OUTI SARAMÄKI

Gene Copy Number Alterations in Prostate Cancer

■

ACADEMIC DISSERTATION
To be presented, with the permission of
the Faculty of Medicine of the University of Tampere,
for public discussion in the auditorium of Finn-Medi 1,
Biokatu 6, Tampere, on August 18th, 2006, at 12 o'clock.

UNIVERSITY OF TAMPERE

ACADEMIC DISSERTATION
University of Tampere, Institute of Medical Technology
Tampere University Hospital, Laboratory Centre
Tampere Graduate School in Biomedicine and Biotechnology (TGSBB)
Finland

Supervised by
Professor Tapio Visakorpi
University of Tampere

Reviewed by
Docent Akseli Hemminki
University of Helsinki
Docent Outi Monni
University of Helsinki

Distribution
Bookshop TAJU
P.O. Box 617
33014 University of Tampere
Finland

Tel. +358 3 3551 6055
Fax +358 3 3551 7685
taju@uta.fi
www.uta.fi/taju
http://granum.uta.fi

Cover design by
Juha Siro

Printed dissertation
Acta Universitatis Tamperensis 1162
ISBN 951-44-6678-0
ISSN 1455-1616

Electronic dissertation
Acta Electronica Universitatis Tamperensis 540
ISBN 951-44-6679-9
ISSN 1456-954X
http://acta.uta.fi

Tampereen Yliopistopaino Oy – Juvenes Print
Tampere 2006

YHTEENVETO

Yksi syövän syntyyn ja etenemiseen vaikuttavista tekijöistä on kromosomaalisten muutosten, kuten geenien häviämien ja monistumien, kertyminen genomiin. Eturauhassyövistä on löydetty monia usein esiintyviä kromosomaalisia muutoksia, mutta vain muutamien muutosten kohdegeeni on tunnistettu. Kohdegeenien tunnistaminen voisi johtaa uusien diagnostisten ja prognostisten menetelmien, tai uusien hoitojen kehitykseen.

Vertailevalla genomisella hybridisaatiolla (VGH) on saatu selville, että HIF1A-geenin lokus, 14q23.2, on monistunut PC-3-eturauhassyöpäsolulinjassa ja EZH2-geenin lokus, 7q36.1, LuCaP41-eturauhassyöpävierassiirteessä. Lisäksi EIF3S3-geenin lokuksen, 8q23.3–24.11, ylimääräisiä kopioita esiintyy jopa 80% pitkälle edenneissä eturauhassyövissä. Fluoresenssi in situ hybridisaatiolla (FISH) pyrittiin selvittämään, kuinka yleisiä em. geenien monistumat ovat eturauhassyövässä ja ovatko ne monistumien kohdegeenejä. Lisäksi EZH2-geenin ilmentymistä tutkittiin kvantitatiivisella käänteiskopiointipolymeraasiketjureaktiolla (Q-RT-PCR) ja immunohistokemiallisella värjäyksellä.

EIF3S3- ja EZH2-geenien ylimääräisiä kopioita oli huomattavasti enemmän pitkälle edenneissä syövissä, jopa yli 50%:ssa hormoniriippumattomista eturauhassyövistä. Muutoksilla oli heikko yhteys lyhyempään tautivapaaseen aikaan prostatektomialla hoidetuilla potilailla. EZH2-proteiinin ilmentyminen oli voimakkaampaa edenneissä syövissä, erityisesti sellaisissa, joissa oli geenin monistuma ($p<0.05$). Kahdeksassa eturauhassyöpävierassiirteessä kymmenestä oli EZH2-geenin monistuma. Yksi näistä oli ns. korkea-asteinen monistuma, 7-10 kopiota. Q-RT-PCR:n mukaan EZH2 myös ilmentyi siinä voimakkaasti mRNA-tasolla. Ilmentyminen näytti voimakkaammalta myös muissa monistuman sisältävissä vierassiirteissä, vaikkakaan ei tilastollisesti merkittävästi. Sekä EZH2- että EIF3S3-geeniä voidaan pitää lokuksiensa mahdollisina kohdegeeneinä. Sen sijaan HIF1A-geeni ei ollut monistunut yhdessäkään tutkitussa eturauhassyöpäkasvaimessa. Ainoa HIF1A-monistuma löytyi PC-3-solulinjasta, joten yleisesti eturauhassyövässä esiintyvä HIF1A-proteiinin yli-ilmentyminen ei johdu geenin monistumasta.

Eturauhassyöpävierassiirteistä ja -solulinjoista seulottiin myös koko genomin kattavasti geenien kopioluku- ja ilmentymismuutoksia cDNA-mikrosirujen avulla. Havaitut kopiolukumuutokset olivat enimmäkseen yhteneväisiä aikaisempien tutkimusten kanssa. cDNA-mikrosiruille tehtävän VGH:n resoluutio on kuitenkin parempi kuin vanhempien menetelmien, joten joidenkin muuttuneiden kromosomialueiden voitiin osoittaa olevan pienempiä tai koostuvan useasta erillisestä muutoksesta. Lisäksi löydettiin muutamia aiemmin tuntemattomia kro-

mosomaalisia muutoksia, kuten alueiden 1q21.2–23.1, 9p13–q21 ja 16p monistumat. 9p13-alueen monistuma varmennettiin FISH:lla. Geenien kopioluvuilla myös näyttäisi olevan suurempi vaikutus geenien ilmentymistasoon kuin aiemmin on uskottu. Geenien ilmentymistä tutkittaessa tunnistettiin myös joitakin mahdollisia kohdegeenejä, kuten POGZ (1q21.3, monistuma), ITGA4 (2q31.3, häviämä), FZD6 (8q22.3, monistuma), UBE2R2 (9p13.3, monistuma) ja RBBP6 (16p13.3, monistuma).

Yhteenvetona voidaan todeta, että EIF3S3- ja EZH2- geenit ovat mahdollisesti lokuksiensa monistumien kohdegeenejä. Lisäksi EIF3S3-geenin monistumaa voidaan pitää ennusteellisena tekijänä. cDNA-mikrosiruille tehdyllä VGH:lla voidaan havaita pienempiä kromosomialueiden muutoksia kuin vanhemmilla menetelmillä. Kohdegeenien suora tunnistaminen on mahdollista tutkittaessa yhtä aikaa kopioluku- ja ilmentymismuutoksia. Vähäinenkin kopioluvun muutos näyttäisi vaikuttavan geenin ilmentymiseen.

CONTENTS

YHTEENVETO ***3***

CONTENTS ***5***

LIST OF ORIGINAL COMMUNICATIONS ***7***

ABBREVIATIONS ***8***

ABSTRACT ***10***

INTRODUCTION ***12***

REVIEW OF THE LITERATURE ***13***

Chromosomal aberrations in prostate cancer **13**

Losses 15

8p: NKX3-1 15

10q: PTEN and MXI1 16

16q: HSD17B2 and CDH1 17

Gains and amplifications 18

8q: MYC and EIF3S3 18

7q: MCM7 20

Xq: AR 21

14q: HIF1A 22

Translocations 22

Microarrays **23**

General properties of microarrays 23

Expression profiling of prostate cancer 24

Hepsin (HPN) 24

α-methylacyl-CoA racemase (AMACR) 25

Enhancer of zeste homologue (Drosophila) 2 (EZH2) 26

Profiling by clustering and meta-analysis 27

Copy number analyses 28

AIMS OF THE STUDY ***30***

MATERIALS AND METHODS ***31***

Cell lines and xenografts [I, III, IV] **31**

Clinical tumour specimens [I, II, III] **31**

Fluorescence *in situ* hybridisation (FISH) [I, II, III, IV] **33**

cDNA microarrays [IV] 34
Array comparative genomic hybridisation 34
Expression arrays 35
Data processing 35
Quantitative reverse transcription polymerase chain reaction (Q-RT-PCR) [III, IV] 36
Immunohistochemistry (IHC) [III] 37
Statistical analyses [II, III, IV] 37
RESULTS *38*
Analyses of *HIF1A*, *EIF3S3*, and *EZH2* 38
Copy numbers in cell lines and xenografts 38
Copy numbers in clinical samples 38
Expression of EZH2 40
Survival analyses 41
Array-CGH 41
cDNA microarray 43
DISCUSSION *46*
Copy numbers of *HIF1A*, *EIF3S3*, and *EZH2* 46
Expression of EZH2 48
aCGH 49
Expression analyses 51
Future prospects 52
CONCLUSIONS *53*
ACKNOWLEDGEMENTS *54*
REFERENCES *56*
ORIGINAL COMMUNICATIONS *72*

LIST OF ORIGINAL COMMUNICATIONS

This thesis is based on the following papers, referred to in the text by their Roman numerals.

I. Saramäki OR, Savinainen KJ, Nupponen NN, Bratt O, Visakorpi T (2001): Amplification of hypoxia-inducible factor 1α gene in prostate cancer. Cancer Genet Cytogenet 128:31–34.

II. Saramäki O, Willi N, Bratt O, Gasser TC, Koivisto P, Nupponen NN, Bubendorf L, Visakorpi T (2001): Amplification of EIF3S3 gene is associated with advanced stage in prostate cancer. Am J Pathol 159:2089–2094.

III. Saramäki OR, Tammela TLJ, Martikainen P, Vessella RL, Visakorpi T (2006): The gene for polycomb group protein enhances of zeste homolog 2 (EZH2) is amplified in late stage prostate cancer. Genes Chromosomes Cancer 45:639-645.

IV. Saramäki OR, Porkka KP, Vessella RL, Visakorpi T (2006): Genetic aberrations in prostate cancer by microarray analysis. Int J Cancer Published Online: Apr 26 2006.

ABBREVIATIONS

aCGH	array-CGH
AMACR	α-methylacyl-CoA racemase
AR	androgen receptor
BAC	bacterial artificial chromosome
BPH	benign prostatic hyperplasia
cDNA	complementary DNA
CGH	comparative genomic hybridisation
CNA	copy number alteration
CNP	copy number polymorphism
DAPI	4,6-diamidino 2-phenylindole
DNA	deoxyribonucleic acid
EIF3S3	eukaryotic translation initiation factor 3, subunit 3
EST	expressed sequence tag
EZH2	enhancer of zeste homologue 2 (*Drosophila*)
FISH	fluorescence *in situ* hybridisation
FITC	fluorescein isothiocyanate
HGPIN	high-grade prostatic intraepithelial neoplasia
HIF1A	hypoxia-inducible factor 1 alpha
HPN	hepsin
IHC	immunohistochemistry
LOH	loss of heterozygosity
MCM	minichromosome maintenance
M-FISH	multiplex fluorescence *in situ* hybridisation
mRNA	messenger RNA
MYC	v-myc myelocytomatosis viral oncogene homologue (avian)
P:C:I	phenol:chloroform:isoamyl alcohol
PAC	P1-vector derived artificial chromosome
PCR	polymerase chain reaction
PIN	prostatic intraepithelial neoplasia
PRC	polycomb repressive complex
PTEN	phosphatase and tensin homologue 1
Q-RT-PCR	quantitative reverse transcription PCR
RNA	ribonucleic acid
siRNA	small interfering RNA
SKY	spectral karyotyping
SNP	single nucleotide polymorphism

SSC	standard saline citrate
TBP	TATA-box binding protein
TMA	tissue microarray
UTR	untranslated region

ABSTRACT

Chromosomal aberrations, including deletions, gains and amplifications are thought to be a mechanism for the development and progression of cancer. Many frequent alterations have been described in prostate cancer, but only a few definitive target genes have been identified. The identification of target genes could lead to the development of diagnostic and/or prognostic markers as well as new targets for therapy.

According to comparative genomic hybridisation (CGH), the *HIF1A* locus (14q23.2) is amplified in the prostate cancer cell line PC-3, and the *EZH2* locus (7q36.1) in the prostate cancer xenograft LuCaP41. The locus of *EIF3S3* (8q23.3–24.11) is gained in up to 80% of advanced prostate cancers. In order to determine whether these genes could be the target genes of the amplifications, fluorescence *in situ* hybridisation (FISH) was used to assess their amplification frequencies. In addition, the expression of EZH2 was studied by quantitative real-time reverse transcription polymerase chain reaction (Q-RT-PCR) and immunohistochemistry (IHC).

No amplifications of *HIF1A* were found in clinical prostate cancer samples. Only PC-3 had an amplification of the gene, indicating that the generally observed overexpression of the protein in prostate cancer is not due to gene amplification. The frequencies of *EIF3S3* and *EZH2* gains/amplifications were significantly higher in advanced disease, reaching over 50% in hormone-refractory prostate cancer. The alterations were weakly associated with poor progression-free survival in prostatectomy-treated patients. The expression of EZH2 was higher in advanced disease and especially in samples with high-level amplification of the gene ($p<0.05$). Eight out of ten xenografts contained a gain of *EZH2*, including one with a high-level amplification of 7–10 copies. This sample also showed markedly higher mRNA expression by Q-RT-PCR. A trend towards increased expression was seen in the xenografts with gain of *EZH2*, although it was not statistically significant. Both *EIF3S3* and *EZH2* may be considered putative target genes for the gains of their respective loci.

Finally, array comparative genomic hybridisation (aCGH) and cDNA microarrays were used to screen prostate cancer cell lines and xenografts for genome wide copy number and expression alterations. The copy number alterations (CNAs) and their frequencies were generally consistent with earlier data obtained by CGH of the same samples, although due to the better resolution some aberrations were narrowed down or shown to consist of several smaller aberrations interrupted by regions of normal copy number. Previously unreported fre-

quent copy number alterations were also found, for example, gains in 1q21.2–23.1, 9p13–q21 and 16p. The amplicon in 9p13 was verified by FISH. cDNA microarrays showed that even a modest increase in copy number significantly affects the expression of the altered genes, indicating that simple gains may have a larger impact in prostate cancer than previously thought. Several putative target genes for copy number aberrations were also identified by cDNA microarray analysis, including *POGZ* (gain 1q21.3), *ITGA4* (loss 2q31.3), *FZD6* (gain 8q22.3), *UBE2R2* (gain 9p13.3), and *RBBP6* (gain 16p13.3).

In conclusion, *EIF3S3* and *EZH2* may be considered putative target genes of the amplifications at 8q23–24 and 7q36.1, respectively. In addition, *EIF3S3* could be used as prognostic marker. Array-CGH (aCGH) detects smaller regions of copy number alterations than CGH and may be used to narrow down known alterations and detect novel CNAs. When it is used together with expression arrays, putative target genes of the CNAs may be directly identified. Even a low-level copy number alteration appears to affect the expression of the altered genes.

INTRODUCTION

The age-adjusted incidence of prostate cancer has steadily increased in the past decades and it has become the most common male malignancy in Finland (Finnish Cancer Registry, 2005). In 2003, over 4,200 new cases were diagnosed and almost 750 men died of the disease in Finland. The steady increase cannot be accounted for by improved diagnostics alone, and the reasons for it remain, for the most part, unknown.

Prostate cancer afflicts predominantly old men. The mean age at diagnosis is around 70 years, and for this reason the impact of prostate cancer on the public health system is expected to rise as the population ages (Finnish Cancer Registry, 2005). The late onset of the disease, often combined with a slow rate of progression, results in most patients dying of other causes before the cancer progresses to a fatal stage. Nevertheless, about a third of prostate cancer patients die of their cancer, regardless of treatment. Although screening the population for prostate cancer has allowed diagnosis at earlier stages of the disease, its benefits have not been proven by randomised trials. Unfortunately, reliable identification of aggressive forms of prostate disease at the time of diagnosis is impossible with present day diagnostics: there are no molecular markers for prostate cancer aggressiveness in clinical use and the prognoses are based on histological grade and clinical stage.

Prostate cancer is a heterogeneous disease, up to the point that a single patient may have multiple tumour lesions with different properties. Sometimes the largest lesion is not the one to lead to the demise of the patient: another one may shed micrometastatic cells into the bloodstream and colonise other organs (Jenkins *et al*., 1997). Diagnostic and prognostic molecular markers for prostate cancer are needed to define which patients a) are at risk of getting prostate cancer, b) have prostate cancer, c) have clinically insignificant cancer that can be left untreated, d) have aggressive cancer that needs aggressive treatment, and e) have aggressive cancer that is likely to recur after initial treatment by prostatectomy and would therefore require adjuvant therapy to prevent, or at least delay, progression.

Like other cancers, prostate cancer arises as the result of the breakdown of cellular control mechanisms of proliferation and/or apoptosis. The reason for the breakdown is deregulation of key genes governing these processes, which may happen, for example, at the chromosomal level as a result of deletions, gains, amplifications, or translocations of the genes. The identification of the genes involved in the alterations could lead to improved diagnostics, identification of aggressive cases, and new therapies.

REVIEW OF THE LITERATURE

Chromosomal aberrations in prostate cancer

Classical cytogenetic analyses of prostate tumours are difficult, because good-quality metaphases are not easy to obtain due to low mitotic indexes of primary cultures. Even if metaphases of cancer cells were obtained in primary culture, they would be scarce on the preparations because of overgrowth by normal epithelial cells. Despite this, classical cytogenetics have been able to identify, for example losses in 8p and 10q in prostate cancer (Lundgren *et al*., 1992).

Multiplex fluorescence *in situ* hybridisation (M-FISH) and spectral karyotyping (SKY) enable the simultaneous identification of copy number changes and translocations (Speicher *et al*., 1996; Schröck *et al*., 1996; Macville *et al*., 1997). The methods are reliant on cancer cell metaphases, and cannot therefore be readily used to study clinical prostate cancers. However, M-FISH and SKY are useful in studying cell lines. SKY analysis has revealed, for example, recurrent breakpoints in chromosome arms 5q11, 8p11, and 10q22 in prostate cancer cell lines (Pan *et al*., 2001).

Loss of heterozygosity (LOH) analysis, a polymorphism-based method that requires normal and tumour DNA from the same patient, has been widely used to detect losses of polymorphic DNA sequences. LOH data cannot always be interpreted as physical copy number losses, since the remaining allele may be duplicated after the loss of the first allele (de Nooij-van Dalen *et al*., 1998; Varella-Garcia *et al*., 1998; White *et al*., 1998). Nonetheless, LOH analyses have been helpful in determining regions of allelic loss in prostate cancer**.** Frequent regions of LOH in prostate cancer include 5q, 6q, 8p, 10q, 13q, 16q, and 18q (Bergerheim *et al*., 1991; Kagan *et al*., 1995; Vocke *et al*., 1996; Cooney *et al*., 1996, 1996b; Carter *et al*., 1990; Elo *et al*., 1997; Cunningham *et al*., 1996; Ittmann and Wieczorek, 1996).

Before the invention of comparative genomic hybridisation (CGH), first described in 1992 by Kallioniemi *et al*., knowledge of the genomic composition of solid tumours was scarce and no genome-wide method to study copy number alterations was known. CGH eliminates the need for metaphases of tumour cells and allows the identification of copy number alterations from a relatively small amount of tumour DNA. The labelled DNA is hybridised together with differentially labelled normal control DNA to metaphases of normal peripheral blood

lymphocytes and the ratios of the hybridised DNA signals are analysed with computer software.

Chromosomal aberrations in prostate cancer have been identified in numerous CGH studies since the mid-1990s, and the inherent heterogeneity of the disease has been revealed. A single chromosomal aberration responsible for most of the cases has not been found in prostate cancer. The most common chromosomal alterations found by CGH in early stage clinical prostate cancer are losses in 5q, 6q, 8p, 13q, 16q, and 18q, and gains of 7p/q, 8q, and 16p (Visakorpi *et al.*, 1995b; Alers *et al.*, 2000). These are found in 10–50% of untreated primary prostate cancers and to some extent in pre-malignant lesions, such as high-grade prostatic intraepithelial neoplasia (HGPIN) (Zitzelsberger *et al.*, 2001). The minimal regions of these alterations have been narrowed down by LOH and aCGH studies and include, for example, 6q16.3–21, 6q22, 8q21.13, 8q22.1, 8q22.2–3, 8q24.13, 8q24.21, 13q14, 13q21–22, 13q33, 16q21.1, and 16q24.3 (Latil *et al.*, 1997; Elo *et al.*, 1999; Hyytinen *et al.*, 1999, 2002; Srikantan *et al.*, 1999; van Duin *et al.*, 2005b; Watson *et al.*, 2004; Hermans *et al.*, 2004).

As prostate cancer progresses to hormone-refractory disease and/or spreads to lymph nodes or distant organs, chromosomal aberrations become more abundant and additional recurrent aberrations appear. In addition to the alterations found already at early stages of the disease, losses in 10q, 15q, 17p, 19p/q, and 22q, as well as gains in 1q, 3q, and Xq are frequently found in locally recurrent hormone-refractory prostate cancer (Visakorpi *et al.*, 1995b; Nupponen *et al.*, 1998b; El Gedaily *et al.*, 2001). Untreated lymph node metastases contain aberrations frequently in more or less the same regions as the locally recurrent hormone-refractory tumours, although the gain in Xq appears to be specific to hormone-refractory disease (Cher *et al.*, 1996; Visakorpi *et al.*, 1995b).

Prostate cancer cell lines are difficult to establish, and less than ten are available commercially. The classical prostate cancer cell lines, PC-3, LNCaP, and DU145 have been most widely profiled for chromosomal alterations. CGH profiles have been published by Nupponen *et al.* (1998a), M-FISH profiles by Pan *et al.* (1999), SKY by van Bokhoven *et al.* (2003), and aCGH profiles by several groups (Clark *et al.*, 2003; Wolf *et al.*, 2004; Zhao *et al.*, 2005). The widely distributed newer cell line 22Rv1/CWR22R has also been profiled with CGH, M-FISH, and aCGH (Laitinen *et al.*, 2002; Wolf *et al.*, 2004; Zhao *et al.*, 2005). The findings have generally been concordant between the studies, with minor differences that may be attributed to different methods of analysis and interpretation of copy number alterations, and allowing the differences in resolution.

Human prostate cancers have also been grown in immunocompromised mice as xenografts and they have become valuable supplementary research tools to the prostate cancer cell lines. Fifteen xenografts (LAPC-4AD, LAPC-4AI, LAPC-9AD, and LAPC-9AI, as well as LuCaPs 23.1, 23.8, 23.12, 35, 41, 49, 58, 69, 70, and 73) have been characterised with CGH (Williams *et al.*, 1997; Laitinen *et al.*, 2002). Most of these models are derived from metastatic prostate cancer and hence represent advanced stages of the disease (reviewed by Sobel and Sadar, 2005a, 2005b). Thus it is not surprising that the most commonly found copy

number alterations in cell lines and xenografts have been the same as for advanced clinical samples.

Losses

Losses are more prevalent in early stage prostate cancer than gains (Alers *et al*., 2001; Chu *et al*., 2003; Visakorpi *et al*., 1995b). This implies that inactivation of tumour suppressor genes may be more important in prostate cancer initiation than oncogene activation. Attempts to identify target genes have been frustrating as somatic mutations in the remaining alleles have rarely been found. Therefore, it is now believed that haploinsufficiency, where the loss of a single gene copy is enough to cause an altered phenotype, or epigenetics, such as hypermethylation of promoter regions, play significant roles in prostate cancer (Chaib *et al*., 2003; reviewed by Santarosa and Ashworth, 2004; and by Li *et al*., 2005).

Despite the high frequency of deletions in 6q, 13q and 18q, target genes for these regions have not been identified. The *BRCA2* and *RB1* genes at 13q13.1 and 13q14.2, respectively, do not seem to play significant roles in sporadic prostate cancer (Tricoli *et al*., 1996; Li *et al*., 1998; Latil *et al*., 1999).

8p: NKX3-1

The most common chromosomal deletion in prostate cancer is the loss of 8p. This alteration is found already in early stage prostate cancer and also in HGPIN (Visakorpi *et al*., 1995b; Zitzelsberger *et al*., 2001). Independent loss of three separate regions in 8p has been identified (Macoska *et al*., 1995; Paris *et al*., 2004). Putative target genes for these regions include *NKX3-1* (8p21.2), *LZTS1* (8p22), and *MSR1* (8p22) (He *et al*., 1997**;** Ishii *et al*., 1999; Hawkins *et al*., 2002).

NKX3-1 is an androgen-regulated homeobox gene that controls the development of the prostate during embryonic development and the differentiation of prostate epithelial cells in adulthood (He *et al*., 1997). In adults it is expressed mainly in the prostate. The loss of a single copy of *NKX3-1* has been shown to cause prostatic intraepithelial neoplasia (PIN) and dysplasia in mice, and its expression is decreased already in the early stages of disease (Abdulkadir *et al*., 2002). Further reduction in expression or mislocalisation of the protein happens during cancer progression (Bowen *et al*., 2000). Haploinsufficiency of *Nkx3-1* has been demonstrated in *Nkx3-1* mutant mice by measuring the expression levels of Nkx3-1 target genes. Some of them were as much deregulated in the heterozygous mutants as they were in the homozygous mutants (Magee *et al*., 2003). Apart from homozygous deletions, inactivating mutations of the *NKX3-1* coding sequence have not been detected in sporadic prostate cancer (Voeller *et al*., 1997; Ornstein *et al*., 2001). In hereditary prostate cancer, however, twenty-one germ-line variants of the gene have recently been identified in 159 probands,

and some of them were shown to be linked to prostate cancer (Zheng *et al*., 2006). One of these variants, a rare mutation, was shown to decrease the binding of the protein to its DNA recognition sequence and co-segregate completely with prostate cancer in a family with three affected brothers and one unaffected brother. Although three CpG sites in the promoter region of *NKX3-1* have been shown to be more methylated in cancer cells compared to adjacent normal cells, widespread methylation of the promoter has not been found (Asatiani *et al*., 2005). The expression of NKX3-1 may be regulated post-transcriptionally: protein levels in mice are low despite normal levels of mRNA (Kim *et al*., 2002a).

10q: PTEN and MXI1

Allelic imbalance in 10q is frequently observed in prostate cancer (Carter *et al*., 1990). A pattern of loss of distal 10p, gain of regions around the centromere, and loss of distal 10q (loss – gain – loss) has been identified in CGH and aCGH studies (Nupponen *et al*., 1998b; Laitinen *et al*., 2002; Hermans *et al*., 2004).

The most studied candidate target gene for 10q is the *PTEN* (phosphatase and tensin homologue 1) tumour suppressor gene at 10q23.3. In addition to frequent hemi- and homozygous deletions, mutations of the gene have been reported in aggressive late-stage prostate cancer, making *PTEN* a case of classical tumour suppressor gene (Suzuki *et al*., 1998). PTEN is essential in early development, since a double knock-out is embryonic lethal (Di Cristofano *et al*., 1998; Podsypanina *et al*., 1999). It dephosphorylates phosphoinositide substrates and negatively regulates the AKT oncoprotein. Haploinsufficiency of *PTEN* has been shown to promote prostate cancer progression in mice and shorten their survival (Trotman *et al*., 2003; Kwabi-Addo *et al*., 2001). Inactivation of both *PTEN* and *NKX3-1* in mice has been shown to lead to HGPIN/early carcinoma by 6 months of age and aggressive prostatic carcinoma after 12 months (Kim *et al*., 2002b; Abate-Shen *et al*., 2003). In contrast, introduction and expression of *PTEN* in PC-3 has been shown to slow their growth and reduce their metastatic potential in mice (Davies *et al*., 2002).

