temperatures (Table 1).

Table 1: Dynazyme PCR reactions

Cycle step	Temperature	Time	Cycles
Initial denaturation	95°C	5 minutes	1
Denaturation	95°C	30 seconds	
Annealing	56°C	30 seconds	35
Extension	72°C	60 seconds	
Final extension	72°C	10 seconds	1
Cooling	4°C	hold	1

PCR-products were run on 1 % agarose gel and visualized with ethidium bromide and the size of the PCR product was verified. *MIIP* gene was also sequenced from the plasmid according to the sequencing protocol described later.

To generate a control vector, the insert was digested with EcoRI and MluI restriction enzymes and the empty plasmid was isolated from agarose gel. The empty plasmid was purified with QIAquick Gel Extraction Kit (Qiagen, Venlo, Netherlands) according to the manufacturer's protocol. The ends of the plasmid were blunted with Klenow's fragment (Fermentas Inc., Burlington, Ontario, Canada) and ligated together with T4 ligase (New England Biolabs Inc., Ipswich, MA) according to manufacturer's protocols.

One Shot[®] chemically competent TOP10 E. coli cells (Invitrogen Life Technologies, Carlsbad, CA) were transformed with pCMV6-AC-HA-MIIP- and empty pCMV6-AC-HA- plasmids using heat-shock transformation according to manufacturer's instructions. Cells were cultured overnight on LB plates containing ampicillin and individual colonies were further grown in suspensions to achieve sufficient amount of plasmids for transfections. Plasmids were isolated from the bacterial culture using Qiagen plasmid Maxi Kit (Qiagen, Venlo, Netherlands) according to manufacturer's protocol.

Commercially available small interfering RNAs (siRNAs) SI00392000 (named MIIP siRNA1 during the study), SI00392007 (MIIP siRNA2), SI00392014 (MIIP siRNA3) and SI04271365 (MIIP siRNA4) for MIIP (Qiagen, Venlo, Netherlands) were used in siRNA transfections. AllStars Negative Control siRNA SI0650318 (Qiagen, Venlo, Netherlands) was used as a control.

8.3 Cell culture and transfections

DU145 and LAPC-4 cells were maintained, respectively, in DMEM and IMDM (Lonza, BioWhittaker[®], Basel, Switzerland) supplemented with 10% and 15% fetal bovine serum (FBS; Lonza, BioWhittaker[®], Basel, Switzerland) and 1% L-glutamine (Lonza, BioWhittaker[®], Basel, Switzerland) and incubated in a humidified incubator at 37°C with 5% CO₂.

Plasmid transfections were performed using Lipofectamine LTX and Plus reagents and their protocol (Invitrogen Life Technologies, Carlsbad, CA). Briefly, DU145 cells were plated 50 000 cells per well on 24-well plates and transfected 2 days later when cells were approximately 70% confluent. For each well, **0.5** μg of plasmid DNA was diluted into 100 μl of Opti-MEM I Reduced Serum Medium without serum. PLUS reagent was gently mixed and **0.5** μl of PLUS reagent per every **0.5** μg of DNA was added to the diluted DNA. Solution was gently mixed and incubated at room temperature for 5 minutes. For each well **1.5** μl of Lipofectamine LTX reagent was added to the diluted DNA solution, mixed gently and incubated at room temperature for 25 minutes to form DNA-Lipofectamine LTX complexes. Growth medium was removed from the cells and replaced with **0.5** ml of complete growth medium. 100 μl of the DNA-Lipofectamine LTX complexes were added dropwise directly to each well

containing cells and mixed gently by rocking the plate back and forth. Transfected cells were then incubated in normal incubating conditions. Two wells were transfected with pCMV6-AC-HA-MIIP plasmid and two wells with pCMV-AC-HA empty plasmid.

To generate cell lines stably expressing MIIP, transfected DU145 cells were selected in the presence of 400 μ g/ml geneticin (G418; Gibco[®], Life Technologies, Carlsbad, CA) for 3 weeks and after that maintained in 200 μ g/ml of G418. Transfected DU145 cells were seeded sparsely on 10 ml petri dishes and individual colonies were ringcloned into 96-well plates to achieve populations originating from a single cell. Colonies were further grown in normal cell culturing conditions to gain sufficient amount of cells for the functional tests.

siRNA transfections for LAPC-4 cells were done using INTERFERin (Polyplustransfection SA; Illkirch, France) and its reverse transfection protocol. In short, for each well, 30 pmol (in the optimizing phase also 6 pmols) of siRNA duplexes were diluted into 100 µl of Opti-MEM. 3 µl of INTERFERin was added to the diluted siRNAs and the solution was mixed by pipetting up and down. The mixture was incubated for 15 minutes at room temperature to form transfection complexes. For proliferation assays, LAPC-4 cells were trypsinazed and plated 100 000 cells per well on 24-well plates. 100 µl of the siRNA solution was pipetted on each well and mixed gently by moving the plate in a figure of 8. After transfection, cells were incubated at 37°C in normal cell culturing conditions. 8 wells were transfected with each siRNA; MIIP siRNA1, MIIP siRNA2 and Qiagen All Stars negative control. Proliferation assays were started the day after transfection (day 0).

Transfections for migration analysis were done in a similar manner, except LAPC-4 cells were plated 300 000 cells per well and 350 000 cells per well on 24-well plates. 3 days later scratches was drawn across (from 11 o'clock to 5 o'clock) the confluent cell layer on the bottom of the wells with a 10 µl pipette tip. 100 µl of the incubated siRNA solution was pipetted dropwise on each well. 4 parallel samples (one of 300 000 cells per well and three of 350 cells per well) was used for each siRNA; MIIP siRNA1, MIIP siRNA2, MIIP siRNA3 and control siRNA. First images were taken the day of transfection (day 0).

8.4 Sequencing

Genomic DNA from cell line and xenograft samples was also amplified with Dynazyme II as with plasmid DNA, using 400 ng of DNA for cell lines and 200 ng of DNA for xenografts. Cell line and xenograft PCR reactions were also purified with QIAQuick PCR purification kit (Qiagen, Venlo, Netherlands) according to manufacturer's protocol prior to sequencing reactions. For full length transcript sequencing, complementary cDNA from cell lines was amplified with Phusion DNA Polymerase (Finnzymes, Espoo, Finland) and purified with QIAQuick PCR purification kit (Qiagen, Venlo,

Netherlands) prior to sequencing with the protocol described in tables 2 and 3. Primer sequences can be found from Table 4.

