KIMMO SAVINAINEN

Amplification and Overexpression of ERBB2, uPA, TRPS1, EIF3S3 and MYC Genes in Prostate Cancer

ACADEMIC DISSERTATION

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ERBB2, *uPA*, *TRPS1*, *EIF3S3* ja *MYC* geenimonistumat ja ilmentyminen eturauhassyövässä

Geneettisten muutosten, kuten syöpägeenien (onkogeenien) aktivaation ja kasvurajoitegeenien inaktivaation uskotaan muuttavan hyvänlaatuisia soluja pahanlaatuisiksi. Tässä työssä on keskitytty viiden mahdollisen onkogeenin (*ERBB2*, *uPA*, *TRPS1*, *EIF3S3* ja *MYC*) osuuden selvittämiseen eturauhassyövän kehityksessä ja etenemisessä.

ERBB2 geenin kopiolukumuutoksia ja ilmentymistä tutkittiin sekä hoitamattomissa syövissä ja etäpesäkkeissä, kuten myös uusiutuneissa, hormonihoidolle vastustuskykyisissä (hormonirefraktorisissa) kasvaimissa. Käytettäessä kromogeenista in situ hybridisaatiota, tutkituista näytteistä löytyi ainoastaan yksi tapaus, jota voidaan pitää *ERBB2* geenimonistumana (6-8 kopiota). Kyseinen näyte, kuten myös kaikki muut tutkitut näytteet, osoittautuivat immunohistokemiallisissa värjäyksissä negatiivisiksi. *ERBB2* lähetti-RNA:n (mRNA) ilmentymistä eturauhassyöpäkasvaimissa ja -solulinjoissa tutkittiin käyttämällä kvantitatiivista RT-PCR – menetelmää (Q-RT-PCR). Eturauhassyöpänäytteiden mRNA määrissä ei todettu eroja eri kasvaintyyppien välillä ja mRNA-tasot eturauhassyövissä olivat vastaavat kuin rintasyövissä, joissa ei ole *ERBB2* monistumaa. Tulokset osoittavat ERBB2 ilmentymisen olevan eturauhassyövässä matala ja riippumaton levinneisyysasteesta. Onkin epätodennäköistä, että ERBB2 ilmentymiseen perustuvat hoitomuodot olisivat tehokkaita eturauhassyövän hoidossa.

uPA geenin kopiolukumuutoksia tutkittiin fluoresenssi in situ hybridisaatiolla (FISH). Näytteinä käytettiin eturauhassyöpään kuolleiden potilaiden hormonirefraktorisia etäpesäkenäytteitä sekä paikallisesti uusiutuneita hormonirefraktorisia kasvaimia. Kohonneita kopiolukuja (ei korkea-asteinen monistuma) löytyi 21 %:ssa paikallisesti uusiutuneissa kasvaimissa. 31 %:ssa etäpesäkkeistä löytyi myös kohonneita kopiolukuja, ja yksi korkea-asteinen monistuma. uPA-inhibiittoreiden vaikutusta eturauhassyöpäsolujen invaasiokykyyn tutkittiin Matrigel – menetelmällä. Tulokset osoittivat, että solujen invaasiota voidaan estää tietyillä uPA inhibiittoreilla (p-aminobentzamidine ja B428), silloin kun soluista löytyy myös *uPA* geenimonistuma.

8q monistuman kohdegeenin tunnistamista varten tutkittiin kolmea mahdollista kohdegeeniä: *TRPS1, EIF3S3* ja *MYC*. FISH -analyysissä huomattiin kaikkien kolmen geenin olevan samanaikaisesti monistuneina noin 30 %:ssa hormonirefraktorisia eturauhassyöpäkasvaimia. Geenien kopioluvut ja mRNA määrät analysoitiin lisäksi rintasyöpä- ja eturauhassyöpäsolulinjoista. SK-Br-3 rintasyöpäsolulinjasta, jossa oli korkein kopioluku kaikista kolmesta geenistä, ainoastaan *EIF3S3* mRNA:n ilmentyminen oli koholla. *TRPS1*, *EIF3S3* ja *MYC*

mRNA-määrät analysoitiin myös näytteistä joissa oli havaittu hyvänlaatuista liikakasvua (hyperplasia), hoitamattomista eturauhassyövistä ja hormonirefraktorisista eturauhassyövistä. Verrattaessa syöpänäytteitä hyperplasioihin, huomattiin *EIF3S3*:n mRNA määrän olevan koholla, kun taas *TRPS1* ja *MYC* mRNA määrät pysyivät samalla tasolla kasvaintyypistä riippumatta. Tulokset osoittavat *EIF3S3*:n ilmentymisen olevan koholla eturauhassyövässä ja eräs mekanismi tälle näyttäisi olevan geenimonistuma.

Lopuksi *EIF3S3*:n ilmentymisen vaikutusta tutkittiin hiiren fibroblastisolulinjassa (NIH 3T3). Kohonneella EIF3S3:n ilmentymisellä oli positiivinen vaikutus kasvualustaan kiinnittymättömien solujen kasvuun ja elinkykyyn. RNA-inhibition vaikutusta tutkittiin eturauhas- ja rintasyöpäsolulinjoissa. Neljän päivän jälkeen *EIF3S3*:n ilmentymisen estolla oli merkittävä negatiivinen vaikutus kaikkien tutkittujen solulinjojen kasvuun. Tulokset osoittavat *EIF3S3*:lla olevan merkitystä solujen kasvun säätelyssä.

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LIST OF ORIGINAL COMMUNICATIONS

This thesis is based on the following communications, which are referred to in the text by their Roman numerals:

I. Savinainen KJ, Saramäki OR, Linja MJ, Bratt O, Tammela TL, Isola JJ and Visakorpi T (2002): Expression and gene copy number analysis of *ERBB2* oncogene in prostate cancer. Am J Pathol 160:339-345.

II. Helenius MA, Savinainen KJ, Bova GS and Visakorpi T (2006): Amplification of the urokinase gene and the sensitivity of prostate cancer cells to urokinase inhibitors. BJU Int 97:404-409.

III. Savinainen KJ, Linja MJ, Saramäki OR, Tammela TL, Chang GT, Brinkmann AO and Visakorpi T (2004): Expression and copy number analysis of TRPS1, EIF3S3 and MYC genes in breast and prostate cancer. Br J Cancer 90:1041-1046.

IV. Savinainen KJ, Helenius MA, Lehtonen HJ and Visakorpi T: Overexpression of EIF3S3 promotes cell growth. Submitted for publication.

ABBREVIATIONS

AR	Androgen receptor
ABL	V-abl Abelson murine leukemia viral oncogene homolog 1
BCL2	B-cell CLL/lymphoma 2
BPH	Benign prostatic hyperplasia
CAV1	Caveolin 1
CD44	CD44 antigen (homing function and Indian blood group system)
DAPI	4, 6-diamino-2-phenylindole
CDH1	Cadherin 1 (E-cadherin)
CGH	Comparative genomic hybridization
CISH	Chromogenic in situ hybridisation
CpG island	Short region of DNA in which the frequency of the CG sequence is higher than other regions
dUTP	Deoxyuridine triphosphate
ECM	Extra cellular matrix
EIF3S3	Eukaryotic translation initiation factor 3, subunit 3 (eIF3-p40)
ERBB2	V-erb-b2 erythroblastic leukemia viral oncogene homolog 2 (HER-2/neu)
ERG	V-ets erythroblastosis virus E26 oncogene like
esiRNA	Endoribonuclease-prepared short interfering RNA
ETV	Ets variant gene 1
EZH2	Enhancer of zeste homolog 2
FISH	Fluorescence in situ hybridisation
FITC	Fluorescein isothiosyanate
GSTP1	Glutathione S-transferase pi
HGPIN	High-grade PIN
HR	Hormone refractory
IHC	Immunohistochemical
KAI1	Kangai 1
KLF5	Kruppel-like factor 5 (intestinal)
KLF6	Kruppel-like factor 6
LOH	Loss of heterozygosity
MET	Met proto-oncogene (hepatocyte growth factor receptor)
MXI1	MAX interactor 1

MSR1	Macrophage scavenger receptor gene 1
MYC	V-*MYC* myelocytomatosis viral oncogene homolog
NKX3-1	NK3 transcription factor related, locus 1
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PSCA	Prostate stem cell antigen
PIA	Proliferative inflammatory atrophy
PIN	Prostatic intraepithelial neoplasia
PSA	Prostate specific antigen
PTEN	Phosphatase and tensin homolog
RAD21	RAD21 homolog
RASSF1A	Ras association (RalGDS/AF-6) domain family 1
RB1	Retinoblastoma gene 1
RNAi	RNA interference
RT-PCR	Reverse transcriptase PCR
siRNA	Small interfering RNA
TBP	TATA box binding protein
TBS	Tris buffered saline
TMA	Tissue microarray
TP53/p53	Tumor protein p53
TRPS1	Trichorhinophalangeal syndrome type I; GC79
TSG	Tumor suppressor gene
TURP	Transurethral resection of prostate
uPA	Urokinase type plasminogen activator, alias PLAU
uPAR	Plasminogen activator, urokinase receptor, alias PLAUR
Q-RT-PCR	Quantitative real-time RT-PCR

ABSTRACT

Genetic alterations, including activation of proto-oncogenes and inactivation of tumor suppressor genes, are involved in transforming benign cells into malignant ones. In this study we wanted to investigate the role of five possible oncogenes, *ERBB2*, *uPA*, *TRPS1*, *EIF3S3* and *MYC* in the development and progression of prostate cancer.

ERBB2 gene copy number and expression was analyzed in both androgen-dependent primary and metastatic tumors, as well as recurrent hormone-refractory tumors. Using chromogenic in situ hybridization only one borderline amplification of *ERBB2* (6-8 copies) was found in the prostate tumors studied. Immunohistochemical staining of ERBB2 protein was negative in all prostate samples, including the sample with the borderline amplification of *ERBB2* gene. The expression level of *ERBB2* mRNA in prostate tumors as well as in prostate cell lines was analyzed using real-time quantitative RT-PCR (Q-RT-PCR). No differences in the expression levels between tumor types were found, and the expression levels in prostate cancer corresponded to levels in breast carcinomas without *ERBB2* amplification. The data suggest that the expression of *ERBB2* is low in prostate cancer, regardless of the stage. Thus, it is unlikely that therapies based on overexpression of the *ERBB2* gene will be effective in the treatment of prostate cancer.

Hormone-refractory metastases and locally recurrent hormone-refractory tumors from patients who died of prostate cancer were analyzed for *uPA* gene copy number by using fluorescence in situ hybridization. Increased copy number but no high-level amplifications, of *uPA* was found in 21% of the locally recurrent hormone-refractory tumors. 31% of metastases showed increased copy number of *uPA*, and one case with a high-level amplification was also observed. The effect of *uPA* inhibitors on the invasion potential of prostate cancer cell lines was studied using Matrigel invasion assay. The data indicated that invasion of prostate cancer cells containing *uPA* amplification was inhibited with specific *uPA* inhibitors (p-aminobentzamidine and B428), whereas this was not the case in cells without amplification.

To identify the target gene for the amplification on 8q, 3 candidate genes, *TRPS1*, *EIF3S3*, and *MYC*, were studied. All 3 genes were found co-amplified in about 30% of hormone-refractory prostate carcinoma tumors, analyzed by FISH. Copy number and mRNA expression (by Q-RT-PCR) of *TRPS1*, *EIF3S3* and *MYC* genes were also analyzed in breast and prostate cancer cell lines. Only *EIF3S3* mRNA was overexpressed in SK-Br-3 breast cancer cell line, which contained the highest copy number of all three genes. Next, the expression levels

of *TRPS1*, *EIF3S3* and *MYC* mRNAs were analyzed in benign prostate hyperplasia (BPH), untreated and hormone-refractory prostate tumors. *EIF3S3* mRNA expression was higher in prostate carcinomas compared to BPH, but *TRPS1* and *MYC* mRNA levels were similar in all prostate tumor types. The data suggest that expression of *EIF3S3* mRNA is increased in prostate cancer, and gene amplification seems to be one mechanism for the overexpression.

Finally, the effect of EIF3S3 overexpression on cell growth was studied in NIH 3T3 murine fibroblasts using pTet-Off expression system. Overexpression had a positive effect on growth rate and survival in soft agar. The effect of *EIF3S3* inhibition was studied in prostate and breast cancer cell lines using the siRNA method. After four days, the reduction in cell growth was significant in all four cell lines. The results imply that *EIF3S3* has a significant role in regulating cell growth, and its overexpression may give rise to improved cancer cell survival.

INTRODUCTION

Prostate is the largest accessory gland of the male reproductive system. It is located in front of the rectum, just below the bladder and is of nearly the same size and shape as a walnut. The main function of the prostate is to produce seminal fluid (i.e. semen), which transports sperm. Because the prostate surrounds the urethra, it also helps to control the flow of urine (Moore et al., 1999). The prostate has been anatomically divided into 4 zones: anterior fibromuscular stroma, central zone, peripheral zone, and preprostatic region, which include the periurethral ducts and transition zone (Srodon et al., 2002).

Prostatic adenocarcinoma is the most common malignancy among men in many Western industrialized countries. In Finland, 4,225 new cases were diagnosed in 2003 (Finnish Cancer Registry 2005). The etiology of prostate cancer is not well understood. Androgens are believed to play an important role in normal prostate development, in benign prostatic hyperplasia (BPH) and in prostate carcinogenesis (reviewed by Parnes et al., 2005). Over 60 years ago, Huggins and Hodges (1941) demonstrated that castration is an effective treatment for prostate cancer. For advanced cancer the only effective treatment is still androgen deprivation, either by surgical or chemical castration or antiandrogens. Recently, the importance of androgens in the development of prostate cancer was once again demonstrated in a cancer prevention trial with finasteride (Thompson et al., 2003). Finasteride inhibits 5-alpha reductase, the enzyme that converts testosterone into dihydrotestosterone, which is the most active form of androgens in the prostate. In the trial, ~25% reduction in the number of prostate cancers was found with the administration of finasteride indicating directly the involvement of androgens in the development of this disease. Epidemiological studies have shown that a family history of prostate cancer is associated with an elevated relative risk for the disease. In addition to family history, major risk factors for prostate cancer include age and race (reviewed by Ostrander et al., 2004). Other factors such as smoking, alcohol consumption, vasectomy, and physical activity have been investigated in several studies, but the overall conclusion is that they do not affect risk of prostate cancer (reviewed by Grönberg, 2003).

The development of cancer, including prostate carcinoma, is a multi-step process requiring a large number of mutations to initiate, promote and allow a tumor to progress through a series of morphologically defined states. High-grade prostatic intraepithelial neoplasia (HGPIN) preferentially develops in the peripheral zone of the prostate, which is also the site of origin for most adenocarcinomas, and is thought to represent the precursor of invasive

carcinomas. However, the lesion is not a necessary precursor because many early cancers do not have adjacent HGPIN (reviewed by DeMarzo et al., 2003). More recently, proliferative inflammatory atrophy (PIA) has been proposed as a precursor to PIN. PIAs are often directly adjacent to PIN lesions or adenocarcinomas, and contain chromosomal abnormalities similar to PIN and prostate cancer cells (Uzgare et al., 2005). The development of prostate cancer via different stages is illustrated in Figure 1.