Frequent loss, including homozygous deletion, of parts of 10q around the *PTEN* locus, has been detected by aCGH in eleven prostate cancer xenografts and three cell lines (Hermans *et al*., 2004). The distal flanking gene, *FLJ11218*, was almost always co-deleted with *PTEN*, in four cases homozygously. In most cases the closest proximal genes *MINPP1* (multiple inositol polyphosphate histidine phosphatase, 1), *PAPSS2* (3'-phosphoadenosine 5'-phosphosulfate synthase 2), and *ATAD1* (ATPase family, AAA domain containing 1, aka *FLJ14600*) were also lost. In addition to *PTEN* inactivation, *FLJ11218* and *PAPSS2* were significantly down-regulated, and inactivating mutations or total loss of the remaining allele were found in *PAPSS2*. These findings suggest that *PTEN* may not be the only target gene of 10q23 deletions.

There is also evidence of independently deleted regions distal to *PTEN*, at 10q25–qter, implying additional tumour suppressor genes on 10q (Leube *et al*.,

2002). A suggested candidate gene is *MXI1* (MAX interactor 1, isoform b, at 10q25.2), whose product is a transcription factor and an antagonist of MYC (v-myc myelocytomatosis viral oncogene homologue [avian]), (Prochownik *et al*., 1998; Zervos *et al*., 1993). Inactivating mutations of the remaining *MXI1* gene have been detected in prostate tumours with deletion at 10q24–25 (Prochownik *et al*., 1998). Forced expression of *MXI1* in DU145 has been shown to suppress their proliferation and colony forming potential (Taj *et al*., 2001).

16q: HSD17B2 and CDH1

LOH studies have defined at least four independently deleted regions in 16q: at 16q21.1, 16q22.1–22.3, 16q23.2–24.1, and 16q24.3–qter (Suzuki *et al*., 1996; Latil *et al*., 1997; Elo *et al*., 1999; Härkönen *et al*., 2005). Loss at 16q24.3 is associated with progression of prostate cancer (Härkönen *et al*., 2005). A small deletion in 16q21 and 13 separate regions of frequent loss in 16q22.2–qter have been defined with a high-resolution chromosome 16q specific BAC/PAC/cosmid array of 326 clones from a pre-selected set of 16 samples with deletions in 16q (Watson *et al*., 2004). The regions were in agreement with the LOH studies, whose resolution is not as good. Six genes located in these regions – *FOXF1* (forkhead box F1), *MAF* (v-maf musculoaponeurotic fibrosarcoma oncogene homolog [avian]), *MVD* (mevalonate [diphospho] decarboxylase), *WFDC1* (WAP four-disulfide core domain 1), *WWOX* (WW domain containing oxidoreductase), and a predicted transcript *Q9H0B8* (now known as *CRISPLD2*, cysteine-rich secretory protein LCCL domain containing 2) – have also been shown to be consistently down-regulated in cancer compared to matched benign tissue, indicating them as putative tumour suppressor genes (Watson *et al*., 2004).

HSD17B2 (hydroxysteroid [17-beta] dehydrogenase 2), located at 16q23.3, and involved in hormone metabolism, has recently been shown to be deleted in 29% (14/48) prostate cancers (Härkönen, 2005). The deletion was associated with metastasis and tumour stage. Mutations of *HSD17B2* have not been found (Elo *et al*., 1999). The expression of this gene has been shown to decrease dramatically in LNCaP cells as they become androgen-independent (Härkönen *et al*., 2003). Others, however, have shown that *HSD17B2* expression is increased in hormone-refractory prostate cancer (Fromont *et al*., 2005).

CDH1 (ECAD, E-cadherin) has been suggested as the target gene of 16q22.1. The encoded protein is a cell–cell adhesion molecule and it has been proposed that the gene could be a metastasis suppressor gene (Umbas *et al*., 1992). Loss of *CDH1* expression is more frequent in advanced prostate cancer than in early stage disease and may contribute to tumour progression, rather than initiation (Umbas *et al*., 1992). Decreased CDH1 protein expression could also be used as a prognostic marker for prostate cancer progression (Umbas *et al*., 1994). Somatic mutations in the coding region of the remaining allele have not been detected but aberrant methylation at the promoter region has been shown in advanced prostate cancer occasionally (Suzuki *et al*., 1996; Graff *et al*., 1995; Ma-

ruyama *et al*., 2002; Li *et al*., 2001). A polymorphism in the promoter region of *CDH1* has been shown to be associated with increased risk of prostate cancer (Jonsson *et al*., 2004).

Gains and amplifications

Gains or low-level amplifications are found in some of the early prostate cancers and in the majority of advanced prostate cancers. The most common gains, 7p/q and 8q, which are found in 20% and 36% untreated prostate cancers, respectively, have been found to be effective in predicting eventual progression in prostatectomy-treated patients (Alers *et al*., 2000; Skacel *et al*., 2001).

High-level amplifications are found mainly in hormone-refractory prostate cancer (El Gedaily *et al*., 2001; Laitinen *et al*., 2002; Alers *et al*., 2000). The most commonly observed amplifications are from the distal 8q (8q23–qter) and proximal Xq (Xq11–13). They are found at frequencies of 73% and 35%, respectively, by CGH (Nupponen *et al*., 1998b). FISH studies have shown that at least *MYC* (at 8q24.21) and *AR* (androgen receptor, at Xq12) are truly amplified in these regions and, taking into account their functions, they are so far the best candidates for the target genes of these amplicons (Jenkins *et al*., 1997; Visakorpi *et al*., 1995a; Nupponen *et al*., 1999).

8q: MYC and EIF3S3

Gain of chromosome arm 8q is the most common copy number increase in prostate cancer and has been associated with poor outcome (Visakorpi *et al.*, 1995b; Alers *et al.*, 2000; van Dekken *et al.*, 2003). Due to its high frequency, it is perhaps the most studied gain in prostate cancer. In many cases, the whole arm is affected, but sometimes smaller gains are observed. This suggests that there are more than one target genes in 8q. Suggested target genes include *TPD52* (tumour protein D52, at 8q21.13), *TCEB1* (transcription elongation factor B [SIII], polypeptide 1 [15kDa, elongin C], at 8q21.11), *EIF3S3* (eukaryotic translation initiation factor 3, subunit 3, at 8q23.3–24.11), *RAD21* (RAD21 homologue [S. pombe], at 8q24.11), *PSCA* (prostate stem cell antigen, at 8q24.3), and *KIAA0196* (Wang *et al*., 2004; Rubin *et al*., 2004; Porkka *et al*., 2002, 2004; Nupponen *et al*., 1999; Savinainen *et al*., 2004; Reiter *et al*., 1998).

Two minimal regions of gain in 8q have been identified by CGH in hormone-refractory prostate cancer: 8q21 and 8q23–24 (Visakorpi *et al*., 1995b; Cher *et al*., 1996; Nupponen *et al*., 1998b). van Duin *et al*. (2005b) identified five separate minimal regions of frequent copy number increase from 34 prostate cancer samples, including cell lines, xenografts, and clinical samples, with a chromosome 8q-specific array of 702 BACs. Based on previous CGH analysis, most of the samples were known to harbour 8q gains. The minimal regions ranged from 81 to 129Mb in size and were situated in bands 8q21.13, 8q22.1, 8q22.2–22.3,

8q24.13, and 8q24.21. For three of these regions, also putative target genes were identified: *PDP* (aka *PPM2C*, protein phosphatase 2C, magnesium-dependent, catalytic subunit) at 8q22.1, *PABPC1* (poly(A) binding protein, cytoplasmic 1) at 8q22.3, and *KIAA0196* at 8q24.13 (van Duin *et al*., 2005b). The expression of *KIAA0196* has been shown to be significantly elevated in hormone-refractory prostate cancer compared to benign prostatic hyperplasia (BPH) and associated with gene amplification (Porkka *et al*., 2004).

The most obvious candidate target gene for 8q23–24 gain/amplification is the oncogene *MYC* at 8q24.21**.** MYC is a transcription factor with thousands of known and suspected target genes, including most RNA genes (Fernandez *et al*., 2003; reviewed by Patel *et al*., 2004). MYC participates in activating translocations in B cell lymphomas and overexpression of MYC has been shown to induce genomic instability, including amplification of some target genes and the gene for MYC itself (reviewed by Mai and Mushinski, 2003). In many cases, the amplifications have been shown to be transient, as the copy number of the genes has returned to normal if MYC expression has ceased. Reversibility of MYC-induced tumours has been shown in a mouse breast cancer model that conditionally expresses human MYC. As long as *Kras2* was not mutated, the *MYC*-induced tumours regressed completely after removal of *MYC* expression (D'Cruz *et al*., 2001). Recently, overexpression of MYC was shown to be sufficient to immortalise normal human prostate epithelial cells by up-regulating hTERT (telomerase reverse transcriptase) and overriding the cell cycle inhibitory INK4a (aka CDKN2A, cyclin-dependent kinase inhibitor 2A) (Gil *et al*., 2005). The immortalised cells retained a normal-like molecular phenotype apart from a deficient Rb/INK4a checkpoint. Another recent report, however, showed that forced overexpression of MYC leads to a cancer phenotype in human prostate cells grown in mice (Williams *et al*., 2005). A major difference that could explain the discrepancy between the two studies may be the source of the primary prostate cells. Gil *et al*. (2005) used prostate epithelium from a bladder cancer patient, whilst Williams *et al*. (2005) harvested histologically normal cells from prostatectomy-treated prostate cancer patients. Expression of human MYC in transgenic mice has been shown to lead to murine PIN and adenocarcinoma in a dose-dependent manner (Ellwood-Yen *et al*., 2003). As well as apparently being involved in prostate tumour initiation, MYC may play a role in hormone-independence of prostate cancer. Ectopic expression of MYC has been shown to lead to androgen-independent growth of the androgen-dependent prostate cancer cell line LNCaP (Bernard *et al*., 2003).

EIF3S3 was identified as overexpressed by suppression subtractive hybridisation in the breast cancer cell line, Sk-Br-3, which contains an 8q amplification (Nupponen *et al*., 1999). The study subsequently showed that the gene was amplified and overexpressed in about 30% of hormone-refractory prostate cancers, thus making it a candidate target gene for 8q amplification in prostate cancer. *EIF3S3* has also been shown to be amplified and overexpressed in 26% of hepatocellular carcinomas (Okamoto *et al*., 2003).

Inhibition of *EIF3S3* expression in HeLa cells by siRNA has been shown to lead to cell death upon entry into mitosis (Kittler *et al.*, 2004). In contrast, *EIF3S3* overexpression in 3T3 cells has been shown to increase the proliferation rate and enhance the survival of the cells compared to control cells, although the cells were unable to form colonies in soft agar (Savinainen *et al.*, 2006). Inhibition of *EIF3S3* expression by siRNA in cancer cell lines has been shown to reduce their growth rate. Since overexpression of *EIF3S3* does not transform cells, it is more likely to be involved in progression rather than initiation of prostate cancer.

The protein encoded by *EIF3S3* is the 40kDa subunit of the eukaryotic translation initiation factor 3 (eIF3) which binds to the 40S ribosomal subunit and keeps it from associating inappropriately with the 60S ribosomal subunit. The location and function of EIF3S3 in the complex are not known (Asano *et al.*, 1997). EIF3S3 is not the only eIF3 subunit that has been implicated in cancer: *EIF3S8* and EIF3S10 have been shown to be overexpressed in cancer, although EIF3S10 overexpression is limited to early stages of disease and associated with good prognosis (Rothe *et al.*, 2000; Chen and Buerger, 1999, 2004; Dallas *et al.*, 1998). Other eukaryotic translation initiation factors have also showed overexpression in cancer. eIF4E and eIF4G, which are components of eIF4F, have been shown to possess oncogenic properties when overexpressed in 3T3 cells or rat embryo fibroblasts (Lazaris-Karatzas *et al.*, 1990; Lazaris-Karatzas and Sonenberg, 1992; Fukuchi-Shigomori *et al.*, 1997).

7q: MCM7

Although gain of chromosome 7 is one of the earliest and most frequent genomic alterations in prostate cancer, only a few candidate target genes have been proposed.

MCM7 (minichromosome maintenance 7, at 7q21.3), was recently shown to be amplified (≥2 times as many copies as centromeres) in 45–50% untreated primary prostate cancers by FISH and Q-RT-PCR (Ren *et al.*, 2005). The cancers that were considered aggressive had a significantly higher copy number of MCM7 than non-aggressive tumours. Overexpression of MCM7 was also shown in most of the tumours with amplification. However, overexpression was observed also in some tumours without amplification, implying that amplification is not the only mechanism for MCM7 overexpression. Increased protein levels were associated with higher tumour stage and Gleason score. Both amplification and overexpression were associated with disease recurrence within five years of prostatectomy. DU145 cells transfected with an *MCM7* expression vector had 50% faster proliferation and invaded Matrigel twice as well as controls. Xenografts created from the transfected cells were 12 times larger than control xenografts and 50% of the hosts died within 6 weeks of grafting. All these results indicate a role for *MCM7* in prostate cancer. MCM7 has been suggested a more accurate marker for proliferation than Ki67, as immunostaining by MCM7 anti-

bodies can be seen not only in proliferating cells, but also in cells that are about to proliferate (Padmanabhan *et al*., 2005).

MCM7 is a component of the minichromosome maintenance (MCM) complex which binds DNA replication origins and prepares them for initiation of replication, (replication licensing) (reviewed by Lei and Tye, 2001; and Lei, 2005). MCM proteins are not expressed in fully differentiated cells, which do not proliferate. Cancer cells and pre-malignant cells in the process of transformation, on the other hand, express MCM proteins at high levels, resulting in chromosomal defects. Given the function of MCM7 in DNA replication licensing, its dysregulation is easy to accept as cancer-causing and -promoting.

Xq: AR

The amplification of the *AR* gene at Xq12 is observed in 20–50% of hormone-refractory prostate cancers and the gene is also overexpressed (Visakorpi *et al*., 1995a; Bubendorf *et al*., 1999; Linja *et al*., 2001; Brown *et al*., 2002). AR is a nuclear steroid receptor and is expressed in normal and malignant prostate. It mediates the effects of androgens which are essential for normal development of the prostate and the differentiation of secretory epithelial cells. The removal of androgens from circulation is an effective treatment for prostate cancer (Huggins and Hodges, 1941). Although about 80% of prostate cancers initially regress after androgen withdrawal or antiandrogen treatment, patients eventually relapse and die. The cancers with *AR* amplification have been shown to respond better to second line maximal androgen blockade than tumours without the amplification, although only for a short period of time (Palmberg *et al*., 2000).

The amplification explains the overexpression of AR a subset (*ca* 30%) of hormone-refractory prostate cancers but the reason for the overexpression of the gene in the rest of the cases remains unknown. Mutations in the promoter and untranslated regions (UTR) of the transcript do not seem to play a part in *AR* overexpression, as Waltering *et al*. (2006) found only 10 non-recurrent mutations in five prostate cancer cell lines and 19 xenografts, and none in 30 untreated and 14 hormone-refractory prostate cancer samples. The mutations were not located in known functional regions of the promoter or 5’ UTR.

The effect of *AR* amplification on gene expression has been demonstrated in prostate cancer xenografts, where one additional copy of *AR* has been shown to increase the mRNA expression of *AR* (Linja *et al*., 2001). Recently Chen *et al*. (2004) demonstrated that increased expression of AR is necessary and sufficient to convert androgen-sensitive growth of prostate cancer xenografts to hormone-refractory growth. However, the xenografts had been derived from patients with hormone-refractory prostate cancer. More importantly, they showed that the hormone-refractory growth is ligand-dependent and requires the nuclear action of AR. These findings may pave the way for new antiandrogen therapies, including blocking the nuclear localisation of the activated receptor.

In addition to amplifications in hormone-refractory prostate cancer, polymorphisms and mutations of the *AR* gene have been found, and some of them have been linked to increased prostate cancer risk or failure of antiandrogen treatment (reviewed by Linja and Visakorpi, 2004). The mutations leading to treatment failure are most often located in the ligand-binding domain and alter the ligand-specificity of the protein. The mutant receptors may be stimulated by estradiol, progesterone, adrenal corticosteroids, glucocorticoids, or the antiandrogens flutamide or bicalutamide (Veldscholte *et al*., 1990; Hara *et al*., 2003; Taplin *et al*., 1995, 1999; Zhao *et al*., 2000). Although earlier studies have found mutations of *AR* in 25–50% of prostate cancers, including untreated samples, later studies have shown that they are rare in untreated prostate cancers as well as castration-treated recurrent prostate cancers (Gaddipati *et al*., 1994; Tilley *et al*., 1996; Marcelli *et al*., 2000; Wallén *et al*., 1999; reviewed by Linja and Visakorpi, 2004).

14q: HIF1A

The cell lines PC-3 and DU145 contain a gain of chromosome 14q (Nupponen *et al*., 1998a). Gains in 14q have been found in about 10% of hormone-refractory prostate cancers (Nupponen *et al*., 1998b).

HIF1A (hypoxia-inducible factor 1, alpha subunit) is located in 14q21–24, and the protein encoded by it has been shown to be overexpressed in the majority of prostate tumours, including HGPIN (Zhong *et al*., 1999). HIF1A is the regulatory subunit of the HIF-1 transcription factor, which activates the transcription of genes involved in adaptation to oxidative stress (reviewed by Semenza, 1999). Although the mRNA is constitutively transcribed, the protein is normally expressed only under hypoxic conditions. In PC-3 cells, however, HIF1A is expressed also under normoxic conditions (Zhong *et al*., 1998). Although *HIF1A* missense mutations that may affect the stability of the protein have been found in hormone-refractory prostate cancer, the gene is not mutated in PC-3 (Anastasiadis *et al*., 2002; Fu *et al*., 2004).

Translocations

To date, one recurrent translocation in prostate cancer has been described. Tomlins *et al*. (2005) applied a bioinformatics method, cancer outlier profile analysis (COPA), to ONCOMINE, a cancer microarray database and data-mining platform, to initially identify genes that were overexpressed in a subset of prostate cancer cases instead of the majority of cases. Two related transcription factors, *ERG* (v-ets erythroblastosis virus E26 oncogene like (avian), at 21q22.3) and *ETV1* (ets variant gene 1, at 7p21.2), were found to be substantially overexpressed in a mutually exclusive way in a subset of cases, and ranked in the top 10 outlier genes in 6 out of 10 independent prostate cancer gene expression profil-

ing studies. No consistent amplification of the genes was found in cell lines and clinical samples overexpressing the genes, so further studies were conducted to see whether the genes were translocated. Both *ERG* and *ETV1* are known to participate in oncogenic translocations in Ewing's sarcoma and myeloid leukaemias (reviewed by Oikawa and Yamada, 2003). By exon-walking quantitative PCR, it was determined that the expression of the first exons of both genes was diminished compared to the overexpressed latter exons, and RLM-RACE (RNA ligase-mediated rapid amplification of cDNA ends) revealed that the later exons of the genes were fused to the untranslated beginning of the *TMPRSS2* gene (transmembrane protease, serine 2, at 21q22.2). *TMPRSS2* is androgen-induced and expressed in normal and neoplastic prostate (Lin *et al.*, 1999) and the translocation renders the transcription factors androgen-inducible. Therefore the overexpression of translocated *ERG* and *ETV1* should be limited to androgen receptor positive prostate cancers.

Further confirmation of the translocations was obtained by FISH analysis. *ETV1* was confirmed to be translocated to *TMPRSS2* in 7/29 cases. The *ERG* gene was shown to split in 16/29 cases. Due to the proximity (*ca*. 3Mb) of *ERG* and *TMPRSS2*, the authors did not prove that the FISH signal of *ERG* was translocated specifically to *TMPRSS2*. The translocation of *ERG* or *ETV1* to *TMPRSS2* was nevertheless reported in 79% (23/29) of prostate cancers. The translocations remain to be confirmed.

Microarrays

The amount of data that has been produced with different microarray technologies in the past five to ten years is overwhelming. Genome-wide expression profiling is commonly used to distinguish tumour categories from each other, and the effect of pharmacological treatments on gene expression can be studied in *in vitro* models of disease. Data on copy number alterations in cancer and in phenotypically normal individuals has accumulated and single nucleotide polymorphisms (SNPs) have been screened for in a high-throughput manner, widening the knowledge on genetic variation.

General properties of microarrays

The target, or probe, sequences on DNA microarrays can be of several types. The first DNA microarrays consisted of cDNA sequences spotted onto a glass slide and the soluble samples were hybridised to the immobilised probes (Schena *et al*., 1995, 1996). Later, it was noted that a more uniform set of targets was preferable in terms of hybridisation reliability. This led to the development of oligonucleotide microarrays, where specifically designed oligonucleotides of a

certain size, usually 40–70bp, are synthesised *in situ* directly onto the microarray slide (Lockhart *et al*., 1996). SNP arrays are oligonucleotide microarrays where four oligos, with a single difference in sequence, are designed to cover known SNPs (Wang *et al.*, 1998).

When methods for hybridising genomic DNA to microarray slides were first described, large targets such as bacterial artificial chromosomes (BACs), P1-vector derived artificial chromosomes (PACs) or cosmid clones were spotted onto the slides (Solinas-Toldo *et al.*, 1997; Pinkel *et al.*, 1998). cDNA microarrays can also be used for copy number analyses (Pollack *et al.*, 1999). The complexity of large targets is a problem also in aCGH. Ligation-mediated PCR has been used to produce representations of the probes and reduce their complexity, and an even further reduction in target complexity has been achieved by designing oligonucleotide arrays specifically for aCGH analysis (Lucito *et al.*, 2000, 2003; Snijders *et al*., 2001; Barrett *et al*., 2004).

The advantages of microarray technologies in profiling of gene expression, gene copy number and SNPs are clear. They are high-throughput methods and allow the characterisation of the whole transcriptome/genome at once, providing the density of targets is sufficient. The implementation of public repositories for microarray data makes it possible for the unreported information to be data mined and reassessed by other investigators.

At present, the major limitation for the widespread use of microarrays is the cost of microarray slides, especially commercial ones, which limits the number of samples used. Custom-made slides are not easily produced and their reliability may be questionable, as it has been reported that 20–40% of cDNA clones in libraries that are used to create the microarrays do not represent the sequence implied by their annotation (Taylor *et al*., 2001; Halgren *et al*., 2001). Data-analysis may also prove cumbersome for investigators not familiar with bioinformatics methods.

Expression profiling of prostate cancer

Both spotted cDNA microarrays and oligonucleotide microarrays have been used to profile gene expression in prostate cancer. Microarray studies have identified new putative markers of prostate cancer (diagnostic markers) and of prostate cancer progression (prognostic markers). Depending on the content of the microarrays used, the samples, and the data-analyses, the most over- and underexpressed genes may vary between studies. Regardless of this, some genes have come up consistently in independent studies using different platforms.

Hepsin (HPN)

Several studies have identified *HPN* (aka *TMPRSS1*) as an overexpressed gene in prostate cancer compared to BPH (Dhanasekaran *et al.*, 2001; Luo *et al.*, 2001;

Magee *et al.*, 2001; Welsh *et al.*, 2001). The gene encodes a membrane bound serine protease, hepsin, which activates the hepatocyte growth factor/c-Met (HGF/c-Met) pathway by cleaving HGF (Kirchhofer *et al.*, 2005). This pathway leads to enhanced cell migration of prostate cancer cells via the production of matrix metalloproteinases (MMPs) and urokinase type plasminogen activator (uPA) (Fujiuchi *et al.*, 2003). Hepsin overexpression in non-metastatic prostate cancer of the mouse causes basement membrane disorganisation in the epithelium, which promotes invasion and metastasis (Klezovitch *et al.* 2004). However, *HPN* expression is lower in metastatic prostate cancer, and forced overexpression of the gene in metastatic prostate cancer cell lines actually weakens the proliferation and colony-forming potential of the cells (Srikantan *et al.*, 2002). This indicates that hepsin's role in disease progression is over once dissemination has happened (Vasioukhin, 2004). Fromont *et al.*, (2005) have also shown decreased expression of *HPN* in hormone-refractory prostate cancer compared to prostate cancers treated by prostatectomy, but the finding was not addressed further.

α-methylacyl-CoA racemase (AMACR)

Another gene implicated by more than one microarray study is the α-methylacyl-CoA racemase (*AMACR*) (Xu *et al.*, 2000; Dhanasekaran *et al.*, 2001; Welsh *et al.*, 2001; Luo *et al.*, 2001; Luo *et al.*, 2002). Compared to benign samples, it is significantly up-regulated in neoplastic prostate samples, including HGPIN, indicating that it has a role in the early stages of prostate cancer development (Luo *et al.*, 2002; Xu *et al.*, 2000; Ashida *et al.*, 2004). Subsequent studies with tissue microarrays (TMAs) and antibodies against AMACR have shown that the protein is expressed almost exclusively in malignant and pre-malignant cells (Rubin *et al.*, 2002; Luo *et al.*, 2002; Jiang *et al.*, 2001). Normal prostate cells located near cancerous cells have shown higher expression of AMACR than normal cells further away from malignant cells, suggesting a cancer field effect (Ananthanarayanan *et al.*, 2005). The expression in these cells was, however, clearly weaker than in HGPIN and prostate cancer.

The sensitivity and specificity of AMACR as a prostate cancer marker have ranged between 79–100% and 82–100%, respectively (Jiang *et al.*, 2001, 2002; Beach *et al.*, 2002; Rubin *et al.*, 2002). Since there would be false positive and false negative diagnoses when using AMACR on its own, supplementation by staining with a basal cell marker is advised (reviewed by Evans, 2003). AMACR expression does not seem to be affected by radiation treatment, and detection of residual cancer post-radiation is as sensitive and specific as that of untreated cancer (Yang *et al.*, 2003).

AMACR staining is weaker in hormone-refractory prostate cancer than in untreated prostate cancer which led to the assumption that *AMACR* might be regulated by androgens (Luo *et al.*, 2002; Rubin *et al.*, 2002). This is not the case, however, as antiandrogen treatment of LNCaP cells had no effect on the expres-

sion levels of the protein (Kuefer *et al*., 2002). This result was confirmed in studies using other cell lines, where neither androgen withdrawal (LAPC-4), nor androgen stimulation (22Rv1 and LNCaP) had any effect on AMACR expression (Zha *et al*., 2003). Another important finding by Zha *et al*., (2003), was that siRNA-mediated knockdown of AMACR expression in LAPC-4 cells had a direct negative effect on cell proliferation, arresting the cells in G_2-M. This growth inhibition was additive to the inhibitory effect of androgen ablation. The implications are that LAPC-4 cells are to some extent dependent on AMACR and that the growth inhibition by AMACR knockdown is androgen-independent.