Table 2: Phusion PCR Program outline

Phusion PCR Program				
Cycle step	Temperature	Time	Number of cycles	
Initial denaturation	98°C	3 min	1	
Denaturation	98 ℃	10s		
Annealing	56,3/57 °C	20s	40	
Extension	72 ℃	30s		
Final extension	72 ℃	10 min	1	
Cooling	4 °C	hold	1	

Table 3: Phusion PCR components and pipetting order

Phusion PCR components and pipetting order		
Component	μl/20 μl reaction	
H ₂ O	11.72	
5x Phusion GC Buffer	4	
dNTPs	0.4	
DMSO	0.6	
MgCl ₂	0.08	
Phusion DNA polymerase	0.2	
forward-primer 10 μM	1	
reverse-primer 10 μM	1	
template DNA	1	

Sequencing was performed using the Big-Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) and the ABI PRISM 3100 sequencer (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. Each sequencing reaction included 20 ng of DNA, 5 pmol of primers, 1.5 μl of 5x sequencing buffer and 1.0 μl of BigDye Terminaton ready Reaction mix (Applied Biosystems, Foster City, CA). The volume of the reactions was adjusted to 10 μl with sterile water. Sequences for the primers used can be seen in Table 4. The sequencing reactions were denatured at 96 °C for 1 minute, followed by 30 cycles of 10 s at 96 °C, 10 s at 50 °C and 4 min at 60 °C.

Amplified DNA was precipitated by adding 26 µl of a mixture containing one part of 3 M sodium acetate and 25 parts of 96% ethanol and incubating for 15 minutes at room temperature. DNA was pelleted by centrifugation at 2000 g for 45 minutes and the supernatants were discarded. Pellets were washed with 125 µl of 70 % ethanol and

DNA was re-pelleted by centrifugation at 2000 g for 15 minutes. Supernatants were again discarded and the DNA pellet was suspended into 12.5 µl of Hi-DiTM formamide (Applied Biosystems, Foster City, CA). Suspensions were denatured by incubating the samples at 95 for 3 minutes and after that cooled on ice. Sequences were analyzed with DNAStar Software (DNASTAR Inc., Madison, WI, USA).

8.5 Determination of MIIP mRNA levels

8.5.1 RNA isolation

Total RNA from cultured cells was isolated using TRIZOL (Invitrogen Life Technologies, Carlsbad, CA) reagent according to manufacturer's protocol. Shortly, cells were lysed by adding 1 ml of TRIZOL reagent per 10 cm² (200 µl per 24-well) directly to the culture dish and the cell lysate was passed through the pipette several times. Lysate was pipetted to a plastic tube and stored in the freezer at -70°C if the isolation was not continued right away.

Samples were then incubated at room temperature for 5 minutes. 0.2 ml of chloroform was added per 1 ml of TRIZOL reagent (40 µl for one 24-well), tubes were capped, shaked vigorously by hands for 15 seconds and incubated for 3 minutes. Samples were centrifuged at 12000 x g for 15 minutes at 2-8°C for phase separation. The colourless upper aqueous phase containing the RNA was transferred to a fresh tube and the rest was discarded. RNA was precipitated from the aqueous phase by adding 0.5 ml of isopropyl alcohol per 1 ml of TRIZOL reagent (100 µl for one 24-well). Samples were incubated at room temperature for 10 minutes and centrifuged at 2-8°C at 12000 x g for 10 minutes. The supernatant was discarded and the gel-like pellet formed in the bottom of the tube was washed once with 75 % ethanol using at least 1 ml of ethanol per 1 ml of TRIZOL reagent used in the beginning. Samples were mixed by vortexing and centrifuged at 7500 x g for 5 minutes at 2-8°C. The ethanol was removed and the RNA pellet was air dried. The dried RNA pellet was dissolved in RNase-free water by passing the solution a few times through the pipette tip.

8.5.2 Reverse transcription and quantitative RT-PCR

In order to carry out complementary DNA (cDNA) reverse transcription, concentrations of total RNA were determined using NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA).

1-5 micrograms of total RNA from cell samples was reverse transcribed to cDNA with AMV-reverse transcriptase (Finnzymes, Espoo, Finland) according to the enzyme manufacturer's protocol using random hexamer primers.

Quantitative real-time PCR (Q-RT-PCR) was done with the LightCycler equipment (Roche Diagnostics, Mannheim, Germany) using FastStart DNA Master SYBR Green I Kit (Roche Diagnostics, Mannheim, Germany) for xenograft samples. For the rest of the

samples Q-RT-PCR was done with CFX96 real-time PCR detection system (Bio-Rad Laboratories Inc., Hercules, CA) using TaqMan Gene Expression assay (Applied Biosystems, Foster City, CA) for *MIIP* expressions and MaximaTM SYBR Green (Fermentas Inc., Burlington, Ontario, Canada) for *TBP* expressions. The expression levels of *MIIP* were normalized by the expression level of the housekeeping gene for TATA box binding protein (*TBP*). The sequences for the PCR-primers were designed with Primer3-program (http://bioinfo.ut.ee/primer3-0.4.0/primer3/input.htm) and can be seen in Table 4. TaqMan probe Hs00976263_m1 (Applied Biosystems, Foster City, CA) was used for detection of MIIP. The specificity of the reactions was confirmed with 1% agarose gel electrophoresis in addition to the melting curve analysis. Standard curve was done using LNCaP + universal prostate cDNA (stock 14.6-10) by using 5-fold dilution series.

Table 4: Primer sequences.