The molecular mechanisms underlying the progression described in Figure 1 are incompletely understood. It is generally known that the development of cancer requires alterations in critical genes that are usually defined either as tumor suppressor genes (TSGs) or proto-oncogenes. Of these, proto-oncogenes, when activated either by point mutations, translocations or amplifications, predispose to cancer. In this study we focused on elucidating the role of a couple of candidate proto-oncogenes in prostate cancer, namely the v-erb-b2 erythroblastic leukemia viral oncogene homolog 2 (*ERBB2*), urokinase type plasminogen activator (*uPA*), trichorhinophalangeal syndrome type I (*TRPS1*), eukaryotic translation initiation factor 3, subunit 3 (*EIF3S3*) and v-*MYC* myelocytomatosis viral oncogene homolog (*MYC*).

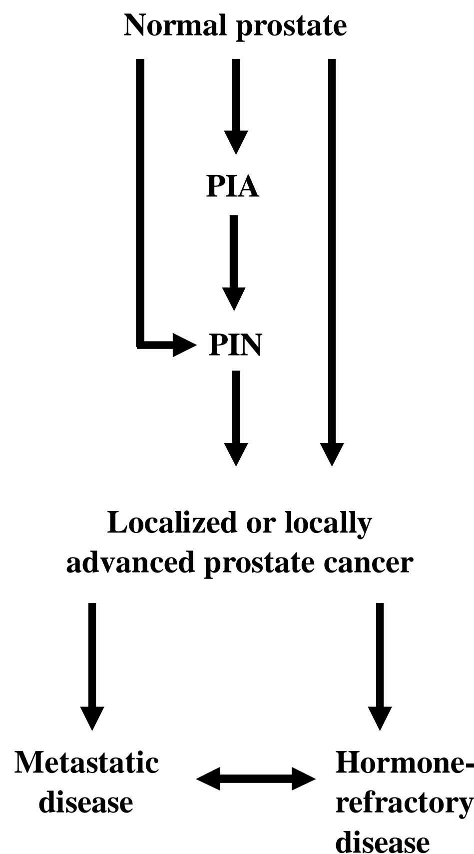


Figure 1. *Development of prostate cancer from normal prostate to metastatic or androgen-independent, hormone-refractory disease.*

REVIEW OF THE LITERATURE

1. Proto-oncogenes and tumor suppressor genes

Proto-oncogenes are derived from normal cellular genes, which upon constitutive activation can cause a cell to become tumorigenic or even metastatic. They are often involved in signal transduction pathways important in the regulation of cell proliferation. Activation of proto-oncogene can occur, for example, by point mutation, amplification or translocation leading to overexpression of the gene product (Figure 2).

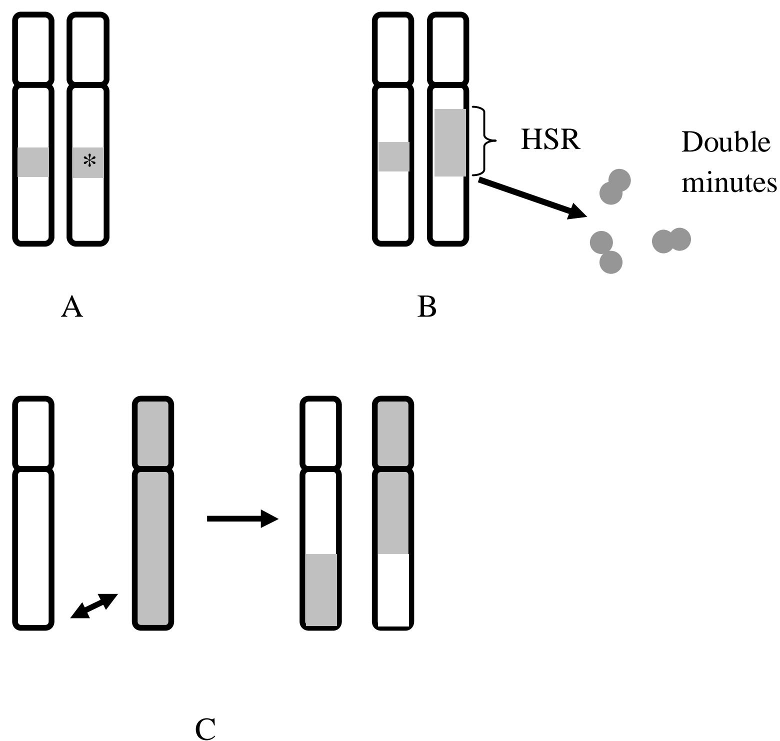
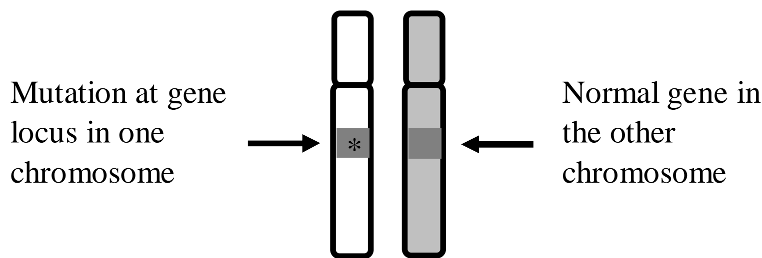


Figure 2. *Possible ways of activating proto-oncogene. A) Activating point mutation (*), B) amplification, which is seen either as extra chromosomal double minutes or as an integrated, homogenous staining region (HSR), and C) translocation of the gene.*

Oncogenes act dominantly, i.e. only one allele has to be altered for phenotypic consequences. Overall many proto-oncogenes have been identified, but only a few have been implicated in prostate cancer (rewieved by Porkka and Visakorpi, 2004).

Tumor suppressor genes, on the other hand, are genes that normally control cellular proliferation or the integrity of the genome. Most of them belong to one of two subclasses, gatekeepers or caretakers (Kinzler et al., 1997). Gatekeepers are capable of inhibiting the proliferation of cancer cells by permanently arresting growth or inducing apoptosis, whereas caretakers can prevent or repair genomic damage (reviewed by Campisi, 2003). Inactivation of gatekeeper genes (for example: *p53*, *RB1*, *VHL* and *APC*) contributes to neoplastic growth, while inactivation of caretaker genes (for example, *XPB*, *MSH2* and *MLH1*) results in a greatly increased mutation rate (Kinzler et al., 1998). According to the Knudson “two-hit” theory, both alleles of the tumor suppressor gene must be inactivated for its activity to be lost (Knudson et al., 1971). This may take place by mutation, deletion or chromosomal loss (reviewed by Knudson, 2001). Chromosomal mechanisms leading to inactivation of tumor suppressor gene are illustrated in Figure 3.

I. First ”hit” (inherited or somatic mutation)



II. Second ”hit”

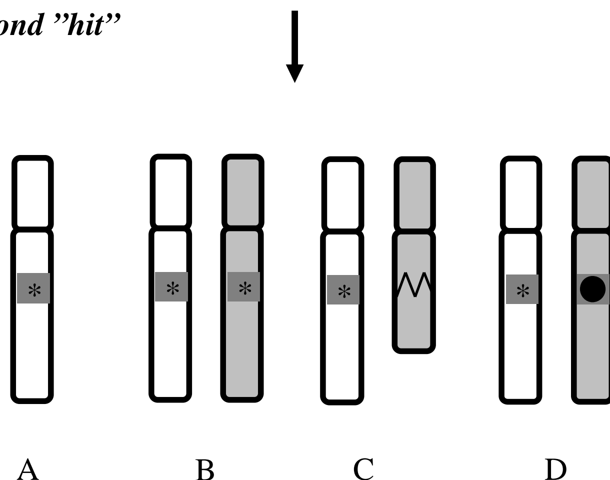


Figure 3. *Chromosomal mechanisms for inactivation of TSG. A) Chromosome loss, B) gene conversion, C) deletion, D) hypermethylation(•).*

Recently it has been suggested that there are other mechanisms which can inactivate tumor suppressor genes, and probably the best-characterized mechanism is DNA hypermethylation (reviewed by Jones and Baylin, 2002; Herman and Baylin, 2003). Classical tumor suppressor genes are thought to require inactivation of both alleles to facilitate tumor phenotype. However, it has been suggested that for some tumor suppressor genes (for example *PTEN* and *TP53)*, loss of only one allele (haploinsufficiency), may lead to cancer formation. In knock-out mouse models tumorigenesis has been attributed to haploinsufficiency in various malignancies, for example gastrointestinal cancer, sarcomas, colorectal cancer, breast and prostate cancer (reviewed by Santarosa and Ashworth, 2004).

2. Molecular basis of prostate cancer

The molecular basis of prostate cancer can include both in heritable and somatic genetic changes that drive the formation and aggressiveness of the disease. Every carcinoma is presumed to arise from a single cell that accumulates genomic changes affecting regulatory genes resulting in a growth or survival advantage. Additional changes lead to local invasion and metastasis (reviewed by DeMarzo et al., 2003). It has been suggested that in about 5-10% of prostate cancers, individuals have inherited a strong predisposition to the disease (Carter et al., 1992). So far, three high-penetrance prostate cancer susceptibility genes, *RNASEL*/*HPC1*, *MSR1* and *ELAC2*/*HPC2,* have been identified (Carpten et al., 2002; Tavtigian et al., 2001; Xu et al., 2002). However, the significance of these genes seems to be limited to only a small subset of familial prostate cancers. Polymorphisms have also been widely studied in prostate cancer. The risk associated with these polymorphisms may be low, however, their population frequencies is often remarkably high. Several risk polymorphisms have been suggested, for example, in the androgen receptor (*AR*), *SRD5A2*, *BRCA2*, *CHECK2*, *VDR*, *PON1* and *CYP17* genes (reviewed by Schaid et al., 2004, and by Porkka and Visakorpi, 2004). However, because of conflicting data, none of these polymorphisms can be considered to be incontestably associated with prostate cancer. Therefore, additional studies are obviously needed. Also, the present data on somatic genetic alterations is limited and often contradictory.

2.1 Chromosomal alterations

Chromosomal aberrations in prostate cancer have been studied using traditional cytogenetic analysis (G-banding), loss of heterozygosity (LOH) and molecular cytogenetic techniques, especially comparative genomic hybridization (CGH) and fluorescence in situ hybridisation (FISH) (reviewed by Brothman et al.,

1997). G-banding is a technique for producing banding patterns in metaphase chromosomes. Each chromosome pair has a unique pattern of bands enabling recognition of a particular chromosome. Since prostate cancer cells do not grow well in vitro, the results from traditional cytogenetic studies have not been very informative. LOH studies have been used to detect allelic imbalances in prostate cancer, but only few genome-wide LOH analyses have been performed. LOH analysis needs "normal" DNA from the same patient and is usually used for scrutinizing smaller regions than the whole genome. Both CGH and FISH are methods for analyzing DNA copy number alterations. Using CGH one can see relative physical copy number changes from the whole genome, whereas FISH is used when analyzing a single locus. So far, CGH has been the most informative tool for the genome-wide analysis of prostate cancer. Studies using CGH have identified the most commonly altered chromosomal regions. These studies have shown that losses of DNA segments are much more common than gains in untreated prostate cancers, suggesting that inactivation of recessive tumor suppressor genes (TSG) is more important in early prostate cancer than amplification of oncogenes. On the other hand, hormone-refractory prostate cancers often contain gains and more extensive amplifications, suggesting that the late-stage progression of prostate cancer is also characterized by activation of proto-oncogenes (reviewed by Porkka and Visakorpi, 2004).

2.1.1 Loss of genetic material

According to the results obtained from CGH analysis, the most common chromosomal losses in prostate cancer are found at 6q, 8p, 10q, 13q and 16q (reviewed by Elo and Visakorpi, 2001, and by DeMarzo et al., 2003).

Deletion of the short arm (p) of chromosome 8 is may be the most frequent genetic alteration in prostate cancer. Loss of 8p has been detected in over 70 % of cases with hormone-refractory prostate carcinoma (Nupponen et al., 1998a) and in 80% of the metastatic cases (Cher et al., 1996). LOH at 8p21 appears to be an early event in prostate tumorigenesis, as it has been detected in PIN lesions (reviewed by Dong, 2001a). The NK3 transcription factor related locus 1 (*NKX3-1*), which maps to 8p21, is currently the most promising target gene for the deletion (He et al., 1997). *NKX3-1* encodes a prostate-specific homeobox protein that is likely to be essential for normal prostate development (reviewed by Nelson et al., 2003). NKX3-1 is expressed in prostate luminal cells (Asatiani et al., 2005) and loss of NKX3-1 expression is strongly associated with hormone-refractory disease and advanced tumor stage (Bowen et al., 2000). However, no mutations in *NKX3-1* have been found in prostate cancer specimens (Kim et al., 2002). Studies in mice have shown that *NKX3-1* haploinsufficiency can predispose to prostate epithelial dysplasia and can cooperate with other oncogenic mutations to augment tumorigenesis (Asatiani et al., 2005).

Another common region of deletion is at 8p22, where the *N33*, *FEZ1*, *PRTLS* and *MSR1* genes are located (MacGrogan et al., 1996; Ishii et al., 1999; Fujiwara

et al., 1995; Emi et al., 1993). Of these, the most interesting one is the macrophage scavenger receptor 1 (*MSR1*). An association between germline mutations in the *MSR1* gene and prostate cancer has been reported (Xu et al., 2002; Miller et al., 2003). However, somatic mutations in *MSR1* are very rare if indeed they exist at all (Nupponen et al., 2004). Thus, it is unlikely that *MSR1* is a target gene for the 8p deletion.

The long arm (q) of chromosome 13 is the second most commonly deleted region in prostate cancer. Loss of 13q14-q22 has been found in 39% of the tumors studied. Although deletion at 13q has been detected in PIN lesions, many studies have shown that these deletions are related to the clinical aggressiveness of the disease (reviewed by Dong, 2001a). There are at least three distinct regions of allelic loss in prostate cancer: 13q14, 13q21-22 and 13q33, (Hyytinen et al., 1999). The following candidate tumor suppressor genes map to chromosome 13: *BRCA2* at 13q12, retinoblastoma 1 (*RB1*) at 13q14, *DBM* at 13q14, *LEU1* and *LEU2* at 13q14 and *EDNRB* at 13q21 (Dong et al., 2001b). Allelic loss and alterations in *RB1* mRNA expression have been detected (Brooks et al., 1995; Kubota, 1995; Tricoli et al., 1996; Mack et al., 1998). However, somatic mutations seem to be rare, and no correlation between LOH and mutation or absence of expression of the *RB1* gene has been reported. Not has mutation in the other candidate TSGs been seen (Li et al., 1998; Latil et al., 1999).

Loss of 10q is generally considered a late step in prostate cancer progression (Hermans et al., 2004). Allelic loss has been found in several separate regions of 10q22-q26, suggesting inactivation of more than one TSG (Komiya et al., 1996; Feilotter et al., 1998; Leube et al., 2002). Suggested TSGs for the 10q loss are the phosphatase and tensin homolog (*PTEN*) at 10q23 (Li et al., 1997a; Steck et al., 1997), and MAX interactor 1 (*MXI1*) at 10q25 (Edelhoff et al., 1994; Schreiber-Agus et al., 1998). *PTEN* encodes a multifunctional phosphatase that is frequently mutated or deleted in sporadic human tumors (Di Vizio et al., 2005), and its role is discussed in Section 2.3. MXI1 has been shown to suppress prostate cancer cell proliferation in vitro (Taj et al., 2001). However, *MXI1* is rarely mutated in prostate tumors (Kawamata et al., 1996; Kuczyk et al., 1998; Hermans et al., 2004). Thus it seems that *MXI1* is not a common target for 10q loss.