Enhancer of zeste homologue (Drosophila) 2 (EZH2)

Overexpression of enhancer of zeste homologue (*Drosophila*) 2 (*EZH2*) in localised prostate cancer and prostate cancer metastasis compared to BPH and normal prostate was discovered by microarray analysis and expression profiling of 74 samples (Varambally *et al*., 2002). The result was confirmed by Q-RT-PCR with 22 samples and IHC with 197 patients. Protein overexpression was associated with recurrence in prostatectomy-treated patients and it was also a better predictor of outcome than Gleason score, surgical margin status, tumour dimension, or pre-operative PSA. Positive EZH2 immunostaining (staining intensity 3–4) combined with a decreased (<4) ECAD (E-cadherin) staining intensity ("EZH2:ECAD status positive") has been shown to be statistically significantly associated with disease recurrence (Rhodes *et al*., 2003).

EZH2 is essential for proliferation, as inhibition of *EZH2* by siRNA has been shown to result in a marked decrease in proliferation of the human papillomavirus 18-immortalised prostate cell line, RWPE, and PC-3 prostate cancer cells, with cell-cycle arrest in G_2 (Varambally *et al*., 2002). An association between EZH2 overexpression and increased proliferation rate in prostate cancer has been shown by Bachmann *et al*. (2006). Forced overexpression of *EZH2* has been shown to change the expression levels of a defined set of genes, indicating its role in regulating gene expression (Varambally *et al*., 2002).

EZH2 is a polycomb group protein and the histone methyltransferase component of polycomb repressive complexes 2, 3, and 4 (PRC2/3/4) (Kuzmichev *et al*., 2004, 2005). These complexes play a crucial role in the maintenance of transcriptional repression of *Hox* genes, in X-chromosome inactivation, and in stem cell pluripotency (reviewed by Cao and Zhang, 2004). PRC2/3/4 methylate lysine 27, and possibly lysine 9, on histone H3, and lysine 26 on histone H1d (Kuzmichev *et al*., 2004, 2005). PRC2/3/4 is predominantly located in the nucleus, as implicated by the substrate-specificities. Recently EZH2 has been detected in complex also in the cytosol of T cells, primary mouse embryonic fibroblasts (MEFs), and human embryonic kidney (HEK-293) cells, and the complex has been shown to have methyltransferase activity *in vitro* (Su *et al*., 2005). The *in vivo* substrates, if any, of this cytosolic PRC remain unidentified. However, cytosolic Ezh2 has been shown to be necessary for TCR (T cell receptor) and

PDGF (platelet derived growth factor) -mediated actin polymerization in murine T cells and fibroblasts, respectively, as well as T cell activation and differentiation (Su *et al.*, 2005).

In addition to being overexpressed in (prostate) cancer, the substrate specificity of EZH2 may be altered through PTEN inactivation. PTEN inactivates AKT, which otherwise appears to phosphorylate EZH2, thus decreasing methylation of the primary substrate of EZH2, H3K27 (Cha *et al.*, 2005). Again, the preferred substrate, if any, of the phosphorylated EZH2, remains unknown. Since phosphorylation of EZH2 does not alter the critical composition of the PRC complex, it may well have targets relevant to tumorigenesis or metastasis.

EZH2 has also been shown to be overexpressed in other cancers and one possible explanation for the overexpression may be gene amplification. Amplification of *EZH2* has been shown in multiple primary cancers, including breast, lung, bladder, and colon cancer (Bracken *et al.*, 2003). The copy number of *EZH2* was elevated in approximately 15% of the cancer cases studied and the frequency varied from 0–45% between cancers of different origin.

Profiling by clustering and meta-analysis

Many studies have shown that by applying hierarchical clustering to microarray data, prostate cancer can be distinguished from normal samples and organ-confined cancer from metastatic disease (Dhanasekaran *et al.*, 2001; Luo *et al.*, 2001; Lapointe *et al.*, 2004; Welsh *et al.*, 2001; Vanaja *et al.*, 2003). Furthermore, primary prostate cancer samples can be clustered into subtypes with distinct clinical properties even when the pathological determinants, such as Gleason score and tumour stage, are similar (Luo *et al.*, 2002; Lapointe *et al.*, 2004). HGPIN lesions and adenocarcinoma share a number genes that are up- or down-regulated compared to benign tissue, and the transition from the pre-neoplastic lesion HGPIN to prostate cancer leads to changes in the expression of specific genes (Ashida *et al.*, 2004). This supports the hypothesis that HGPIN is a precursor of carcinoma. Glinsky *et al.* (2004) identified several molecular signatures consisting of small clusters of genes (four to five genes), which were 88% accurate in predicting which patients would progress within one year of radical prostatectomy. The differences between gene expression patterns in healthy donor prostates, prostate cancer, and normal prostate adjacent to prostate cancer implied that histologically normal prostate tissue from cancer patients shares many molecular characteristics of prostate cancer (Yu *et al.*, 2004). The same study identified a set of 70 genes that could be used to predict the aggressiveness of cancer with high accuracy (93% sensitivity and 87% specificity). For comparison, the sensitivity and specificity of Gleason score were 37% and 88%, respectively.

Since only few genes are selected for further studies from microarray studies, a lot of potentially meaningful data are unreported. These data can be salvaged from public microarray repositories and re-examined by independent investiga-

tors. Several groups are now doing meta-analysis to compare microarray studies and assess the validity and relevance of the original findings. Meta-analysis can also be used to identify genes that have not been found important in individual analyses because of small sample size and/or data filtering. Larger sample sizes decrease the error rate of results and increase the statistical power of microarray studies (Mukherjee *et al.*, 2003). Meta-analysis can sometimes find completely new sets of significant genes. For example, a pair of genes, *HPN* and *STAT6* (signal transducer and activator of transcription 6), was identified by meta-analysis of five microarray studies as a robust marker pair for distinguishing primary prostate cancer from benign samples (Xu *et al.*, 2005). A similar analysis on the individual datasets produced different marker pairs whose performance in separating cancers from benign samples was tested and found to be inferior to the *HPN/STAT6* pair.

Copy number analyses

Methods of genomic profiling of solid tumours have been limited due to difficulties in obtaining good-quality metaphases for karyotypic analysis. The only widely used method has been CGH, which unfortunately has a limited resolution of about 5–10Mb (Kallioniemi *et al*., 1992; du Manoir *et al*., 1995). The general idea of genomic profiling by microarrays was first published by Solinas-Toldo *et al*., (1997). Since then, the variety of platforms has been widened to include BAC/PAC/cosmid arrays, cDNA microarrays, oligo arrays, and SNP arrays (Solinas-Toldo *et al.*, 1997; Pinkel *et al.*, 1998; Pollack *et al.*, 1999; Snijders *et al*., 2001; Lucito *et al.*, 2003; Bignell *et al*., 2004; Barrett *et al*., 2004). SNP arrays have also been used specifically for detection of LOH (Lindblad-Toh *et al*., 2000; Dumur *et al*., 2003). The resolution of aCGH depends on the genomic distribution, size, and number of the features on the array. Sub-megabase resolution has been reached with a tiling resolution DNA microarray constructed of over 30,000 overlapping BAC clones (Ishkanian *et al.*, 2004), and even higher resolution may be possible within a few years with oligo arrays.

Clinical samples of untreated prostate cancer have been studied by genomic microarrays consisting of BAC clones (Paris *et al*., 2003, 2004; van Dekken *et al.*, 2004). The most commonly found copy number alterations, such as losses of 6q, 8p, 13q, 16q, and 18q, and gains of chr 7 and 8q are supported by numerous CGH studies of prostate cancer samples (*e.g.* Visakorpi *et al.*, 1995b; Alers *et al.*, 2000; El Gedaily *et al*., 2001; Nupponen *et al*., 1998b). Novel recurrent aberrations have also been identified, for example gains of 2p25 and 11p15.4 (Paris *et al*., 2004).

Analogously to gene expression profiling, a comparison of aCGH profiles from 32 primary prostate tumours that never progressed and 32 tumours that did, showed several chromosomal regions whose copy number alterations could be used to distinguish the two groups (Paris *et al*., 2004). Approximately 40 loci that were seldom (<20%) altered in the non-progressors but often (20–45%) in

the tumours that did progress, were found. 20–90% of metastatic samples had gain in these same loci. For example, a gain in distal 13q was seen far more often in the non-progressors than in the tumours that eventually recurred. Gain at 11q13.1 predicted progression independent of other clinical parameters and a small deletion in 8p23.2 was associated with advanced stage.

The heterogeneity of prostate cancer has been addressed by comparing aCGH profiles of paired Gleason grade 3 and 4 samples from ten patients with organ confined prostate cancer of Gleason score 7 (van Dekken *et al*., 2004). The samples were not from separate foci, but from large lesions containing areas of differing Gleason grades. Losses were more often shared by the paired samples than gains (46% vs. 13%), indicating that losses occur earlier in prostate cancer development than gains. However, the majority of differences between the Gleason grades were single BAC copy number alterations, which may well be due to poor quality hybridisation and/or mismapped clones.

The superior resolution of aCGH compared to CGH has enabled the identification of smaller regions of copy number alterations, as has been demonstrated by comparing the aCGH profiles of 20 prostate tumours to CGH profiles of the same tumours (Paris *et al*., 2003). On the whole, the profiles were 90% concordant, although the aCGH found more small aberrations than CGH. High-resolution aCGH analyses of chromosome arms 8q, 10q, and 16q have confirmed and refined frequent CNAs as discussed earlier (van Duin *et al*., 2005b; Hermans *et al*., 2004; Watson *et al*., 2004).

The prostate cancer cell lines PC-3, LNCaP, DU145, and 22Rv1 have been profiled by several groups with aCGH on a cDNA platform (Clark *et al*., 2003; Wolf *et al*., 2004; Zhao *et al*., 2005). The profiles have revealed some genomic alterations that had not been identified by CGH. For example, Clark *et al*. (2003) found a homozygous deletion of about 500kb in 17q21.31 of PC-3, and Wolf *et al*. (2004) were able to define the breakpoints of two distinct regions of amplification, 1q24.2-25.1 and 1q32.1 of PC-3, that flanked a deletion. When aCGH results were compared to CGH data, the concordance was found to be 92% for gains and 82% for losses (Wolf *et al*., 2004). The chromosome arm 8q of PC-3 has been profiled with an 8q-specific BAC array, and the high-resolution of the array allowed the identification of a small, 0.6Mb, single copy loss at 8q22.2 (three copies of locus, four of centromere) (van Duin *et al*., 2005a). This loss was also visible in the aCGH profile by Zhao *et al*., (2005), but not in the profiles by Wolf *et al*., (2004) and Clark *et al*., (2003). The 8q-array has also been used to profile 8q of DU145, which, as expected, had a simple gain, and LNCaP, which had no copy number alterations relative to the rest of its near-tetraploid genome (van Duin *et al*., 2005b).

Loss of heterozygosity in clinical prostate cancers has been studied with a SNP array and a selected set of P1, BAC, and PAC clones (Dumur *et* al., 2003; Yano *et al*., 2004). The concordance with CGH or LOH analysis by microsatellite markers was high, indicating that microarrays are suitable also for detection of LOH.

AIMS OF THE STUDY

The general aim of this thesis was to assess the frequency and significance of genomic copy number alterations in prostate cancer. Specifically, the aims of this thesis were:

1. to determine whether *HIF1A* and *EZH2* genes are amplified in prostate cancer;
2. to evaluate the amplification frequencies of *EIF3S3* and *MYC* genes in prostate cancer;
3. to assess the prognostic significance of *EIF3S3* and *EZH2* gene amplifications;
4. to screen prostate cancer cell lines and xenografts for amplified and overexpressed, as well as deleted and underexpressed, genes; and
5. to identify (novel) regions of recurrent genomic copy number aberrations in prostate cancer.

MATERIALS AND METHODS

Cell lines and xenografts [I, III, IV]

The prostate cancer cell lines LNCaP, DU145, PC-3, 22Rv1, and NCI-H660 were obtained from American Type Culture Collection (ATCC, Rockville, MD, USA) (studies I, III, and IV). Dr. Charles Sawyers (Jonsson Cancer Center, UCLA, Los Angeles, USA) kindly provided the prostate cancer cell line LAPC-4 for study IV. Tsu-Pr and JCA-1, derivatives of the bladder cancer cell line T24 and previously thought to be distinct prostate cancer cell lines (van Bokhoven *et al*, 2001), were kindly provided by Dr. U. Bergerheim (Karolinska Institut, Stockholm, Sweden) (study I). The cell lines were grown in recommended media. Interphase and metaphase spreads were prepared following standard laboratory protocols. Genomic DNA was extracted and purified according to routine laboratory protocols and total RNA was extracted with the TRIZol®-reagent (Invitrogen Life Technologies, Carlsbad, CA, USA), following the manufacturer's protocols.

Material from 17 prostate cancer xenografts (LuCaPs 23.1, 23.8, 23.12, 35, 35V, 41, 49, 58, 69, 70, 73, 77, 86.2, 93, 96, 105, and 115), was made available by Dr. Robert Vessella (Department of Urology, University of Washington, Seattle, WA, USA) and used in studies III and IV. Genomic DNA and total RNA of the xenografts were extracted from snap frozen pieces of tissue. DNA extraction was done with the DNAzol reagent following the manufacturer's protocol (Molecular Research Center, Inc. Cincinnati, OH, USA) and RNA was isolated with the Tri-Pure reagent and protocol (Roche Diagnostics, Mannheim, Germany). 5 µm sections were cut from snap frozen tissue blocks for FISH analysis.

Clinical tumour specimens [I, II, III]

The clinical samples used in studies I, II, and III are summarised in Table 1. The material was used with consent of the patients and with the approvals of the ethical committee of the Tampere University Hospital and the National Authority for Medicolegal Affairs (TEO).

Paraffin-embedded samples of 21 (7+14) benign prostatic hyperplasia (BPH), 221 primary untreated prostate carcinomas, and 146 locally recurrent hormone-refractory prostate cancers were obtained from the Tampere University Hospital. Tissue microarrays were constructed of the cancer samples according to published guidelines (Kononen *et al*., 1998). The BPH samples were used in studies I and III, and subsets of the cancer samples in studies I, II, and III.

Fifty-four paraffin-embedded untreated pelvic lymph node metastases from patients operated at Lund University Hospital were provided by Dr. Ola Bratt (Department of Surgery, Helsingborg Hospital, Helsingborg, Sweden). Tissue microarrays of the samples were used in studies I and II.

Two tissue microarrays containing a total of 559 prostate samples were provided by Dr. Lukas Bubendorf (Institute for Pathology, University of Basel, Basel, Switzerland). The first array was used together with the samples from the Lund University Hospital and some of the samples from the Tampere University Hospital to study the frequency of *EIF3S3* and *MYC* copy number alterations in different stages of prostatic disease (study II). It contained 21 BPH, 42 PIN, 85 radical prostatectomy specimens, 20 Tru-cut needle biopsies of T3/T4 prostate tumours, 95 locally recurrent hormone-refractory tumours, and 39 hormone-refractory distant metastases. The second array, which was used for the assessment of the prognostic utility of the copy number status of *EIF3S3*, contained 145 radical prostatectomy samples and 112 incidentally found stage pT1a/b prostate cancers.

Table 1. *Summary of clinical samples used in the studies*

	HIF1A	**EIF3S3**			**EZH2**		
Type of sample		Group I	Group II	Group III	IHC [6]	FISH [6]	Q-RT-PCR
BPH [1]	7	21			14		9
HGPIN [2]		42					
Prostatectomy/TURP [3]**:**							
pT1/pT2	35	183	112	145 [7]	103	76	27
pT3/pT4	17	20			61	45	
Lymph node metastases	37	54					
Locally recurrent HR [4]	28	95			49	46	12
HR [4] **metastases**		39					
Prostatectomy/TURP [3]**:**							
Gleason score [5] **<7**		115	80	100	57	42	
=7					81	60	
>7		78	21	33	24	19	
not known			4	2			

[1] benign prostatic hyperplasia; [2] high-grade prostatic intraepithelial neoplasia; [3] transurethral resection of the prostate; [4] hormone-refractory; [5] Gleason scores of successfully hybridised samples; [6] number of successfully hybridised/stained samples; [7] radical prostatectomies, pT-stage unknown for 38.

Freshly frozen samples of 9 benign prostatic hyperplasia, 27 untreated primary prostate cancers, and 12 locally recurrent hormone-refractory prostate cancers were obtained from the Tampere University Hospital. Total RNA was extracted from the samples as described and used in study III (Linja *et al*., 2001).

Fluorescence *in situ* hybridisation (FISH) [I, II, III, IV]

Locus-specific BAC and PAC probes were labelled with digoxigenin-dUTP (Roche) or AlexaFluor 594 (Molecular Probes, Eugene, OR, USA) by nick translation. Centromeric probes, which were used as reference, were labelled with fluorescein isothiocyanate (FITC)-dUTP (Roche). Table 2 lists the probes.

The interphase and metaphase slides, and Carnoy-fixed (3:1 methanol:acetic acid glacial; 50%, 75%, 2×100%, 2 min each) frozen sections of the xenografts were denatured in 70% formamide/2×SSC for 2–3 min, followed by dehydration in an ascending ethanol series (70%, 85%, 100%, 2 min each) and air-drying. The probes were denatured in the hybridisation mix containing 50% formamide/2×SSC for 5 min at 75°C and applied to the slides. After hybridisation in a humid chamber for 1 to 3 days at 37°C, the slides were washed in 0.4×SSC/0.3% NP-40 at 72°C, 2×SSC/0.1% NP-40, at room temperature, and 2×SSC, stained with anti-digoxigenin-rhodamine (Roche), and counterstained with 0.1 M 4,6-diamidino-2-phenylindole (DAPI) in an antifade solution (Vectashield, Vector Laboratories, Burlingame, CA, USA).

Table 2. *FISH probes used in the analyses*

Gene/locus	clone	Accession no(s)	label
EZH2	RP5-1151M5	AC006323	digoxigenin
EIF3S3	RPCI1-49K5		digoxigenin
MYC	RMC08P001		digoxigenin
HIF1A	RPCI1-106C2		digoxigenin, AlexaFluor 594
9p13.3	RP11-165H19	AQ382511, AQ415409	digoxigenin
cen1	pUC177		FITC
cen7	p7alphaTET		FITC
cen8	pJM128		FITC
cen9	pHUR98		FITC
RAD21	CTD-3071K10	AC104391	digoxigenin
KIAA0196	RP11-300I14	AF216671	digoxigenin
TCEB1	RP11-367E12	AC022868	digoxigenin
14q23–24	RMC14POO5		digoxigenin

For FISH on the TMAs, deparaffinised slides were pre-treated in 1 M NaSCN for 10 min at 80°C. The slides were then rinsed in H_2O and incubated in 3.25 or 4 mg/ml pepsin (Sigma-Aldrich Co., St. Louis, MO, USA) in 0.9% NaCl (pH 1.5) for 8 min, followed by washes in 2×SSC, and dehydration. The hybridisation mix containing the probes and Human CotI DNA (Roche) was applied to the slides and co-denatured on an 80°C water bath for 8–10 min. Hybridisation was carried out in a humid chamber for 2 to 3 days at 37°C, after which the slides were stringently washed in 50% formamide/2×SSC at 45°C, stained with anti-digoxigenin-rhodamine or anti-digoxigenin-FITC (Roche), and counterstained.

The probe signals from non-overlapping nuclei were scored with an Olympus BX50 epifluorescence microscope (Tokyo, Japan). The definitions of amplification varied slightly. The presence of three or four copies of the gene in question was scored as a gain (HIF1A and EZH2) or a low-level amplification (EIF3S3 and MYC, from now on called a gain). EIF3S3 and MYC were considered amplified if more than 5 copies of the gene were detected. For *EZH2*, two categories of amplification were defined. A minimum of five copies of the gene was considered an amplification, and a gene/centromere ratio ≥2 indicated a high-level amplification. Otherwise, the amplification was considered a low-level amplification.

cDNA microarrays [IV]

cDNA microarrays were used to screen for amplified and overexpressed genes in prostate cancer cell lines and xenografts. The slides were obtained from the Finnish Microarray Consortium (http://www.microarrays.btk.utu.fi, University of Turku and Åbo Akademi University, Turku, Finland) and contained, in duplicate, approximately 16,000 annotated clones from the sequence verified I.M.A.G.E. Consortium cDNA library. Chromosomal locations of the genes represented by the clones were retrieved from the UCSC website (http://hgdownload.cse.ucsc.edu/goldenPath/hg16/database/, July 2003 freeze). The slides were pre-treated with succinic-anhydride according to the slide manufacturer's protocols.

Array comparative genomic hybridisation

Genomic DNA from the cell lines LNCaP, DU145, PC-3, LAPC-4, and 22Rv1 as well as 13 xenografts (LuCaPs 23.1, 35, 49, 58, 69, 70, 73, 77, 86.2, 93, 96, 105, and 115) were restriction-digested with RsaI (Fermentas UAB, Vilnius, Lithuania). 2–10 μg of digested and phenol:chloroform:isoamyl alcohol (P:C:I) purified DNA was labelled with Cy5-dCTP (Amersham Biosciences UK Ltd., Little Chalfont, United Kingdom) as described by Pollack *et al*. (1999). RsaI-

digested and P:C:I purified normal male DNA, extracted from peripheral blood lymphocytes, was labelled with Cy3-dCTP (Amersham) and used as reference.

The labelled sample and reference DNAs were concentrated with Microcon columns (Millipore, Billerica, MA, USA), and co-hybridised under cover slips to the cDNA microarrays in a humid chamber o/n at +65°C. The final volume of 38.5 µl of hybridisation mix contained 3.4×SSC, 0.3% SDS, 1.3×Denhardt's (Sigma-Aldrich, St. Louis, MO, USA), and 0.5×DIG Blocking Buffer (Roche). After stringent washes, the slides were scanned with ScanArray4000 confocal laser scanner (Perkin Elmer, Boston, MA, USA).

Expression arrays

30 µg of total RNA from the cell lines LNCaP, DU145, PC-3, and LAPC-4 as well as from 7 xenografts (LuCaPs 35, 49, 58, 73, 77, 86.2, and 105) were labelled with Cy3-dUTP (Amersham) by oligo dT-primed SuperScriptII reverse transcriptase (Invitrogen) according to the slide manufacturer's protocols. A mixture of LNCaP total RNA and Human Universal Reference Total RNA (60/40, BD Biosciences Clontech, Palo Alto, CA, USA) was similarly labelled with Cy5-dUTP (Amersham) and used as reference in the hybridisation. The LNCaP RNA was used in the mixture to ensure sufficient signals on the control channel for genes expressed in the prostate. The hybridisation was carried out as for the aCGH.

Data processing

Visually identified bad spots were flagged and the signal intensities of the spots were quantitated with QuantArray software (Packard BioScience, Billerica, MA, USA). Kensington Discovery Edition 1.8 (InforSense, London, United Kingdom) was used for further data processing. The raw data was filtered to exclude the bad spots, as well as spots with the weakest signal intensities (<3.0 arbitrary units) or with greater local background than spot intensity in either channel. The sample to control signal ratio values were calculated and $\log_2$ transformed. Normalisation was done sub-arraywise to the median value. Mean $\log_2$ ratios from clones that showed adequate signals in both duplicate spots were calculated and used in the analyses. Lowess curves of individual chromosomes were created and plotted in a whole genome plot from the averaged $\log_2$ ratios with GraphPad Prism 4.00 for Windows (GraphPad Software Inc. San Diego, CA, USA). To pinpoint regions of gains and losses, cut-off values for Lowess curves for each hybridisation were defined as mean ±0.5×SD of the $\log_2$ ratio values (Clark *et al.*, 2003). The regions showing gain or deletion by the Lowess curves were analysed in more detail. Only regions where four or more adjacent clones showed $\log_2$ ratio values beyond the cut-off values, and regions with six adjacent clones with one clone showing $\log_2$ ratio within the cut-offs were ultimately defined

altered. Chromosome Y was excluded from the analyses due to scarcity of clones from chr Y on the array.

Quantitative reverse transcription polymerase chain reaction (Q-RT-PCR) [III, IV]

mRNA expression was studied with quantitative reverse transcription PCR. The LC FastStart DNA SYBR Green I Kit (Roche) and the LC-FastStart DNA Master Hybridization Probes Kit for LightCycler (Roche) were used according to previously published guidelines (Linja *et al*., 2001, 2004). The primer and probe sequences are listed in Table 3. The primers were designed for different exons to avoid amplification from genomic contamination.

A mixture of LNCaP/Universal Total Human RNA (60/40, Clontech) or Normal Human Prostate RNA (BD Biosciences Clontech, Mountain View, CA, USA) was used to create the standard curve for the analysis. The expression values obtained were normalised to the expression values of the housekeeping gene TATA-box Binding Protein (TBP). With the SYBR Green Kit, a melting curve analysis and a 1% agarose gel were run to ensure that only the correct size product had been amplified.

Table 3. *Primer and probe sequences for Q-RT-PCR*

Gene	Primer sequences (5'–3')	Hybridisation probe sequences (5'–3')
EZH2	TCTATTGCTGGCACCATCTG	
	TGCATCCACCACAAAATCAT	
BM009	TGGCAAATCGAATGTCTTTG	
	ACACCCACCATTACCCTCAA	
KIAA0196	ATCGCTCAGTTGCCAAAACT	
	CTGGCTTGTACACTGCTCCA	
EXT1	CATCCTGGAGGATTGTTCGT	
	GGCTGCTCATAACCTTGCTC	
RAD21	GAGGCCAGCAGAACAAACAT	
	TGCTGAGTCCTTTTGTTCCA	
TCEB1	ACCTATGGTGGCTGTGAAGG	
	AGGTGCAATTGGGAATTCAG	
EIF3S3	GCCCAGGCTCTTCAAGAATAC	GCTGAATCTCCCGAGCCGCCTTT–Fluorescein
	ATAGCCAAAATCGGCAATGA	Red640–CCTTTGCCTTTCCCTGCTGCGC
TBP	GAATATAATCCCAAGCGGTTTG	TTTCCCAGAACTGAAAATCAGTGCC–Fluorescein
	ACTTCACATCACAGCTCCCC	Red640–TGGTTCGTGGCTCTCTTATCCTCATG

Immunohistochemistry (IHC) [III]

Immunohistochemistry with two rabbit polyclonal antibodies against EZH2 was done on TMAs and routine sections of 14 BPH samples. One antibody recognised the internal region of EZH2 (1:1000 dilution, Zymed Laboratories, South San Francisco, CA, USA), while the other recognised the N-terminus of the EZH2 protein (1:100 dilution, Abgent, San Diego, CA, USA).