Sequencing primers for plasmids			
	Primer sequence 5'-3'		
forward vector primer T7	TAATACGACTCACTATAGGG		
reverse vector primer XL39	ATTAGGACAAGGCTGGTGGG		
forward MIIP	AAAGAGCTTTGACGCCTCTG		
reverse MIIP	GTCAGTCCTCAGGGCTTCTG		
SNP sequencing primers for cell lines and xenografts			
	Primer sequence 5'-3'		
forward MIIP	AAGAGGCTCCTGGGAGATAC		
reverse MIIP	GAGGATGGCACGGTCAGA		
PCR primers for Dynazyme PCR for plasmids			
	Primer sequence 5'-3'		
forward MIIP	AAAGAGCTTTGACGCCTCTG		
reverse MIIP	GTCAGTCCTCAGGGCTTCTG		
PCR primers for Dynazyme F	PCR for cell lines and xenografts		
	Primer sequence 5'-3'		
forward MIIP	AAAGAGCTTTGACGCCTCTG		
reverse MIIP	GTCAGTCCTCAGGGCTTCTG		
RT-qPCR primers			
	Primer sequence 5'-3'		
forward MIIP	AAAGAGCTTTGACGCCTCTG		
reverse MIIP	GTCAGTCCTCAGGGCTTCTG		
forward TBP	GAATATAATCCCAAGCGGTTTG		
reverse TBP	ACTTCACATCACAGCTCCCC		
	Phusion PCR and transcript sequencing primers		
forward 1 st MIIP	TCACCTGACCAATCAAGACG		
reverse 1 st MIIP	AGGCGCCAGCTCCTCTTA		
forward 2 nd MIIP	CGAGACCCCACTCAGCAC		
reverse 2 nd MIIP	TGTCAGAGGCGTCAAAGCTC		
forward 3 rd MIIP	TGCGTGTACTGTTACCGTGTC		
reverse 3 rd MIIP	CAGACAAGCTTGGGTCCAC		

8.6 Determination of MIIP protein levels

8.6.1 Protein isolation

25 ml cell culture flasks were placed on ice and the cells were washed three times with 10 ml of ice cold phosphate buffered saline (PBS). Cells were then collected into

1.5 ml of PBS with rubber policeman and the tubes were centrifuged gently. Any excess PBS was removed from the tubes and the tubes were stored at -70 °C.

Nuclear and cytoplasmic proteins were isolated using modified Dignam's method (Dignam et al. 1983). The samples were melted on ice and possible excess PBS was removed. The volume of the cell pellet was estimated and it was suspended to three times the volume of the pellet of hypotonic buffer (for detailed composition, see Appendix 1). The solution was homogenized by pipetting back and forth for 10 seconds and incubated on ice for 10 minutes. The samples were centrifuged at 3300 x g at 4 °C for 15 minutes. The supernatant containing cytoplasmic proteins was carefully collected and stored at -80 °C.

The pellet containing nuclear proteins was diluted to low-salt buffer (for detailed composition, see Appendix 1) to a volume half of that of the hypotonic buffer used for the same sample. The solution was mixed by pipetting back and forth a few times before adding high-salt buffer (for detailed composition, see Appendix 1) in a dropwise manner. The volume of the high-salt buffer used was half the volume of low-salt buffer used for the same sample. The samples were incubated on ice for 30 minutes and mixed gently during the incubation. After incubation, the samples were centrifuged at 2500 x g for 30 minutes at 4 °C. Supernatant was collected and stored at-80 °C.

Protein samples were diluted 1:100 with water for concentration measurements. The standard curve was prepared from bovine serum albumin (BSA) with concentrations of 8, 26, 44, 62 and 80 μ g/ml. Protein concentrations were measured with Bio-Rad protein assay reagent (Bio-Rad Laboratories Inc., Hercules, CA). 40 μ l of Bio-Rad reagent was added to 160 μ l of standard or diluted sample. The samples were incubated at room temperature for 15 minutes and after that absorbances were measured at 595 nm with Multiskan GO Microplate Spectrophotometer (Thermo Scientific, Thermo Fisher Scientific, Inc., Waltham, USA).

8.6.2 SDS-PAGE

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out in a 10% separating gel and a stacker gel (for details, see Appendix 2). Protein samples were diluted with water to two different concentrations: 1 µg/µl and 0.65 µg/µl to a total volume of 10 µl. 10 µl of a mixture containing 9/10 parts of 3 x SDS reaction sample buffer (Bio-Rad Laboratories Inc., Hercules, CA) and 1/10 parts of 1.25 M dithiothreitol (DTT) was added to samples, mixed and the samples were then denaturated at 97°C for three minutes. Samples were then loaded in the wells of the gel. Kaleidoscope Prestained Standard (Bio-Rad Laboratories Inc., Hercules, CA) was used as a marker. The samples were run with Bio-Rad Mini-PROTEAN® 3 Cell, 67S/09185 (Bio-Rad Laboratories Inc., Hercules, CA) with 100 V to the interface of the gels (about 15 minutes) and after that the voltage was raised to 200 V. Samples were run until the dye of the SDS sample buffer reached the bottom of the gel (about one hour).

8.6.3 Western blotting

Western blotting was done with Bio-Rad Trans-Blot® SD Cell, 221BR 18151 (Bio-Rad Laboratories Inc., Hercules, CA). Immobilon-P PVDF membrane (Millipore, Billerica, MA, USA) was cut to appropriate size and prepared for blotting by soaking it in methanol for 15 seconds, in water for 5 seconds and transferring it then into the blotting buffer (see Appendix 2). First, three pieces of chromatography paper (Whatman, Brentford, UK) were soaked in the blotting buffer and placed on the blotting cell. Then, PVDF membrane was placed on top of the papers and the SDS-PAGE gel on top of the membrane. At last, three pieces of chromatography paper soaked in the blotting buffer were laid on top. The blotting was carried out at 20 V for one hour.

The PVDF membrane was carefully moved to mixture made of 2.5 grams of powdered milk and 50 ml of PBS and blocked overnight at 4°C. The primary monoclonal antibody HA.11 Clone 16B12 (Covance Inc., Delaware, USA) was diluted 1:1000 in a 1% BSA, PBS-0.1% Tween-20 mixture. After blocking, the membrane was placed to the primary antibody solution and incubated for two hours at room temperature. The membrane was washed five times for 5 minutes with PBS-Tween20. The secondary polyclonal antibody, rabbit anti-mouse immunoglobulins (Dako, Glostrup, Denmark) was diluted 1:1000 in a 1% BSA, PBS-0.1% Tween-20 mixture. The membrane was placed to the secondary antibody solution and incubated for one hour at room temperature. The membrane was then washed again two times for 10 minutes and once for 20 minutes with PBS-Tween20.