Both primary and metastatic tumors have been found to contain allelic loss at 16q, and the occurrence of LOH has also been associated with aggressive and metastatic behavior of the disease and poor differentiation of the tumor (Elo et al., 1999). At least three regions for 16q losses have been indicated: 16q22.1, 16q23.2 and 16q24.3, (Latil et al., 1997). LOH at 16q22, where E-cadherin gene (*CDH1*) is located, has been detected in some prostate cancers and reduced E-cadherin expression also occurs in prostate cancer (reviewed by Dong, 2001a). Umbas et al. (1992) reported reduced E-cadherin expression in 50% of primary or metastatic prostate cancer specimen and they also found a correlation between the decreased expression of E-cadherin and loss of tumor differentiation. However, neither correlation between *CDH1* deletion and reduced E-cadherin

expression nor mutations in *CDH1* has been found, suggesting that *CDH1* is not a target gene for deletions at 16q (Li et al., 1999). However, *CDH1* may still have a role in the tumorigenesis of the prostate. *CDH1* belongs to the cadherin gene family, which encodes membrane-anchored cell adhesion molecules. They are involved in Ca2+-mediated cell-cell interactions and they also transmit external signals into the cells. Cadherins also play important roles during embryonic development, in cell differentiation, and in the maintenance of adult tissue integrity (Thedieck et al., 2005). The mechanism for reduction in E-cadherin expression has not been ascertained, but one possible way could be through hypermethylation of the promoter region (Li et al., 2001). Other candidate TSGs for 16q loss includes *WWOX* at 16q23.1, *WFDC1* at 16q24.1, *CDH13* at 16q24.2 and *CBFA2T3* at 16q24.3. However none of these have been shown to have altered structure or expression in prostate cancer (Watson et al., 2004). *ATFB1* at 16q22 is also a possible candidate TSG for deletions at 16q. In a recent study Sun et al. (2005) found *ATBF1* to be frequently mutated in human prostate cancer. They also found an inhibitory function for ATBF1 in cell proliferation, suggesting a tumor suppressing role.

Deletions of the long arm of chromosome 6, with the minimal shared region at 6q15 –q22, are frequent events in prostate cancer (Visakorpi et al., 1995a). Metastases and recurrent tumors have more frequently deletions at 6q than do primary tumors, and studies have suggested that one or more suppressor genes important for prostate cancer development map the indicated chromosome region (reviewed by Dong et al., 2001a). Unfortunately, no promising target genes for the 6q deletion have yet been identified.

2.1.2 Gains of genetic material

According to analysis by CGH, the most common chromosomal regions displaying gains in prostate cancer are at 7p, 7q, 8q and Xq (reviewed by Elo and Visakorpi, 2001, and DeMarzo et al., 2003).

Gain of both arms of chromosome 7 has been found by FISH and CGH (Visakorpi et al., 1995a; Jenkins et al., 1998), and aneusomy of chromosome 7 has been shown to be associated with cancer progression and poor prognosis (Alcaraz et al., 1994; Bandyk et al., 1994; Alers et al., 2000). Nupponen et al. (1998a) showed that in several tumors there were extra copies of the entire chromosome 7. In addition, three separate regions showing gains were observed: 7p15-p21, 7q21 and 7q31. 7q31 contains several possible target genes such as *TES*, caveolin 1 (*CAV1*), *CAV2*, met proto-oncogene (*MET*), *CAPZA2* and *WNT2* (Chene et al., 2004).

CAV1 is one of the putative target genes for 7q gain and encodes a major structural component of caveolae, specialized plasma membrane invaginations that are involved, for example, in molecular transport, cell adhesion and signal transduction (Yang et al., 2005). CAV1 expression is increased in primary and metastatic human prostate cancer with highest levels observed after androgen

ablation therapy (reviewed by Mouraviev et al., 2002). It has also been shown that the combined expression of MYC and CAV1 predicts prostate cancer progression (Yang et al., 2005).

The well known *MET* proto-oncogene is another candidate for gain on 7q. *MET* belongs to the tyrosine kinase family of proto-oncogenes and appears to be closely related in sequence to the human insulin receptor and v-abl Abelson murine leukemia viral oncogene homolog 1 (*ABL*) genes (Dean et al., 1985). *MET* encodes the receptor for hepatosyte growth factor/scatter factor (HGF/SF) (MacDougall et al., 2005) and overexpression of the MET protein is frequently detected in PIN, high-grade prostate cancers and metastatic disease (Pisters et al., 1995; Humphrey et al., 1995; Knudsen et al., 2002). The role of alterations on 7q31 for prostate cancer tumorigenesis is controversial, because LOH on 7q31 has also been detected (Chene et al., 2004; Zenklusen et al., 1994; Takahashi et al., 1995). Huang et al. (1998) located the second most common aphihicolin-inducible fragile site in the human genome (FRA7G) to region 7q31. This is an alternative explanation for the deletion of this chromosomal region.

Yet another putative target for 7q gain is enhancer of zeste homolog 2 (*EZH2*), which is a member of the polycomb group protein family. It has an essential role in the regulation of embryonic development and is involved in the regulation of the cell cycle and gene silencing (Sudo et al., 2005; Kleer et al., 2003). EZH2 overexpression in B cell-derived Ramos cell line has been reported to cause an increase in the proliferation rate (Visser et al., 2001), whereas inhibition of *EZH2* by RNA interference (RNAi) leads to growth inhibition (Varambally et al., 2002). EZH2 overexpression has been reported in hormone-refractory, metastatic prostate cancer (Varambally et al., 2002). In a recent study, amplification of *EZH2* was found to be rare in early prostate cancer; whereas it was found in ~20% of late stage disease (Saramäki et al., personal communication). The gene amplification was associated with overexpression of the gene, strengthening the suspicion that EZH2 is associated with the progression of prostate cancer.

Gain of the long arm of chromosome 8 is one of the most common chromosomal alterations in hormone-refractory and metastatic prostate carcinomas (Nupponen et al., 1998a). The significance of 8q gain for prostate cancer is discussed in Section 2.1.2.1.

Gain of the X chromosome was detected in 56% of hormone-refractory tumors (Visakorpi et al., 1995a). The target for this amplification is androgen receptor (*AR*) gene located at Xq11-q12. The significance of AR for prostate cancer is discussed in Section 2.2.3.

2.1.2.1 Gain of chromosome 8q

Chromosome 8q gain is one of the most frequent alterations in advanced prostate cancer detected by CGH (Visakorpi et al., 1995a; Cher et al., 1996; Nupponen et al., 1998a). It is found in about 80% of hormone-refractory tumors and distant metastases but only in ~5% of untreated primary prostate carcinomas (Visakorpi et al., 1995a; Nupponen et al., 1998a). Gain of chromosome 8q has also been shown to be associated with early progression of prostate cancer after radical surgery (Van Dekken et al., 2003) or hormonal treatment (Steiner et al., 2002). Although the gain usually covers the whole arm, CGH analysis has also identified two independently amplified subregions, 8q21 and 8q23-q24, suggesting the involment of several target genes (Nupponen et al., 1998a).

Putative target genes in the 8q23-24 region are e.g. *TRPS1* at 8q24.12 *EIF3S3* at 8q24.11 and *MYC* at 8q24.12-13 (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?CMD=search&DB=gene). They are discussed more specifically in Sections 2.1.2.1.1 to 2.1.2.1.3. The prostate stem cell antigen (*PSCA*) located at 8q24 is another putative target gene for 8q23-q24 amplification (Reiter et al., 1998). *PSCA* encodes a glycosyl phosphatidylinositol anchored cell surface protein related to the lymphocyte antigen 6 complex/ Thymus cell antigen 1, theta (Ly-6/Thy-1) family of cell surface antigens (Lam et al., 2005). PSCA is expressed in the normal human prostate and overexpressed in human prostate cancers. Overexpression of PSCA has been demonstrated to correlate with increased Gleason score, advanced stage and bone metastasis (reviewed by Jalkut and Reiter, 2002). *PSCA* has been shown to co-amplify with *MYC* in locally advanced prostate cancer. However, *MYC* has also been found to be independently amplified in a subset of tumors without *PSCA* amplification that are overexpressing PSCA. Thus, *PSCA* is probably not the target gene for the 8q24 amplification (Reiter et al., 2000).

In a recent study, 2 new putative target genes for 8q24 amplification were discovered. Using microarray method, Porkka et al. (2004) observed 68 overexpressed genes in the PC-3 prostate cancer cell line. Expression and copy number of 29 selected genes was further analyzed by Q-RT-PCR and FISH in prostate cancer cell lines, xenografts, BPHs, untreated and hormone-refractory prostate cancer specimens. From region 8q24, the *S.pombe* RAD 21 homolog (*RAD21*) and *KIAA0196* were both overexpressed and amplified in clinical prostate carcinomas, suggesting that they are putative target genes for the common amplification of 8q23-q24. At the region 8q21 there are only a few suggested target genes for amplification. One putative target gene is *PrLZ*, which is a novel member of the TPD52 family, whose overexpression is found in early stage prostate cancer (Wang et al., 2004). Another putative target gene is *Elongin C*, which is amplified and overexpressed in the PC-3 prostate cancer cell line as well as in hormone-refractory disease (Porkka et al., 2002; Porkka et al. personal communication).

2.1.2.1.1 TRPS1

TRPS1, alias *GC79*, encodes a 160 kD zinc finger GATA-type protein. GATA transcription factors have been reported to be involved in cell proliferation, differentiation and oncogenesis. They have been found to be expressed during tumorigenesis of endocrine tissues and bind to promoters of several hormone-responsive genes (reviewed by Chang et al., 2002). By using LNCaP-FGC (androgen-dependent) and LNCaP-LNO (androgen-independent) human prostate cancer sublines Chang et al. (1997) cloned and identified several differentially expressed genes, of one which was *TRPS1*. The expression of *TRPS1* was higher in LNCaP-FGC than in LNCaP-LNO cells and physiological levels (0.1 nM) of androgens repressed the expression of *TRPS1* mRNA in LNCaP-FGC cell line. Next, Chang et al. (2000) studied *TRPS1* expression in various adult and fetal human tissues and in the prostate glands of castrated rats. *TRPS1* was also transfected to LNCaP and COS-1 cell lines to study the association of *TRPS1* expression and apoptosis. Androgen withdrawal was found to increase the expression of *TRPS1* mRNA in the regressing rat ventral prostate. In cell culture studies, induced expression of *TRPS1* was found to elevate the number of apoptotic cells compared to non-induced transfected cells. The results indicate that the expression of *TRPS1* is repressed by androgens and might be involved in prostate cancer apoptosis. The TRPS1 protein has also been shown to be down-regulated in vivo by androgens in prostate cancer xenograft models (Chang et al., 2004). TRPS1 itself represses the expression of PSA (van den Bemd et al., 2003).

2.1.2.1.2 EIF3S3

Eukaryotic initiation factor 3 (EIF3) is a protein multimere consisting of at least ten subunits. It has central role in translation initiation. In the absence of other initiation factors EIF3 binds to 40 S ribosomal subunits and helps to maintain 40 and 60 S ribosomal subunits in a dissociated state. It also has a role in the formation of 40 S initiation complex by interacting with the ternary complex of EIF2-GTP-Met-tRNA and promoting DNA binding (Asano et al., 1997). EIF3S3, alias eIF3-p40, is a subunit of EIF3.

Nupponen et al. (1999) used suppression subtractive hybridization (SSH) to identify overexpressed genes in the SK-Br-3 breast cancer cell line containing high-level amplification of 8q23-q24. The subtracted cDNA clones were then directly sequenced and an expressed sequence tag (EST) for *EIF3S3* was redundantly found. Subsequently, FISH, Southern and Northern blotting confirmed amplification and overexpression of EIF3S3 in SK-Br-3 cell line. High-level amplification was found in 30% of hormone-refractory tumors and in 18% of primary tumors, and amplification of the gene was associated with overexpression of its mRNA as detected by in situ hybridization. By using tissue microarray method (TMA), Saramäki et al. (2001) showed that the *EIF3S3* gene

was amplified in 9% of early-stage prostate tumors and in 33% of hormone-refractory, locally recurrent tumors and in 50% of hormone-refractory, metastatic lesions. High-level amplification of *EIF3S3* gene was also associated with androgen independence, advanced stage, and poor differentiation of prostate cancer. Amplification of *EIF3S3* was recently also demonstrated in hepatocellular carcinoma (Okamoto et al., 2003).

2.1.2.1.3 MYC

The well-known proto-oncogene *MYC* is implicated in various opposite physiological processes, such as cell proliferation, differentiation, and apoptosis (Williams et al., 2005). Constitutive or deregulated expression of MYC is associated with many human cancers often with poor prognosis (reviewed by Pelengaris et al., 2003). In Burkitt lymphoma, *MYC* is activated through translocation (8;14 or 8;22) placing *MYC* under the control of the regulatory element of immunoglobulin or T cell receptor genes (reviewed by Boxer and Dang, 2001). Amplification of *MYC* has also been demonstrated in many malignancies (Blancato et al., 2004). Thus, *MYC* is also a strong candidate gene for the 8q24 amplification in prostate cancer. Various studies have indicated that *MYC* mRNA is commonly overexpressed in hyperplasic and malignant prostate tissue (Visakorpi et al., 1995a; Nupponen et al., 1998b; Kaltz-Wittmer et al., 2000). Recently, forced overexpression of MYC was found to immortalize primary prostate epithelial cells (Gil et al., 2005). Some research groups have reported that *MYC* amplification is found in up to 50% of HGPIN and over 70% of primary prostate cancer (reviewed by Quinn et al., 2005). On the other hand, Saramäki et al. (2001) reported that no *MYC* amplification was found in BPH or HGPIN. In some studies amplification of *MYC* has been found to correlate with overexpression of the protein (Reiter et al., 2000). However, Kaltz-Wittmer et al. (2000) found no correlation between *MYC* amplification and survival. In addition, overexpression of MYC protein detected by immunohistochemistry (IHC) has not been demonstrated to have prognostic value (reviewed by Quinn et al., 2005). Nevertheless, growth-inhibitory effects of the *MYC* antisense oligonucleotide have been demonstrated using the PC-3 prostate cancer xenograft model (Iversen et al., 2003). Recently it was shown in the transgenic mouse expressing MYC in the mouse prostate that mice develop PIN and subsequently invasive adenocarcinoma. Expression profiling of the tumors indicated for similar molecular features with human prostate cancer (Ellwood-Yen et al., 2004). The transgenic mouse study is the most common evidence suggesting that *MYC* could also function as an oncogene in prostate cancer. However, whether it is a target for 8q gain still needs further investigation.

2.2. Proto-oncogenes in prostate cancer

As mentioned in Section 1, proto-oncogens and TSGs have an important role in the development of cancer. In the following sections, proto-oncogenes and TSGs that have been shown to be involved in the development of prostate cancer are described.

2.2.1 ERBB2

ERBB2, also known as *HER-2/neu*, was initially identified as a transforming gene in chemically induced rat neuroblastomas (Schechter et al., 1984). *ERBB2* gene, located at 17q21, encodes for a 185-kD transmembrane glycoprotein (Popescu et al., 1989) with tyrosine kinase activity and belongs to the epidermal growth factor receptor family (Akiyama et al., 1986). ERBB2 is amplified and overexpressed in a wide variety of human tumors, mainly from the epithelial origin (reviewed by Scholl et al., 2001). The frequency of amplification is about 15-30% in breast cancer (reviewed by Ross and Fletcher, 1999), and amplification and/or overexpression are associated with poor prognosis (Reese et al., 1997). The finding of frequent amplification of *ERBB2* in breast cancer led to the development of anti-*ERBB2* antibody (trastuzumab) based therapy for breast cancer (Slamon et al., 2001; Pegram et al., 1999). It is known that only tumors overexpressing the *ERBB2* gene respond to trastuzumab (Seidman et al., 2001; Ross et al., 2003, Slamon et al., 2001).