The sections were deparaffinized and the antigens were retrieved by autoclave cooking at 121°C for 2 minutes in 10 mmol/L of sodium citrate buffer, pH 6.0. PowerVision+^{TM} Poly-HRP IHC Detection Kit (ImmunoVision Technologies Corporation, Brisbane, CA, USA) was used for antibody visualisation. The sections were counterstained with hematoxylin and the nuclear staining was evaluated on a scale from 0 to 3 (0=no staining, 1= weak staining, 2=moderate staining, 3=strong staining).

Statistical analyses [II, III, IV]

The association of gene amplifications of *EIF3S3* and *EZH2* with clinicopathologic variables, such as clinical stage and Gleason score, were tested with χ^2 test. Fisher's exact test was used to test the association between *EZH2* amplification and EZH2 protein expression. The significance of differences in *EZH2* mRNA expression between BPH, untreated prostate cancer, and hormone-refractory disease were shown with Kruskal-Wallis test.

Survival analysis was done with Kaplan-Meier method together with Wilcoxon rank test or Mantel-Cox and Breslow tests.

The association of global gene expression and DNA sequence copy number was tested by variance analysis. Mann-Whitney-U test was used for testing the association between copy number and expression level of individual genes. The concordance of cDNA microarray and Q-RT-PCR results was tested with Spearman rank test. aCGH and CGH were compared with κ-statistics.

RESULTS

Analyses of *HIF1A*, *EIF3S3*, and *EZH2*

Copy numbers of *HIF1A*, *EIF3S3*, *MYC*, and *EZH2* were studied by FISH on TMAs of clinical samples. FISH was also used for copy number analyses on metaphase spreads of the cell lines and on freshly frozen sections of the xenografts. The expression of EZH2 was studied by Q-RT-PCR and IHC.

Copy numbers in cell lines and xenografts

The copy number of *HIF1A* was studied in five cell lines. The prostate cancer cell lines DU145, LNCaP, and NCI-H660 all had four copies of *HIF1A*, whereas the prostate cancer cell line PC-3 had a high-level amplification with 10–30 copies of the gene. Both T24 derivatives (TsuPr-1 and JCA-1) showed three copies of *HIF1A*.

EZH2 was studied in four cell lines and ten prostate cancer xenografts. Three copies of *EZH2* were seen in DU145, PC-3, and 22Rv1, while LNCaP had four copies of the gene. Eight of the xenografts had an increased copy number of the gene: gain (3-4 copies) was observed in LuCaPs 23.1, 35, and 70, amplifications ($\geq$ 5 copies) in 23.8, 23.12, 41, 58, and 69. LuCaP41 had the only high-level amplification ($\geq$ 5 copies and a gene to centromere ratio $\geq$ 2), 8–10 copies. LuCaP49 and LuCaP73 had two copies of *EZH2*.

Copy numbers in clinical samples

The results of the most important copy number analyses of the clinical samples are summarised in Table 4.

All seven BPHs hybridised with the probe containing the *HIF1A* gene showed two copies of the gene. No amplifications of *HIF1A* were found in a total of 117 prostate cancers, but 42 (36%) samples showed increased copy number. The gains were equally distributed among all the samples of untreated primary prostate cancers, untreated lymph node metastases and locally recurrent hormone-refractory cancers.

Table 4. *Summary of FISH analyses*

	HIF1A		EIF3S3				EZH2					
										amplifications		
	normal	gain	normal	gain	amplification	*p*-value [5]	loss	normal	gain	low-level	high-level	*p*-value [5]
BPH [1]	7/7	0/7	19/19	0/19	0/19							
HGPIN [2]			36/36	0/36	0/36							
Untreated primary CaP [3]:												
pT1/pT2	33/52 [7]	19/52 [7]	74/125	40/125	11/125		1/76 [9]	56/76	18/76	1/76	0/76	
pT3/pT4			18/44	14/44	12/44		0/45	30/45	15/45	0/45	0/45	
Lymph node metastases	23/37	14/37	10/37	19/37	8/37							
HR [4], local	19/28	9/28	20/78	32/78	26/78		0/46	21/46	15/46	7/46	3/46 [11]	<0.0001 [12]
HR [4] metastases			4/30	11/30	15/30	<0.001						
Gleason score [6] <7			79/115 [8]	31/115 [8]	5/115 [8]		0/42	35/42	7/42	0/42	0/42	
=7							1/60 [9]	40/60	18/60	1/60 [10]	0/60	
>7			22/78	33/78	23/78	<0.001	0/19	12/19	7/19	0/19	0/19	0.1464

[1] benign prostatic hyperplasia; [2] high-grade prostatic intraepithelial neoplasia; [3] carcinoma of the prostate; [4] hormone refractory; [5] χ^2 test; [6] Gleason scores from untreated primary prostate cancer samples; [7] pT1/2 and pT3/4 samples grouped; [8] Gleason score ≤7 grouped together for analysis; [9] grouped with "normal" in analyses; [10] grouped with "gain" in analysis; [11] grouped with "low-level amplification" in analysis; [12] pT1/pT2 and pT3/pT4 grouped together for analysis, difference between untreated primary prostate cancer and locally recurrent hormone-refractory prostate cancer.

In the set of prostate samples that was used to assess the amplification frequency of *EIF3S3* in different stages of prostatic disease, all 19 non-malignant (BPH) and 36 pre-malignant (PIN) prostate samples contained a normal copy number of *EIF3S3*. Gains (3-4 copies) were found in 32% untreated (T1/2) prostate cancer specimens, 32% locally advanced (T3/4) hormone-naïve tumours, 51% untreated lymph node metastases, 41% locally recurrent hormone-refractory carcinomas, and 37% hormone-refractory metastases. The gene was amplified in 9% prostatectomy samples, 27% locally advanced tumours, 22% lymph node metastases, 33% local, and 50% metastatic hormone-refractory prostate carcinomas. The gene amplification was statistically significantly associated with advanced stage of the disease ($p<0.001$, χ^2 test).

In the sample set used to study the prognostic utility of *EIF3S3* amplification in incidentally found prostate cancer, gains were found in 26% and amplifications in 5.7% of the T1a/b tumours studied. In the prostatectomy samples used for evaluating the prognostic value of *EIF3S3* alterations, 22% and 8.1% of samples contained gains and amplifications, respectively. *EIF3S3* and *MYC* were co-amplified in 68/461 (15%) prostate cancer samples and gained in 157/461 (34%). One sample had an amplification of *EIF3S3* with only two copies of *MYC*, whereas *MYC* was never amplified without additional copies of *EIF3S3*.

One low-level amplification and 33 gains of *EZH2* were detected in 125 untreated primary prostate cancers. One untreated sample had only one copy of the gene. Fifteen gains, seven low-level amplifications and three high-level amplifications were found in 46 locally recurrent hormone-refractory prostate cancers. The frequency of increased copy number was significantly higher in the recurrent tumours ($p<0.0001$, χ^2 test).

Expression of EZH2

EZH2 mRNA expression was studied in 48 fresh clinical samples and was significantly higher in hormone-refractory carcinomas than untreated carcinomas and BPH ($p=0.0009$, Kruskal-Wallis test). The cell lines DU145, LNCaP, PC-3, and 22Rv1 expressed nearly equal amounts of *EZH2*. LuCaP41 had the highest *EZH2* expression of the xenografts.

Immunohistochemical analysis of EZH2 was done on TMAs and on 14 BPH samples. Both antibodies stained mainly the nuclear compartment. However, in some samples, also cytoplasmic staining was seen with the antibody against the N-terminus of EZH2 (Abgent). The EZH2 staining in all BPH samples was moderate or strong. The cancer specimens showed heterogeneous nuclear staining, which was stronger in the hormone-refractory tumours than in the untreated tumours with both antibodies ($p=0.1003$ (Zymed), $p=0.0021$ (Abgent), χ^2 test). The protein expression was associated with high-level amplification of *EZH2* ($p=0.0283$ and $p=0.0045$, Fischer's exact test), but not with Gleason score ($p=0.1201$, $p=0.515$, χ^2 test) or pT-stage ($p=0.0554$, $p=0.1804$, χ^2 test).

Survival analyses

Kaplan-Meier survival analyses showed that the amplification of *EIF3S3* is associated with poor disease-specific survival of patients with incidentally found prostate cancer (p=0.023). Progression-free survival of prostatectomy-treated patients with *EIF3S3* amplification was not statistically significantly poorer than that of patients without the gene amplification.

The gain of 7q/*EZH2* was not statistically significantly associated with disease recurrence. No difference was seen in progression-free survival between groups expressing higher (staining intensity 2–3) or lower (intensity 0–1) levels of EZH2 protein.

Array-CGH

aCGH was done with five prostate cancer cell lines and thirteen prostate cancer xenografts. A normal female vs. normal male hybridisation was done to control the hybridisation conditions. The control hybridisation distinguished the X-chromosomal genes from autosomal genes. The mean (±SD) $\log_2$ ratio was 0.0027 (±0.155) for autosomal genes and -0.3 (±0.220) for X-chromosomal genes. The difference was highly significant (p<0.0001, unpaired t-test).

The most common gains were found in chromosome arms 1q (44%), 7p (33%), 8q (67%), 9p/q (39%), 14q (39%), 16p (44%), and 17q (39%), and the most common losses were in 2q (44%), 4p/q (44%), 6q (44%), 8p (61%), 13q (66%), 16q (44%), 17p (39%), 18q (50%), and 22q (56%). The minimal regions of the most commonly (found in >30% of cases) gains were: 1q21.2–23.1, 1q24.2–q25.1, 7p22, 7q36.1, 8q13.3–q21.11, 8q22.3, 8q24.13–qter, 9p13.3, 9p13.1–q21.11, 14q32.3, 16p13.3, 16p12.2–p11.2, 17q23.2, and 17q25.3. The minimal regions of most common losses were: 2q14.2–q14.3, 2q21.3–q32.1, 2q37.3, 4p16, 4p15.1–p14, 4q25–q27, 4q28.2–q31.1, 4q32.3–q34.1, 4q35.1, 6q13, 6q15–16.1, 6q21, 6q25.2, 8p21.2, 13q12.11, 13q14.13, 13q32.1, 16q12.1–q12.2, 16q22.1, 16q23.1–q24.1, 17p13.3, 18q12.1, 18q21.33, 18q23, and 22q11.21. All alterations are depicted in Fig 1.

Ten aCGH-profiles were compared with published CGH profiles of the same samples (Laitinen *et al*., 2002; Nupponen *et al*., 1998a). At 850 chromosome band resolution, the agreement between the two methods in identifying copy number alterations was 88% (κ=0.54) for all aberrations, 91% (κ=0.57) for gains, and 86% (κ=0.52) for losses. aCGH identified some small amplicons and deletions that were missed by CGH. In addition, some large copy number alterations detected by CGH were seen as two or more separate aberrations in the aCGH profiles. Some of the whole chromosome arm copy number changes seen in the CGH profiles were missed by aCGH.

Figure 1. *Copy number alterations detected in 5 cell lines and 13 xenografts by array-CGH. The lines on the left of each chromosome ideogram represent losses and the lines on the right represent gains.*

The copy numbers of *EIF3S3*, *RAD21*, *KIAA0196*, *MYC*, and *TCEB1*, as well as the BAC clone RP11-165H19 (at 9p13.3), were studied by FISH in LuCaP35 to confirm the aCGH results. *EIF3S3* and *RAD21* were present in two copies, while *TCEB1* and RP11-165H19 showed more than ten signals. *KIAA0196* and *MYC* were seen in tight clusters of uncountable signals.

cDNA microarray

Expression of approximately 12,000 genes was studied by cDNA microarray analysis of PC-3, LNCaP, DU145, and LAPC4, and seven xenografts (LuCaPs 35, 49, 58, 73, 77, 86.2, and 105) to study the association of gene copy number and gene expression. The genome-wide expression levels of the genes were significantly associated with the copy numbers of the genes ($p<0.0001$, ANOVA and χ^2 test, Table 5). The association was also evident by visual inspection of expression and copy number profiles, which were surprisingly similar.

The association of expression of individual genes and copy number of the chromosomal loci harbouring the genes was analysed. From a list of genes of the 10% most and least expressed in each sample, the ones that were located in the minimal regions of the most common (found in >30% of cases) gains and losses were picked. The proportion of samples with concomitant overexpression and gain of a gene to the total number of samples with gain of the gene was calculated (samples with gain and overexpression / samples with gain). The same was done for the samples with concomitant underexpression and loss. Genes with a proportion of >0.4 were picked and Mann-Whitney-U test was used to test the association between $\log_2$ ratio expression values and copy number status of the loci. 32 of the selected genes showed significant ($p<0.05$) association between copy number status and expression, 22 of these were up, and 10 down. The genes whose expression correlated significantly with their copy number status are listed in Table 6.

Table 5. *Correlation of gene copy number and gene expression*

		Gene copy number			
		Deleted	**Normal**	**Gained/ amplified**	**Total**
Gene expression	**Underexpressed**	2.0 %	7.0 %	1.0 %	10 %
	Normal	13.2 %	56.9 %	9.9 %	80 %
	Overexpressed	1.2 %	6.9 %	1.9 %	10 %
	Total	16.4 %	70.8 %	12.8 %	100 %

Table 6. *Genes whose expression correlated with copy number status*

GenBank Accession no	Gene name	Official Gene Symbol	Chromosome band	p-value [1]
Gained and overexpressed				
AA775828	pogo transposable element with ZNF domain	*POGZ*	1q21.3	0.0043
AA186895	HBxAg transactivated protein 2	*BAT2D1*	1q24.3	0.0043
AA719064	EST	*na*	16p11.2	0.0061
AA434435	ubiquitin-conjugating enzyme E2R 2	*UBE2R2*	9p13.3	0.0061
R98205	KIAA1892 protein	*WDR40A*	9p13.3	0.0061
R88741	retinoblastoma binding protein 6	*RBBP6*	16p12.1	0.0087
N36176	chromosome 1 open reading frame 9	na	1q24.3	0.0087
AA704613	EST	na	16p11.2	0.0095
AA449463	EST	na	16p12.2	0.0118
AA167728	CD27-binding (Siva) protein	na	14q32.33	0.0121
AA156461	pituitary tumour-transforming 1 interacting protein	*PTTG1IP*	21q22.3	0.0121
AI038391	breast carcinoma amplified sequence 3	*BCAS3*	17q23.2	0.0159
AI371514	DKFZP564O0463 protein	*na*	8q22.3	0.0167
T68333	frizzled homolog 6 (Drosophila)	*FZD6*	8q22.3	0.0242
AA291486	PTK2 protein tyrosine kinase 2	*PTK2*	8q24.3	0.0303
T65790	farnesyl diphosphate synthase	*FDPS*	1q22	0.0303
AA629849	Siah-interacting protein	*CACYBP*	1q25.1	0.0317
AA887530	regulator of G-protein signalling 11	*RGS11*	16p13.3	0.0381
AA070434	KIAA0683 gene product	na	16p13.3	0.0381
AI017242	dynactin 3 (p22)	*DCTN3*	9p13.3	0.0424
AA706041	RAB GTPase binding effector protein 2	*RABEP2*	16p11.2	0.0424
AA487588	ATPase, H+ transporting, lysosomal interacting protein 1	*ATP6IP1*	Xq28	0.0424
Deleted and underexpressed				
W73004	integrin, alpha 4	*ITGA4*	2q31.3	0.0121
R38669	tumour necrosis factor receptor superfamily, member 10b	*TNFRSF10B*	8p21.3	0.0121
H73591	cytochrome b5 outer mitochondrial membrane precursor	na	16q22.1	0.0173
AA156571	alanyl-tRNA synthetase	*AARS*	16q22.1	0.0173
AA448641	E2F transcription factor 4, p107/p130-binding	*E2F4*	16q22.1	0.0173
AI016999	catechol-O-methyltransferase	*COMT*	22q11.2	0.0238
H17943	ring finger protein 4	*RNF4*	4p16.3	0.0242
AA196638	KIAA0853 protein	*KIAA0853*	13q14.13	0.0381
AA485676	SH3 domain containing ring finger	*SH3MD2*	4q32.3–33	0.0424
W42849	amyloid beta (A4) precursor protein	*APP*	21q21	0.0424

[1] Mann-Whitney U test

The expression of *TCEB1*, *EIF3S3*, *RAD21*, *EXT1*, *KIAA0196*, and *BM009* was measured by Q-RT-PCR to evaluate the reliability of the microarrays for expression analyses. The correlation coefficients (Spearman rank correlation) ranged from 0.3273 to 0.8000.

DISCUSSION

Copy numbers of *HIF1A*, *EIF3S3*, and *EZH2*

Overexpression of HIF1A has been reported in the majority of prostate tumours and HGPIN (Zhong *et al*., 1999, 2004). Since the region harbouring the gene for HIF1A is amplified in PC-3 according to CGH, and the protein is also constitutively expressed, it was of interest to see whether its amplification is a common phenomenon and perhaps the cause of the observed overexpression (Nupponen *et al*., 1998a; Zhong *et al*., 1998). The high-level amplification of *HIF1A* found in PC-3 by FISH is supported also by the aCGH-profiles of chr 14 of the cell line (Wolf *et al*., 2003; Clark *et al*., 2003; Zhao *et al*., 2005). The aCGH profile created in study IV also corroborates the high-level amplification at the *HIF1A* locus. In addition, the localisation of the signals to several different derivative chromosomes is supported by SKY data on the cell line (Pan *et al*., 1999). Although *HIF1A* is amplified and overexpressed in PC-3, it may not be the only target gene of the amplification at 14q12-24, as the amplicon is about 48Mb in size and contains over 430 genes.

The gain of *HIF1A* was as common (*ca* 35%) in the untreated primary cases as in the untreated lymph node metastases and locally recurrent hormone-refractory prostate cancers. Gain in 14q has never been reported in the literature at these frequencies. No amplifications were detected in the clinical samples, so it seems that PC-3 is a rare exception in harbouring *HIF1A* amplification, and the observed high protein levels under normoxia may be due to gene amplification in this case only. Since HIF1A is frequently overexpressed in clinical prostate cancer, the mechanisms of overexpression must be other than gene amplification (Zhong *et al*., 1999). It has been shown that *HIF1A* mRNA is expressed regardless of oxygen conditions (Gradin *et al*., 1996). The protein, however, is not present under normoxia, so regulation of HIF1A happens post-transcriptionally (Jiang *et al*., 1996). HIF1A has, in fact, been shown to be degraded quickly via ubiquitinylation and targeting to the proteasomes (Kallio *et al*., 1999).

The copy number increases of *EIF3S3* and *EZH2* genes were significantly more common in advanced disease, and in the case of *EIF3S3* the shift towards more copies was shown to be gradual. This is consistent with CGH and aCGH studies which have shown that gains in both 7q and 8q become more frequent as the cancer advances (Alers *et al*., 2000; Nupponen *et al*., 1999; Visakorpi *et al*.,

1995b; Cher *et al*., 1996). The frequencies observed were also in line with the earlier studies, where the gains of 7q and 8q have been found in up to 20% of untreated prostate cancers, and in 20–30% and up to almost 90% of locally recurrent hormone-refractory prostate cancers, respectively (Chu *et al*., 2003; Nupponen *et al*., 1998b).

The association of the *EIF3S3* amplification with advanced stage, Gleason score, and androgen-independence indicates a role for *EIF3S3* in aggressive prostate cancer. As a prognostic marker for incidentally found prostate cancers, the gain/amplification of *EIF3S3* was statistically significantly associated with poor disease-specific survival, and could be useful in determining which cancers are clinically significant and require aggressive treatment. In prostatectomy-treated prostate cancers, the difference in progression-free survival time was not statistically significant, however.

EIF3S3 was almost always co-amplified with *MYC*, as has also been shown by Nupponen *et al*., (1999) and Tsuchiya *et al*., (2002). The 8q23–24 region is the most studied of the 8q amplicons and several target genes have been proposed, including *RAD21*, *TRPS1* (trichorhinophalangeal syndrome 1), and *KIAA0196* (Porkka *et al*., 2004; Savinainen *et al*., 2004; Chang *et al*., 2000; van Duin *et al*., 2005b), of which *RAD21* and *KIAA0196* are located between *EIF3S3* and *MYC*, and *TRPS1* slightly proximal to *EIF3S3*. *TRPS1*, like *MYC*, has been shown to be co-amplified with *EIF3S3*, indicating that the gained region in 8q is usually quite large, ranging at least from *TRPS1* to *MYC* (Savinainen *et al*., 2004). The minimal region of 8q23–24 gain/amplification seems to lie at 8q24.13–q24.21, distally to the *EIF3S3* gene at 8q23.3–24.11 (Tsuchiya *et al*., 2002; van Duin *et al*., 2005b). However, the general co-amplification with other genes makes *EIF3S3* a putative marker for prostate cancer aggressiveness. Furthermore, overexpression of *EIF3S3*, unlike that of *TRPS1* and *MYC*, has been shown to be associated with the gene amplification in hormone-refractory prostate cancer (Nupponen *et al*., 1999; Savinainen *et al*., 2004). It is therefore unlikely that the gene is co-amplified by chance alone, but contributes in a yet unknown way to cancer progression. Some other components of the translation initiation complex have been shown to promote tumorigenesis and a similar effect is conceivable with EIF3S3 (Lazaris-Karatzas *et al*., 1990).

The chromosomal region harbouring the *EZH2* gene has been shown to be gained/amplified in all the xenografts that showed increased copy number of the gene by FISH (Laitinen *et al*., 2002). The xenograft with the highest peak in the CGH profile, LuCaP41, exhibited 8–10 *EZH2* signals in FISH analysis. Two xenografts had a normal copy number of *EZH2* and the cell lines had gains of the gene. These were all to be expected, judging by the CGH profiles.

In clinical samples, the significance of *EZH2* amplification was shown by the highly increased protein expression in samples with high-level amplification. Simple gain of *EZH2* did not affect the protein levels statistically significantly. Although EZH2 expression may be increased also in tumours without gene amplification, it is always increased when the gene is amplified. The patients, who had a gain of *EZH2*, had a slightly shorter progression-free survival after

prostatectomy. These results suggest that amplification is one mechanism for increased expression of EZH2, and that *EZH2* is a target gene for 7q35–36 gain/amplification.

Expression of EZH2

Expression of *EZH2* was studied in cell lines and xenografts by Q-RT-PCR. There were no significant differences between the cell lines. In the xenografts, a trend towards higher levels of mRNA could be seen with increasing copy number. The differences were not statistically significant, but this could be due to the small sample size (n=10).

mRNA expression was also studied in clinical samples and it was higher in untreated prostate cancer than in BPH, and higher still in the hormone-refractory samples. One untreated sample showed notably higher expression than the rest of the sample group. The sample was obtained by transurethral resection from a patient whose cancer had metastasised by the time of sample acquisition. This is in agreement with the study by Varambally *et al*., (2002), who found that hormone-refractory metastatic disease had higher levels of *EZH2* than benign lesions and localised tumours. However, these results show that *EZH2* expression is increased already in locally recurrent hormone-refractory prostate cancer.

Immunohistochemical analysis of clinical samples provided further evidence for the increase in EZH2 expression in advanced prostate cancer. The staining in hormone-refractory prostate cancer was statistically significantly stronger than in untreated prostate cancer. Contrary to studies by others, all BPH samples studied by IHC were stained at least moderately with the EZH2 antibody (Varambally *et al*., 2002; Rhodes *et al*., 2003). This unexpected result may be because of the different antibodies used, or because the dynamic range of the analysis may be reduced due to the highly sensitive detection method used. The antibodies used in study III were both polyclonal. Monoclonal antibodies may be more specific and reveal a stronger association between EZH2 expression and cancer (Laitinen and Visakorpi, personal communication).

Consistent with previous studies, there was no association between EZH2 protein expression and Gleason score or tumour stage (Varambally *et al*., 2002; Rhodes *et al*., 2003). These earlier studies did, however, find a weak association between high EZH2 expression and shorter progression-free survival, which was not the case with this sample set. Again, the reason may lie in the sensitivity of the detection system which may not detect the subtler differences in expression. On the other hand, an association between WHO (World Health Organisation) histologic grade (1=well differentiated carcinoma, 2=moderately differentiated, and 3=poorly differentiated) and EZH2 expression has been found using a monoclonal antibody against EZH2 (Bachmann *et al*., 2006).

Increased expression of EZH2 in prostate cancer may lead to methylation of histones at as yet unidentified target genes and/or increased repression of PRC2/3/4 target genes. It was also recently shown that EZH2 directly interacts with and controls DNA methyl transferases, which methylate and thus inactivate DNA (Vire *et al*., 2006). The effects of EZH2 overexpression may also take place in the cytoplasm, as one of the antibodies used in this study stained some samples in the cytoplasm. Cytoplasmic EZH2 has been reported in hepatocellular carcinoma, in thymocytes and fibroblasts (Sudo *et al*., 2005; Su *et al*., 2005). Not all of the components of the cytoplasmic PRC are known, and the distinct recognition sites of the antibodies may be differently exposed in the complex. EZH2 has been shown to be required for circular ruffle formation in fibroblasts and actin polymerisation in thymocytes (Su *et al*., 2005). Both these processes are cytoplasmic and may be of importance in proliferation and cell motility also in prostate cancer cells.

aCGH

The copy number profiles obtained by aCGH were in good agreement with CGH profiles of the same samples (Nupponen *et al*., 1998a; Laitinen *et al*., 2002). Previous aCGH studies of the cell lines have also produced similar profiles (Wolf *et al*., 2004; Clark *et al*., 2003; Zhao *et al*., 2005). As expected, the observed differences in the copy number profiles were mainly due to aCGH's better resolution, as additional, smaller aberrations were identified by aCGH, and some large alterations were shown to be comprised of two or more separate aberrations. Large alterations identified by CGH were also narrowed down by the aCGH. However, CGH profiles showed some whole chromosome (arm) gains that were not seen by aCGH, which could be due to the normalisation procedures and sample-specific cut-offs applied.

The most commonly found copy number alterations (CNAs) in advanced prostate cancer, such as loss of 2q, 5q, 6q, 8p, 13q, 16q, and 18q as well as gains of 1q, 7, 8q, 16p, and 17q have been described by previous CGH and aCGH studies (Visakorpi *et al*., 1995b; Nupponen *et al*., 1998b; Cher *et al*., 1996; Paris *et al*., 2004). These recurrent CNAs were also found in this set of samples, which was mainly of metastatic origin.

aCGH has previously been shown to have superior resolution compared to CGH (Paris *et al*., 2003). In this study, an example of this is 8q of LuCaP35. In the CGH profile, one large peak is evident, whereas the aCGH profile of the same chromosome shows three separate peaks in 8q13.3–q21.1, 8q22.1–23.1, and 8q24.12–q24.3. A recent study on a chromosome 8q-specific BAC array showed that in 32 clinical samples as many as five minimal regions (8q21.13, 8q22.1, 8q22.2–3, 8q24.13, and 8q24.21) of frequent gain can be seen (van Duin *et al*., 2005b). The peaks in LuCaP35 roughly coincide with these gains. The

most proximal and distal peaks were confirmed by FISH with *TCEB1*, *KIAA0196*, and *MYC*, which are located in the amplified regions, and the dip in the profile at 8q23–q24.12 was confirmed by FISH with *EIF3S3* and *RAD21*. The resolution of the chromosome 8q-specific BAC array was better than the resolution of the cDNA array used here, so it is possible that additional information is still hidden in the profile of LuCaP35.