The detection was done with Western Blotting Luminol Reagent, cat# sc-2048 (Santa Cruz Biotechnology, Santa Cruz, CA) for one minute. The films (Kodak BioMax MR Film, Sigma-Aldrich Co., Missouri, USA) were exposed for 15 seconds.

8.7 Proliferation assays

Proliferation of transfected cells was measured with alamarBlue and image analysis methods. For proliferation assays, stable DU145 clones and parental DU145 cells were plated 5000 cells per well on 24-well plates. Four parallel samples were used in each experiment. Proliferation assays were initiated the next day from plating (day 0).

LAPC-4 cells were plated 100 00 cells per well and transfected as previously described for proliferation assays. Four parallel samples were used in each experiment.

8.7.1 AlamarBlue

The amount of cells in the beginning of the experiment and in the end of the experiment was analyzed using alamarBlue reagent (Invitrogen Life Technologies, Carlsbad, CA). 10% alamarBlue reagent was added to every well and the plates were incubated at 37°C and 5% CO₂ for 3 hours for DU145 cells and 2 hours for LAPC-4 cells. After incubation, 100µl of alamarBlue containing medium from each well was pipetted to a 96-well plate. The fluorescence was measured with ELISA plate reader,

(Wallac 1420 Victor, PerkinElmer, Fremont, CA). Medium with 10% added alamarBlue reagent without cells was used as a control. For relative growth analysis, the luminometric values of the cells in each well at the end of the experiment were divided by the mean values at the beginning of the experiment.

8.7.2 Imaging and image analysis

25 images from each well were taken at regular intervals using motorized, inverted fluorescence imaging microscope system (Märzhäuser micro-positioning systems, Germany). The amount of cells in each well was analyzed by calculating the area of the images covered by living cells using freely available ImageJ image analysis software (http://imagej.nih.gov/ij/). Values were normalized with the area covered by cells in each well at the first day of imaging (day 0).

8.8 Migration assays

For LAPC-4 cells, scratches were drawn to the cell layer in the bottom of the wells prior to siRNA transfections as previously described. For stable DU145 clones and parental DU145 cells, 100 000 cells were plated on each 24-well plate well. The next day, scratches was drawn across (from 11 o'clock to 5 o'clock) the confluent cell layer on the bottom of the wells with a 10 µl pipette tip in the same manner as for LAPC-4 cell, growth medium was exchanged and cells were imaged.

25 images from each well were taken at regular intervals using motorized, inverted fluorescence imaging microscope system (Märzhäuser micro-positioning systems, Germany). The size of the scratch was analyzed by manually drawing the edges of the scratch and calculating the surface area of it with ImageJ image analysis software. Values of each well were compared to the area of the scratch in that well at the first day of imaging (day 0).

9. RESULTS

9.1 Sequencing

A segment of *MIIP* gene containing SNP rs2295283 previously shown to affect the risk of breast cancer was sequenced from seven prostate cancer cell line and 17 prostate cancer xenograft genomic DNA samples. Cell line copy numbers and most xenograft copy numbers (23.1, 35, 49, 58, 69, 70, 73, 77, 86.2, 93, 96, 105 and 115) were obtained from Saramäki et al. (2006) data and the rest of the xenograft copy numbers from Kivinen, Saramäki et al. (unpublished). The results are shown in Table 5.

Table 5: Copy numbers and single nucleotide polymorphism forms of MIIP in prostate cancer cell lines and LuCaP xenografts

Cell line/	Copy	SNP
Xenograftt	nro	
DU145	1	A-
VCaP	2	GG
PC-3	2	AA
LNCaP	2	AG
DuCaP	2	GG
22Rv1	2	GG
LAPC-4	3	AG/n/a
LuCaP 23.1	2	GG
LuCaP 23.1 AI	1	n/a
LuCaP 23.8	n/a	GG
LuCaP 23.12	1	G-
LuCaP 35	2	n/a
LuCaP 35 AI	1	G-
LuCaP 41	1	n/a
LuCaP 49	1	A-
LuCaP 58	2	GG
LuCaP 69	1	G-
LuCaP 70	2	GG
LuCaP 73	2	AG
LuCaP 77	2	GG
LuCaP 78	2	AG
LuCaP 81	2	n/a
LuCaP 86.2	2	AA
LuCaP 92.1	n/a	GG
LuCaP 93	2	AA
LuCaP 96	1	G-
LuCaP 96AI	1	n/a
LuCaP 105	2	AG
LuCaP 115	1	G-

AA means both alleles contain A, AG means one allele contains A and the other G and GG means both alleles contain G. If information of the copy number or SNP status was not available, it is marked as n/a.

Of the 24 cell line and xenograft samples sequenced, 58% carried GG (or G-), 21% AA (or A-) and 21% AG form of the functional SNP in *MIIP* in their genome. Genomic DNA and copy number information was not available for some samples (indicated n/a).

The full-length transcript of *MIIP* was also sequenced in three parts from 7 cell line cDNA samples to identify possible mutations. Mutations were not detected in the samples screened.

9.2 Expression levels of MIIP

9.2.1 MIIP mRNA levels

Relative expression of *MIIP* in prostate cancer cell lines and xenografts was measured with quantitative RT-PCR to evaluate the connection of the level of expression of *MIIP* with SNP results and to determine which cell lines to use for functional experiments. The expressions are plotted in Figures 5 and 6.

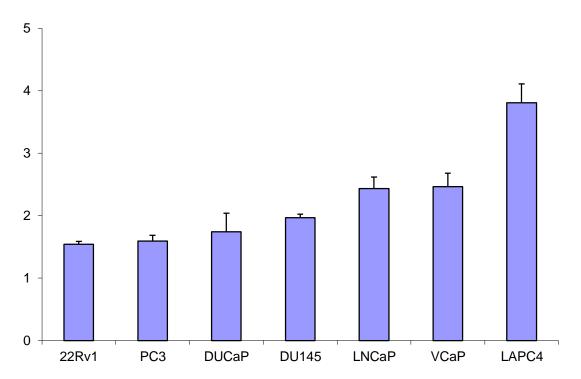


Figure 5: The relative expression of MIIP (MIIP/TBP) in different prostate cancer cell lines.