ERBB2 protein has been detected in LNCaP, PC-3 and DU-145 cell lines (Zhau et al., 1992). In addition, ERBB2 is also expressed in androgen independent 22Rv1 prostate cancer cell line, which derives from a human prostatic carcinoma xenograft, CWR22R (Sramkoski et al., 1999).

Although ERBB2 overexpression has been extensively investigated in clinical prostate tumors, the results have been contradictory. Some studies have reported that ERBB2 protein is overexpressed in prostate cancer (Myers et al., 1994; Gu et al., 1996; Signoretti et al., 2000), while other studies have failed to detect ERBB2 overexpression (Visakorpi et al., 1992; Reese et al., 2001). It seems that the main problem in the evaluation of ERBB2 expression is the definition of overexpression and the vast amount of different antibodies used for immunostaining (Sanchez et al., 2002; Calvo et al., 2003). Another way to study expression of ERBB2 in clinical samples is to analyze mRNA levels by Q-RT-PCR. So far, only one such study has been published (Calvo et al., 2003). It indicated no overexpression of *ERBB2* in prostate cancer. A summary of the studies analyzing ERBB2 expression in prostate cancer is shown in Table 1.

Table 1. *Summary of the ERBB2 expression studies in prostate cancer*

Author	Year	Method	Tumor type	Number of samples	%OE.	Comments
Calvo et al.	2003	IHC	AD	50	18	17/50 (1+), 8/50 (2+) and 1/50 (3+)
		IHC	HR	25	0	1/25 (1+)
Calvo et al.	2003	RT-PCR	benign	15	0	
		RT-PCR	AD	19	0	no overexpression in either AD or HR
		RT-PCR	HR	14	0	
Di Lorenzo et al.	2002	IHC	AD	58	36	21/58 (+2 or +3)
		IHC	HR	16	56	9/16 (+2 or +3)
Fossa et al.	2002	IHC	AD	112	37	41/112 showed *ERBB2* expression
Jorda et al.	2002	IHC	AD	216	15	31/216 (weak positive; 2+); 2/216 (strong positive; 3+)
Lara et al.	2002	IHC	AD	62	8	4/62 (2+) and 1/62 (3+)
Morris et al	2002	IHC	AD	84	7	
		IHC	HR	13	0	
		IHC	AD Met.	8	12	
		IHC	HR Met.	12	42	
Sanchez et al.	2002	IHC	AD	38	50	modified DAKO protocol. 10/38 (2+) and 9/38 (3+)
		IHC	AD	38	3	standard DAKO method. 1/38 (2+) and 0/38 (3+)
Liu et al.	2001	IHC/IF	PIN	6	0	
		IHC/IF	AD	30	0	0% by DAKO protocol; 3% by monoclonal antibody
		IHC/IF	Met.	5	20	1/5 (3+) by IHC and IF
Osman et al.	2001	IHC	AD	83	39	32/83 (2+)
		IHC	Met.	20	80	bone metastases;10/20 (2+) and 6/20 (3+)
Reese et al.	2001	IHC	HR	39	36	9/39 (1+), 2/39 (2+), 2/39 (3+)
Shi et al.	2001	IHC	AD	31	29	
		IHC	AD	30	50	short-term androgen ablation therapy before surgery
		IHC	HR	20	85	
Signoretti et al.	2000	IHC	AD	67	25	
		IHC	AD	34	59	short-term androgen ablation therapy before surgery
		IHC	HR	18	78	
Haussler et al.	1999	IHC	Adenosis	48	2	moderate/strong 1/48
		IHC	BPH	20	40	moderate/strong 8/20
		IHC	PIN	30	60	moderate/strong 20/30
		IHC	AD	38	0	

continued

Table 1. *Summary of the ERBB2 expression studies in prostate cancer cont´d*

Morote et al.	1999	IHC	HR	70	64	
Mydlo et al.	1998	IHC	AD	14	0	2 out of 13 revealed 20-50% of cells stained
		IHC	HR	3	0	1 out of 3 revealed 20-50% of cells stained
Ross et al.	1997a	IHC	AD	113	29	
Ross et al.	1997b	IHC	AD	62	29	
		IHC	PIN	6	17	
Gu et al.	1996	IHC	BPH	10	10	1/10 weak and 1/10 moderate
		IHC	AD	39	62	24/39 strong, 10/39 moderate and 5/39 weak
Fox et al.	1994	IHC	AD	45	36	16/45 positively stained
Myers et al.	1994	IHC	BPH (basal)	23	100	23/23 moderate to strong
		IHC	BPH (luminal)	23	13	14/23 weak and 3/23 moderate to strong
		IHC	PIN	22	100	moderate to strong both basal and luminal
		IHC	AD	29	93	2/29 weak and 27/29 moderate to strong
		IHC	AD Met.	16	94	1/16 weak and 15/16 moderate to strong
Veltri et al.	1994	IHC	AD	124	78	
Giri et al.	1993	IHC	BPH	36	94	34/36 positive stained
		IHC	AD	7	100	Moderate to strong immunnoreactivity
Kuhn et al.	1993	IHC	BPH	9	0	
		IHC	AD	53	34	18/53 positive stained
Sadasivan et al.	1993	IHC	BPH	15	0	
		IHC	AD	25	36	9/25 positive staining
Mellon et al.	1992	IHC	BPH	34	18	6/34 positive staining
		IHC	AD	29	21	6/29 strong staining
Visakorpi et al.	1992	IHC	BPH	17	0	
		IHC	AD	147	0	11/147 showed low-level immunoreactivity
Zhau et al.	1992	IHC/WB	BPH	6	0	2 by IHC and 4 by WB
		IHC/WB	AD	16	75	12/15 showed positive staining by IHC and 11/16 reacted positively by WB
Zhau et al.	1992	IHC/WB	BPH	6	0	2 by IHC and 4 by WB
		IHC/WB	AD	16	75	12/15 showed positive staining by IHC and 11/16 reacted positively by WB

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Amplification of *ERBB2* has also been widely studied. Signoretti et al. (2000) studied 67 tumors from patients treated by surgery alone, 34 from patients treated with neoadjuvant combined androgen ablation with surgery and 18 from patients who developed bone metastasis after failed androgen ablation therapy. No *ERBB2* gene amplification was detected in any of the tumors. Bubendorf et al. (1999) also found no *ERBB2* amplifications among the 262 prostate tumors. However, there are a few published studies reporting *ERBB2* amplification detected by FISH in prostate cancer. Ross et al. (1997a, 1997b) reported high-level amplification (>5 copies/nucleus), and Liu et al. (2001) and also Kaltz-Wittmer et al. (2000) low-level amplification of *ERBB2* gene. The significance of low-level amplifications of *ERBB2* is considered to be less important than high-level amplifications, since low-level amplifications of *ERBB2* are typically not correlated with ERBB2 protein expression (Tanner et al., 2000). Ross et al. (1997a) studied 113 men who underwent radical retropubic prostatectomy. They found *ERBB2* amplification in 41% of these tumors, but it was not associated with ERBB2 overexpression detected by IHC. Later the same year Ross et al. (1997b) published a study where they analyzed 62 prostate cancer patients for ERBB2 amplification and overexpression. They found that 44% of the tumors contained amplification. Comparison of FISH and IHC results from the same tumors revealed no association between amplification and overexpression of ERBB2. The majority of the studies analyzing *ERBB2* amplification, either by Southern blotting or FISH, show clearly that *ERBB2* amplification is absent or at least very rare in prostate cancer (Fournier et al., 1995; Bubendorf et al., 1999). Only one research group has reported high-level amplification *ERBB2* gene in a substantial fraction of prostate cancers (Ross et al., 1997a, b). The studies in which *ERBB2* copy number has been investigated in prostate cancer are listed in Table 2.

Table 2. *Summary of the ERBB2 gene copy number studies in prostate cancer*

Author	Year	Method	Type	Number of samples	Amp. %	Comments
Calvo et al.	2003	FISH	AD	20	0	
		FISH	HR	19	0	
Lara et al.	2002	FISH	AD	7	0	
Oxley et al.	2002	FISH	AD	114	0	2/114 aneuploid
Liu et al.	2001	FISH	PIN	15	0	
		FISH	AD	30	0	16/30 low-level amplification
		FISH	AD Met.	5	0	4/5 low-level amplification
Osman et al.	2001	FISH	AD	66	0	2/66 had *ERBB2* amplification

continued

Table 2. *Summary of the ERBB2 gene copy number studies in prostate cancer cont´d*

Author	Year	Method	Type	Number of samples	Amp. %	Comments
Reese et al.	2001	FISH	HR	36	6	2/36 had *ERBB2* amplification
Skacel et al.	2001	FISH	AD	39	0	10/39 aneuploid
Kaltz-Wittmer et al.	2000	FISH	AD	22	0	
		FISH	HR	63	3	19/63 low-level amplification
Signoretti et al.	2000	FISH	AD/HR/Met.	21	0	all scorable tumor samples together
Bubendorf et al.	1999	FISH	BPH	31	0	
		FISH	AD/HR	262	0	all evaluable tumors together
Mark et al.	1999	FISH	AD	86	9	1/86 moderate and 7/86 low-level amplified
Kallakury et al.	1998	FISH	AD	106	42	44/106 amplified tumors
Ross et al.	1997a	FISH	AD	113	41	46/113 amplified tumors
Ross et al.	1997b	FISH	AD	62	44	27/62 amplified tumors
		FISH	PIN	6	17	1/6 amplified
Fournier et al.	1995	Southern	AD	15	0	
Latil et al.	1994	Southern	AD	21	0	
Zhau et al.	1992	Southern	AD	10	0	

AD, androgen dependent; HR, hormone-refractory; BPH, benign prostatic hyperplasia; PIN, prostatic intraepithelial neoplasia; Met, metastases; FISH fluorescence in situ hybridisation; CISH, chromogenic in situ hybridisation; Amp, amplification

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2.2.2 uPA

Mammalian cells contain two different forms of plasminogen activators: urokinase-type (uPA) and tissue-type (tPA) (Tripputi et al., 1985). The *uPA* (alias *PLAU*) gene is located at 10q22. It is a member of the serine protease family that catalyzes the conversion of inactive zymogen plasminogen to its active form plasmin (Helenius et al., 2001; Pakneshan et al., 2004). One of the major functions of plasmin is to degrade extracellular matrix (ECM) components (reviewed by Sheng, 2001).

The uPA system plays a key role in tumor-associated tissue remodeling by initiating proteolytic cascades leading to the activation of multiple proteases and growth factors and degradation of surrounding ECM (reviewed by Dano et al., 1999; reviewed by Andreasen et al., 1997). uPA is highly expressed in the most

aggressive PC-3 cell line. In DU145 cell line the expression is lower and LNCaP cell line does not express uPA at all (Van Veldhuizen et al., 1996; Helenius et al., 2001). Hoosein et al. (1991) found that unlike LNCaP, PC-3 and DU145 cell lines were invasive in Matrigel assays in vitro. This behavior was enhanced by the addition of plasminogen and suppressed by antiurokinase antibodies. Hollas et al. (1992) studied mRNA levels of urokinase in PC-3, DU145 and LNCaP cell lines. They found the highest urokinase mRNA levels in PC-3 cell line whereas in the LNCaP cell line no detectable amount of urokinase mRNA was observed. They also studied the amplification of urokinase gene by Southern blotting in the three cell lines. No evidence of gene amplification or deletion was found in DU145 and LNCaP cell lines, whereas a 3-fold amplification of the gene was detected in PC-3 cell line. Gaylis et al. (1989) studied association of plasminogen activators with aggressiveness of prostate cancer. They found that an aggressive variant cell line (PC-3CALN) showed significantly greater invasive behavior, than the unselected PC-3 line. They also found that plasminogen activators secreted by PC-3CALN cells had much higher activity than unselected PC-3 cells. In addition, metastases derived from intrasplenic injection of PC-3 cells had greater plasminogen activator activities than the corresponding primary tumors. The data implies that uPA may have a role in the migration and invasion of prostate cancer cells and it might provide a marker for the aggressive phenotype. Using IHC, Van Veldhuizen et al. (1996) found that ~70 % of cancer specimens with extracapsulal extension showed increased expression of uPA. In specimens without capsular invasion the percentage was ~27%.

The expression of uPA is known to be increased in many different cancer types (reviewed by Look and Foekens 1999; Skriver et al., 1984; Pyke et al., 1991) including prostate cancer (Gaylis et al., 1989; Van Veldhuizen et al., 1996), and it has been found to have prognostic value (Miyake et al., 1999a; Yang et al., 2000; Meo et al., 2004). Elevated serum levels of uPA and uPAR have been reported in patients with prostate cancer (Miyake et al., 1999b; Van Veldhuizen 1996). However the levels were comparable between patients with and without metastatic disease (Miyake et al., 1999b). Serum levels of uPA and uPAR appear to have prognostic significance as the survival rate of prostate cancer patients with elevated serum levels of uPA or plasminogen activator, urokinase receptor (uPAR) is lower than that among patients with normal serum levels (Miyake et al., 1999b).

Helenius et al. (2001) studied the frequency of *uPA* gene amplification in hormone-refractory prostate cancer by FISH and expression of the gene by Q-RT-PCR and Northern blot. Association between *uPA* gene amplification and effect of uPA inhibitor for inhibition of three prostate cancer cell lines was studied by Matrigel invasion assay. Increased copy number of *uPA* was found in 3 out of 13 hormone-refractory tumors, and one of these cases was high-level amplification. Expression of *uPA* was also increased in two cases. Matrigel invasion assay showed that PC-3 cells containing *uPA* amplification were more sensitive to uPA inhibitor (amiloride) than non-amplified LNCaP and DU145

cell lines. The results suggest that one mechanism for *uPA* overexpression is amplification of the gene. Both clinical and experimental evidence suggests that uPA/uPAR system may have an important role in prostate cancer invasion and metastasis, which can be inhibited either by manipulating gene expression or using uPA inhibitors (reviewed by Sheng et al., 2001).

2.2.3 AR

AR is a member of the steroid hormone receptor family and is responsible for mediating the physiological effects of androgens by binding to specific DNA sequences that influence the transcription of androgen-responsive genes (reviewed by Gelmann, 2002). Overexpression of AR has been detected in the majority of hormone-refractory prostate cancers. In addition, microarray profiling of prostate cancer models has shown that androgen-regulated genes are re-activated at the time of progression despite the androgen ablation (reviewed by Linja and Visakorpi, 2004). To date however the most direct evidence of the oncogenic property of AR was reported by Han et al., (2005) who showed that transgenic mouse expressing mutated form of AR (E231G) developed prostate cancer in all mice studied. Chen et al. (2004a) showed in prostate cancer xenografts that overexpression of AR was sufficient to transform androgen-sensitive prostate cancer to hormone-refractory disease. This all indicated that AR signalling is important through the progression of prostate cancer from early carcinogenesis into the emergence of hormone-refractory disease.

Visakorpi et al. (1995b) detected amplification of *AR* in 30 % of hormone-refractory tumors but not in specimens taken from the same patients prior to therapy. This finding has been confirmed by several other studies (Koivisto et al., 1997; Bubendorf et al., 1999; Linja et al., 2001). The *AR* gene amplification leads to high expression levels of AR. Patients with *AR* gene amplification respond better to second-line combined androgen ablation than patients without the amplification (reviewed by Linja and Visakorpi, 2004). Point mutations in *AR* have widely been studied in prostate cancer. The data have been somewhat contradictory. While a few studies have suggested that mutations are present in a substantial fraction of untreated prostate cancers (Gaddipati et al., 1994; Tilley et al., 1996), the majority of studies have found no frequent mutations (Newmark et al., 1992; Marcelli et al., 2000). *AR* mutations in prostate cancers treated with androgen ablation alone also seem to be rare (Wallén et al., 1999), whereas tumors treated with antiandrogens, such as flutamide or bicalutamide, contain activating mutations in 10-30% of cases (Taplin et al., 2003; Taplin et al., 1999; Haapala et al., 2001).