In the whole data set, four minimal regions of gain in 8q were identified, the two proximal ones defined mainly by LuCaP35. The 8q24 amplicon was usually amplified as one, but there were also cases where part of the most distal band, 8q24.3, was amplified by itself, and a case where the amplicon did not include 8q24.3. These data, taken together with data from other studies, including studies of gene expression, show that patterns of gain/amplification in 8q can be complex and involve more than one target gene.

In addition to the well known frequently gained chromosomal regions, several previously unreported frequent CNAs, such as gain in 9p13–q21, were detected. This gain may have been overlooked in CGH studies because it contains the large heterochromatic region in proximal 9q, and such regions are usually omitted from CGH analyses due to difficulties in interpreting repetitive sequences and copy number variations between individuals (Kallioniemi *et al.*, 1993). The array used here only contained expressed sequences, which are not as variable. However, there were naturally no clones from the heterochromatic region, and the profiles were created based on the clones flanking it. The gain was found in over a third of the samples and the minimal region spanned only 3.1Mb in 9p13.3. This stretch of the genome is gene-rich and contains almost 30 known or predicted genes, including interleukin 11 receptor alpha (*IL11RA*), carbonic anhydrase 9 (*CA9*), and valosin containing protein (*VCP*), whose overexpression has been reported in prostate cancer (Zurita *et al.*, 2004; Tsujimoto *et al.*, 2004; Potter and Harris, 2003).

The gain in 9p13.3 of LuCaP35 was confirmed by FISH and it is, in fact, a high-level amplification, as shown by 7–10 copies of the BAC-clone RP11-165H19. The proximal boundary of the amplification in this xenograft lies between BACs RP11-165H19 and RP11-201P13 (unpublished results). Validating FISH studies have also been done on some of the other LuCaPs and cell lines. DU145 and LuCaPs 58, 69, 70, 77, 86.2, 93, and 115 contain a gain in 9p13.3 by FISH, although in LuCaPs 58, 69, and 70 it was not detected by the aCGH analyses. The cell line 22Rv1 contains a gain in 9p slightly distal to the minimal region identified, which was detected by the aCGH, and has been confirmed by FISH.

Stretches of 16p (in 16p13.3 and 16p11.2–12.2) were found to be gained in about 30% of the samples. Some CGH studies have reported gains of 16p (El Gedaily *et al.*, 2001; Joos *et al.*, 1995; Kasahara *et al.*, 2002). Most of the studies do not report which of their samples were hormone-refractory or metastatic, and the higher frequency of 16p gains may be restricted to metastatic and/or hormone-refractory disease. There may be several target genes in 16p, as the gained regions are gene-rich.

Recently, concerns on the reliability of (a)CGH have been raised, based on findings of frequent copy number polymorphisms (CNPs). Two articles claim that CNPs occur frequently in the normal healthy population and constitute a hitherto unknown level of genomic variation (Sebat *et al*., 2004; Iafrate *et al*., 2004). The size of these polymorphic regions can be as large as several megabases and the existence of such polymorphisms could cause problems in interpretation of aCGH results. For instance, a frequently occurring CNP present in a sample used as normal reference could lead to false discoveries of disease-specific CNAs. Alternately, a common CNP present in a disease sample could lead to the false assumption that this is a disease-specific CNA. Methods to minimise the effects of CNPs on interpretation of CGH results are clearly needed.

Expression analyses

The global effect of copy number on gene expression was determined and was found to be significant. The same conclusion has been reached by Wolf *et al*., (2003) in a microarray study of prostate cancer cell lines, and by Phillips *et al*., (2000), who have shown that the average expression level of genes residing in gained chromosome arms in a prostate cancer progression model is significantly elevated. This implies that the simple gains often found in prostate cancer may be more important than previously thought. More genes showed significant up-regulation when amplified than down-regulation when deleted, which may be because very low expression levels may not have been reliably detected, and the spots were filtered out during data analysis.

The eight genes whose expression was most significantly ($p<0.01$) associated with increased copy number were from three chromosome arms: 1q, 9p, and 16p. The function of four of the genes, *WDR40A* (WD repeat domain 40A, at 9p13.3) and three ESTs (expressed sequence tags, two at 16p11.2, one at 1q24.3), is currently unknown. The information on the others is also limited. *BAT2D1* (BAT2 domain containing 1, at 1q24.3) amplification and overexpression has been reported in bladder cancer (Huang *et al*., 2002) and *RBBP6* (retinoblastoma binding protein 6, at 16p12.1) binds the unphosphorylated form of the retinoblastoma protein (Sakai *et al*., 1995). *UBE2R2* (ubiquitin-conjugating enzyme E2R 2, at 9p13.3) is a ubiquitin conjugating enzyme which in its activated form influences β-catenin degradation (Semplici *et al*., 2002). *POGZ* (pogo transposable element with ZNF domain, at 1q21.3) contains a zinc finger domain that is usually associated with DNA-binding activity.

The decreased expression of ten genes was associated ($p<0.05$) with deletion of the gene. At least three of these, *ITGA4* (integrin α 4, at 2q31.3), *TNFRSF10B* (tumour necrosis factor receptor superfamily member 10b, at 8p21.3), and *COMT* (catechol-O-methyl transferase, at 22q11.2) have potentially important

functions related to (prostate) cancer. *ITGA4* has been suggested a metastasis suppressor gene in oral squamous cell carcinoma and its loss of expression via hypermethylation has been shown in gastric cancer (Zhang *et al*., 2004; Park *et al*., 2004). In addition, restored expression of *ITGA4* has been shown to reduce the invasive potential of gastric cancer cells through Matrigel (Park *et al*., 2004). According to the microarray data, *ITGA4* may play a role also in prostate cancer metastasis, as most of the samples analysed in this study are derived from metastatic lesions. The results also implicate *ITGA4* as a putative target gene of the frequent (39%) 2q31.3 loss.

TNFRSF10B is located in almost the centre of the minimally deleted region in 8p21.2. It can be activated by TRAIL (tumour necrosis factor [TNF]-related apoptosis inducing ligand) and transduces the extrinsic apoptosis pathway (Özören *et al*., 2003). Inactivating mutations of the gene have been detected in human cancers, although the frequencies have been low (Pai *et al*., 1998; Lee *et al*., 1999; Park *et al*., 2001; Adams *et al*., 2005). As the 8p21 deletion still lacks a confirmed target gene in prostate cancer, *TNFRSF10B* should be further investigated.

COMT is an enzyme that inactivates the potentially carcinogenic catecholestrogens. It is a polymorphic gene, and the variant with lower activity has been linked to oestrogen-induced breast cancer (Lavigne *et al*., 1997). 4-hydroxyestradiol, a carcinogen and a substrate for COMT, has been shown to induce tumours in Noble rat prostates, particularly in regions with low activity of COMT (Cavalieri *et al*., 2002).

Future prospects

DNA array technologies are continually being developed, and further refinement of amplicon structure and regions of loss in cancer is to be expected. 200,000 element oligo arrays are already being used for expression analyses and tiling resolution BAC arrays will provide uninterrupted genomic information on CNAs. Together these will ultimately lead to the identification of putative target genes of chromosomal amplifications and deletions. The relevance of the findings of this and future microarray studies will need to be tested with functional studies in animal or cell models.

CONCLUSIONS

HIF1A is not a frequently amplified gene in prostate cancer, but a gain of the gene was found in over 30% of the clinical samples studied. Although the increased copy number may explain overexpression of the gene in cases containing extra copies, other mechanisms must be responsible for its generally observed overexpression. In PC-3, where the only high-level amplification of the gene was found, it may contribute significantly to the overexpression of the protein.

EIF3S3 is frequently gained or amplified in prostate cancer and is likely to contribute to tumour progression, since the alteration is associated with advanced disease. The results also indicate that increased copy number of *EIF3S3* could be used as a prognostic marker with incidentally found prostate cancer.

EZH2 is gained or amplified in over half of locally recurrent hormone-refractory prostate cancers and high-level amplification seems to have a significant impact on protein expression. *EZH2* should be considered a putative target gene for 7q35–36 gain in prostate cancer.

Chromosomal alterations in prostate cancer cell lines and xenografts determined by aCGH analysis are generally consistent with CGH analyses. The frequencies of alterations were comparable to the frequencies found by other methods. Regions that are not identifiable by methods with poorer resolution can be identified with aCGH. Novel frequent amplicons were detected, for example, in 9p13 and 16p and further investigations into the significance of these alterations are warranted.

Gene copy number aberrations affect the expression levels of the altered genes even when the copy number increase is small. Although the effect is in most individual cases small, the combined effect is considerable and may contribute to tumorigenesis and/or metastasis. Several genes in previously described, as well as the new, regions of copy number alteration have significantly altered expression levels. These genes should be considered putative target genes of the copy number alterations.

Although this microarray study is not sufficient to determine whether any of the implicated genes are the target genes for the amplifications or deletions, it does point out genes that should be studied further. When the putative target genes are confirmed by clinical or functional studies, they may be used as diagnostic or prognostic markers, or targeted therapies may be developed against them or their products.

ACKNOWLEDGEMENTS

The laboratory work required for this thesis was carried out in the Laboratory of Cancer Genetics (SGY), Institute of Medical Technology (IMT), University of Tampere and Tampere University Hospital during the years 1999-2005.

I owe humongous thanks to my supervisor, Prof. Tapio Visakorpi, M.D., Ph.D., without whom this thesis would never have been started, let alone finished. I must also thank him for allowing me to take my time with things. I like to believe I have taught him something about the wonderful worlds of some computer software and the verbal abuse they may require to work adequately.

Prof. Olli Silvennoinen, M.D., Ph.D., the director of the Institute of Medical Technology is acknowledged for providing the research facilities for this study.

I wish to thank my steering committee members, Prof. Jorma Isola, M.D., Ph.D., and doc. Minna Tanner, M.D., Ph.D., who have steered my thesis and sometimes me.

Docents Outi Monni, Ph.D., and Akseli Hemminki, Ph.D., are greatly appreciated for reviewing this thesis and for their constructive suggestions to make it better.

I am thankful to Joel Kuortti, Ph.D., who reviewed the language of this thesis and was the first to admit that it was good.

All co-authors, Finnish and foreign, of the papers required for this thesis are acknowledged for their contribution. I could not have done the work alone.

I am grateful for having been helped in the lab by numerous laboratory technicians, most importantly, Ms. Arja Alkula, Ms. Mariitta Vakkuri, Heli Lehtonen, M.Sc., and especially Ms. Maarit Ohranen.

I am indebted to everyone who’s ever been a member of our lab, as they have helped me in the lab and/or outside it. I especially need to mention Marika Linja, Ph.D., for taking me to rock festivals, Kimmo Savinainen, Ph.D., for taping TV-programs for me when I forget to do so myself, and Mika Wallén, Ph.D., for sharing the evening shift. Kati Waltering, M.Sc., is enormously thanked for endless talks on the phone at night on topics regarding work, life, the universe, and everything, and for lending her car when mine is broken.

Of the supporting crew at IMT, I especially wish to thank Mr. Heimo Koskinen and Ms. Kaisu Pekonen, our attendants (past and present), who have sometimes gone out of their way to do something for me.

I owe thanks to my parents, Pirjo Saramäki, M.Sc. and Jussi Saramäki, Ph.D., without whom I wouldn't be here. They have supported me in their own ways, never actually demanding anything, yet doing so. I also wish to thank my sisters

Kaija Saramäki, M.Sc., and Varpu Saramäki-Snape, B.A., for occasionally listening to me and not telling everything to our parents.

My absolutely greatest, loving thanks go to my daughter, Helmi Saramäki, 6 years, for being the purpose of my life. She finished her väitöskirja before me, but I'm not bitter. I would also like to express my sincere thanks to her father, Petteri Ojala, M.Sc., for looking after the purpose of life in order for me to concentrate on a lesser thesis.

This study was financially supported by grants from the Sigrid Juselius Foundation, the Pirkanmaa Cultural Foundation, the Reino Lahtikari Foundation, the Foundation for Finnish Cancer Institute, Medical Research Fund of Tampere University Hospital, the Finnish Cancer Society, the Pirkanmaa Cancer Society, the Ida Montin Foundation, and the Scientific Foundation of the city of Tampere.

Tampere, June 2006

Outi Saramäki

REFERENCES

Abate-Shen C, Banach-Petrosky WA, Sun X, Economides KD, Desai N, Gregg JP, Borowsky AD, Cardiff RD, and Shen MM (2003): *Nkx3.1;Pten* mutant mice develop invasive prostate carcinoma and lymph node metastases. Cancer Res 63:3886–3980.

Abdulkadir Magee JA, Peters TJ, Kaleem Z, Naughton CK, Humphrey PA, and Milbrandt J (2002): Conditional loss of Nkx3.1 in adult mice induces prostatic intraepithelial neoplasia. Mol Cell Biol 22:1495–1503.

Adams J, Cuthbert-Heavens D, Bass S, and Knowles MA (2005): Infrequent mutation of TRAIL receptor 2 (TRAIL-R2/DR5) in transitional cell carcinoma of the bladder with 8p21 loss of heterozygosity. Cancer Lett 220:137–144.

Alers JC, Rochat J, Krijtenburg P-J, Hop WCJ, Kranse R, Rosenberg C, Tanke HJ, Schröder FH, and van Dekken H (2000): Identification of genetic markers for prostatic cancer progression. Lab Invest 80:931–942.

Alers JC, Krijtenburg PJ, Vis AN, Hoedemaeker RF, Wildhagen MF, Hop WC, van der Kwast TT, Schröder FH, Tanke HJ, and van Dekken H (2001): Molecular cytogenetic analysis of prostatic adenocarcinomas from screening studies: early cancers may contain aggressive genetic features. Am J Pathol 158:399–406.

Ananthanarayanan V, Deaton RJ, Yang XJ, Pins MR, and Gann PH (2005): Alpha-methyl-CoA racemase (AMACR) expression in normal prostatic glands and high-grade prostatic intraepithelial neoplasia (HGPIN): association with diagnosis of prostate cancer. Prostate 63:341–346.

Anastasiadis AG, Ghafar MA, Salomon L, Vacherot F, Benedit P, Chen M-W, Shabsigh A, Burchardt M, Chopin DK, Shabsigh R, and Buttyan R (2002): Human hormone-refractory prostate cancers can harbor mutations in the O_2-dependent degradation domain of hypoxia inducible factor-1α (HIF-1α). J Cancer Res Clin Oncol 128:358–362.

Asano K, Vornlocher H-P, Richter-Cook NJ, Merrick WC, Hinnebusch AG, and Hershey JWB (1997): Structure of cDNAs encoding human eukaryotic initiation factor 3 subunits: Possible roles in RNA binding and macromolecular assembly. J Biol Chem 272:27042–27052.

Asatiani E, Huang WX, Wang A, Rodriguez Ortner E, Cavalli LR, Haddad BR, and Gelmann EP (2005): Deletion, methylation, and expression of the NKX3.1 suppressor gene in primary human prostate cancer. Cancer Res 65:1164–1173.

Ashida S, Nakagawa H, Katagiri T, Furihata M, Iizumi M, Anazawa Y, Tsunoda T, Takata R, Kasahara K, Miki T, Fujioka T, Shuin T, and Nakamura Y (2004): Molecular features of the transition from prostatic intraepithelial neoplasia (PIN) to prostate cancer: genome-wide gene-expression profiles of prostate cancers and PINs. Cancer Res 64:5963–5972.

Bachmann IM, Halvorsen OJ, Collett K, Stefansson IM, Straume O, Haukaas SA, Salvesen HB, Otte AP, and Akslen LA (2006): EZH2 expression is associated with high proliferation rate and aggressive tumor subgroups in cutaneous melanoma and cancers of the endometrium, prostate, and breast. J Clin Oncol 24:268–273.

Barrett MT, Scheffer A, Ben-Dor A, Sampas N, Lipson D, Kincaid R, Tsang P, Curry B, Baird K, Meltzer PS, Yakhini Z, and Bruhn L (2004): Comparative genomic hybridisation using oligonucleotide microarrays and total genomic DNA. Proc Natl Acad Sci USA 101:17765–17770.

Beach R, Gown AM, De Peralta-Venturina MN, Folpe AL, Yaziji H, Salles PG, Grognon DJ, Fanger GR, and Amin MB (2002): P504S immunohistochemical detection in 405 prostatic specimens including 18-gauge needle biopsies. Am J Surg Pathol 26:1588–1596.

Bergerheim US, Kunimi K, Collins VP, and Ekman P (1991): Deletion mapping of chromosomes 8, 10, and 16 in human prostatic carcinoma. Genes Chromosomes Cancer 3:215–220.

Bernard D, Poutier-Manzanedo A, Gil J, and Beach DH (2003): Myc confers androgen-independent prostate cancer cell growth. J Clin Invest 112:1724–1731.

Bignell GR, Huang J, Greshock J, Watt S, Butler A, West S, Grigorova M, Jones KW, Wei W, Stratton MR, Futreal PA, Weber B, Shapero MH, and Wooster R (2004): High-resolution analysis of DNA copy number using oligonucleotide microarrays. Genome Res 14:287–295.

van Bokhoven A, Varella-Garcia M, Korch C, and Miller GJ (2001): TSU-Pr1 and JCA-1 are derivatives of T24 bladder carcinoma cells and are not of prostatic origin. Cancer Res 61:6340–6344.

van Bokhoven A, Caires A, Maria MD, Schulte AP, Lucia MS, Nordeen SK, Miller GJ, and Varella-Garcia M (2003): Spectral karyotype (SKY) analysis of human prostate carcinoma cell lines. Prostate 57:226–244.

Bowen C, Bubendorf L, Voeller HJ, Slack R, Willi N, Sauter G, Gasser TC, Koivisto P, Lack EE, Kononen J, Kallioniemi O-P, and Gelmann EP (2000): Loss of NKX3.1 expression in human prostate cancers correlates with tumor progression. Cancer Res 60:6111–6115.

Bracken AP, Pasini D, Capra M, Prosperini E, Colli E, and Helin K (2003): *EZH2* is downstream of the pRB-E2F pathway, essential for proliferation and amplified in cancer. EMBO J 22:5323–5335.

Brown RSD, Edwards J, Dogan A, Payne H, Harland SJ, Bartlett JMS, and Masters JRW (2002): Amplification of the androgen receptor gene in bone metastases from hormone-refractory prostate cancer. J Pathol 198:237–244.

Bubendorf L, Kononen J, Koivisto P, Schraml P, Moch H, Gasser TC, Willi N, Mihatsch MJ, Sauter G, and Kallioniemi O-P (1999): Survey of gene amplifications during prostate cancer progression by high-throughput fluorescence *in situ* hybridization on tissue microarrays. Cancer Res 59:803–806.

Cao R and Zhang Y (2004): The functions of E(Z)/EZH2-mediated methylation of lysine 27 in histone H3. Curr Opin Genet Dev 14:155–164.

Carter BS, Ewing CM, Ward WS, Treiger BF, AaldersTW, Schalken JA, Epstein JI, and Isaacs WB (1990): Allelic loss of chromosomes 16q and 10q in human prostate cancer. Proc Natl Acad Sci USA 87:8751–8755.

Cavalieri EL, Devanesan P, Bosland MC, Badawi AF, and Rogan EG (2002): Catechol estrogen metabolites and conjugates in different regions of the prostate of Noble rats treated with 4-hydroxyestradiol: implications for estrogen-induced initiation of prostate cancer. Carcinogenesis 23:329–333.

Cha T-L, Zhou BP, Xia W, Wu Y, Yang C-C, Chen C-T, Ping B, Otte AP, and Hung M-C (2005): Akt-mediated phosphorylation of EZH2 suppresses methylation of lysine 27 in histone H3. Science 310:306–310.

Chaib H, MacDonald JW, Vessella RL, Washburn JG, Quinn JE, Odman A, Rubin MA, and Macoska JA (2003): Haploinsufficiency and reduced expression of genes local-

ized to the 8p chromosomal region in human prostate tumors. Genes Chromosomes Cancer 37:306–313.

Chang GT, Steenbeek M, Schippers E, Blok LJ, van Weerden WM, van Alewijk DC, Eussen BH, van Steenbrugge GJ, and Brinkmann AO (2000): Characterization of a zinc-finger protein and its association with apoptosis in prostate cancer cells. J Natl Cancer Inst 92:1414–1421.

Chen CD, Welsbie DS, Tran C, Baek SH, Chen R, Vessella R, Rosenfeld MG, and Sawyers CL (2004): Molecular determinants of resistance to antiandrogen therapy. Nat Med 10:33–39.

Chen G and Burger MM (1999): p150 expression and its prognostic value in squamous-cell carcinoma of the esophagus. Int J Cancer 84:95–100.

Chen G and Burger MM (2004): p150 overexpression in gastric carcinoma: the association with p53, apoptosis and cell proliferation. Int J Cancer 112:393–398.

Cher ML, Bova GS, Moore DH, Small EJ, Carroll PR, Pin SS, Epstein JI, Isaacs WB, and Jensen RH (1996): Genetic alterations in untreated metastases and androgen-independent prostate cancer detected by comparative genomic hybridization and allelotyping. Cancer Res 56:3091–3102.

Chu LW, Troncoso P, Johnston DA, and Liang JC (2003): Genetic markers useful for distinguishing between organ-confined and locally advanced prostate cancer. Genes Chromosomes Cancer 36:303–312.

Clark J, Edwards S, Feber A, Flohr P, John M, Giddings I, Crossland S, Stratton MR, Wooster R, Campbell C, and Cooper CS (2003): Genome-wide screening for complete genetic loss in prostate cancer by comparative genomic hybridization onto cDNA microarrays. Oncogene 22:1247–1252.

Cooney KA, Wetzel JC, Merajver SD, Macoska JA, Singleton TP, and Wojno KJ (1996): Distinct regions of allelic loss on 13q in prostate cancer. Cancer Res 56:1142–1145.

Cunningham JM, Shan A, Wick MJ, McDonnell SK, Schaid DJ, Tester DJ, Qian J, Takahashi S, Jenkins RB, Bostwick DG, and Thibodeau SN (1996): Allelic imbalance and microsatellite instability in prostatic adenocarcinoma. Cancer Res 56:4475–4482.

Dallas A, Torhorst J, Bachmann F, Banziger R, Schulheiss E, and Burger MM (1998): Expression of p150 in cervical neoplasia and its potential value in predicting survival. Cancer 83:1376–1383.

Davies MA, Kim SJ, Parikh NU, Dong Z, Bucana CD, and Gallick GE (2002): Adenoviral-mediated expression of MMAC/PTEN inhibits proliferation and metastasis of human prostate cancer cells. Clin Cancer Res 8:1904–1914.

D'Cruz CM, Gunther EJ, Boxer RB, Hartman JL, Sintasath L, Moody SE, Cox JD, Ha SI, Belka GK, Golant A, Cardiff RD, and Chodosh LA (2001): c-MYC induces mammary tumorigenesis by means of a preferred pathway involving spontaneous Kras2 mutations. Nat Med 7:235–239.

van Dekken H, Alers JC, Damen IA, Vissers KJ, Krijtenburg PJ, Hoedemaeker RF, Wildhagen MF, Hop WC, van der Kwast TH, Tanke HJ, and Schröder FH (2003): Genetic evaluation of localized prostate cancer in a cohort of forty patients: gain of distal 8q discriminates between progressors and nonprogressors. Lab Invest 83:789–796.

van Dekken H, Paris PK, Albertson DG, Alers JC, Andaya A, Kowbel D, van der Kwast TH, Pinkel D, Schröder FH, Vissers KJ, Wildhagen MF, and Collins C (2004): Evaluation of genetic patterns in different tumor areas of intermediate-grade prostatic adenocarcinomas by high-resolution genomic array analysis. Genes Chromosomes Cancer 39:249–256.

Dhanasekaran SM, Barrette TR, Ghosh D, Shah R, Varambally S, Kurachi K, Pienta KJ, Rubin MA, and Chinnaiyan AM (2001): Delineation of prognostic biomarkers in prostate cancer. Nature 412:822–826.

Di Cristofano A, Pesce B, Cordon-Cardo C, and Pandolfi PP (1998): Pten is essential for embryonic development and tumour suppression. Nat Genet 19:348–355.

van Duin M, van Marion R, Watson JEV, Paris PL, Lapuk A, Brown N, Oseroff VV, Albertson DG, Pinkel D, de Jong P, Nacheva EP, Dinjens W, van Dekken H, and Collins C (2005a): Construction and application of a full-coverage, high-resolution, human chromosome 8q genomic microarray for comparative genomic hybridization. Cytometry A 63A:10–19.

van Duin M, van Marion R, Vissers K, Watson JEV, van Weerden WM, Schröder FH, Hop WCJ, van der Kwast TH, Collins C, and van Dekken H (2005b): High-resolution array comparative genomic hybridization of chromosome arm 8q: evaluation of genetic progression markers for prostate cancer. Genes Chromosomes Cancer 44:438–449.

Dumur CI, Dechsukmun C, Ware JL, Cofield SS, Best AM, Wilkinson DS, Garrett CT, and Ferreira-Gonzalez A (2003): Genome-wide detection of LOH in prostate cancer using human SNP microarray technology. Genomics 81:260–269.

El Gedaily A, Bubendorf L, Willi N, Fu W, Richter J, Moch H, Mihatch MJ, Sauter G, and Gasser TC (2001): Discovery of new DNA amplification loci in prostate cancer by comparative genomic hybridization. Prostate 46:184–190.

Ellwood-Yen K, Graeber TG, Wongvipat J, Iruela-Arispe ML, Zhang JF, Matusik R, Thomas GV, and Sawyers CL (2003): Myc-driven murine prostate cancer shares molecular features with human prostate tumors. Cancer Cell 4:223–238.

Elo JP, Härkönen P, Kyllönen AP, Lukkarinen O, Poutanen M, Vihko R, and Vihko P (1997): Loss of heterozygosity at 16q24.1–q24.2 is significantly associated with metastatic and aggressive behavior of prostate cancer. Cancer Res 57:3356–3359.

Elo JP, Härkönen P, Kyllönen AP, Lukkarinen O, and Vihko P (1999): Three independently deleted regions at chromosome arm 16q in human prostate cancer: allelic loss at 16q24.1–q24.2 is associated with aggressive behaviour of the disease, recurrent growth, poor differentiation of the tumour and poor prognosis for the patient. Br J Cancer 79:156–160.