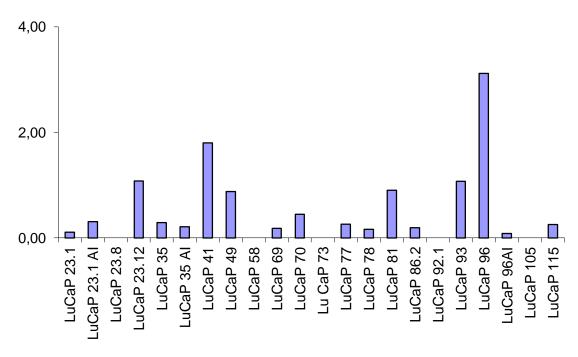


Figure 6: The relative expression of MIIP (MIIP/TBP) in prostate cancer LuCaP xenografts

The lowest expression of *MIIP* in cell lines was found in 22Rv1 and PC3 and in LuCaP xenografts in 96AI, 23.1, 78 and 69.

LAPC-4 cell line with the highest expression of *MIIP* was chosen for experiments with small interfering RNAs (siRNAs) to properly see the effects of gene silencing. LAPC-4 cells were transfected with four different siRNAs targeting *MIIP* (indicated with numbers 1,2,3 and 4), a pool of aforementioned containing equal amount of each siRNA and a control siRNA in two different concentrations to determine the optimal transfection conditions. Relative expression of *MIIP* was measured from the samples with quantitative RT-PCR and the results can be seen in Figure 7.

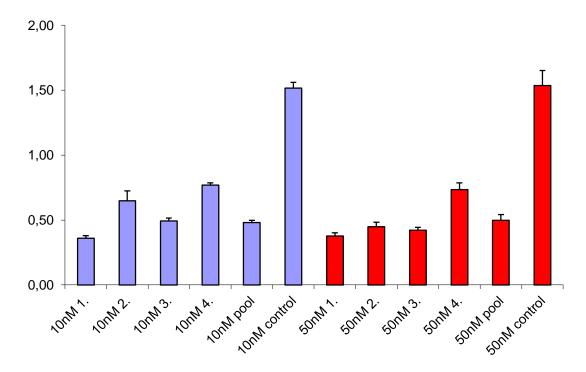


Figure 7: Relative expression of MIIP (MIIP/TBP) in LAPC-4 after different siRNA transfections.

MIIP was found to be most efficiently silenced with 50 nM siRNAs 1 and 3. In those samples, the expression of MIIP was less than one third of that of the control. The difference in expression between siRNAs 2 and 3 was so small, that siRNA 1 and 2 were used for proliferation assays. In the migration assay, also siRNA 3 was included.

Interestingly, DU145, a cell line derived from PC brain metastasis, was found to express *MIIP* in relatively low levels, and to carry only one copy of *MIIP* and the more risk prone A-genotype of the SNP rs2295283. For these reasons, and due to fast growth of cells, DU145 cells were chosen for *MIIP* overexpression studies. DU145 cells were transfected with a pCMV6-AC-HA plasmid containing *MIIP* and an empty pCMV6-AC-HA control plasmid. Stable clones were created and their relative expression of *MIIP* was measured. Results are shown in Figure 8.

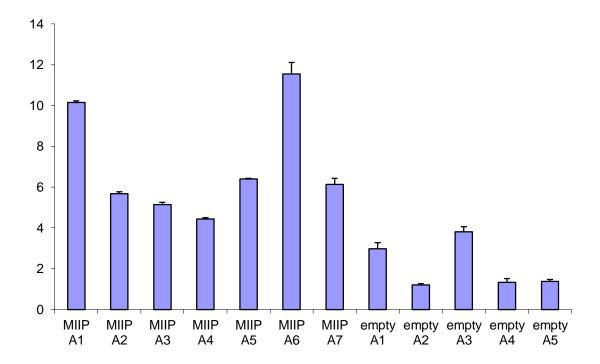


Figure 8: Relative expression of MIIP (MIIP/TBP) in stable DU145 clones.

Over eightfold expression was seen in clones MIIP A1 and MIIP A6 transfected with the plasmid containing *MIIP* when compared to controls empty A2 and empty A4 with the lowest expression of *MIIP* (clones indicated MIIP A1, MIIP A6, empty A2 and empty A4 according to the wells they were first ringcloned to in 96-wellplates).

9.2.2 MIIP protein level

Due to technical difficulties, MIIP protein level could not be measured. Protein concentrations after isolation were low or could not be measured for most nuclear protein components.

9.3 Proliferation assays

Relative growth of transfected cells was assessed with image analysis method and with alamarBlue method as described in sections 8.7.1 and 8.7.2.

Relative growth of siRNA transfected LAPC-4 cells measured with both methods are shown in Figures 10 and 11.

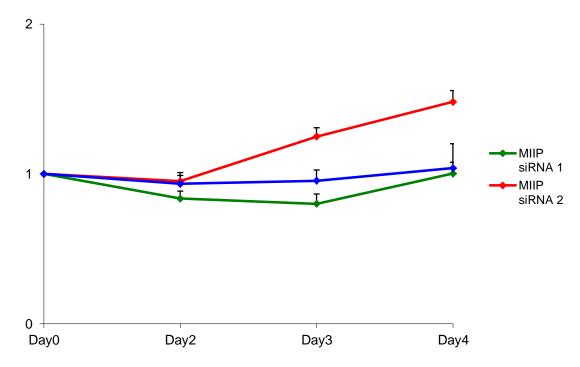


Figure 10: Relative growth of siRNA transfected LAPC-4 cells obtained by image analysis

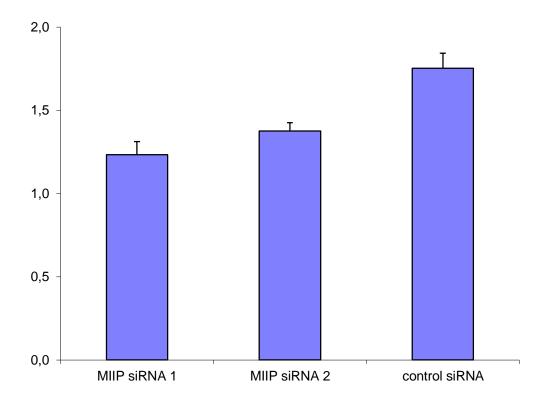


Figure 11: Relative growth of siRNA transfected LAPC-4 obtained with alamarBlue

Transfections with MIIP siRNA 1 caused the cells to eventually detach from the bottom of the wells, so no growth was observed. In Figure 10, it seems that some

growth was seen with MIIP siRNA 1. However, the misguided information is due to floating cells that the image analysis software incorrectly interpreted as growing cells.