2.2.4 BCL2

B-cell CLL/lymphoma 2 (BCL2) is an inhibitor of apoptosis (Popescu et al., 1998). The gene is translocated to the immunoglobulin heavy-chain locus in over 50% of all non-Hodgkins lymphomas, which results elevated levels of BCL2 mRNA and protein (reviewed by Kitada et al., 2002). BCL2 overexpression has also been found in malignancies of the prostate, breast, lung, colorectal and pancreas. In some of these cancers BCL2 overexpression has been suggested to be associated with chemo- and radioresistance (Fahy et al., 2005). Radioresistance has also been found in prostate cancer (Rosser et al., 2003). Expression of BCL2 has been reported to be more frequent in high-grade prostate tumors and nodal metastases than in lower grade primary prostate cancer tumors (Krajewska et al., 1996). Several other studies have reported that increased BCL2 expression in prostate cancer may facilitate progression of androgen independence (reviewed by Quinn et al., 2004). Increased copy number of *BCL2* in prostate cancer has been reported, but no high-level amplifications have been found (Nupponen et al., 1998a). Although the mechanisms of the oncogenic function of BCL2 have been quite well characterized, its significance in prostate cancer remains unclear.

2.2.5 ERG, ETV1

In a recent study, Tomlins et al. (2005) found a recurrent fusion of *TMPRSS2* to two ETS transcription factors, v-ets erythroblastosis virus E26 oncogene-like (*ERG*) or ets variant gene 1 (*ETV1*) in prostate cancer. They used a method called cancer outlier profile analysis (COPA) and analyzed 132 gene expression data sets representing 10, 486 microarray experiments. They identified strong outlier profiles in prostate cancer for *ERG* (21q22.3) and *ETV1* (7p21.2), and ranked them among the top 10 outlier genes in six independent prostate cancer profiling studies. The genes were found to be highly expressed in primary or metastatic prostate cancer, but not in PIN or adjacent benign tissue. No consistent amplification with overexpression of the genes was found, but *TMPRSS2:ERG* and *TMPRSS2:ETV1* fusions were found in over 90 % of cases that overexpressed *ERG* or *ETV1*, implying the importance of the fusion for the overexpression. Using FISH, rearrangement of *ERG* or *ETV1* was found in 23 of 29 samples. Both *ERG* and *ETV1* have previously been found to be translocated in Ewing's sarcoma (reviewed by Oikawa and Yamada, 2003; reviewed by Hsu et al., 2004; Jeon et al., 1995). This finding is interesting but needs to be confirmed in an independent study.

2.3 Tumor suppressor genes in prostate cancer

PTEN tumor suppressor gene (Li et al., 1997b), also known as *MMAC1* (Steck et al., 1997) or *TEP-1* (Li et al., 1997b), encodes a phosphatase whose dysregulation is implicated in the pathogenesis of a number of familial and sporadic cancers including sporadic prostate carcinoma (Zysman et al., 2002). PTEN negatively controls the phosphoinositide 3-kinase signaling pathway by dephosphorylating the phosphatidylinositol 3,4,5-triphosphate (reviewed by Bonneau and Longy, 2000). Tumor suppressor activity of *PTEN* is thought to be primary due to its ability to control this pathway (Kwabi-Addo et al., 2001). *PTEN* is the most frequently found mutated gene in metastases of prostate cancer (reviewed by Dong, 2001a) and deletions and mutations of the *PTEN* gene have been reported in primary prostate carcinomas (reviewed by Porkka and Visakorpi, 2004). LOH frequency of *PTEN* locus averages in 40% of the cultured or noncultured prostate cancers (Fernandez and Eng, 2002). Mutations in *PTEN* have been found in 10% of the primary prostate cancer and 63% of the metastatic prostate cancers (Xu et al., 2004). Thus, the rate of LOH is often much higher than the apparent rate of inactivation of the remaining allele and it has been suggested that inactivation of a single allele (i.e. haploinsufficiency) is sufficient to promote tumor growth (Kwabi-Addo et al., 2001).

Glutathione S-transferase pi (*GSTP1*) promoter hypermethylation is the most frequent somatic genome alteration in prostate cancer. It has been detected in over 90% of cancers and also in a high proportion of HGPIN (reviewed by Henrique and Jerónimo, 2004). Since HGPIN is thought to be a precursor of prostate cancer, *GSTP1* promoter methylation appears to be an early event in prostate tumorigenesis. Although the cells with inactivated *GSTP1* alleles seemed to be selected during carcinogenesis, *GSTP1* does not function as a tumor suppressor gene but may rather act as a “caretaker” gene (Lin et al., 2001b). GSTP1 belongs to the glutathione S-transferase family that participates in detoxification reactions thus protecting cells from carcinogenic factors (reviewed by Elo and Visakorpi, 2001). Inactivation of *GSTP1* may thus lead to additional somatic genome alterations that promote tumor growth (Lin et al., 2001b). *GSTP1* promoter methylation is infrequently observed in BPH, suggesting that it might be a useful biomarker for prostate cancer (reviewed by Henrique and Jerónimo, 2004). *GSTP1* promoter methylation has been detected in body fluids, tissues and needle biopsies (Harden et al., 2003; Goessl et al., 2000; Cairns et al., 2001).

Tumor protein p53 (*TP53*) is the most commonly mutated gene in human cancers (reviewed by de Moura Gallo et al., 2005). *TP53* encodes for 53 kD nuclear phosphoprotein (p53), which functions by regulating the transcription of genes involved in G1 phase growth arrest in response to DNA damage. TP53 also has roles in the regulation of the spindle checkpoint, centrosome homeostasis and G2 – M phase transition as well as in regulation of apoptosis and tumor angiogenesis (reviewed by Quinn et al., 2005). Mutations in *TP53* are rare in primary prostate tumors, but more common in metastases or hormone-

refractory prostate cancers, found in about 20-40% of cases (Bookstein et al., 1993; reviewed by Elo et al., 2001; reviewed by Porkka et al., 2004).

There are a number of other suggested but less studied putative tumor suppressor genes in prostate cancer, such as *p27/Kip*, kruppel-like factor 5 (*KLF5*), kruppel-like factor 6 (*KLF6*), kangai 1 (*KAI1*), CD44 antigen (*CD44*), Ras association (RalGDS/AF-6) domain family 1 (*RASSF1A*), and EPH receptor B2 (*EPHB2*). The tumor suppressor role of *p27/Kip*, also known as *CDKN1B*, has been demonstrated in knockout mice models (reviewed by Dong, 2001a). Reduced levels of p27 are also common in prostate cancers with poor prognosis (Yang et al., 1998; Cote et al., 1998).

KLF5 is a transcription factor with a potential tumor suppressor function in prostate cancer (Chen et al., 2004b). Frequent hemizygous deletions of *KLF5* as well as loss of expression and growth suppression have been reported (Chen et al., 2003a). In both *p27/Kip* and *KLF5* haploinsufficiency seems to be the primary mechanism of gene inactivation (reviewed by Dong, 2001a).

KLF6 has been shown to be mutated in a subset of human prostate cancer. Narla et al. (2001) found in LOH analyses that one *KLF6* allele was deleted in 77 % of primary prostate tumors. Sequence analysis of the retained *KLF6* allele revealed mutations in 71% of these tumors. However, later studies have indicated that *KLF6* mutations are rare in prostate cancer (Muhlbauer et al., 2003; Chen et al., 2003b). Li et al. (2005) found that decreased *KLF6* mRNA expression correlates with clinical outcome in prostate cancer, and that somatic LOH and DNA mutations may result in functional deletion of the *KLF6* gene in ~60 % of prostate tumors. It has also been reported that an alternative splicing of *KLF6* caused by germline polymorphism is associated with increased risk for prostate cancer (Narla et al., 2005).

KAI1 was shown to suppress metastasis when introduced into rat prostate cancer cells (Dong et al., 1995). *KAI1* protein expression is down-regulated in more than 70% of the primary prostate cancers of untreated patients and is reversely correlated to clinical stage, Gleason score and metastatic stage (Gao et al., 2003). However, LOH at the *KAI1* locus, mutations or promoter hypermethylation were not detected (Marreiros et al., 2005). Thus, the mechanism of the down-regulation of *KAI1* remains unclear.

CD44 encodes transmembrane glycoprotein that functions as an extracellular matrix receptor involved in cell–cell and cell–matrix interactions and may also participate in growth regulation (Alam et al., 2004). *CD44*, as well as *KAI1*, belongs to a subgroup of metastasis suppressor genes and down-regulation of *CD44* is associated with the progression of prostate cancer (Kito et al., 2001). Hypermethylation of *CD44* has been reported to result in decreased expression of *CD44* in prostate cancer and is associated with the progression and metastasis of prostate cancer (Kito et al., 2001; Verkaik et al., 2000; Lou et al., 1999). No studies on mutations of *CD44* in prostate cancer have been published.

RASSF1A hypermethylation is frequent in prostate cancer (Liu et al., 2002; Kuzmin et al., 2002). Methylation status of *RASSF1A* is associated with aggressiveness of tumors (Liu et al., 2002). *RASSF1A* also suppresses the growth

of prostate carcinoma cells (Kuzmin et al., 2002), but mutations of the gene have not yet been reported. *EPH2B* belongs to a family of receptor tyrosine kinase and may have role in cell migration and maintenance of normal tissue architecture (Huusko et al., 2004). Mutational inactivation of *EPHB2* has been reported in prostate cancer cell lines and primary as well as metastatic prostate cancers. (Huusko et al., 2004). However, the presence and frequency of the mutations requires further investigations.

AIMS OF THE STUDY

The overall aim of the study was to elucidate the significance of *ERBB2*, *uPA, TRPS1, EIF3S3* and *MYC* gene amplifications in prostate cancer. The specific aims were:

1. To find out whether *ERBB2* is amplified or overexpressed in prostate cancer.
2. To evaluate the frequency of *uPA* amplification.
3. To study the influence of uPA inhibitors on the invasion potential of prostate cancer cell lines.
4. To study the expression and copy number alterations of the putative 8q target genes *TRPS1*, *EIF3S3* and *MYC* in prostate and breast cancer cell lines as well as in prostate cancer tumors.
5. To study the effect of *EIF3S3* on cell growth.

MATERIALS AND METHODS

1. Cell lines (Studies I-IV)

Prostate cancer cell lines LNCaP, DU145, PC-3, 22Rv1, NCI-H660 and breast cancer cell lines SK-Br-3, ZR75-1, MDA-436, MDA-361, MDA-134, MDA-415, MDA-157, MCF-7, MCF-A10, BT-474, UACC-893, DU-4475, CAMA-1, HBL-100, HCC-1419 were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA), mouse fibroblast cell line, NIH 3T3 transfected with Tet-Off^{TM} expression plasmid, from Clonetech (BD Biosciences-Clonetech, Mountain View, CA, USA) and cultured according to the recommended conditions.

2. Clinical tumor samples (Studies I-III)

Freshly frozen and formalin-fixed paraffin-embedded specimens (Studies I, II and III) of BPHs, primary untreated prostate carcinomas and locally-recurrent hormone-refractory prostate carcinomas, as well as untreated breast cancers, were obtained from Tampere University Hospital. Untreated lymph-node metastases of prostate cancer (Study I) were obtained from Lund University Hospital. Formalin-fixed and paraffin-embedded prostate hormone-refractory tumor metastases (Study II) collected during autopsy of patients who died of prostate cancer and normal control samples of various tissues were kindly made available by one of the authors (Dr. G. S. Bova, PELICAN Laboratory, Johns Hopkins University, Baltimore, USA).

The specimens were histologically examined for the presence of more than 60% of cancerous or hyperplastic tissue using haematoxylin and eosin-stained slides. The BPH samples were obtained from prostatectomy specimens from cancer patients. However, the specimens were histologically verified not to contain any cancer cells.

The use of clinical tumor material was approved by the Ethical Committee of Tampere University Hospital, The National Authority for Medicolegal Affairs (Finland), and the Johns Hopkins Medicine IRB.

3. Chromogenic in situ hybridization (CISH) (Study I)

CISH was done on 5 µm thick archival formalin-fixed paraffin-embedded tissue sections with minor modifications to the previously described protocol (Tanner et al., 2000). Briefly, the sections were de-paraffinized and incubated in 0.1 M Tris-HCl (pH 7.3) in a temperature-controlled microwave oven (at 92°C for 10 min). The sections were then allowed to cool down for 20 min and washed with phosphate buffered saline (PBS). Enzymatic digestion was done by applying 100µl of digestion enzyme (Digest-All III solution, Zymed Inc., South San Francisco, CA, USA) to slides for 10-15 min at room temperature (RT). The slides were then washed with PBS and dehydrated with graded ethanol. The ready-to-use digoxigenin-labeled HER-2/neu probe (Zymed Inc., consisting of two contig bacterial artificial chromosome (BAC) clones) was applied to sample. The slides were denatured on a hot plate for 3 min, and the hybridization was carried out overnight at 37°C. After hybridization, the slides were washed with 0.5 x standard saline citrate (SSC) for 5 min at 75°C, followed by three washes in PBS/0.2%Tween20 at room temperature.The HER-2/neu probe was detected with sequential incubations with mouse anti-digoxigenin (diluted 1:300; Roche Biochemicals, Mannheim, Germany), anti-mouse-peroxidase polymer (Powervision, Immunovision Technologies, Daly City, CA, USA) and diaminobenzidine chromogen (DAB). Tissue sections were lightly counterstained with hematoxylin and embedded.

The CISH hybridizations were evaluated using 40X and 60X dry objectives and using 10x22 widefield oculars. Amplification was defined as 6 or more signals per nucleus in over 50% of cancer cells, or when a gene copy cluster was seen. Cases with other type of copy number alterations were considered as aneuploid.

4. Fluorescence in situ hybridization (FISH) (Studies II and III)

Locus-specific BAC probe for human *uPA* (BAC 417O11 GenBank accession # AL596247), as well as locus-specific PAC probes for human *TRPS1* (Chang et al., 2000), *EIF3S3* (Nupponen et al., 1999), and *MYC* (Nupponen et al., 1998a) were labeled with digoxigenin-dUTP (Roche Diagnostics) by nick translation. A pericentromeric probe for chromosome 8 (pJM128) was labeled with Fluorescein isothiosyanate (FITC)-dUTP (NEN, Boston, MA, USA), whereas FITC labeled pericentromeric probe for chromosome 10 was obtained from Vysis Inc. (Downers Grove, IL, USA). The metaphase preparations from the cancer cell lines were prepared using standard techniques. The dual-color hybridization was done essentially as described previously (Hyytinen et al., 1994). The formalin-fixed paraffin-embedded TMAs were pretreated and hybridized by using

modifications of previously described procedures (Saramäki et al., 2001). Briefly, the slides were deparaffinized followed by dehydration in 100% ethanol, and incubated in a 0.2 M HCl solution at RT for 20 min, and washed. Subsequently, the slides were incubated in citric acid buffer (pH 6.0) at 80ºC for 60 min and washed, followed by digestion with pepsin (0.05% pepsin in 10 mM HCl) at 37ºC for 5 min, and washed. Next, the samples were fixed in 10% buffered formalin at RT for 10 min, washed and dehydrated. Probes and samples were denatured together on the slide at 93°C for 3 minutes on a heatblock. Hybridization was done overnight at 37°C in a humid chamber. After stringent washes, the slides were stained with anti-digoxigenin-rhodamine (Roche Diagnostics, Mannheim, Germany) and counterstained with an antifade solution (Vectashied, Vector Laboratories, Burlingame, CA, USA) containing 4,6-diamidino-2-phenylindole (DAPI).