Evans AJ (2003): Alpha-methylacyl-CoA racemase (P504S): overview and potential uses in diagnostic pathology as applied to prostate needle biopsies. J Clin Pathol 56:892–897.

Fernandez PC, Frank SR, Wang L, Schroeder M, Liu S, Greene J, Cocito A, and Amati B (2003): Genomic targets of the human c-Myc protein. Genes Dev 17:1115–1129.

Finnish Cancer Registry (2005): Cancer in Finland 2002 and 2003: Cancer statistics of the National Research and Development Centre for Welfare and Health.

Fromont G, Chene L, Vidaud M, Vallancien G, Mangin P, Fournier G, Validire P, Latil A, and Cussenot O (2005): Differential expression of 37 selected genes in hormone-refractory prostate cancer using quantitative taqman real-time RT-PCR. Int J Cancer 114:174–181.

Fu XS, Choi E, Bubley GJ, and Balk SP (2004): Identification of hypoxia-inducible factor-1alpha (HIF-1alpha) polymorphism as a mutation in prostate cancer that prevents normoxia-induced degradation. Prostate 63:215–221.

Fujiuchi Y, Nagakawa O, Murakami K, Fuse H, and Saiki I (2003): Effect of hepatocyte growth factor on invasion of prostate cancer cell lines. Oncol Rep 10:1001–1006.

Fukuchi-Shigomori T, Ishii I, Kashiwagi K, Mashiba H, Ekimoto H, and Igarashi K (1997): Malignant transformation by overproduction of translation initiation factor eIF4G. Cancer Res 57:5041–5044.

Gaddipati JP, McLeod DG, Heidenberg HB, Sesterhenn IA, Finger MJ, Moul JW, and Srivastava S (1994): Frequent detection of codon 877 mutation in the androgen receptor gene in advanced prostate cancers. Cancer Res 54:2861–2864.

Gil J, Kerai P, Lleonart M, Bernard D, Cigudosa JC, Peters G, Carnero A, and Beach D (2005): Immortalization of primary human prostate epithelial cells by c-Myc. Cancer Res 65:2179–2185.

Glinsky GV, Glinskii AB, Stephenson AJ, Hoffman RM, and Gerald WL (2004): Gene expression profiling predicts clinical outcome of prostate cancer. J Clin Invest 113:913–923.

Gradin K, McGuire J, Wenger RH, Kvietikova I, Whitelaw, MI, Toftgård R, Tora L, Gassmann M, and Poellinger L (1996): Functional interference between hypoxia and dioxin signal transduction pathways: competition for recruitment of the Arnt transcription factor. Mol Cell Biol 16:5221–5231.

Graff JR, Herman JG, Lapidus RG, Chopra H, Xu R, Jarrard DF, Isaacs WB, Pitha PM, Davidson NE, and Baylin SB (1995): E-cadherin expression is silenced by DNA hypermethylation in human breast and prostate cancer. Cancer Res 55:5195–5199.

Halgren RG, Fielden MR, Fong CJ, and Zacharewski TR (2001): Assessment of clone identity and sequence fidelity for 1189 IMAGE cDNA clones. Nucleic Acid Res 29:582–588.

Hara T, Miyazaki J, Araki H, Yamaoka M, Kanzaki N, Kusaka M, and Miyamoto M (2003): Novel mutations of androgen receptor: a possible mechanism of bicalutamide withdrawal syndrome. Cancer Res 63:149–153.

Härkönen P (2005): 17β-hydroxysteroid dehydrogenases/17-ketosteroid reductases (17HSD/KSRS) in prostate cancer – The role of 17HSD/KSR types 2, 5, and 7 in steroid hormone action and loss of heterozygosity at chromosome region 16q. Oulu University Press, Oulu.

Härkönen P, Törn S, Kurkela R, Porvari K, Pulkka A, Lindfors A, Isomaa V, and Vihko P (2003): Sex hormone metabolism in prostate cancer cells during transition to an androgen independent state. J Clin Endocrinol Metab 88:705–712.

Härkönen P, Kyllönen AP, Nordling S, and Vihko P (2005): Loss of heterozygosity in chromosomal region 16q24.3 associated with progression of prostate cancer. Prostate 62:267–274.

Hawkins GA, Mychaleckyj JC, Zheng SL, Faith DA, Kelly B, Isaacs SD, Wiley KE, Chang BL, Ewing CM, Bujnovszky P, Bleecker ER, Walsh PC, Meyers DA, Isaacs WB, and Xu J (2002): Germline sequence variants of the LZTS1 gene are associated with prostate cancer risk. Cancer Genet Cytogenet 137:1–7.

He WW, Sciavolino PJ, Wing J, Augustus M, Hudson P, Meissner PS, Curtis RT, Shell BK, Bostwick DG, Tindall DJ, Gelmann EP, Abate-Shen C, and Carter KC (1997): A novel human prostate-specific, androgen-regulated homeobox gene (NKX3.1) that maps to 8p21, a region frequently deleted in prostate cancer. Genomics 43:69–77.

Hermans KG, van Alewijk DC, Veltman JA, van Weerden W, van Kessel AG, and Trapman J (2004): Loss of a small region around the *PTEN* locus is a major chromosome 10 alteration in prostate cancer xenografts and cell lines. Genes Chromosome Cancer 39:171–184.

Huang WC, Taylor S, Nguyen TB, Tomaszewski JE, Libertino JA, Malkowicz SB, and McGarvey TW (2002): KIAA1096, a gene on chromosome 1q, is amplified and overexpressed in bladder cancer. DNA Cell Biol 21:707–715.

Huggins C and Hodges CV (1941): Studies on prostatic cancer: I. The effect of castration, of estrogen and of androgen injection on serum phosphatases in metastatic carcinoma of the prostate. Cancer Res 1:293–297.

Hyytinen ER, Frierson HF, Jr., Boyd JC, Chung LW, and Dong JT (1999): Three distinct regions of allelic loss at 13q14, 13q21–22, and 13q33 in prostate cancer. Genes Chromosomes Cancer 25:108–114.

Hyytinen ER, Saadut R, Chen C, Paull L, Koivisto PA, Vessella RL, Frierson HF, Jr., and Dong JT (2002): Defining the region(s) of deletion at 6q16–q22 in human prostate cancer. Genes Chromosomes Cancer 34:306–312.

Iafrate AJ, Feuk L, Rivera MN, Listewnik ML, Donahoe PK, Qi Y, Scherer SW, and Lee C (2004): Detection of large-scale variation in the human genome. Nat Genet 36:949–951.

Ishii H, Baffa R, Numata S-H, Murakumo Y, Rattan S, Inoue H, Mori M, Fidanza V, Alder H, and Croce CM (1999): The *FEZ1* gene at chromosome 8p22 encodes a leucine-zipper protein, and its expression is altered in multiple human tumors. Proc Natl Acad Sci USA 96:3928–3933.

Ishkanian AS, Malloff CA, Watson SK, de Leeuw RJ, Chi B, Coe BP, Snijders A, Albertson DG, Pinkel D, Marra MA, Ling V, MacAulay C, and Lam WL (2004): A tiling resolution DNA microarray with complete coverage of the human genome. Nat Genet 36:299–303.

Ittmann MM and Wieczorek R (1996): Alterations of the retinoblastoma gene in clinically localized, stage B prostate adenocarcinomas. Hum Pathol 27:28–34.

Jenkins RB, Qian J, Lieber MM, and Bostwick DG (1997): Detection of c-myc oncogene amplification and chromosomal anomalies in metastatic prostatic carcinoma by fluorescence in situ hybridization. Cancer Res 57:524–531.

Jiang BH, Semenza GL, Bauer C, and Marti HH (1996): Hypoxia-inducible factor 1 levels vary exponentially over a physiologically relevant range of O2 tension. Am J Physiol 271:C1172–1180.

Jiang Z, Woda BA, Rock KL, Xu Y, Savas L, Khan A, Pihan G, Cai F, Babcook JS, Rathanaswami P, Reed SG, Xu J, and Fanger GR (2001): P504S: a new molecular marker for the detection of prostate carcinoma. Am J Surg Pathol 25:1397–1404.

Jiang Z, Wu CL, Woda BA, Dresser K, Xu J, Fanger GR, and Yang XJ (2002): P504S/alpha-methyl-CoA racemase: a useful marker for diagnosis of small foci of prostatic carcinoma on needle biopsy. Am J Surg Pathol 26:1169–1174.

Jonsson BA, Adami HO, Hägglund M, Bergh A, Göransson I, Stattin P, Wiklund F, and Grönberg H (2004): -160C/A polymorphism in the E-cadherin gene promoter and risk of hereditary, familial and sporadic prostate cancer. Int J Cancer 109:348–352.

Joos S, Bergerheim US, Pan Y, Matsuyama H, Bentz M, du Manoir S, and Lichter P (1995): Mapping of chromosomal losses and gains in prostate cancer by comparative genomic hybridization. Genes Chromosomes Cancer 14:267–276.

Kagan J, Stein J, Babaian RJ, Joe YS, Pisters LL, Glassman AB, von Eschenbach AC, and Troncoso P (1995): Homozygous deletions at 8p22 and 8p21 in prostate cancer implicate these regions as the sites of candidate tumor suppressor genes. Oncogene 11:2121–2126.

Kallio PJ, Wilson WJ, O'Brien S, Makino Y, and Poellinger L (1999): Regulation of the hypoxia-inducible transcription factor 1 alpha by the ubiquitin-proteasome pathway. J Biol Chem 274:6519–6525.

Kallioniemi A, Kallioniemi O-P, Sudar D, Rutowitz D, Gray JW, Waldman F, and Pinkel D (1992): Comparative genomic hybridization for molecular cytogenetic analysis of solid tumors. Science 258:818–821.

Kallioniemi OP, Kallioniemi A, Sudar D, Rutowitz D, Gray JW, Waldman F, and Pinkel D (1993): Comparative genomic hybridization: a rapid new method for detecting and mapping DNA amplifications in tumors. Semin Cancer Biol 4:41–46.

Kasahara K, Taguchi T, Yamasaki I, Kamada M, Yuri K, and Shuin T (2002): Detection of genetic alterations in advanced prostate cancer by comparative genomic hybridization. Cancer Genet Cytogenet 137:59–63.

Kim MJ, Cardiff RD, Desai N, Banach-Petrosky WA, Parsons R, Shen MM, and Abate-Shen C (2002a): Cooperativity of *Nkx3.1* and *Pten* loss of function in a mouse model of prostate carcinogenesis. Proc Natl Acad Sci USA 99:2884–2889.

Kim MJ, Bhatia-Gaur R, Banach-Petrosky WA, Desai N, Wang Y, Hayward SW, Cunha GR, Cardiff RD, Shen MM, and Abate-Shen C (2002b): Nkx3.1 mutant mice recapitulate early stages of prostate cancer carcinogenesis. Cancer Res 62: 2999–3004.

Kirchhofer D, Peek M, Lipari MT, Billeci K, Fan B, and Moran P (2005): Hepsin activates pro-hepatocyte growth factor and is inhibited by hepatocyte growth factor inhibitor-1B (HAI-1B) and HAI-2. FEBS Lett 579:1945–1950.

Kittler R, Putz G, Pelletier L, Poser I, Heninger AK, Drechsel D, Fischer S, Konstantinova I, Habermann B, Grabner H, Yaspo ML, Himmelbauer H, Korn B, Neugebauer K, Pisabarro MT, and Buchholz F (2004): An endoribonuclease-prepared siRNA screen in human cells identifies genes essential for cell division. Nature 432:1036–1040.

Klezovitch O, Chevillet J, Mirosevitch J, Roberts RL, Matusik RJ, and Vasioukhin V (2004): Hepsin promotes prostate cancer progression and metastasis. Cancer Cell 6:185–195.

Kononen J, Bubendorf L, Kallioniemi A, Bärlund M, Schraml P, Leighton S, Torhorst J, Mihatsch MJ, Sauter G, and Kallioniemi O-P (1998): Tissue microarrays for high-throughput molecular profiling of tumor specimens. Nat Med 4:844–847.

Kuefer R, Varambally S, Zhou M, Lucas PC, Loeffler M, Wolter H, Mattfeldt T, Hautmann RE, Gschwend JE, Barrette TR, Dunn RL, Chinnaiyan AM, and Rubin MA (2002): Alpha-Methylacyl-CoA racemase: expression levels of this novel cancer biomarker depend on tumor differentiation. Am J Pathol 161:841–848.

Kuzmichev A, Jenuwein T, Tempst P, and Reinberg D (2004): Different EZH2-containing complexes target methylation of histone H1 or nucleosomal histone H3. Mol Cell 23:183–193.

Kuzmichev A, Margueron R, Vaquero A, Preissner TS, Scher M, Kirmizis A, Ouyang X, Brockdorff N, Abate-Shen C, Farnham P, and Reinberg D (2005): Composition and histone substrates of polycomb repressive complexes change during cellular differentiation, Proc Natl Acad Sci USA 102:1859–1864.

Kwabi-Addo B, Giri D, Schmidt K, Podsypanina K, Parsons R, Greenberg N, and Ittmann M (2001): Haploinsufficiency of the Pten tumor suppressor gene promotes prostate cancer progression. Proc Natl Acad Sci USA 98:11563–11568.

Laitinen S, Karhu R, Sawyers CL, Vessella RL, and Visakorpi T (2002): Chromosomal aberrations in prostate cancer xenografts detected by comparative genomic hybridization. Genes Chromosomes Cancer 35:66–73.

Lapointe J, Li C, Higgins JP, van de Rijn M, Bair E, Montgomery K, Ferrari M, Egevald L, Rayford W, Bergerheim U, Ekman P, DeMarzo AM, Tibshirani R, Botstein D, Brown PO, Brooks JD, and Pollack JR (2004): gene expression profiling identifies clinically relevant subtypes of prostate cancer. Proc Natl Acad Sci USA 101:811–816.

Latil A, Cussenot O, Fournier G, Driouch K, and Lidereau R (1997): Loss of heterozygosity at chromosome 16q in prostate cancer. Cancer Res 57:1058–1062.

Latil A, Bieche I, Pesche S, Volant A, Valeri A, Fournier G, Cussenot O, and Lidereau R (1999): Loss of heterozygosity at chromosome arm 13q and RB1 status in human prostate cancer. Hum Pathol 30:809–815.

Lavigne JA, Helzlsouer KJ, Huang HY, Strickland PT, Bell DA, Slmin O, Watson MA, Hoffman S, Comstock GW, and Yager JD (1997): An association between the allele

coding for a low activity variant of catechol-O-methyltransferase and the risk for breast cancer. Cancer Res 57:5493–5497.

Lazaris-Karatzas A and Sonenberg N (1992): The mRNA 5’ cap-binding protein, eIF-4E, cooperates with v-myc or E1A in the transformation of primary rodent fibroblasts. Mol Cell Biol 12:1234–1238.

Lazaris-Karatzas A, Montine KS, and Sonenberg N (1990): Malignant transformation by a eukaryotic initiation factor subunit that binds to mRNA 5' cap. Nature 345:544–547.

Lee SH, Shin MS, Kim HS, Lee HK, Park WS, Kim SY, Lee JH, Han SY, Park JY, Oh RR, Jang JJ, Han JY, Lee JY, and Yoo NJ (1999): Alterations of the DR5/TRAIL receptor 2 gene in non-small cell lung cancers. Cancer Res 59:5683–5686.

Lei M (2005): The MCM complex: its role in DNA replication and implications for cancer therapy. Curr Cancer Drug Targets 5:365–380.

Lei M and Tye BK (2001): Initiating DNA synthesis: from recruiting to activating the MCM complex. J Cell Sci 114:1447–1454.

Leube B, Drechsler M, Mühlman K, Schäfer R, Schulz W, Santourlidis S, Anastasiadis A, Ackermann R, Visakorpi T, Müller W, and Royer-Pokora B (2002): Refined mapping of allele loss at chromosome 10q23–26 in prostate cancer. Prostate 50:135–144.

Li C, Larsson C, Futreal A, Lancaster J, Phelan C, Aspenblad U, Sundelin B, Liu Y, Ekman P, Auer G, and Bergerheim US (1998): Identification of two distinct deleted regions on chromosome 13 in prostate cancer. Oncogene 16:481–487.

Li LC, Zhao H, Nakajima K, Oh BR, Ribeiro Filho LA, Carroll P, and Dahiya R (2001): Methylation of the E-cadherin gene promoter correlates with progression of prostate cancer. J Urol 166:705–709.

Li LC, Carroll PR, and Dahiya R (2005): Epigenetic changes in prostate cancer: implication for diagnosis and treatment. J Natl Cancer Inst 97:103–115.

Lin B, Ferguson C, White JT, Wang S, Vessella R, True LD, Hood L, and Nelson PS (1999): Prostate-localized and androgen-regulated expression of the membrane-bound serine protease *TMPRSS2*. Cancer Res 59:4180–4184.

Lindblad-Toh K, Tanenbaum DM, Daly MJ, Winchester E, Lui W-O, Villapakkam A, Stanton SE, Larsson C, Hudson TJ, Johnson BE, Lander ES, and Meyerson M (2000): Loss-of-heterozygosity analysis of small-cell lung carcinomas using single-nucleotide polymorphism arrays. Nat Biotechnol 18:1001–1005.

Linja MJ and Visakorpi T (2004): Alterations of androgen receptor in prostate cancer. J Steroid Biochemistry Mol Biol 92:255–264.

Linja MJ, Savinainen KJ, Saramäki OR, Tammela T, Vessella RL, and Visakorpi T (2001): Amplification and overexpression of androgen receptor gene in hormone-refractory prostate cancer. Cancer Res 61:3550–3555.

Linja MJ, Porkka KP, Kang Z, Savinainen KJ, Jänne OA, Tammela TLJ, Vessella RL, Palvimo JJ, and Visakorpi T (2004): Expression of androgen receptor coregulators in prostate cancer. Clin Cancer Res 10:1032–1040.

Lockhart DJ, Dong H, Byrne MC, Follettie MT, Gallo MV, Chee MS, Mittmann M, Wang C, Kobayashi M, Horton H, and Brown EL (1996): Expression monitoring by hybridization to high-density oligonucleotide arrays. Nat Biotechnol 14:1675–1680.

Lucito R, West J, Reiner A, Alexander J, Esposito D, Mishra B, Powers S, Norton L, and Wigler M (2000): Detecting gene copy number fluctuations in tumor cells by microarray analysis of genomic representations. Genome Res 10:1726–1736.

Lucito R, Healy J, Alexander J, Reiner A, Esposito D, Chi M, Rodgers L, Brady A, Sebat J, Troge J, West JA, Rostan S, Nguyen KCQ, Powers S, Ye KQ, Olshen A, Venkatraman E, Norton L, and Wigler M (2003): Representational oligonucleotide

microarray analysis: a high-resolution method to detect genome copy number variation. Genome Res 13:2291–2305.

Lundgren R, Mandahl N, Heim S, Limon J, Henrikson H, and Mitelman F (1992): Cytogenetic analysis of 57 primary prostatic adenocarcinoma. Genes Chromosomes Cancer 4:16–24.

Luo J, Duggan DJ, Chen Y, Sauvageot J, Ewing CM, Bittner ML, Trent JM, and Isaacs WB (2001): Human prostate cancer and benign prostatic hyperplasia: molecular dissection by gene expression profiling. Cancer Res 61:4683–4688.

Luo J-H, Yu YP, Cieply K, Lin F, Deflavia P, Dhir R, Finkelstein S, Michalopoulos G, and Becich M (2002): Gene expression analysis of prostate cancers. Mol Carcinogenesis 33:25–35.

Macoska JA, Trybus TM, Benson PD, Sakr WA, Grignon DJ, Wojno KD, Petruk T, and Powell IJ (1995): Evidence for three tumor suppressor gene loci on chromosome 8p in human prostate cancer. Cancer Res 55:5390–5395.

Macville M, Veldman T, Padilla-Nash H, Wangsa D, O'Brien P, Schröck E, and Ried T (1997): Spectral karyotyping, a 24-colour FISH technique for the identification of chromosomal rearrangements. Histochem Cell Biol 108:299–305.

Magee JA, Araki T, Patil S, Ehrig T, True L, Humphrey PA, Catalona WJ, Watson MA, and Milbrandt J (2001): Expression profiling reveals hepsin overexpression in prostate cancer. Cancer Res 61:5692–5696.

Magee JA, Abdulkadir SA, and Milbrandt J (2003): Haploinsufficiency at the Nkx3.1 locus: A paradigm for stochastic, dosage-sensitive gene regulation during tumor initiation. Cancer Cell 3:273–283.

Mai S and Mushinski JF (2003): c-Myc-induced genomic instability. J Environ Pathol Toxicol Oncol 22:179–199.

du Manoir S, Schrok E, Bentz M, Speicher MR, Joos S, Ried T, Lichter P, and Cremer T (1995): Quantitative analysis of comparative genomic hybridization. Cytometry 19:27–41.

Marcelli M, Ittmann M, Mariani S, Sutherland R, Nigam R, Murthy L, Zhao Y, DiConcini D, Puxeddu E, Esen A, Eastham J, Weige NL, and Lamb DJ (2000): Androgen receptor mutations in prostate cancer. Cancer Res 60:944–949.

Maruyama R, Toyooka S, Toyooka KO, Virmani AK, Zochbauer-Muller S, Farinas AJ, Minna JD, McDonnell J, Frenkel EP, and Gazdar AF (2002): Aberrant promoter methylation profile of prostate cancers and its relationship to clinicopathological features. Clin Cancer Res 8:514–519.

Mukherjee S, Tamayo P, Rogers S, Rifkin Engle A, Campbell C, Golub TR, and Mesirov JP (2003): Estimating dataset size requirements for classifying DNA microarray data. J Comput Biol 10:119–142.

de Nooij-van Dalen AG, van Buuren-van Seggelen VH, Lohman PH, and Giphart-Gassler M (1998): Chromosome loss with concomitant duplication and recombination both contribute most to loss of heterozygosity in vitro. Genes Chromosomes Cancer 21:30–38.

Nupponen NN, Hyytinen ER, Kallioniemi AH, and Visakorpi T (1998a): Genetic alterations in prostate cancer cell lines detected by comparative genomic hybridization. Cancer Genet Cytogenet 101:53–57.

Nupponen NN, Kakkola L, Koivisto P, and Visakorpi T (1998b): Genetic alterations in hormone-refractory recurrent prostate carcinomas. Am J Pathol 153:141–148.

Nupponen NN, Porkka K, Kakkola L, Tanner M, Persson K, Borg Å, Isola J, and Visakorpi T (1999): Amplification and overexpression of p40 subunit of eukaryotic translation initiation factor 3 in breast and prostate cancer. Am J Pathol 154:1777–1783.

Oikawa T and Yamada T (2003): Molecular biology of the Ets family of transcription factors. Gene 303:11–34.

Okamoto H, Yasui K, Zhao C, Arii S, and Inazawa J (2003): PTK2 and EIF3S3 genes may be amplification targets at 8q23–q24 and are associated with large hepatocellular carcinomas. Hepatology 38:1242–1249.

Ornstein DK, Cinquanta M, Weiler S, Duray PH, Emmert-Buck MR, Vocke CD, Linehan WM, and Ferretti JA (2001): Expression studies and mutational analysis of the androgen regulated homeobox gene NKX3.1 in benign and malignant prostate epithelium. J Urol 165:1329–1334.

Özören N and El-Deiry WS (2003): Cell surface Death Receptor signaling in normal and cancer cells. Semin Cancer Biol 13:135–147.

Padmanabhan V, Callas P, Philips G, Trainer TD, and Beatty BG (2004): DNA replication regulation protein Mcm7 as a marker of proliferation in prostate cancer. J Clin Pathol 57:1057–1062.

Pai SI, Wu GS, Özören N, Wu L, Jen J, Sidransky D, and El-Deiry WS (1998): Rare loss-of-function mutation of a death receptor gene in head and neck cancer. Cancer Res 58:3513–3518.

Palmberg C, Koivisto P, Kakkola L, Tammela TL, Kallioniemi O-P, and Visakorpi T (2000): Androgen receptor gene amplification at primary progression predicts response to combined androgen blockade as second line therapy for advanced prostate cancer. J Urol 164:1992–1995.

Pan Y, Kytölä S, Farnebo F, Wang N, Lui WO, Nupponen N, Isola J, Visakorpi T, Bergerheim US, and Larsson C (1999): Characterization of chromosomal abnormalities in prostate cancer cell lines by spectral karyotyping. Cytogenet Cell Genet 87:225–232.

Pan Y, Lui W-O, Nupponen N, Larsson C, Isola J, Visakorpi T, Bergerheim USR, and Kytölä S (2001): 5q11, 8p11, and 10q22 are recurrent chromosomal breakpoints in prostate cancer cell lines. Genes Chromosomes Cancer 30:187–195.

Paris PL, Albertson DG, Alers JC, Andaya A, Carroll P, Fridlyand J, Jain AN, Kamkar S, Kowbel D, Krijtenburg P-J, Pinkel D, Schröder FH, Vissers KJ, Watson JEV, Wildhagen MF, Collins C, and van Dekken H (2003): High-resolution analysis of paraffin-embedded and formalin-fixed prostate tumors using comparative genomic hybridization to genomic microarrays. Am J Pathol 162:763–770.

Paris PL, Andaya A, Fridlyand J, Jain AN, Weinberg V, Kowbel D, Brebner JH, Simko J, Watson JEV, Volik S, Albertson DG, Pinkel D, Alers JC, van der Kwast TH, Vissers KJ, Schröder FH, Wildhagen MF, Febbo PG, Chinnaiyan AM, Pienta KJ, Carroll PR, Rubin MA, Collins C, and van Dekken H (2004): Whole genome scanning identifies genotypes associated with recurrence and metastasis in prostate tumors. Hum Mol Genet 13:1303–1313.

Park J, Song S-H, Kim TY, Choi M-C, Jong H-S, Kim T-Y, Lee JW, Kim NK, Kim W-H, and Bang Y-J (2004): Aberrant methylation of integrin α4 gene in human gastric cancer cells. Oncogene 23:3474–3480.

Park WS, Lee JH, Shin MS, Park JY, Kim HS, Kim YS, Park CH, Lee SK, Lee SH, Lee SN, Kim H, Yoo NJ, and Lee JY (2001): Inactivating mutations of KILLER/DR5 gene in gastric cancers. Gastroenterology 121:1219–1225.

Patel JH, Loboda AP, Showe MK, Showe LC, and McMahon SB (2004): Analysis of genomic targets reveals functions of MYC. Nat Rev Cancer 4:562–568.