Image analysis results show, that the growth of the cells transfected with MIIP siRNA 2 was faster than with the cells transfected with control siRNA (p=0.03<0.05, Mann-Whitney U-test). On the contrary, alamarBlue results show that the metabolism in MIIP siRNA 2 transfected cells is lower than in the control (p=0.03<0.05, Mann-Whitney U-test) It should be noted that transfections with the control siRNA caused changes in the phenotype of the cells. LAPC-4 cells grow clustered together also in normal conditions but the transfection with control siRNA caused the cell clusters to be more round and distinct from each other.

Relative growth of stable, *MIIP* overexpressing DU145 clones was also assessed with both methods and the results are seen in Figures 12 and 13.

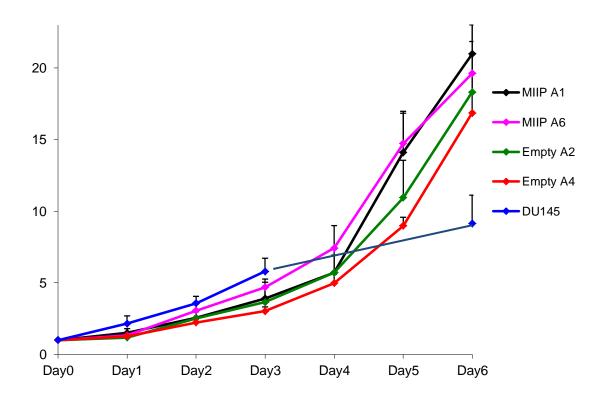


Figure 12: Relative growth of stable DU145 clones obtained by image analysis.

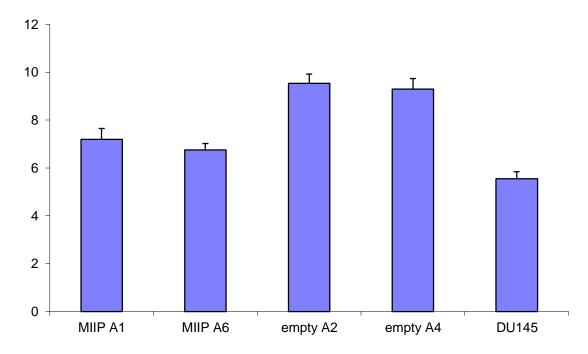


Figure 13: Relative growth of stable DU145 clones obtained with alamarBlue.

With image analysis, it was seen that the growth was only a little faster with the clones with the higher expression of *MIIP* (MIIP A1 and MIIP A6) than with the empty controls (empty A2 and empty A4) (p=0.19>0.05, Mann-Whitney U-test). The growth of all transfected clones (*MIIP* and empty control) was significantly faster than with parental DU145 (p=0.0004<0.05, Mann-Whitney U-test). AlamarBlue showed that the metabolic activity was significantly higher in controls empty A2 and empty A4 than in MIIP A1 and MIIP A6 clones with higher expression of *MIIP* (p=0,0002<0,05, Mann-Whitney U-test). The lowest metabolic activity in the transfected clones was observed in MIIP A6 which had the highest expression of *MIIP*.

9.4 Migration assays

The effect of *MIIP* expression on cell migration was studied with scratch assay. Figure 14 representing relative scratch size in siRNA transfected LAPC-4 shows that significant difference was not seen between migration in cells transfected with MIIP siRNAs and cells transfected with control siRNA (p=0,33>0,05, Mann-Whitney U-test). Transfection with MIIP siRNA 1 did not cause the cells to completely detach from the bottom of the wells as in the proliferation assay, but it prevented the cells from migrating. Transfection in general with all siRNAs inhibited migration which was seen when compared to parental LAPC-4 (data not shown).

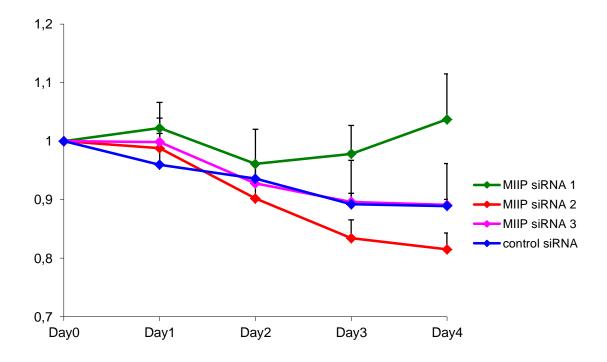


Figure 14: Relative scratch size in siRNA transfected LAPC-4 cells.

Results of the effect of *MIIP* overexpression on cell migration in stable DU145 clones are shown in Figure 15.

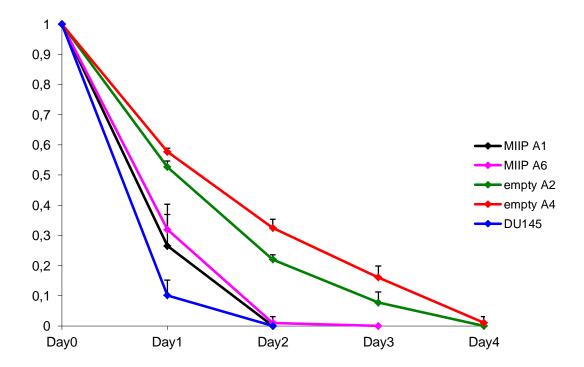


Figure 15: Relative scratch size in stable DU145 clones and in parental DU145.

The migration of parental DU145 was very significantly faster than that of transfected clones (p=0,000075<0,05, Mann-Whitney U-test) and clones MIIP A1 and MIIP A6 migrated significantly faster than clones empty A2 and empty A4 (p=0,000018<0,05, Mann-Whitney U-test).

10. DISCUSSION

In this study, we examined the effect of MIIP on prostate cancer cell proliferation and migration by overexpressing and silencing MIIP in two prostate cancer cell lines. In addition, the gene was screened for mutations and SNP rs2295283 previously proven to affect the risk of breast cancer. Previous studies have shown overexpression of MIIP to inhibit proliferation and migration in glioma cell lines (Song et al. 2003). This gene and its effects in prostate cancer have not been previously studied. Due to its significance in other cancer types, mechanisms of action of the protein and the location of the gene in a commonly deleted area, the effect of MIIP in prostate cancer is a topic worth studying.