The metaphase preparations from the cancer cell lines were prepared using standard techniques. The dual-color hybridization was done essentially as described previously (Hyytinen et al., 1994). Briefly, the slides were denatured in a 70% formamide/2×SSC solution (pH 7.0) at 70°C for 3 min and dehydrated in an ascending ethanol series. Hybridization was done over two nights at 37°C. After stringent washes, the slides were stained with anti-digoxigenin-rhodamine (Roche Diagnostics) and counterstained with an antifade solution (Vectashied, Vector Laboratories, Burlingame, CA) containing DAPI. The formalin-fixed paraffin-embedded TMAs were pretreated and hybridized as described previously (Saramäki et al., 2001).

The FISH signals were scored from non-overlapping epithelial cells using an Olympus BX50 epifluorescence microscope (Tokyo, Japan). Tumors with a tight cluster of signals or at least two-fold higher copy number of the locus-specific probe signals versus centromeric signals or ≥5 copies of locus specific probe signals were considered to contain a high-level amplification of the gene. Tumors with 3-4 copies of the gene signals were considered to have a gain of the gene.

5. Immunohistochemistry (IHC) (Studies I and IV)

The sections were first de-paraffinized, followed by antigen retrieval (autoclave cooking at 121°C for 2 min in 10 mM sodium citrate buffer, pH 6.0). $p185^{HER2}$ protein was detected with the monoclonal antibody CB11 (Novocastra Laboratories, Newcastle, UK). The antibody was diluted 1:200 in Powervision blocking solution (ImmunoVision Technologies, Daly City, CA, USA) and incubated overnight at 4°C. A conjugate of anti-mouse antibody and horseradish peroxidase (HRP) was used for visualization (30 min at RT) with diaminobenzidine as a chromogen (PowerVision, ImmunoVision Technologies, Daly City, CA, USA). Sections were counterstained with hematoxylin. Evaluation of the staining was done as described previously (Tanner et al.,

2000). Briefly, intense membranous staining in >50% of cancer cells was considered as 3+. Staining present in a smaller proportion of the cells or lower intensity was considered as 2+ staining. No staining or faint staining in a few cells was scored as 0 and 1+ respectively.

After harvesting with trypsin, the cells were centrifuged. Pellets were suspended in PBS and dropped onto the slide (Super-Frost^{TM} Plus, Menzel Gläser, Germany). The slides were dehydrated at RT and fixed with methanol and acetone at -20ºC. After fixation the slides were washed with tris buffered saline (TBS). The cells were incubated without pre-blocking in 1:500 dilution of cleaved-caspase-3 antibody (Cell Signalling Technology Inc., Beverly, MA, USA) for 60 minutes at RT and rinsed with TBS (0.1xTBS+0.05% Tween). The cells were then incubated with Poly-HRP anti-Rabbit IgG (from PowerVision+^{TM} Poly-HRP IHC Detection Kit) at RT for 30 minutes and rinsed with TBS. The cells were incubated with DAB solution for six minutes, and rinsed with ddH_2O. After washing, the cells were incubated with $CuSO_4$ at RT for five minutes and rinsed again with ddH_2O. The cells were counterstained with hematoxylin. The rate of apoptosis was defined by counting 200 cells in a randomly chosen field and determining the proportion of cleaved-caspase-3 positive cells.

6. Real-time quantitative reverse-transcriptase PCR (Q-RT-PCR) (Studies I-IV)

One to three 20 µm frozen sections of clinical tumor samples were used in Studies I and III. Total RNAs were isolated from the sections using Qiagen RNeasy MiniKit (Qiagen Inc, Valencia, CA, USA), and used for first-strand cDNA synthesis with Superscript™ II reverse transcriptase and oligo $d(T)_{12\text{-}18}$ primer according to the manufacturer's protocol (Invitrogen Corporation, Carlsbad, California, USA).

Total RNA from cancer cell lines in Studies I, II and III was isolated using Trizol reagent (Life Technologies) according to the manufacturer's instructions, and reverse transcribed as described above. In Study IV total RNA from cancer cell lines was isolated using Qiagen RNeasy Minikit (Qiagen Inc, Valencia, CA, USA) according to the manufacturer's instructions, and reverse transcribed using AMV reverse transcriptase (Finnzymes, Finland) and oligo $d(T)_{12\text{-}18}$ primer according to the manufacturer's protocol.

For preparing the standard curve, 5 µg of total RNA from LNCaP (Studies I and III), UACC-812 (Study II) or PC-3 (Study IV) was reverse transcribed as described above. After the first strand cDNA synthesis serial dilutions (1 to 5) were made to correspond cDNA transcribed from 500, 100, 20, 4, 0.8, and 0.16 ng of total RNA.

Primers for the genes were designed with the assistance of the Primer3 program (available at http://www-genome.wi.mit.edu/cgi-bin/primer3.www.cgi).

The probes for the *ERBB2*, *uPA*, *EIF3S3*, *MYC*, and TATA box binding protein (*TBP*) genes were designed and synthesized by TIBMolBiol (Berlin, Germany). The PCR reactions were performed with a LightCycler™ instrument using the LightCycler – FastStart DNA Master Hybridization Probes Kit (Studies I, II, III and IV) or FastStart DNA Master SYBR Green I Kit (Studies III and IV) (Roche Diagnostics, Mannheim, Germany). The sizes of PCR products were designed to be under 400 base pairs (bp) to optimize sensitivity of the Q-RT-PCR. Primer and probe sequences for the genes are given in Table 3.

Table 3. *Primer and probe sequences used in the Q-RT-PCR.*

Gene	Primer sequences (5´-3´)	Probe sequences (5´-3´)
ERBB2	AGCCGCGAGCACCCAGT TTGGTGGGCAGTAGGTGAGTT	CCTGCCAGTCCCGAGACCCACCT-*FL* *Red640*-CATGCTCCGCCACCTCTACCA GGG
uPA	TCACCACCAAAATGCTGTGT AGGCCATTCTCTTCCTTGGT	TCCCCCTGAGTCTCCCTGGCA-*FL* *Red640*-AATCTGTTTTCCACTGTGGGT CAGCAG
TRPS1	GTATCCTGCATCGGGAGAAA AGCTTCTGGTAGAGGCCACA	*No probes used*
EIF3S3	GCCCAGGCTCTTCAAGAATAC ATAGCCAAAATCGGCAATGA	GCTGAATCTCCCGAGCCGCCTTT-*FL* *Red640*-CCTTTGCCTTTCCCTGCTGCGC
MYC	CCTACCCTCTCAACGACAGC CGCCTCTTGACATTCTCCTC	GCCTCCCTCCACTCGGAAGGACT-*FL* *Red640*-TCCTGCTGCCAAGAGGGTCAA GTT
TBP	GAATATAACCCAAGCGGTTTG ACTTCCATCACAGCTCCCC	TTTCCCAGAACTGAAAATCAGTGCC-*FL* *Red640*-TGGTTCGTGGCTCTCTTATCCTC ATG
RAD21	GAGGCCAGCAGAACAAACAT TGCTGAGTCCTTTTGTTCCA	*No probes used*
KIAA0196	ATCGCTCAGTTGCCAAAACT CTGGCTTGTACACTGCTCCA	*No probes used*

FL = fluorescein; *Red640*= LightCycler Red640 acceptor fluorophore

The housekeeping gene *TBP* was chosen for normalization of the expression level of the genes, because there are no known retropseudogenes for it and the expression of *TBP* is lower than in many commonly used abundantly expressed reference genes (Bieche et al., 1999). After the PCR, every sample was also run in a 1.5% agarose gel electrophoresis to ensure that a product of the right size was amplified in the reaction.

7. Matrigel invasion assay (Study II)

The assays were done using Matrigel (BD Biosciences, Palo Alto, CA, USA) invasion method in a 24-well cell culture plate containing inserts having a PET membrane with 8 µm pores (BD Biosciences). Matrigel was diluted in a concentration of 1.25 mg/ml in PBS and 100 µl of this was pipetted into each insert and allowed to solidify (37 °C, 4-5 hours). 750 µl of culture medium containing 5% fetal bovine serum (FBS) and fibronectin (5 µg/ml) as an attractant was pipetted into each well. Finally, 1 x 10^5 cells in culture medium containing 1% FBS in the volume of 100 µl was added to an insert and urokinase inhibitors, either 250 µM of p-aminobenzamidine (Sigma A-7148) or 10 µM or 20 µM B428, kindly provided by Dr. Bruce A. Littlefield (Eisai Research Institute, Andover, MA, USA), were added to the inserts. The cells were incubated for 22 hours (37 °C, 5% CO_2). Subsequently, Matrigel, medium, and cells inside the inserts were carefully removed and the invaded cells were fixed with methanol and stained with crystal-violet. The number of invaded cells in each of the inserts was counted under the microscope. The experiment was repeated five times with B428 and six times with p- aminobenzamidine.

8. RNA interference (RNAi) (Study IV)

Potential siRNA target sites present in the *EIF3S3* mRNA (GenBank™ accession number U54559) were identified following the recommendations of Dr. Thomas Tuschl (http://www.rockefeller.edu/labheads/tuschl/sirna.html) for siRNA design. siRNA oligos were screened against the GenBank™ database by the BLAST (http://www.ncbi.nlm.nih.gov/BLAST) program to ensure that the selected target sequences did not exhibit similarity to other known gene sequences available in the database. Two control siRNAs, siLUC and siCONT, were also used. siCONT is a non-targeting siRNA for human genes (at least four mismatches to all known human genes) and siLUC targets firefly luciferase gene (Accession number, M15077). Selected 21-nucleotide siRNA duplexes (Table 4) were obtained from Proligo (Proligo France SAS, Paris, France). Transfections of siRNA duplexes were done by using Oligofectamine reagent (Invitrogen Corporation, Carlsbad, California, USA) according to published guidelines (http://www.rockefeller.edu/labheads/tuschl/sirna.html). Forty-eight hours following the transfection, cell lysates were prepared and tested for silencing of the expression of the *EIF3S3* gene by Q-RT-PCR.

Table 4. *The siRNA duplexes used in RNAi assays.*

Name	Sequences (5´-3´)
siRNA_a	AGGCAAAGGCGGCUCGGGATT
	UCCCGAGCCGCCUUUGCCUTT
siRNA_b	AAGUGCCGAUUGUAAUUAATT
	UUAAUUACAAUCGGCACUUTT
siRNA_c	GCAAGUGCAGAUAGAUGGCTT
	GCCAUCUAUCUGCACUUGCTT
siLUC	GAUUUCGAGUCGUCUUAAUTT
	AUUAAGACGACUCGAAAUCTT
siCONT	CGGUAGAUAGACGUGAACGTT
	CGUUCACGUCUAUCUACCGTT

9. Cell counting (Study IV)

The cells were washed with PBS and harvested with trypsin and diluted in Coulter® ISOTON® III dilution buffer (Beckman Coulter Inc, California, USA) and counted with a Z2™ COULTER COUNTER® cell and particle counter (Beckman Coulter Inc, California, USA). Alternatively, the cells were counted with a Bürker chamber.

10. Flow cytometry (FACS) (Study IV)

After 48 hours of transfection, siRNA-treated prostate and breast cancer cells were prepared for flow cytometry (FACS) analysis. Cells were centrifuged and pellet was suspended in 500 µl Hypotonic Staining Buffer (10 mg sodium citrate tribasic dihydrate, 30 µl Triton® X-100, 5.0 mg propidium iodide and 0.2 mg Ribonuclease A added to 100 ml H_2O). After incubation for 30 minutes on ice (protected from light), the amount of propidium iodide incorporated was determined using a flow cytometry (Coulter® EPICS XL-MCL, Beckman Coulter Inc, California, USA). Cell cycle distribution was analyzed using the Cylchred program (http://www.cardiff.ac.uk/medicine/haematology/cytonetuk/ documents/ software.html).

11. Tet-Off™ gene expression system (Study IV)

Tet-Off™ expression system (BD Biosciences-Clonetech) uses a chimeric transactivator to regulate transcripition of the gene of interest from a silent promoter. The transactivator (tTa) is expressed from the consitutive CMV promoter. tTA binds to the Tet Response Element (TRE) in the silent promoter and activates transcription in the absence of inducer (doxycycline). Clonetech pTet-Off system plasmids (BD Biosciences-Clonetech), pTRE2 and pTK-Hyg were used to make stable *EIF3S3* expressing clone in premade NIH 3T3 Tet-Off™ mouse fibroblast cell line. *EIF3S3* was cloned into pTRE2 plasmid in both sense and antisense orientation. pTRE2 plasmids (pTRE2_*EIF3S3*_sense, pTRE2_*EIF3S3*_antisense and pTRE2) were cotransfected with pTK_Hyg plasmid to NIH 3T3 Tet-Off™ cell line using Lipofectamine Plus (Invitrogen Corporation, Carlsbad, CA, USA). Selection was done with 400 µg/ml Geneticin (Invitrogen Corp.) and 100 µg/ml Hygromycin B (BD Biosciences Clonetech). After 3 weeks of selection, pure clones were isolated and the expression of *EIF3S3* was measured using Q-RT-PCR. The size of the product was determined by Northern Blot method and the most suitable cell lines were then selected for further analyses.

12. Soft agar colony assay (Study IV)

The agarose solution (Agarose, low gelling temperature; Electran, BDH Chemicals Ltd, Poole, UK) was mixed with culturing medium to a final concentration of 0.5% or 0.35% agar for the lower and upper layers respectively. The lower layer solution (1.5 ml) was aliquoted into three parallel 6-well plates and allowed to solidify at room temperature. NIH 3T3 cells were trypsinized, and counted by direct microscopic cell counting in a Bürker chamber. 5000 cells were mixed with 1.5 ml of the upper solution and immediately plated onto the solidified lower layer and allowed to solidify on the ice. After the upper layer had solidified, the dishes were transfered to a 37°C incubator. The plates were fed twice a week with 200 µl of culturing medium (with 20% FBS) and colonies were counted microscopically after two and three weeks. Untransfected NIH 3T3 cells were used as negative and PC-3 prostate cancer cells as positive controls. The experiments were done in triplicate

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13. Statistical analyses (Studies I-IV)

The associations of the gene copy numbers, tumor types, histological grades, and clinical stages with expression levels were calculated with non-parametric Kruskal-Wallis and Mann-Whitney U-tests. Outliers were detected by using extreme studentized deviate method (ESD).

RESULTS

1. Gene copy number and expression analysis of *ERBB2* in prostate cancer (Study I)

Fifty-four untreated primary prostate cancers and 50 hormone-refractory tumors were analyzed for gene copy numbers by CISH (Table 5) and expression by IHC. Breast cancer specimens were used as a control (22 specimens for CISH and 8 specimens for IHC).