Phillips JL, Hayward SW, Wang Y, Vasselli J, Pavlovich C, Padilla-Nash H, Pezullo JR, Ghadimi BM, Grossfeld GD, Rivera A, Linehan WM, Cunha GR, and Ried T (2001): The consequences of chromosomal aneuploidy on gene expression profiles in a cell line model for prostate carcinogenesis. Cancer Res 61:8143–8149.

Pinkel D, Segraves R, Sudar D, Clark S, Poole I, Kowbel D, Collins C, Kuo W-L, Chen C, Zhai Y, Dairkee SH, Ljung B-M, Gray JW, and Albertson DG (1998): High resolution analysis of DNA copy number variation using comparative genomic hybridization to microarrays. Nat Genet 20:207–211.

Podsypanina K, Ellenson LH, Nemes A, Gu J, Tamura M, Yamada KM, Cordon-Cardo C, Catoretti G, Fisher PE, and Parsons R (1999): Mutation of Pten/Mmac1 in mice causes neoplasia in multiple organ systems. Proc Natl Acad Sci USA 96:1563–1568.

Pollack JR, Perou CM, Alizadeh AA, Eisen MB, Pergamenschikov A, Williams CF, Jeffrey SS, Botstein D, and Brown PO (1999): Genome-wide analysis of DNA copy-number changes using cDNA microarrays. Nat Genetics 23:41–46.

Porkka K, Saramäki O, Tanner M, and Visakorpi T (2002): Amplification and overexpression of Elongin C gene discovered in prostate cancer by cDNA microarrays. Lab Invest 82:629–637.

Porkka KP, Tammela TLJ, Vessella RL, and Visakorpi T (2004): *RAD21* and *KIAA0196* at 8q24 are amplified and overexpressed in prostate cancer. Genes Chromosomes Cancer 39:1–10.

Potter CP and Harris AL (2003): Diagnostic, prognostic and therapeutic implications of carbonic anhydrases in cancer. Br J Cancer 89:2–7.

Prochownik EV, Eagle Grove L, Deubler D, Zhu XL, Stephenson RA, Rohr LR, Yin X, and Brothman AR (1998): Commonly occurring loss and mutation of the MXI1 gene in prostate cancer. Genes Chromosomes Cancer 22:295–304.

Reiter RE, Gu Z, Watabe T, Thomas G, Szigeti Kinga, Davis E, Wahl M, Nisitani S, Yamashiro J, Le Beau MM, Loda M, and Witte ON (1998): Prostate stem cell antigen: a cell surface marker overexpressed in prostate cancer. Proc Natl Acad Sci USA 95:1735–1740.

Ren B, Yu G, Tseng GC, Cieply K, Gavel T, Nelson J, Michalopoulos G, Yu YP, and Luo J-H (2005): MCM7 amplification and overexpression are associated with prostate cancer progression. Oncogene 25:1090–1098.

Rhodes DR, Sanda MG, Otte AP, and Chinnaiyan AM (2003): Multiple biomarker approach for determining risk of prostate-specific antigen-defined recurrence of prostate cancer. J Natl Cancer Inst 95:661–667.

Rothe M, Ko Y, Albers P, and Wernert N (2000): Eukaryotic initiation factor 3 p110 mRNA is overexpressed in testicular seminomas. Am J Pathol 157:1597–1604.

Rubin MA, Zhou M, Dhanasekaran SM, Varambally S, Barrette TR, Sanda MG, Pienta KJ, Ghosh D, and Chinnaiyan AM (2002): α-methyl coenzymes A racemase as a tissue biomarker for prostate cancer. JAMA 287:1662–1670.

Rubin MA, Varambally S, Beroukhim R, Tomlins SA, Rhodes DR, Paris PL, Hofer MD, Storz-Schweizer M, Kuefer R, Fletcher JA, Hsi BL, Byrne JA, Pienta KJ, Collins C, Sellers WR, and Chinnaiyan AM (2004): Overexpression, amplification, and androgen regulation of TPD52 in prostate cancer. Cancer Res 64:3814–3822.

Sakai Y, Saijo M, Coelho K, Kishino T, Niikawa N, and Taya Y (1995): cDNA sequence and chromosomal localization of a novel human protein RBQ-1 (RBBP6), that binds to the retinoblastoma gene product. Genomics 30:98–101.

Santarosa M and Ashworth A (2004):Haploinsufficiency for tumour suppressor genes: when you don't need to go all the way. Biochim Biophys Acta 1654:105–122.

Savinainen KJ, Linja MJ, Saramäki OR, Tammela TLJ, Chang GTG, 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.

Savinainen KJ, Helenius MA, Lehtonen HJ, and Visakorpi T (2006): Overexpression of EIF3S3 promotes cancer cell growth. Prostate (Published Online: May 1 2006).

Schena M, Shalon D, Davis RW, and Brown PO (1995): Quantitative monitoring of gene expression patterns with a complementary DNA microarray. Science 270:467–470.

Schena M, Shalon D, Heller R, Chai A, Brown PO, and Davis RW (1996): Parallel human genome analysis: microarray-based expression monitoring of 1000 genes. Proc Natl Acad Sci USA 93:10614–10619.

Schröck E, du Manoir S, Veldman T, Schoell B, Wienberg J, Ferguson-Smith MA, Ning Y, Ledbetter DH, Bar-Am I, Soenksen D, Garini Y, and Ried T (1996): Multicolor spectral karyotyping of human chromosomes. Science 273:494–497.

Sebat J, Lakshmi B, Troge J, Alexander J, Young J, Lundin P, Månér S, Massa H, Walker M, Chi M, Navin N, Lucito R, Healy J, Hicks J, Ye K, Reiner A, Gilliam TC, Trask B, Patterson N, Zetterberg A, and Wigler M (2004): Large-scale copy number polymorphism in the human genome. Science 305:525–528.

Semenza GL (1999): Regulation of mammalian O2 homeostasis by hypoxia-inducible factor 1. Annu Rev Cell Dev Biol 15:551–578.

Semplici F, Meggio F, Pinna LA, and Olivieiro S (2002): CK2-dependent phosphorylation of the E2 ubiquitin conjugating enzyme UBC3B induces interaction with beta-TrCP and enhances beta-catenin degradation. Oncogene 21:3978–3987.

Skacel M, Ormsby AH, Pettay JD, Tsiftsakis EK, Liou LS, Klein EA, Levin HS, Zippe CD, and Tubbs RR (2001): Aneusomy of chromosomes 7, 8, and 17 and amplification of HER-2/neu and epidermal growth factor receptor in Gleason score 7 prostate carcinoma: a differential fluorescent in situ hybridization study of Gleason pattern 3 and 4 using tissue microarray. Hum Pathol 32:1392–1397.

Snijders AM, Nowak N, Segraves R, Blackwood S, Brown N, Conroy J, Hamilton G, Hindle AK, Huey B, Kimura K, Law S, Myambo K, Palmer J, Ylstra B, Yue JP, Gray JW, Jain AN, Pinkel D, and Albertson DG (2001): Assembly of microarrays for genome-wide measurement of DNA copy number. Nat Genet 29:263–264.

Sobel RE and Sadar MD (2005a): Cell lines used in prostate cancer research: a compendium of old and new lines — part 1. J Urol 173:342–359.

Sobel RE and Sadar MD (2005b): Cell lines used in prostate cancer research: a compendium of old and new lines — part 2. J Urol 173:360–372.

Solinas-Toldo S, Lampel S, Stilgenbauer S, Nickolenko J, Benner A, Döhner H, Cremer T, and Lichter P (1997): Matrix-based comparative genomic hybridization: biochips to screen for genomic inbalances. Genes Chromosomes Cancer 20:399–407.

Speicher MR, Gwyn Ballard S, and Ward DC (1996): Karyotyping human chromosomes by combinatorial multi-color FISH. Nat Genet 4:368–375.

Srikantan V, Sesterhenn IA, Davis L, Hankins GR, Avallone FA, Livezey JR, Connelly R, Mostofi FK, McLeod DG, Moul JW, Chandrasekharappa SC, and Srivastava S (1999): Allelic loss on chromosome 6q in primary prostate cancer. Int J Cancer 84:331–335.

Srikantan V, Valladares M, Rhim JS, Moul JW, and Srivastava S (2002): *HEPSIN* inhibits cell growth/invasion in prostate cancer cells. Cancer Res 62:6812–6816.

Su I, Dobenecker M-W, Dickinson E, Oser M, Basavaraj A, Marqueron R, Viale A, Reinberg D, Wülfing C, and Tarakhovsky A (2005): Polycomb group protein Ezh2 controls actin polymerization and cell signaling. Cell 121:425–436.

Sudo T, Utsunomiya T, Mimori K, Nagahara H, Ogawa K, Inoue H, Wakiyama S, Fujita H, Shirouzu K, and Mori M (2005): Clinicopathological significance of EZH2 mRNA expression in patients with hepatocellular carcinoma. Br J Cancer 92:1745–1748.

Suzuki H, Komiya A, Emi M, Kuramochi H, Shiraishi T, Yatani R, and Shimazaki J (1996): Three distinct commonly deleted regions of chromosome arm 16q in human primary and metastatic prostate cancers. Genes Chromosomes Cancer 17:225–233.

Suzuki H, Freije D, Nusskem DR, Okami K, Cairns P, Sidransky D, Isaacs WB, and Bova GS (1998): Interfocal heterogeneity of PTEN/MMAC1 gene alterations in multiple metastatic prostate cancer tissues. Cancer Res 58:204–209.

Taj MM, Tawil RJ, Engstrom LD, Zeng Z, Hwang C, Sanda MG, and Wechsler DS (2001): Mxi1, a MYC antagonist, suppresses proliferation of DU145 human prostate cells. Prostate 47:194–204.

Taplin ME, Bubley GJ, Shuster TD, Frantz ME, Spooner AE, Ogata GK, Keer HN, and Balk SP (1995): Mutation of the androgen-receptor gene in metastatic androgen-independent prostate cancer. N Engl J Med 332:1393–1398.

Taplin ME, Bubley GJ, Ko YJ, Small EJ, Upton M, Rajeshkumar B, and Balk SP (1999): Selection for androgen receptor mutations in prostate cancers treated with androgen antagonist. Cancer Res 59:2511–2515.

Taylor E, Codgell D, Coombes K, Hu L, Ramdas L, Tabor A, Hamilton S, and Zhang W (2001): Sequence verification as quality-control step for production of cDNA microarrays. Biotechniques 31:62–65.

Tilley WD, Buchanan G, Hickey TE, and Bentel JM (1996): Mutations in the androgen receptor gene are associated with progression of human prostate cancer to androgen independence. Clin Cancer Res 2:277–285.

Tomlins SA, Rhodes DR, Perner S, Dhanasekaran SM, Mehra R, Sun X-W, Varambally S, Cao X, Tchinda J, Kuefer R, Lee C, Montie JE, Shah RB, Pienta KJ, Rubin MA, and Chinnaiyan AM (2005): Recurrent fusion of TMPRSS2 and ETS transcription factor genes in prostate cancer. Science 310:644–648.

Tricoli JV, Gumerlock PH, Yao JL, Chi SG, D'Souza SA, Nestok BR, and de Vere White RW (1996): Alterations of the retinoblastoma gene in human prostate adenocarcinoma. Genes Chromosomes Cancer 15:108–114.

Trotman LC, Niki M, Dotan ZA, Koutcher JA, Di Cristofano A, Xiao A, Khoo AS, Roy-Burman P, Greenberg NM, Van Dyke T, Cordon-Cardo C, and Pandolfi PP (2003): Pten dose dictates cancer progression in the prostate. PloS Biol 1:E59.

Tsuchiya N, Kondo Y, Takahashi A, Pawar H, Qian J, Sato K, Lieber MM, and Jenkins RB (2002): Mapping and gene expression profile of the minimally overrepresented 8q24 region in prostate cancer. Am J Pathol 160:1799–1806.

Tsujimoto Y, Tomita Y, Hoshida Y, Kono T, Oka T, Yamamoto S, Nonomura N, Okuyama A, and Aozasa K (2004): Elevated expression of valosin-containing protein (p97) is associated with poor prognosis of prostate cancer. Clin Cancer Res 10:3007–3012.

Umbas R, Schalken JA, Aalders TW, Carter BS, Karthaus HF, Schaafsma HE, Debruyne FM, and Isaacs WB (1992): Expression of the cellular adhesion molecule E-cadherin is reduced or absent in high-grade prostate cancer. Cancer Res 52:5104–5109.

Umbas R, Isaacs WB, Bringuier PP, Schaafsma HE, Karthaus HF, Oosterhof GO, Debruyne FM, and Schalken JA (1994): Decreased E-cadherin expression is associated with poor prognosis in patients with prostate cancer. Cancer Res 54:3929–3933.

Vanaja DK, Cheville JC, Iturria SJ, and Young CYF (2003): Transcriptional silencing of zinc finger protein 185 identified by expression profiling is associated with prostate cancer progression. Cancer Res 63:3877–3882.

Varambally S, Dhanasekaran SM, Zhou M, Barrette TR, Kumar-Sinha C, Sanda MG, Ghosh D, Pienta KJ, Sewalt RGAB, Otte A, Rubin MA, and Chinnaiyan AM (2002): The polycomb group protein EZH2 is involved in progression of prostate cancer. Nature 419:624–629.

Varella-Garcia M, Gemmill RM, Rabenhorst SH, Lotto A, Drabkin HA, Archer PA, and Franklin WA (1998): Chromosomal duplication accompanies allelic loss in non-small cell lung carcinoma. Cancer Res 58:4701–4707.

Vasioukhin V (2004): Hepsin paradox reveals unexpected complexity of metastatic process. Cell Cycle 3:1394–1397.

Veldscholte J, Ris-Stalpers C, Kuiper GG, Jenster G, Berrevoets C, Claassen E, van Rooij HC, Trapman J, Brinkmann AO, and Mulder E (1990): A mutation in the ligand binding domain of the androgen receptor of human LNCaP cells affects steroid binding characteristics and response to anti-androgens. Biochem Biophys Res Commun 173:534–544.

Vire E, Brenner C, Deplus R, Blanchon L, Fraga M, Didelot C, Morey L, Van Eynde A, Bernard D, Vanderwinden JM, Bollen M, Esteller M, Di Croce L, de Launoit Y, and Fuks F (2006): The polycomb group protein EZH2 directly controls DNA methylation. Nature 439:871–874.

Visakorpi T, Hyytinen E, Koivisto P, Tanner M, Keinänen R, Palmberg C, Palotie A, Tammela T, Isola J, and Kallioniemi OP (1995a): *In vivo* amplification of the androgen receptor gene and progression of prostate cancer. Nat Genet 9:401–406.

Visakorpi T, Kallioniemi AH, Syvänen A-C, Hyytinen ER, Karhu R, Tammela T, Isola JJ, and Kallioniemi O-P (1995b): Genetic changes in primary and recurrent prostate cancer by comparative genomic hybridization. Cancer Res 55:342–347.

Vocke CD, Pozzatti RO, Bostwick DG, Florence CD, Jennings SB, Strup SE, Duray PH, Liotta LA, Emmert-Buck MR, and Linehan WM (1996): Analysis of 99 microdissected prostate carcinomas reveals a high frequency of allelic loss on chromosome 8p12–21. Cancer Res 56:2411–2416.

Voeller HJ, Augustus M, Madike V, Bova GS, Carter KC, and Gelmann EP (1997): Coding region of NKX3.1, a prostate-specific homeobox gene on 8p21, is not mutated in human prostate cancer. Cancer Res 57:4455–4459.

Wallén MJ, Linja M, Kaartinen K, Schleutker J, and Visakorpi T (1999): Androgen receptor gene mutations in hormone-refractory prostate cancer. J Pathol 189:559–563.

Waltering KK, Wallén MJ, Tammela TLJ, Vessella RL, and Visakorpi T (2006): Mutation screening of the androgen receptor promoter and untranslated regions in prostate cancer. Prostate (in press).

Wang DG, Fan J-B, Siao C-J, Berno A, Young P, Sapolsky R, Ghandour G, Perkins N, Winchester E, Spencer J, Kruglyak L, Stein l, Hsie L, Topaloglou T, Hubbell E, Robinson E, Mittmann M, Morris MS, Shen N, Kilburn D, Rioux J, Nusbaum C, Rozen S, Hudson TJ, Lipshutz R, Chee M, and Lander ES (1998): Large-scale identification, mapping, and genotyping of single-nucleotide polymorphisms in the human genome. Science 280:1077–1082.

Wang R, Xu J, Saramäki O, Visakorpi T, Sutherland WM, Zhou J, Sen B, Lim SD, Mabjeesh N, Amin M, Dong J-T, Petros JA, Nelson PS, Marshall FF, Zhau HE, and Chung LWK (2004): *PrLZ*, a novel prostate-specific and androgen–responsive gene of the TPD52 family, amplified in chromosome 8q21.1 and overexpressed in human prostate cancer. Cancer Res 64:1589–1594.

Watson JEV, Doggett NA, Albertson DG, Andaya A, Chinnaiyan AM, van Dekken H, Ginzinger D, Haqq C, James K, Kamkar S, Kowbel D, Pinkel D, Schmitt L, Simko JP, Volik S, Winberg VK, Paris PL, and Collins C (2004): Integration of high-resolution array comparative genomic hybridization analysis of chromosome 16q with expression array data refines common regions of loss at 16q23–qter and identifies underlying candidate tumor suppressor genes in prostate cancer. Oncogene 23:3487–3494.

Welsh JB, Sapinoso LM, Su AI, Kern SG, Wang-Rodriguez J, Moskaluk CA, Frierson HF, Jr., and Hampton GM (2001): Analysis of gene expression identifies candidate markers and pharmacological targets in prostate cancer. Cancer Res 61:5974–5978.

White VA, McNeil BK, and Horsman DE (1998): Acquired homozygosity (isodisomy) of chromosome 3 in uveal melanoma. Cancer Genet Cytogenet 102:40–45.

Williams BJ, Jones E, Kozlowski JM, Vessella RL, and Brothman AR (1997): Comparative genomic hybridization and molecular cytogenetic characterization of two prostate cancer xenografts. Genes Chromosomes Cancer 18:299–304.

Williams K, Fernandez S, Stien X, Ishii K, Love HD, Lau Y-F, Roberts RL, and Hayward SW (2005): Unopposed c-MYC expression in benign prostatic epithelium causes a cancer phenotype. Prostate 63:369–384.

Wolf M, Mousses S, Hautaniemi S, Karhu R, Huusko P, Allinen M, Elkalhoun A, Monni O, Chen Y, Kallioniemi A, and Kallioniemi O-P (2004): High-resolution analysis of gene copy number alterations in human prostate cancer using CGH on cDNA microarrays: impact of copy number on gene expression. Neoplasia 6:240–247.

Xu L, Tan AC, Naiman DQ, Geman D, and Winslow RL (2005): Robust prostate cancer marker genes emerge from direct integration of inter-study microarray data. Bioinformatics 21:3905–3911.

Xu J, Stolk JA, Zhang X, Silva SJ, Houghton RL, Matsumura M, Vedvick TS, Leslie KB, Badaro R, and Reed SG (2000): Identification of differentially expressed genes in human prostate cancer using subtraction and microarray. Cancer Res 60:1677–1682.

Yang XJ, Laven B, Tretiakova M, Blute RD, Jr., Woda BA, Steinberg GD, and Jiang Z (2003): Detection of alpha-methylacyl-CoA racemase in postradiation prostatic adenocarcinoma. Urology 62:282–286.

Yano S, Matsuyama H, Matsuda K, Matsumoto H, Yoshihiro S, and Naito K (2004): Accuracy of an array comparative genomic hybridisation (CGH) technique in detecting DNA copy number aberrations: comparison with conventional CGH and loss of heterozygosity analysis in prostate cancer. Cancer Genet Cytogenet 150:122–127.

Yu YP, Landsittel D, Jing l, Nelson J, Ren B, Liu L, McDonald C, Thomas R, Dhir R, Finkelstein S, Michalopoulos G, Becich M, and Luo J-H (2004): Gene expression alterations in prostate cancer predicting tumor aggression and preceding development of malignancy. J Clin Oncol 22:2790–2799.

Zervos AS, Gyuris J, and Brent R (1993): Mxi1, a protein that specifically interacts with Max to bind Myc-Max recognition sites. Cell 72:223–232.

Zha S, Ferdinandusse S, Denis S, Wanders RJ, Ewing CM, Luo J, De Marzo AM, and Isaacs WB (2003): Alpha-methylacyl-CoA racemase as an androgen-independent growth modifier in prostate cancer. Cancer Res 63:7365–7376.

Zhang Y, Lu H, Dazin P, and Kapila Y (2004): Functional differences between integrin alpha4 and integrins alpha5/alphav in modulating the motility of human oral squamous carcinoma cells in response to the V region and heparin-binding domain of fibronectin. Exp Cell Res 295:48–58.

Zhao H, Kim Y, Wang P, Lapointe J, Tibshirani R, Pollack JR, and Brooks JD (2005): Genome-wide characterization of gene expression variations and DNA copy number changes in prostate cancer cell lines. Prostate 63:187–197.

Zhao XY, Malloy PJ, Krishnan AV, Swami S, Navone NM, Peehl DM, and Feldman D (2000): Glucocorticoids can promote androgen-independent growth of prostate cancer cells through a mutated androgen receptor. Nat Med 6:703–706.

Zheng SL, Ju J, Chang B, Ortner E, Sun J, Isaacs SD, Sun J, Wiley KE, Liu W, Zemedkun M, Walsh PC, Ferretti J, Gruschus J, Isaacs WB, Gelmann EP, and Xu J (2006): Germ-line mutation of *NKX3.1* cosegregates with hereditary prostate cancer and alters the homeodomain structure and function. Cancer Res 66:69–77.

Zhong H, Agani F, Baccala AA, Laughner E, Rioseco-Camacho N, Isaacs WB, Simons JW, and Semenza GL (1998): Increased expression of hypoxia inducible factor-1alpha in rat and human prostate cancer. Cancer Res 58:5280–5284.

Zhong H, De Marzo AM, Laughner E, Lim M, Hilton DA, Zagzag D, Buechler P, Isaacs WB, Semenza GL, and Simons JW (1999): Overexpression of hypoxia-inducible factor 1α in common human cancers and their metastases. Cancer Res 59:5830–5835.

Zhong H, Semenza GL, Simons JW, and De Marzo AM (2004): Up-regulation of hypoxia-inducible factor 1alpha is an early event in prostate carcinogenesis. Cancer Detect Prev 28:88–93.

Zitzelsberger H, Engert D, Walch A, Kulka U, Aubele M, Höfler H, Bauchinger M, and Werner M (2001): Chromosomal changes during development and progression of prostate adenocarcinomas. Br J Cancer 84:202–208.

Zurita AJ, Troncoso P, Cardo-Vila M, Logothetis CJ, Pasqualini R, and Arap W (2004): Combinatorial screening in patients: the interleukin- 11 receptor alpha as a candidate target in progression of human prostate cancer. Cancer Res 64:435–439.

ORIGINAL COMMUNICATIONS

American Journal of Pathology, Vol. 159, No. 6, December 2001
Copyright © American Society for Investigative Pathology

Amplification of EIF3S3 Gene Is Associated with Advanced Stage in Prostate Cancer

Outi Saramäki,* Niels Willi,† Ola Bratt,‡ Thomas C. Gasser,§ Pasi Koivisto,¶ Nina N. Nupponen,‖ Lukas Bubendorf,† and Tapio Visakorpi*

From the Laboratory of Cancer Genetics, Institute of Medical Technology, and the Department of Clinical Genetics and Laboratory of Cancer Genetics,¶ Tampere University Hospital, Tampere, Finland; the Institute for Pathology† and Urologic Clinics BS/BL,§ University of Basel, Basel, Switzerland; the Department of Surgery,‡ Helsingborg Hospital, Helsingborg, Sweden; and the Cancer Genetics Branch,‖ National Human Genome Research Institute, National Institutes of Health, Bethesda, Maryland*

Gain of the long arm of chromosome 8 (8q) is one of the most common gains found in the advanced prostate cancer by comparative genomic hybridization. We have previously identified a putative target gene for the 8q gain, EIF3S3, that encodes a p40 subunit of eukaryotic translation initiation factor 3 (eIF3). Here, we studied the frequency of the EIF3S3 amplification in different stages of prostate cancer and co-amplification of EIF3S3 and oncogene MYC. In addition, prognostic utility of the EIF3S3 copy number alteration was evaluated. The analyses were done with fluorescence *in situ* hybridization and tissue microarray technology. High-level amplification of EIF3S3 was found in 11 of 125 (9%) of pT1/pT2 tumors, 12 of 44 (27%) of pT3/pT4 tumors, and 8 of 37 (22%) of lymph node metastases as well as in 26 of 78 (33%) and 15 of 30 (50%) of hormone refractory locally recurrent tumors and metastases, respectively. The amplification was associated with high Gleason score ($P < 0.001$). One of the 79 tumors with EIF3S3 amplification had only two copies of MYC, whereas all tumors with amplification of MYC had also amplification of EIF3S3 indicating common co-amplification of the genes. Gain of EIF3S3 was associated with poor cancer-specific survival in incidentally found prostate carcinomas ($P = 0.023$). In the analyses of prostatectomy-treated patients, the amplification was not statistically significantly associated with progression-free time. In conclusion, amplification of EIF3S3 gene is common in late-stage prostate cancer suggesting that it may be functionally involved in the progression of the disease. *(Am J Pathol 2001, 159:2089–2094)*

During the past decades prostate cancer has become the most commonly diagnosed cancer of men in many Western countries.[1] Despite the substantial clinical importance of the disease, the molecular mechanisms underlying the development and progression of the disease are incompletely understood.[2] Chromosomal aberrations in prostate cancer have been studied with several techniques, such as classical cytogenetics, loss of heterozygosity analysis, fluorescence *in situ* hybridization (FISH), and especially, by comparative genomic hybridization (CGH).[2] These studies have implicated several chromosomal regions, such as 6q, 8p, 10q, 13q, 16q, and Xq, that may harbor genes involved in the tumorigenesis of prostate cancer.[3–9]

Using CGH, we and others have previously shown that one of the most common genetic aberrations in advanced prostate cancer is the gain of the long arm (q-arm) of chromosome 8.[3–7] It is found in up to 80% of hormone-refractory tumors and distant metastases but only in ~5% of untreated primary prostate carcinomas.[3,4] In the prostatectomy-treated patients, the gain of 8q seems to be associated with advanced stage and poor prognosis.[7,10] In addition to prostate cancer, gain of 8q is commonly found in several other malignancies, such as breast, bladder, and ovarian cancers.[11] For example, almost half of the breast carcinomas contain gain of 8q. And, the gain seems to be associated also with poor survival.[12]

In most of the prostate tumors gain of 8q comprises the whole q-arm. However, CGH studies have indicated that there are, at least two independently amplified subchromosomal regions, 8q21 and 8q23-q24, suggesting the presence of several target genes.[4,5] The well-known oncogene, MYC, located at 8q24.1, is considered to be a putative target gene for the gain.[4] To identify other possible target genes, we recently used the subtraction hybridization technique to clone overexpressed genes in breast and prostate tumors.[13] We found that EIF3S3, located at 8q23, was amplified and overexpressed in approximately one-third of the hormone-refractory prostate carcinomas.[13,14] The EIF3S3 gene encodes for the

Supported by grants from the Academy of Finland, the Cancer Society of Finland, the Reino Lahtikari Foundation, the Medical Research Fund of Tampere University Hospital, the Sigrid Juselius Foundation, and the CaP CURE.