10.1 Methodological aspects

MIIP protein level could not be discovered. One possible reason for this could lie in the used antibody. The antibody has previously been used in another research group with no problems. However, the received aliquot had been stored for some time before using it again in this assay. Even though it had been stored according to manufacturer's instructions, it is possible that the storage time could have reduced the efficacy of the antibody. Since the antibody used was against the human influenza hemagglutinin (HA) tag of the protein, it is also possible that for some reason the expression of the tag was disrupted and caused the antibody not to recognize the protein.

Image analysis was done using ImageJ software. Measured proliferation was based on the area of the cells covering the well. Since the area of the wells is relatively small, any air bubbles left in the surface of the growth media during plating or media exchange, might affect the amount of cells seen in a specific image.

ImageJ program calculated the area covered by cells based on contrast differences between areas where cells were growing and the background. Since cells were grown in a humidified incubator, condensing water on the lids of the plates made lightning adjustments of the microscope very difficult as the amount of condensed water was different on each well and at each timepoint. Too much lightning caused ImageJ to interpret also areas with cells as background, especially with DU145 cells which seem very transparent in the bottom of the culture vessel. Contrast could be enhanced with ImageJ, but it probably still caused some errors in the calculated values. Also floating cells detached from the bottom were incorrectly interpreted as growing cells by the software and might have had an effect on the achieved results.

Scratch area was measured with ImageJ by manually defining the edges of the scratch. Although the work was done with utmost attention in the accuracy, human errors are still possible. Due to these numerous difficulties, results from image analysis methods in both assays are very unreliable. Results from alamarBlue method should be considered more trustworthy.

Transfections with all siRNAs reduced proliferation and migration when compared to parental LAPC-4 cells. Transfections with all siRNAs also caused changes in the phenotype of the cells and transfection with MIIP siRNA1 caused the cells to detach from the bottom of the wells in the proliferation assay. Transfection in general is a procedure that can disrupt the highly orchestrated balance of cellular functions so these alterations could be due to that. It is also possible that the siRNAs were not completely specific to MIIP and interfered also the expression of other genes. In migration assays, transfection efficiency also suffers from the fact that cell density is high. Maybe by optimizing the transfection conditions even further and possibly using a lower concentration of siRNAs, better results could have been achieved. Therefore, conclusions about the connection of silencing MIIP and cell migration cannot be drawn until the assays have been replicated with different conditions and possibly different siRNAs and the results confirmed.

10.2 Sequencing

The entire *MIIP* gene was sequenced from prostate cancer cell lines and xenografts for mutations and SNPs. Mutations have not been found in previous studies in other cancers and they were not distinguished in this study either (Song et al. 2005, Wang et al. 2010). However, as stated earlier, this does not rule out *MIIP* being a tumor suppressor. Mutation is not the only mechanisms that can inactivate tumor suppressors and since the overall mutation rate in prostate cancer is relatively low (~1 per MB) compared to other cancers, these alternative inactivating mechanisms may play even bigger role in prostate cancer than in other cancers (Barbieri and Tomlings 2014). One possible way could be alternative splicing, which has been seen in gliomas and was not studied in this thesis (Song et al. 2005).

If SNP rs2295283 would have a drastic effect on prostate cancer risk, it could be thought that the more risk prone allele A would be more common among cancer cell lines and xenografts. However, most of the samples carried the protective G allele in their genotype either as homozygous or heterozygous. This would implicate that SNP rs2295283 has no considerable effect on prostate cancer risk. In addition, clear correlation between SNP rs2295283 variant and expression of *MIIP* was not seen, supporting this conclusion.

10.3 Proliferation and migration assays

It could be hypothesized that when a tumor suppressor gene is silenced, the growth of these cells is accelerated. And in turn, when the expression of a tumor suppressor is increased, growth is hindered. However, in reality the situation is not necessarily that simple. Since the regulatory mechanisms of cancer cells are already impaired, silencing yet another protection mechanism could function as the final trigger to induce apoptosis. Then again, cancer cells develop mechanisms to overcome growth obstacles rather readily (Hanahan and Weinberg 2000). Thus, overexpressing a single growth suppressing gene may not have that drastic effect on growth, when growth inhibitory signals are cancelled out with new or excess growth promoting ones.

Transfections with all siRNAs inhibited LAPC-4 cell migration compared to parental cells and significant differences in migration between cells transfected with MIIP siRNAs or with control siRNA were not seen. Image analysis showed that the growth of the cells transfected with MIIP siRNA 2 was faster than with the cells transfected with control siRNA but as stated earlier, results are not very reliable. On the contrary, alamarBlue results show that the metabolism in MIIP siRNA transfected cells is lower than in the control.

In the proliferation assays, image analysis method revealed that stable *MIIP* overexpressing DU145 cells proliferated slightly faster than cells transfected with the control vector and significant differences were not observed. All transfected cells also proliferated faster than parental DU145 cells. However, results of the image analysis method cannot be fully trusted. AlamarBlue assay showed the metabolic activity of cells transfected with *MIIP* to be significantly lower than in cells transfected with control vector. Metabolic activity of parental DU145 cells was found to be lower than that of transfected ones. Transfection in general seemed to decelerate migration since migration of all transfected clones was significantly slower than that of parental DU145. DU145 cells overexpressing *MIIP* also migrated significantly faster that cells transfected with control vector.

In both cell lines and transfection methods, similar contradiction was seen in the proliferation results of image analysis method and alamarBlue method. It is possible that even though overexpression of *MIIP* did not slow DU145 cell growth, it caused changes in cell metabolic activity. The metabolic activity of the transfected clones was seen to be the lowest with the DU145 clone expressing highest levels of *MIIP*, which supports this possibility. However, it is more likely that this contradiction is caused by some systematic error in performing the assays or by the problems with image analysis. Due to numerous technical difficulties in the image analysis method, results of alamarBlue should be considered more reliable and results of image analysis method in both assays should not be fully trusted. Differences between samples transfected with siRNAs were so minor, even though considered statistically significant, and number of samples was so small that conclusions should not be drawn, especially when taking into notice other issues related to the siRNA transfections described earlier.