Table 5. *Summary of the gene amplification studied in prostate cancer.*

Gene	Method	Tumor type	n:o of samples	% Amplification	Comments
ERBB2 (Study I)	CISH	AD	40	0	14/40 gain, 0/40 high-level amplification
	CISH	AD Met.	14	0	8/14 gain, 0/14 high-level amplification
	CISH	HR	32	3	8/32 gain, 1/32 high-level amplification
uPA (Study II)	FISH	HR	63	0	13/63 gain, 0/63 high-level amplification
	FISH	HR Met.	29	4	8/29 gain, 1/29 high-level amplification
	FISH	Normal	13	0	13/13 diploid
TRPS1 (Study III)	FISH	HR	36	28	18/36 gain, 10/36 high-level amplification
EIF3S3 (Study III)	FISH	HR	40	28	19/40 gain, 11/40 high-level amplification

AD, androgen dependent; HR, hormone-refractory; Met., metastase

In gene copy number analysis, none of the untreated primary prostate cancers showed high-level amplification of *ERBB2*, whereas gain (3-5 copies/cell) was found in 26% of cases. In the untreated lymph-node metastases, gain was found in 36% of cases and the rest of them showed 2 copies of the gene. From hormone-refractory tumors gain was found in 34% of cases and one showed 6-8 copies of *ERBB2* gene (/cell), which is considered to be a borderline

amplification. The breast cancer controls showed high-level amplification of the *ERBB2* in 27% of cases.

None of the androgen-dependent primary prostate tumors, lymph-node metastases or locally recurrent hormone-refractory tumors showed positive immunostaining (with 2+ or 3+ intensity). By contrast, all eight breast carcinoma control specimens, containing *ERBB2* amplification showed strong membranous staining indicating overexpression of the ERBB2.

In the expression studies of *ERBB2* mRNA by Q-RT-PCR, 16 breast cancer (as controls) and 3 prostate cancer cell lines (of which none contained *ERBB2* amplification) were used. An almost 20 times greater amount of *ERBB2* mRNA was found in the amplified cell lines (BT-474, UACC-893, HCC-1419, SK-Br-3, MDA-361) (Järvinen et al., 2000; Kallioniemi et al., 1992; Bärlund et al., 1997), compared to the cell lines without amplification. ZR75-1, which has a gain of *ERBB2*, expressed about 5 times more mRNA than cell lines without high-level amplification. Of the 3 prostate cancer cell lines, LNCaP expressed the mRNA at low levels (the highest expression levels of prostate cancer cell lines studied), and in 2 other prostate cancer cell lines the expression level of *ERBB2* was extremely low.

When comparing the expression levels of *ERBB2* mRNA in clinical tumor samples there were no significant differences between androgen-dependent prostate carcinomas, hormone-refractory prostate carcinomas, or the breast tumors without *ERBB2* gene amplification. The expression level was below the lowest standard in 25% of the hormone-refractory prostate carcinomas, 19% of the untreated prostate carcinomas and 33% of the BPH. In 32% of the untreated breast carcinomas, the expression level of *ERBB2* mRNA was also below the lowest standard. The median value of *ERBB2* expression in the breast cancer tumors with the *ERBB2* gene amplification was about 20 times higher than in the breast cancers without the amplification or in prostate tumors.

2. Gene copy number and expression analysis of *uPA* gene in prostate cancer (Study II)

The *uPA* gene copy number was analyzed in PC-3, LNCaP, DU145 and 22Rv1 prostate cancer cell lines. 2 copies *uPA* and chromosome 10 centromere was found in 22Rv1. 3 copies of centromere and about 10 copies of *uPA* were found in PC-3 (high-level amplification of *uPA*). DU145 and LNCaP had 3 and 4 copies of both *uPA* and chromosome 10 centromere respectively.

FISH analysis of locally recurrent hormone-refractory prostate cancers (Table 5) showed that 24% of the samples studied had alteration of *uPA* gene copy number. 21% of tumors showed 3 copies of both *uPA* and chromosome 10 centromere. One case showed 1 copy of *uPA* gene and 2 copies of the centromere and in another case, 3 copies of centromere 10 but only 2 copies of *uPA* gene were found, indicating reduced dosage of *uPA*.

The copy number of *uPA* gene was also analyzed in 78 samples from 29 prostate cancer metastases (Table 5). 62% of them showed 2 copies of both *uPA* and chromosome 10 centromere. 28% of cases contained a gain of *uPA* gene. The most common alteration detected was 3 copies of both *uPA* and centromere 10. *uPA* gene dosage was reduced in one case as evidenced by 5 copies of both *uPA* and centromere 10. 31% of prostate cancer metastases had an increased copy number of *uPA* gene. All normal tissue controls in the same TMA had two copies of *uPA* and chromosome 10.

In expression studies the level of *uPA* was analyzed in the prostate cancer cell lines by Q-RT-PCR. PC-3 cells showed about 6-fold higher mRNA expression than DU145 cells whereas LNCaP and 22Rv1 cells did not express *uPA* mRNA at all.

3. Effect of uPA inhibitors on invasion of prostate cancer cell lines (Study II)

Four prostate cancer cell lines (PC-3, LNCaP, DU145 and 22Rv1) were used to study the effect of 2 different uPA inhibitors (B428 and p-aminobentzamidine) on invasion potential. Of the cell lines PC-3 and DU145 invaded through Matrigel-coated membrane, whereas LNCaP and 22Rv1 displayed only a weak invasion potential. 10 μM B428 decreased the number of invading PC-3 cells by 60%, whereas the number of invading DU145 cells increased by 25%. When 20 μM of the B428 inhibitor was used, the number of invading PC-3 cells decreased 82% compared to controls. In the DU145 cell line B428 decreased the invasion of DU145 cells only 10%. B428 had no effect on the invasion of LNCaP and 22Rv1. The reduction of invasion was statistically significant only in the PC-3 cell line, at both 10 μM ($p=0.0075$), and 20 μM ($p<0.0001$) concentrations of B428.

The influence of p-aminobenzamidine was studied using invasive PC-3 and DU145 prostate cancer cell lines. 250 μM p-aminobenzamidine reduced the number of invading PC-3 cells to one third, whereas in the DU145 cells the reduction was 50 %. The reduction of invasion was statistically significant only in the PC-3 cell line ($p= 0.014$).

4. Gene copy number and expression analyses of *TRPS1, EIF3S3* and *MYC* genes (Study III)

To study the gene copy number of *TRPS1* and *EIF3S3* in hormone-refractory prostate carcinomas, we used TMA and FISH (Table 5). Using FISH it was found that *TRPS1* and *EIF3S3* were co-amplified in about one third of hormone-refractory prostate carcinomas. The co-amplification of the genes was also

verified by hybridizing differentially-labeled gene specific probes (AlexaFluor 594 labeled *TRPS1* probe and biotin labeled *EIF3S3* probe) simultaneously to the TMA. A gain (3-4 copies) of *TRPS1* and *EIF3S3* was found in 50% and 48% of the cases, respectively.

Breast and prostate cancer cell lines were also analyzed for copy number alterations of the *TRPS1*, *EIF3S3* and *MYC* genes. The highest copy number of all the genes was found in the breast cancer cell line SK-Br-3, which showed 21 copies of *EIF3S3* and 47 copies of *TRPS1*, and only 1 copy of chromosome 8 centromere. In addition, a high-level amplification (locus/centromere ratio ≥2) of all 3 genes was found in MDA436 and PC-3 cancer cell lines. *EIF3S3* and *MYC* were also highly amplified in the EFM19 cell line.

The expression of 3 genes was analyzed by Q-RT-PCR. In the breast cancer cell line SK-Br-3 (which contains the highest copy number of all the 3 genes investigated) *EIF3S3* was the only gene showing significantly high mRNA levels (3-10 fold compared to the other cell lines). The relative expression of *TRPS1* mRNA was highest in ZR75-1 cells, which contain a loss of the gene compared to the centromere copy number.

Next, the expression of *TRPS1*, *EIF3S3* and *MYC* was measured in BPH, untreated primary and locally recurrent hormone-refractory carcinomas. *EIF3S3* mRNA level was higher (on average, 3-fold) in carcinomas than in BPH. When hormone-refractory and untreated prostate carcinomas were compared, there was no difference in the mRNA levels of *EIF3S3*. *MYC* and *TRPS1* were equally expressed in BPH, untreated carcinomas and HR tumors. No significant association was found between histological grade or clinical stage (T3-T4 and/or N+ and/or M+ versus T1-2N0M0) and the expression of any of the three genes in the untreated tumors.

5. EIF3S3 overexpression in NIH 3T3 cells (Study IV)

The effect of EIF3S3 overexpression on cell growth and colony formation was studied in NIH 3T3 cells. EIF3S3 overexpression caused a significant increase in NIH 3T3 proliferation, whereas *EIF3S3* in antisense orientation decreased cell growth. NIH 3T3 cells transfected with *EIF3S3* gene had acquired the ability to survive in soft agar, but no effect on colony formation was found. All the control cells died in two weeks, whereas the subset of cells overexpressing EIF3S3 was still alive after three weeks. The data demonstrate that EIF3S3 overexpression has an effect on cell viability in vitro.

6. Effect of *EIF3S3* siRNA treatment on breast and prostate cancer cell lines (Study IV)

Three different siRNA oligos were tested in PC-3 prostate and ZR75-1 breast cancer cell lines for their capacity to silence *EIF3S3* expression. The downregulation of *EIF3S3* mRNA levels was analyzed by Q-RT-PCR. The inhibition efficacy of selected *EIF3S3*-siRNA was 96% in PC-3 prostate cancer cells and 65% in the ZR75-1 breast cancer cell line. The specificity of *EIF3S3* siRNA´s was determined in the PC-3 and ZR75-1 cell lines by measuring the expression of *KIAA0196* and *RAD21* genes. siRNA oligo for *EIF3S3* had a high inhibitory potential and specificity towards *EIF3S3* expression. The relative inhibition efficacy (normalized with siLUC control siRNA) of *EIF3S3*-siRNA was 55% in LNCaP, 96% in PC-3, 70% in SK-Br-3 and 65% in the ZR75-1 cell line.

The effect of *EIF3S3*-siRNA transfection on proliferation was studied by counting the number of cells at various intervals. After 4 days there was a statistically significant decrease in cell number in all 4 cell lines (p-value=0.0022). In cell lines LNCaP and ZR75-1, the inhibition influence was evident earlier than in cell lines PC-3 and SK-Br-3. Apoptosis was studied using IHC and FACS analysis. As no difference in the amount of apoptotic cells between *EIF3S3*-siRNA treated and control cells was found, the data suggest that the inhibitory effect is associated with proliferation rate.

DISCUSSION

1. Overexpression and amplification of *ERBB2* in prostate cancer

Using CISH, we showed that *ERBB2* oncogene is not highly amplified in either androgen-dependent or independent primary or metastatic prostate carcinomas. We found only one case out of 126 with borderline amplification of *ERBB2*. The tumor did not overexpress ERBB2 protein by IHC, suggesting that *ERBB2* is not the target gene of the amplification in this case.

The majority earlier studies analyzing *ERBB2* amplification in prostate cancer either by Southern blotting or FISH (Signoretti et al., 2000; Latil et al., 1994; Fournier et al., 1995; Kuhn et al., 1993; Bubendorf et al., 1999) concur with our findings. Only Ross and co-authors have reported high-level amplification of *ERBB2* in prostate cancer. One reason for the discrepancy could be the definition of gene amplification. In this study we used a definition that has been validated in breast cancer (Seidman et al., 2001; Tanner et al., 2000). The CISH used in this study is based on single color detection of one probe, similarly as in the U.S. Food and Drug Administration-approved FISH test (Tanner et al., 2000). Only cases with more than 5 copies are considered as amplification. Such amplification is associated with overexpression, poor prognosis, and response to trastuzumab in breast cancer. Today it is generally accepted that *ERBB2* is not amplified in prostate cancer.

To study the expression of ERBB2 protein and mRNA we used IHC and Q-RT-PCR respectively. In IHC, the intensity of staining was 0 or 1+ in all prostate tumors studied. Breast carcinomas containing gene amplification showed clear staining. Our results are in agreement with some published reports (McCann et al., 1990; Visakorpi et al., 1992), but different results have also been reported (Signoretti et al., 2000; Ross et al., 1997b; Myers et al., 1994). These discrepancies in the IHC findings are probably due to different antibodies and staining techniques as well as due to inconsistent interpretation of the staining results. In this study, we used antibody (CB11 monoclonal antibody; Novocastra Laboratories), and a staining method that has been shown to correlate with gene amplification by FISH or CISH, as well as with poor prognosis in breast cancer (Tanner et al., 2000; Tanner et al., 2001; Kallioniemi et al., 1992). CB11 monoclonal antibody is widely used in ERBB2 IHC studies. For example Larsimot et al. (2002) used the same antibody, when they examined over 300 breast cancer samples by IHC and FISH. Concordance between FISH and IHC

was 86% when CB11 antibody was used. Gancberg et al. (2002) also evaluated the usefulness of CB11 antibody for *ERBB2* IHC studies. They found that not only the antibody but also the protocol used has a significant effect on results. Their data from breast cancer samples (n=394) in two different laboratories demonstrated that ERBB2 IHC is not a reproducible method if there is no standardization of the procedure.

We used real-time Q-RT-PCR to analyze mRNA levels and confirm our IHC data. In real-time PCR the quantification of the template is based on the detection of the cycle in which the reaction enters the exponential phase, instead of measuring the amount of final PCR product. Thus, none of the reagents are rate limiting in the reaction at the time of measurement of the fluorescence. Several studies have shown that the method is highly quantitative and reliable (Gibson et al., 1996; Bieche et al., 1999; Helenius et al., 2001; Linja et al., 2001).

The level of *ERBB2* mRNA expression was low both in prostate cancer cell lines and tumors, and there were no differences between androgen-dependent or independent tumors. With Q-RT-PCR, the expression levels of *ERBB2* mRNA in prostate cancer tumors were approximately the same as in breast carcinomas without *ERBB2* amplification. In the breast tumors containing *ERBB2* amplification, the expression level was ~20 times higher than in prostate cancer tumors. The significance of the low-level expression of the *ERBB2* gene for the prostate tumors and its functional role for prostate cancer remains to be studied.

Based on published ERBB2 data from breast and prostate cancer studies, it seems that trastuzumab is not a relevant treatment for prostate cancer. It has been shown that only breast carcinomas with *ERBB2* amplification and overexpression respond to trastuzumab (reviewed by Shak, 1999). Although some preclinical studies with prostate cancer xenograft models have suggested that trastuzumab could be useful in treating prostate cancer (Agus et al., 1999) clinical trials have not shown any efficacy. For example, Ziada et al. (2004) demonstrated in a phase II trial that trastuzumab has no effect on hormone-refractory prostate cancer. Still, although ERBB2 is not amplified or overexpressed in prostate cancer, it could have a role in the development of the disease. It has been suggested that *ERBB2* could activate AR in the presence of low levels of androgens (Craft et al., 1999; Yeh et al., 1999). Furthermore Mellinghoff et al. (2004) recently suggested that ERBB2 dimerized with ERBB3 modulates the AR function.

A limitation of trastuzumab is that it is effective only when ERBB2 is highly expressed. What kind of therapies might be useful when ERBB2 level is low or moderate? ERBB2 is believed to function as a coreceptor to form heterodimers with EGFR, ERBB3 or ERBB4. This heterodimerization can be used as a target when trying to influence on ERBB2 mediated signaling. Humanized monoclonal antibody, namely 2C4, binds to a different epitope of ERBB2 than trastuzumab and sterically impedes ERBB2 recruitment in heterodimers with other ERBB receptors (Albanell et al., 2003). Agus et al. (2002) found in both in vitro and in vivo breast and prostate cancer models that 2C4 antibody inhibits growth

regardless of the ERBB2 levels. Mendoza et al. (2002) also demonstrated that rhuMab 2C4 inhibits tumor growth in an androgen-independent xenograft model. The effect of some tyrosine kinase inhibitors in the growth of prostate cancer xenografts has also been tested. For example, ERBB2/EGFR tyrosine kinase inhibitor PKI-166 has been found to inhibit LAPC4 xenograft growth (Mellinghoff et al., 2002). Whether one of these strategies will be a useful treatment in the prostate cancer remains to be seen.

2. Frequency of *uPA* amplification and sensitivity of prostate cancer cells to uPA inhibition

The mechanisms responsible for increased expression of uPA in many invasive cancers are still mainly unknown. Helenius et al. (2001) have previously shown that amplification of the *uPA* gene may be one mechanism for the increased expression of uPA. Amplification was shown to be associated with higher expression of *uPA*.

In this study, we evaluated the frequency of the *uPA* gene copy number alterations in 63 locally recurrent hormone-refractory prostate tumors and 78 metastases from 29 hormone-refractory cases by FISH. These tumors represented advanced stage of the disease, and thus were assumed to contain the highest frequency of genetic aberrations. Increased copy number but no high-level amplification of *uPA* gene was found in 21% of the locally recurrent hormone-refractory prostate cancers. Three copies of both *uPA* gene and chromosome 10 centromere was the main alteration type detected. In total, only one (1.5%) out of 76 (63+13 from the previous study) locally recurrent hormone-refractory tumors contained an amplification of *uPA* gene, suggesting that the amplification of *uPA* gene is rare. The metastases analyzed here were obtained from autopsies of patients who died of prostate cancer. The samples also represented hormone-refractory disease. Of the 29 prostate cancer metastases, 31% had an increased copy number of *uPA*, including one case of high-level amplification. Thus, the amplification of *uPA* gene also seems to be quite rare in prostate cancer metastases, suggesting that the increased level of *uPA* detected in a high percentage of prostate tumors (Gaylis et al. 1989, Van Veldhuisen et al. 1996) is mainly due to mechanisms other than gene amplification.

Today there is no good therapy for patients with invasive prostate cancer. Since uPA has an important role in cell invasion, it is an interesting candidate target for cancer therapy. Helenius et al. (2001) have previously shown that the invasion of PC-3 is greatly reduced with the uPA inhibitor amiloride. Amiloride also inhibits lymph node metastasis in rat (Jankun et al., 2001) and reduces the size of prostate cancer xenografts in mouse (Ray et al., 1988). Amiloride is not a very specific inhibitor and it also inhibits, for example, T-type calcium channels (Tang et al., 1988) and epithelial sodium channels (Jankun et al., 2001). In this study we used two more specific uPA inhibitors: B428 (Billstrom et al., 1995)

and p-aminobenzamidine (Holst-Hansen et al., 1996) for Matrigel invasion analyses.

Of the cell lines studied, LNCaP and 22Rv1 (which did not express uPA) were both non-invasive in the Matrigel assay, suggesting that prostate cancer cells require active uPA for their invasion through the Matrigel. Our finding is in agreement with other studies showing that the expression of uPA in cells and activity of uPA is associated with invasiveness (Pulukuri et al., 2005; Festuccia et al., 1998; Xing et al., 1999; Alonso et al., 1998). As expected, uPA inhibitors did not have any influence on these two cell lines. On the contrary, the PC-3 cell line, which has high-level amplification of *uPA* gene and expresses high levels of *uPA* mRNA, was very sensitive to both of the uPA inhibitors tested. With the B428 inhibitor, statistically highly significant, dose-dependent reduction of PC-3 invasion was seen. This kind of effect has previously also been reported in human breast cancer cells (Evans et al., 2000) as well as the F3II mouse mammary carcinoma cell line (Alonso et al., 1998). The DU145 prostate cancer cell line, which also expresses high levels of *uPA* mRNA and is invasive, showed only a slight reduction in invasion with the higher concentration of B428. p-aminobenzamidine also reduced PC-3 invasion statistically significantly, but in the DU145 cell line the reduction of invasive cells was not statistically significant. It has previously been shown (Billstrom et al., 1995), that p-aminobenzamidine causes dose-dependent inhibition of uPA activity in vitro and also inhibits the growth of DU145 xenografts in severe combined immunodeficiency (SCID) mice. However, our results indicate that neither p-aminobenzamidine nor B428 are able to effectively inhibit the invasion of DU145 cells through Matrigel. Recently, Pulukuri et al. (2005) also demonstrated also uPA-dependent invasion of prostate cancer cells. They found that RNAi-directed knockdown of uPA and uPAR inhibits PC-3 prostate cancer cell invasion as indicated by Matrigel assay. It also had an effect on prostate cancer cell survival and tumorigenecity in vivo. Intratumoral injection of plasmid construct expressing small hairpin RNAs (shRNAs) for *uPA* and *uPA*R almost completely inhibited tumor growth and survival in an orthotopic mouse prostate cancer model. These results indicate the importance of *uPA* for prostate cancer, confirming our results.

Since, both the PC-3 and DU145 cell lines express high levels of *uPA* mRNA, but only PC-3 contains the gene amplification of *uPA*, the finding indicates a strong association between genetic aberration of *uPA* gene and sensitivity of cells to uPA inhibitors. Inhibitors, amiloride, p-aminobenzamidine and B428, most likely inhibit the uPA in both the DU145 and PC-3 cell lines, but the amplification of *uPA* gene in PC-3 suggests that *uPA* is a more important mechanism for invasion in PC-3 than in DU145 cells. A similar association of drug sensitivity and genetic alteration of drug target has been found with some novel drugs, such as trastuzumab, imatinib, and gefitinib. As mentioned previously, trastuzumab (a monoclonal antibody against *ERBB2*) is effective only in patients with *ERBB2* gene amplification (Vogel et al., 2002). Imatinib, which is a protein-tyrosine kinase inhibitor that inhibits ABL, platelet-derived

growth factor receptor, beta polypeptide (*PDGFR*) and v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog (*KIT*) (Buchdunger at al., 2000), has been successfully used in treating chronic myeloid leukemia (CML) and gastrointestinal stromal tumors (GIST). CML is driven by translocated *ABL*-oncogene, whereas activating point mutations in *KIT* or *PDGFR* cause GIST. Imatinib works extremely well in all these three situations (reviewed by Sawyers, 2004). Although imatinib is a frontline therapy for CML, resistance to the treatment is increasingly encountered. The resistance occurs through selection for tumor cells containing point mutation in *ABL* domain, interfering with drug binding (Shah et al., 2004), or by amplification of the translocated *ABL* resulting in an elevated level of the transcript (Gorre et al., 2001). Resistance to imatinib caused by secondary mutations, is also found among GIST patients (Antonescu et al. 2005). In addition, studies have shown that *EGFR* mutations in lung cancer correlate with clinical response to gefitinib therapy (Paez et al. 2004). All this suggests that genetic alterations pinpoint a critical mechanism for a particular tumor, and thus may help in designing therapies for that particular case.

3. *TRPS1*, *EIF3S3* and *MYC* as target genes for 8q amplification

Some oncogenes are activated by overexpression of the gene, and amplification is one of the mechanisms of the overexpression. Amplicons are believed to contain many genes, of which only one or few are the true target genes of the amplification (Brodeur and Hogarty, 1998). Gain or amplification of chromosome 8q is one of the most common chromosomal alterations in breast and prostate cancer (Forozan et al., 1997). In order to evaluate the significance of *TRPS1*, *EIF3S3* and *MYC*, which have been suggested to be putative target genes for amplification in 8q23-q24 (Jenkins et al., 1997; Nupponen et al., 1999; Chang et al., 2000), we analyzed both the gene copy numbers and expressions of the three genes in breast and prostate cancer.

It has previously been shown that *EIF3S3* and *MYC* are co-amplified in about one third of locally recurrent hormone-refractory prostate carcinomas (Saramäki et al., 2001). In this study we found that the *EIF3S3* and *TRPS1* genes are also co-amplified in about 30% of hormone-refractory prostate tumors. The finding that *TRPS1*, *EIF3S3* and *MYC* are commonly co-amplified in hormone-refractory tumors indicates that the size of the amplicon is quite large. The *TRPS1* and *EIF3S3* genes are located about 12 Mb and 11 Mb centromeric from *MYC* respectively. The large size and the relatively low copy number of the amplicon have previously also been implicated by CGH and FISH studies (Visakorpi et al., 1995a; Nupponen et al., 1998b; Cher et al., 1996).

One means of identifying the target gene for amplification is to study the expression of the gene. The target gene for amplification is assumed to be

overexpressed. We first analyzed the expression of *TRPS1*, *EIF3S3* and *MYC* mRNAs in the cell lines using Q-RT-PCR. In SK-Br-3, the *TRPS1* has the highest copy number of these 3 genes, but its mRNA expression was lower than in the ZR75-1 cell line, which contains relative loss of the *TRPS1* gene. The expression of *MYC* mRNA varied among the cell lines and no correlation between *MYC* amplification and expression was found. In SK-Br-3, where the copy numbers of all 3 genes are high, *EIF3S3* is the only gene the expression of which is high. This result also confirms earlier Northern blot data by Nupponen et al. (1999). The data from the cell lines suggest that of the 3 genes, *EIF3S3* is the most likely target gene for amplification of SK-Br-3.

A previous analysis by mRNA in situ hybridization showed that *EIF3S3* is expressed more in hormone-refractory prostate carcinomas than in BPH (Nupponen et al., 1999). In this study, we found that the level of *EIF3S3* mRNA expression was significantly higher (p=0.029) in all prostate cancer than in BPH. Although hormone-refractory tumors have a higher rate of 8q gain (Visakorpi et al., 1995a), there was no difference in expression level of *EIF3S3* between untreated and hormone-refractory tumors. The data imply that *EIF3S3* is generally overexpressed in prostate cancer, but there are also other mechanisms besides gene amplification leading to overexpression of the gene. *TRPS1* and *MYC* mRNA expression levels were similar in BPH, untreated and hormone-refractory specimens, suggesting that the alterations in the expressions of these two genes are rare in prostate cancer. Altogether, the data suggested that of the 3 genes, *EIF3S3* is the most likely target gene for the 8q amplification. Thus we continued our studies only with *EIF3S3* and tried to ascertain the role of EIF3S3 overexpression in cell growth.

Here we showed that the expression of EIF3S3 is associated with cell growth. Normal murine fibroblasts (NIH 3T3) with overexpression of human *EIF3S3* grew significantly faster than cells transfected *EIF3S3* in antisense orientation. The importance of *EIF3S3* for cell growth was also seen in cancer cell lines. The effect of inhibition was tested in breast (SK-Br-3, ZR75-1) and prostate (PC-3, LNCaP) cell lines. The reduction of *EIF3S3* expression with siRNA led to growth inhibition in all four cell lines. In the LNCaP and ZR75-1 cells the inhibition of growth was seen at day 3, whereas in PC-3 and SK-Br-3 the inhibition was seen at day 4. However, the difference was due to the slow growth of the PC-3 and SK-Br-3 cells after transfection, indicating that SK-Br-3 and PC-3 are more sensitive to the transfection method than ZR75-1 and LNCaP. The effect of siRNA treatment on apoptosis was detected using IHC and FACS. The amount of apoptotic cells did not increase during siRNA treatment, suggesting that the function of *EIF3S3* is rather related to proliferation rate than to apoptosis.

Recently, Kittler et al. (2004) used over 5000 endoribonuclease-prepared short interfering RNAs (esiRNAs) to screen for genes required for cell division in HeLa cells. 37 genes required for cell division were identified and two of them were EIF3 subunits: *EIF3S3* and *EIF3S10*. These findings confirm our data that

EIF3S3 is involved in cell growth and may have role in the progression of prostate cancer.

Since the ability to grow without anchorage to a substratum is thought to be typical of the transformed cells, we determined the effect of EIF3S3 overexpression on transformation of cells using a soft agar colony formation assay. EIF3S3 overexpression had no effect on colony formation rate, indicating that *EIF3S3* is not a "classical" transforming oncogene. However, the NIH 3T3 cells transfected with *EIF3S3* gene had acquired the ability to survive in soft agar. The lack of transforming potential indicates that *EIF3S3* could be associated rather with the progression of the disease than early carcinogenesis.

It has recently been shown using microarray profiling of tumors that factors related to the regulation of translation are often overexpressed metastatic diseases (Ramaswamy et al., 2003). It is also known that some of the eukaryotic translation initiation factors (EIFs) have oncogenic properties. Elevated levels of EIF4E and EIF4GI are found in a broad spectrum of transformed cell lines and human cancers and are often associated with aggressive, poorly differentiated tumors (Avdulov et al., 2004). EIF4E expression is elevated in carcinomas of the head and neck (HNSCC), breast and prostate (reviewed by De Bendetti and Harris, 1999). The expression of EIF4E is elevated in lung adenocarcinomas compared to normal lung. It has also been found to be progressively increased during cancer progression from atypical adenomatous hyperplasia to invasive cancer (Seki et al., 2002). Based on published data, it seems that EIF4E overexpression alone can drive metastatic progression by selectively upregulating the translation of key proteins for malignancy (VEGF, cyclin D1 and ODC) (reviewed by Graff and Zimmer, 2003). EIF4G is also one of the translation initiation factors associated with cancer. EIF4G gene has been found to be amplified (Brass et al., 1997) and overexpressed (Bauer et al., 2002) in squamous cell lung carcinomas.

EIF3S3 is one of the subunits of eukaryotic translation initiation factor 3 (EIF3). EIF3 has a prominent role in the translation initiation pathway by binding to 40S ribosomal subunits in the absence of other translational components. It also helps to maintain the 40S and 60S subunits in a dissociated state (Asano et al., 1997). In humans, EIF3 is composed of at least 11 subunits (Browning et al., 2001) and some of them are associated with cancer. The expression level of EIF3S10 is upregulated in many transformed cell lines and also in several human cancers including breast, cervical, esophageal, and stomach carcinomas (Lin et al., 2001a). EIF3S8 is overexpressed in testicular semiomas (Rothe et al., 2000). Since quite little is known about EIF3S3 itself, it is difficult to predict how the protein mechanistically affects cell growth. In a recent study it was shown that EIF3S3 interacts with protein containing SH3-domain, which may link EIF3S3 to signaling pathways (Rual et al., 2005). Obviously more studies are required on the function of EIF3S3. Functional information could also indicate means for targeting EIF3S3 in therapeutic purposes. Since EIF3S3 harbors some characteristics of oncogene, it should be considered as putative drug target.

SUMMARY

The main findings of the study were:

1. Amplification and overexpression of *ERBB2* is absent or at least very infrequent in prostate carcinoma. The expression level of *ERBB2* mRNA and protein do not vary between prostate tumor types (BPH, hormone-naïve or hormone-refractory) and they are on the same level as in breast carcinomas without *ERBB2* amplification. It is likely that treatment strategies based on ERBB2 overexpression are not effective in the treatment of prostate cancer.

2. At least in a small subset of prostate cancer patients, uPA may have a role in the progression of prostate cancer from localized to early metastatic disease. Gain of *uPA* is found in about one third of patients, when high level amplification of *uPA* appears not to be very common ($\leq$ 5% of patients).

3. Sensitivity to uPA inhibition appears to be associated with DNA copy number rather than expression levels. If suitable, non-toxic uPA inhibitors were available, the data suggest that *uPA* copy number status could be important in the selection criteria for clinical trials.

4. The expression of *EIF3S3* mRNA is increased in both untreated and hormone-refractory prostate cancer. Amplification of *EIF3S3* seems to be one mechanism of overexpression of the gene, but the data suggest that there are also another mechanisms affecting *EIF3S3* mRNA expression. Overexpression of *TRPS1* and *MYC* mRNAs seems to be rare in prostate cancer and it is unlikely that alterations of the expression of these two genes have any effect on the progression of prostate cancer.

5. Overexpression of EIF3S3 increases cell growth and survival, whereas inhibition of expression reduces proliferation, suggesting that the gene is associated with cell growth. The results from soft-agar colony assays suggest that overexpression of EIF3S3 alone is incapable of transforming cells.

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