A simple explanation for the results could be that *MIIP* does not have a direct effect on cell proliferation and migration and for that reason overexpression or silencing of the gene does not have an apparent effect on the behaviour of the cells on such short time scale. The observed changes in cell growth and migration can also be due to other factors, such as harmful effects of the transfection process, transfection efficiency or other extrinsic factors. Also, the effect of *MIIP* on proliferation and migration in prostate cancer cells might be dependent on some other, still unknown factor or factors that were not present in sufficient quantities in the studied cells.

It is also possible, that other prostate cancer cell lines would behave differently in these assays. They all have their distinct properties and only LAPC-4 and DU145 cells were tested in this study. It should be noted, that stable transfections with pCMV-AC-HA-MIIP and the control vector was attempted also for LNCaP cell line (data not shown). Despite optimizing the transfection protocol several times and using different transfection reagents, stable clones overexpressing MIIP eventually died. It is possible that overexpression of MIIP had a more notable inhibitory effect on cell growth in LNCaP cells and cell death was at least partly due to that. Then again, also cells transfected with control vector eventually died, although later than those overexpressing MIIP. Thus, the reason for the failure in maintaining transfected cells was more likely that LNCaP cells were more sensitive to transfection conditions and optimal conditions were not found in spite of persistent attempts.

It should also be remembered that prostate cancer cell lines and clinical prostate cancer are very different things. Clinical prostate cancer is genetically remarkably heterogenic and clinically variable (Barbieri and Tomlings 2014). Many changes occur in cancer cells during progression of the disease and cancer cell lines represent quite advanced stages of cancer. The behaviour of cells also changes when they are grown in laboratory conditions and go through multiple passaging series. However, they represent an easy and in some cases also only possibility to model effects of different genetic events on behaviour of cells. It is still possible that *MIIP* could have a more profound effect in earlier stages of prostate cancer.

11. CONCLUSIONS

In conclusion, the observed effect of *MIIP* in prostate cancer cells was small and inconsistent. It seems unlikely that *MIIP* plays a significant role in all or most prostate cancer cases. However, prostate cancer is very heterogeneous and there are numerous different kinds of prostate cancers. It is thus possible, that *MIIP* has an important effect in genesis and/or progression of some of the subtypes, even though tests with the studied cell lines and xenografts did not reveal significant effects. More experiments are needed to fully uncover the effect of *MIIP* in prostate cancer cells.

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APPENDIX 1: SOLUTIONS USED IN DIGNAM'S METHOD

Hypotonic buffer: 10 mM Hepes, pH 7.9 1.5mM MgCl₂ 10mM KCl 0.2mM PMSF 0.5mM dithiothreitol (DTT) Low-salt buffer: 20mM Hepes, pH 7.9 25% glycerol 1.5mM Mgcl₂ 20mM KCl

High-salt buffer:

0.2mM EDTA

0.2mM PMSF

0.5mM DTT

20mM Hepes, pH 7.9

25% glycerol

1.5mM Mgcl₂

1.2M KCl

0.2mM EDTA

0.2mM PMSF

0.5mM DTT

APPENDIX 2: COMPOSITIONS OF GELS AND BUFFERS USED IN SDS-PAGE

Separating gel:

H2O	6,2 ml
Glycerol	2 ml
1.5 M Tris	5 ml
30% Acrylamide-N,N'methylene-bis-acrylamide mix	6.7 ml
10% Ammonium persulfate (APS)	100 μ
N,N,N',N'-tetramethylenediamine (TEMED)	10 µl

Stacker gel:

H2O	6.5 ml
0.5 M Tris	2.5 ml
30% Acrylamide-N,N'methylene-bis-acrylamide mix	1.3 ml
10% Ammonium persulfate (APS)	90 µl
N,N,N',N'-tetramethylenediamine (TEMED)	10 µl

Running buffer:

25mM Tris 190 mM glycine 0.1% SDS

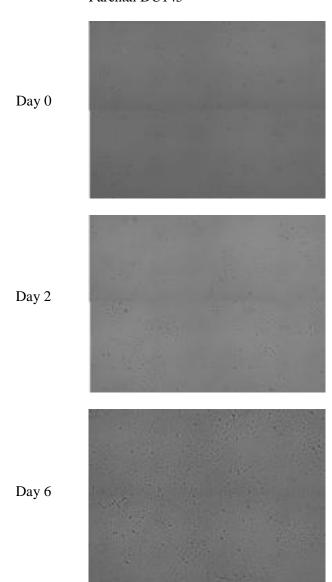
Blotting buffer:

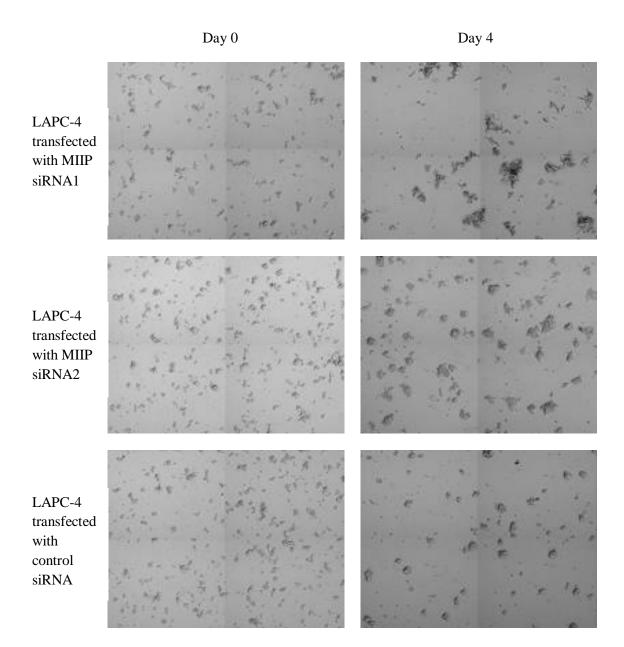
48mM Tris 39mM glycine 0.0375% SDS 10% methanol

APPENDIX 3: IMAGES OF PROLIFERATION ASSAYS

DU145 expressing MIIP (clone A1) DU145 empty control (clone A4) Day 0 Day 2 Day 6

Parental DU145





APPENDIX 4: IMAGES OF MIGRATION ASSAYS