Accepted for publication August 17, 2001.

Address reprint requests to Dr. Tapio Visakorpi, M.D., Ph.D., Laboratory of Cancer Genetics, Institute of Medical Technology, FIN-33014 University of Tampere, Finland. E-mail: tapio.visakorpi@uta.fi.

p40 subunit of the eukaryotic translation initiation factor 3 (eIF3). eIF3 is the largest (~600 kd) translation initiation factor protein complex, which has a central role in the initiation of translation. It binds to 40S ribosomal subunits in the absence of other initiation factors and preserves the dissociated state of 40S and 60S ribosomal subunits. It also stabilizes eIF2 · GTP · Met-tRNA binding to 40S subunits and mRNA binding to ribosomes.[15] However, very little is known about the p40 subunit itself and how it could be functionally involved in tumorigenesis.[13,16] There are, however, suggestions that aberrant regulation of translation could be important in the development of cancer. For example, overexpression of initiation factors EIF4E and EIF4G1 has been shown to transform normal cells.[17,18] In addition, amplification of EIF4G1, and overexpression of EIF4E have been found in squamous cell lung and breast carcinomas, respectively.[19,20] Also, allelic imbalance of INT6, which encodes for p48 subunit of eIF3, has been detected in breast cancer.[21] Recently, a new gene EIF5A2, encoding a putative translation initiation factor was cloned and shown to be amplified in the subset of ovarian cancer.[22,23]

The aim of this study was to investigate the frequency of the EIF3S3 amplification in different stages of prostate cancer and to study the co-amplification of EIF3S3 and MYC. In addition, prognostic utility of the EIF3S3 amplification was evaluated. The analyses were done using FISH and new tissue microarray technology allowing large number of tumors to be rapidly analyzed.

Materials and Methods

Tissue Samples

The material consisted of three sets of prostate tumors. Group I included 21 benign prostatic hyperplasias, 42 prostatic intraepithelial neoplasias, 183 radical prostatectomy specimens, 20 Tru-Cut needle biopsy specimens of stage T3/T4 prostate tumors, 95 hormone refractory prostate tumors, and 39 distant metastases (obtained from University of Basel and Tampere University Hospitals). Fifty-four untreated local lymph node metastases were obtained from Lund University Hospital. Clinical stage and Gleason score of the tumors were available.

Group II included 112 incidentally found T1a/b tumors from transuretral resections for benign prostatic hyperplasia obtained from the University of Basel. The age of the patients at the time of diagnosis varied between 58 and 94 years with a mean of 76 years. FISH analysis was successful in 105 of 112 specimens. Of those 105, there were 20 Gleason 2-4, 60 Gleason 5-7, and 21 Gleason 8-10 tumors. Gleason score of four tumors was not available. The patients had been treated with standard therapies. Overall and prostate cancer-specific survival data were available.

Group III included 145 radical prostatectomy specimens from the University of Basel. The age of the patients at time of diagnosis was between 45 and 82 years with a mean of 65.4 years. The TNM stage distribution of the successfully hybridized cases was: 1 T1N0M0, 26 T2N0M0, 46 T3N0M0, 4 T2N1M0, 11 T3N1M0, and 6 T3N2M0. The TNM distribution was not available for 41 tumors. The Gleason score distribution was 100 Gleason 5-7, 33 Gleason 8-10 tumors, and 2 unknown. The progression-free time of the patients was available. The progression was defined either by increase in prostate-specific antigen levels (86% of cases), a positive finding in bone scan (11% of cases), or by biopsy proven local recurrence (3% of cases). The average recurrence-free time was 4.5 years (range, 0.6 to 15.1 years).

FISH

Multitissue blocks were made from the original formalin-fixed paraffin-embedded tumor blocks according to published guidelines.[24] Routine hematoxylin and eosin-stained slides were used to evaluate the representativeness of the samples. For the FISH analyses 5-μm sections from the multitissue blocks were either cut onto SuperFrost Plus slides (Menzel-Gläser, Braunschweig, Germany) and baked overnight, or an adhesive-coated tape sectioning system (Instrumedics, Hackensack, NJ) was used. A locus-specific PAC probe for EIF3S3 or MYC[13] and pericentromeric probe for chromosome 8 (pJM128) were labeled by nick translation with digoxigenin (locus-specific probes) and fluorescein-isothiocyanate (centromere-specific probe). The deparaffinized slides were treated with 1 mol/L NaSCN for 10 minutes at 80°C, followed by incubation in 4 mg/ml pepsin (P-7012, in 0.9% NaCl, pH 1.5; Sigma Chemical Co., St. Louis, MO) for 15 minutes at 37°C. The slides were then washed in H_2O and 2× standard saline citrate, followed by dehydration in an ethanol series, and air-dried. The probes were applied on the slides in a hybridization mix (50% formamide, 10% dextran sulfate in 1× standard saline citrate, pH 7) and then co-denatured with the samples at 80°C for 8 minutes. After hybridization for 2 to 3 days in a humid chamber, the slides were washed and the locus-specific probes were detected immunohistochemically by anti-digoxigenin rhodamine. The slides were counterstained with 0.1 mol/L 4,6-diamidino-2-phenylindole in Vectashield anti-fade solution (Vector Laboratories Inc., Burlingame, CA).

The FISH signals were scored from nonoverlapping epithelial cells using an Olympus BX50 epifluorescence microscope (Tokyo, Japan). A Photometrics charge-coupled device camera (Photometrics, Tucson, AZ) and IPLab software program (Scananalytics Inc., Fairfax, VA) were used to capture images. The previously published criteria for amplification[13] were slightly modified because tissue sections, instead of isolated nuclei, were analyzed here. Briefly, the tumors were classified into three groups: nonamplified (no increase in EIF3S3 or c-*myc* copy number), low-level amplification (3 to 5 copies per cell), and high-level amplification (≥5 copies of the genes per cell). Tumors that showed >10% of malignant cells with increased copy number of either EIF3S3 or c-*myc* were considered to have copy number alterations.

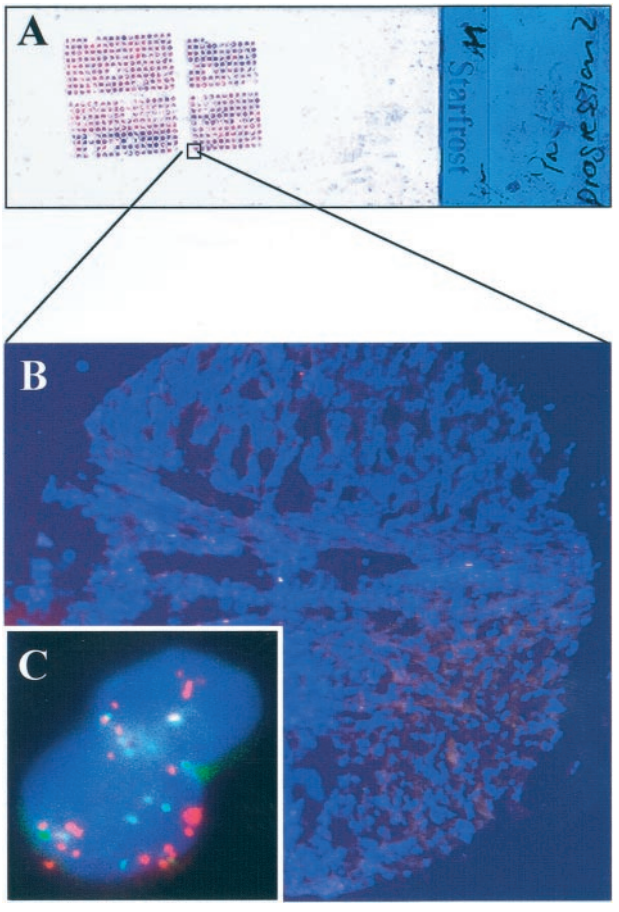

Figure 1. FISH analysis of the copy number of EIF3S3 gene in prostate cancer. The analysis was performed on multitissue array format in which one slide (**A**) contained up to ~500 small (diameter, 0.6 mm) samples (**B**) counterstained with 4,6-diamidino-2-phenylindole. **C:** Red signals indicated >10 copies of the EIF3S3 gene and green color approximately six copies of chromosome 8 centromere in a hormone-refractory prostate tumor.

Statistical Analysis

Statistical analysis of the data were done using BMDP Statistical Software Package.[25] Pearson chi-square test was used to evaluate the associations of the gene copy number and tumor type, clinical stage, and Gleason score. The survival differences of patients were evaluated by Kaplan-Meier method, and the statistical significance of survival differences between the patient groups was determined with Mantel-Cox and Breslow tests.

Results

Figure 1 demonstrates a FISH analysis of a hormone-refractory prostate tumor with high-level amplification of EIF3S3.

Group I: this set of samples was used to study the frequency of EIF3S3 amplification across different grades and stages of prostate tumors. FISH analysis of the EIF3S3 was successful in 369 of 454 (81.3%) of cases. Table 1 summarizes the frequency of the EIF3S3 amplification in prostate tumors. No copy number changes were found in nonmalignant (benign prostatic hyperplasia) or premalignant (prostatic intraepithelial neoplasia) prostate. The high-level amplification of EIF3S3 was found only in <10% of local (pT1 and pT2) prostate cancers, whereas hormone-naïve lymph node metastases as well as hormone-refractory tumors showed the amplification in ~20 to 50% of the cases. Gain (or low-level amplification) of EIF3S3 was found in ~30 to 50% of the prostate cancers. The amplification of the gene was statistically significantly associated with advanced stage of disease ($P < 0.001$) and high Gleason score ($P < 0.001$).

Group II: this set of samples was used to evaluate the prognostic significance of EIF3S3 amplification in prostate carcinomas that were incidentally found in transurethral resection specimens. FISH analysis was successful in 105 of 112 (93%) of cases. High-level amplification of EIF3S3 was found only in 6 of 105 cases (5.7%), whereas low-level amplification was found in 27 of the 105 (26%). Of the six cases with high-level amplification, five (83.3%) had died during the follow-up period. In contrast, 18 of the 27 (55.7%) cases with low-level amplification and 37 of the 72 (51.4%) cases without copy number alterations had died. However, there was no association between

Table 1. Association of EIF3S3 Amplification with Clinicopathological Variables

Variable	EIF3S3 copy number (%)			*P* value
	Normal	Low-level amplification	High-level amplification	
Specimen type				
Androgen-dependent				
BPH	19/19 (100)	0/19 (0)	0/19 (0)	<0.001*
High-grade PIN	36/36 (100)	0/36 (0)	0/36 (0)	
Prostatectomy specimen (T1/T2)	74/125 (59)	40/125 (32)	11/125 (9)	
Locally advanced (T3/4)	18/44 (41)	14/44 (32)	12/44 (27)	
Lymph-node metastases	10/37 (27)	19/37 (51)	8/37 (22)	
Hormone-refractory				
Local	20/78 (26)	32/78 (41)	26/78 (33)	
Metastases	4/30 (13)	11/30 (37)	15/30 (50)	
Gleason score				
≤7	79/115 (69)	31/115 (27)	5/115 (4)	<0.001
≥8	22/78 (28)	33/78 (42)	23/78 (30)	

*Comparison made between all groups under the title "specimen type."

Table 2. Association of EIF3S3 Amplification with Prostate Cancer-Specific Death in Incidentally Found Prostate Cancer

	No. of cases (%)			
	EIF3S3 copy number			
	Normal	Low-level amplification	High-level amplification	Total
Alive or died of some other reason than prostate cancer	55 (73)	18 (24)	2 (3)	75 (100)
Dead	4 (40)	5 (50)	1 (10)	10 (100)

$P = 0.084$.

overall survival and EIF3S3 copy number alterations. The prostate cancer-specific follow-up data were available from 85 of the 105 cases. One tumor from the 10 (10%) patients who had died of prostate cancer showed the gene amplification, whereas only 2 tumors out of the 75 (3%) patients who had not died of prostate cancer showed the amplification (Table 2). The difference was not quite statistically significant ($P = 0.084$). In the disease-specific survival analysis (Figure 2A), the increased copy number (either low- or high-level amplification) of EIF3S3 was associated with poor survival ($P = 0.023$).

Group III: this set of samples was used to study prognostic significance of the EIF3S3 amplification in patients treated by radical prostatectomy. The FISH analysis was successful in 135 of 145 (93.1%) cases. Eleven of 135 (8.1%) and 30 of 135 (22%) of the samples showed high-level and low-level amplification of EIF3S3, respectively. In a single case only one copy of chromosome 8 centromere and EIF3S3 were found. According to Kaplan-Meir analysis, the progression-free survival was slightly poorer for patients with EIF3S3 amplification than in patients without the amplification (Figure 2B). However, the difference was not statistically significant ($P = 0.380$).

By combining the data from all above-mentioned samples ($n = 461$), the co-amplification of EIF3S3 and c-*myc* in almost all cases became evident (Table 3). There was only one case with high-level EIF3S3 amplification with two copies of MYC.

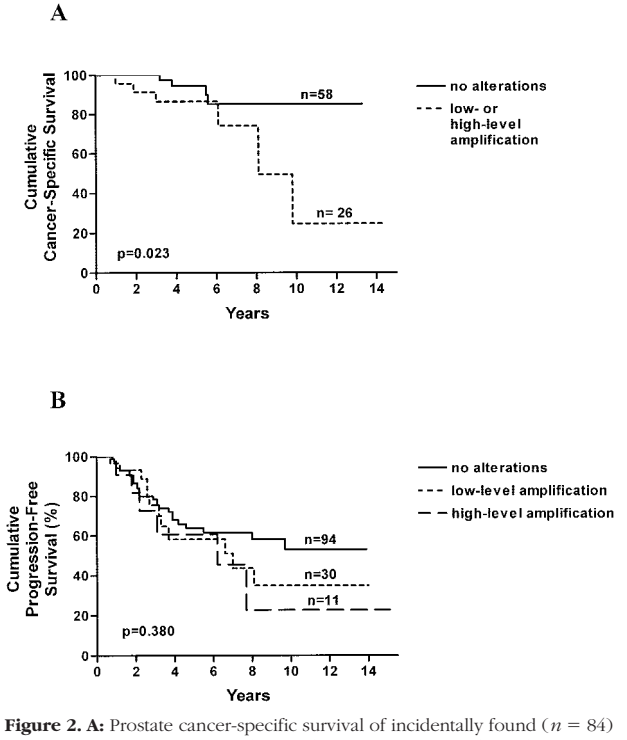

Figure 2. A: Prostate cancer-specific survival of incidentally found ($n = 84$) prostate cancer patients according to the EIF3S3 copy number. The patients had been treated with various forms of therapies according to routine clinical practice. **B:** Progression-free survival of the prostatectomy treated men ($n = 135$) according to the EIF3S3 copy number.

Discussion

Gain of 8q is one of the most common chromosomal alterations in late-stage prostate cancer detected by CGH.[3–5] We have earlier identified a putative target gene for the 8q gain, EIF3S3.[13,14] Here, we analyzed the frequency of the amplification of EIF3S3 in prostate carcinomas of different stages. None of the benign prostatic hyperplasia or prostatic intraepithelial neoplasia lesions showed amplification of the gene. In addition, the high-level amplification was found to be rare in untreated stage pT1/pT2 tumors. However, in locally advanced and metastatic tumors, the amplification was found approximately in one-fourth of the cases. In hormone-refractory tumors the amplification was detected in 30 to 50% of the cases. The findings are in good agreement with our earlier FISH analyses showing high-level amplification of the gene in 13 of 44 (30%) of locally recurrent hormone-refractory prostate carcinomas.[13] The frequency of low-level amplification in the locally recurrent hormone-refractory tumors was somewhat lower (41% *versus* 70%) than we have previously reported.[13] This may be because of the fact that more tumors were analyzed here. In addition, the FISH analysis was performed on tissue sections instead of isolated nuclei as in our previous study.[13] Lost signals because of nuclear slicing may possibly lead to underdetection of low-level amplifications. The amplification was found almost eight times more often in poorly differentiated tumors (Gleason score ≥ 8) than moder-

Table 3. Co-Amplification of EIF3S3 and MYC

	No. of tumors		
	EIF3S3 copy number		
MYC copy number	Normal	Low-level amplification	High-level amplification
Normal	218	2	1
Low-level amplification	2	157	10
High-level amplification	0	3	68

ately or well-differentiated tumors (Gleason score ≤ 7). Altogether, the results suggest that the amplification of EIF3S3 is involved in the late progression of prostate cancer.

The FISH analyses were done using tissue sections making the evaluation of actual copy number of the gene difficult. However, in the case of amplification, typically 5 to 10 signals per nucleus were seen indicating that the level of amplification was quite moderate. The finding is consistent with the earlier CGH studies, which most often have shown gain of the whole q-arm of the chromosome, and only rarely high-level regional amplification.[3–5,9] This feature makes the gain of 8q clearly different from, for example gain of Xq, the second most common gain in hormone-refractory prostate cancers. According to FISH analysis, the amplification of the target gene of the Xq gain, androgen receptor gene, typically consists of 10 or more copies of the gene.[26] However, we have previously shown that even the moderately increased copy number of EIF3S3 may lead to overexpression of the gene in prostate cancer.[13] Therefore, it is possible that amplification of lower level but larger chromosomal region harboring many genes in 8q is selected for during the progression of prostate cancer.

Another putative target gene for the gain of 8q in prostate cancer is MYC located at 8q24.1.[4] We have earlier shown that in a subset of breast carcinomas EIF3S3 and MYC are not co-amplified.[14] Here, in this large series of prostate tumors, we found only one case in which EIF3S3 was amplified without MYC amplification, whereas all cases with MYC amplification had also EIF3S3 amplification. The finding suggests that both EIF3S3 and MYC may be important in the progression of prostate cancer, and therefore they are equally selected for. In addition to MYC and EIF3S3 there are other putative target genes for 8q gain in prostate cancer as well. These include recently cloned prostate stem-cell antigen, and GC79 encoding a zinc-finger protein, both located in the 8q23-24 region.[27,28] The other minimal commonly amplified region in prostate cancer is 8q21 of which target genes are still not known.[4,5,9] Because there are likely to be numerous putative target genes, it will be important to compare the alterations of all of the different putative target genes in large tumor materials as done here for MYC and EIF3S3.

The prognostic significance of the EIF3S3 amplification was retrospectively studied here in two sets of tumors. In the cases of incidental prostate cancers, the amplification was found in only ~5% of cases. The number of tumors with the amplification was too small for prognostic analyses. However, by combining the groups of low- and high-level amplification, we found that the increased copy number of EIF3S3 was associated with poor disease-specific survival. Thus, it may be that the copy number alteration of EIF3S3 could be useful in predicting which incidental cancers are clinically significant. Evidently larger studies are needed to confirm the finding.

In the second set of cases, 135 prostatectomy specimens, from patients whose progression-free survival data were available, were analyzed. Approximately 55% of the patients with the amplification but only ~30% of those without amplification experienced progression. Although the progression-free time curves showed a worse prognosis for the patients with amplification of EIF3S3 than for patients without the amplification, the difference was not statistically significant. This is in some contrast to the findings of Sato and co-workers,[29] who have previously suggested that MYC amplification is associated with poor survival in prostatectomy treated stage C disease. Because MYC and EIF3S3 were almost always co-amplified, it was evident that MYC did not have prognostic value in our material either (data not shown). The major difference between the studies by Sato and colleagues[29] and by us is that Sato and co-workers analyzed high-grade, stage pT3 tumors, whereas our material consisted of both moderately and poorly differentiated pT1-pT3 tumors. Thus, for example, the frequency of 8q gain was higher in the study by Sato and colleagues[29] than in our study (54.2% *versus* 30.4%). The definition of high-level amplification was also different in the two studies. Sato and co-workers[29] found an "additional increase" of MYC in 19.4% of cases, whereas high-level amplification of EIF3S3 (and MYC) was found in only 8% of our prostatectomy series. It may also be that because the cases in our study were lower stage and grade, the prostatectomy had removed these tumors before the effects of EIF3S3 (and/or MYC) on progression had have enough time to affect.

In this study, the FISH analyses were performed in a multitissue section format. The use of multitissue blocks instead of original single tumor blocks has several advantages. It allowed us to screen a large number of tumors in a relatively short period of time. Altogether 609 specimens were analyzed. Because the hybridizations were done on a few slides (five slides per gene), the slide-to-slide variation could also be expected to be low. An additional advantage of multitissue slides is that, at least in our hands, the FISH analyses seemed to work better in this than in the traditional one tumor section per slide format. This may well be because of the fact that in the multitissue blocks the tissue samples are small and equal in size. Therefore, the pretreatment of the slide, which is the most important variable in the FISH analysis, probably affects equally to each specimen on the slide. The disadvantage of the multitissue technology is that only a small proportion of the tumor is analyzed. Because of the known intratumoral heterogeneity of prostate cancer, the absolute frequency of aberrations may thus be somewhat underestimated. However, this possible underestimation of absolute frequencies should not affect the clinicopathological associations based on a large number of specimens, because all specimens on a tissue microarray are subjected to the same sampling limitations.

In conclusion, we have shown here that the high-level amplification of EIF3S3 gene is associated with advanced stage, androgen independence, and poor differentiation of prostate cancer. The gene is in most of the cases co-amplified with MYC. Both association with advanced stage and preliminary prognostic analyses suggest that amplification of EIF3S3 might be important for the progression of prostate cancer. Further studies are

warranted to evaluate the function of the gene as well as possible prognostic utility of the amplification.

Acknowledgments

We thank Mrs. Mariitta Vakkuri and Ms. Heli Lehtonen for technical assistance.

References

1. Hsing AW, Tsao L, Devesa SS: International trends and patterns of prostate cancer incidence and mortality. Int J Cancer 2000, 85:60–67
2. Elo JP, Visakorpi T: Molecular genetics of prostate cancer. Ann Med 2001, 33:130–141
3. Visakorpi T, Kallioniemi AH, Syvanen AC, Hyytinen ER, Karhu R, Tammela T, Isola JJ, Kallioniemi OP: Genetic changes in primary and recurrent prostate cancer by comparative genomic hybridization. Cancer Res 1995, 55:342–347
4. Nupponen NN, Kakkola L, Koivisto P, Visakorpi T: Genetic alterations in hormone-refractory recurrent prostate carcinomas. Am J Pathol 1998, 153:141–148
5. Cher ML, Bova GS, Moore DH, Small EJ, Carroll PR, Pin SS, Epstein JI, Isaacs WB, Jensen RH: Genetic alterations in untreated metastases and androgen-independent prostate cancer detected by comparative genomic hybridization and allelotyping. Cancer Res 1996, 56:3091–3102
6. Cher ML, MacGrogan D, Bookstein R, Brown JA, Jenkins RB, Jensen RH: Comparative genomic hybridization, allelic imbalance, and fluorescence in situ hybridization on chromosome 8 in prostate cancer. Genes Chromosom Cancer 1994, 11:153–162
7. Alers JC, Rochat J, Krijtenburg PJ, Hop WC, Kranse R, Rosenberg C, Tanke HJ, Schroder FH, van Dekken H: Identification of genetic markers for prostatic cancer progression. Lab Invest 2000, 80:931–942
8. Cunningham JM, Shan A, Wick MJ, McDonnell SK, Schaid DJ, Tester DJ, Qian J, Takahashi S, Jenkins RB, Bostwick DG, Thibodeau SN: Allelic imbalance and microsatellite instability in prostatic adenocarcinoma. Cancer Res 1996, 56:4475–4482
9. El Gedaily A, Bubendorf L, Willi N, Fu W, Richter J, Moch H, Mihatsch MJ, Sauter G, Gasser TC: Discovery of new amplification loci in prostate cancer by comparative genomic hybridization. Prostate 2001, 46:184–190
10. Van Den Berg C, Guan X-Y, Von Hoff D, Jenkins R, Bittner M, Griffin C, Kallioniemi O, Visakorpi T, McGill J, Herath J, Epstein J, Sarosdy M, Meltzer P, Trent J: DNA sequence amplification in human prostate cancer identified by chromosome microdissection: potential prognostic implications. Clin Cancer Res 1995, 1:11–18
11. Knuutila S, Bjorkqvist AM, Autio K, Tarkkanen M, Wolf M, Monni O, Szymanska J, Larramendy ML, Tapper J, Pere H, El-Rifai W, Hemmer S, Wasenius VM, Vidgren V, Zhu Y: DNA copy number amplifications in human neoplasms: review of comparative genomic hybridization studies. Am J Pathol 1998, 152:1107–1123
12. Isola JJ, Kallioniemi OP, Chu LW, Fuqua SA, Hilsenbeck SG, Osborne CK, Waldman FM: Genetic aberrations detected by comparative genomic hybridization predict outcome in node-negative breast cancer. Am J Pathol 1995, 147:905–911
13. Nupponen NN, Porkka K, Kakkola L, Tanner M, Persson K, Borg Å, Isola J, Visakorpi T: Amplification and overexpression of p40 subunit of eukaryotic translation initiation factor 3 in breast and prostate cancer. Am J Pathol 1999, 154:1777–1783
14. Nupponen NN, Isola J, Visakorpi T: Mapping the amplification of EIF3S3 in breast and prostate cancer. Genes Chromosom Cancer 2000, 28:203–210
15. Hershey JW, Asano K, Naranda T, Vornlocher HP, Hanachi P, Merrick WC: Conservation and diversity in the structure of translation initiation factor eIF3 from humans and yeast. Biochimie 1996, 78:903–907
16. Asano K, Vornlocher HP, Richter-Cook NJ, Merrick WC, Hinnebusch AG, Hershey JW: Structure of cDNAs encoding human eukaryotic initiation factor 3 subunits. Possible roles in RNA binding and macromolecular assembly. J Biol Chem 1997, 272:27042–27052
17. Fukuchi-Shimogori T, Ishii I, Kashiwagi K, Mashiba H, Ekimoto H, Igarashi K: Malignant transformation by overproduction of translation initiation factor eIF4G. Cancer Res 1997, 57:5041–5044
18. Lazaris-Karatzas A, Sonenberg N: The mRNA 5′ cap-binding protein, eIF-4E, cooperates with v-myc or E1A in the transformation of primary rodent fibroblasts. Mol Cell Biol 1992, 12:1234–1238
19. Brass N, Heckel D, Sahin U, Pfreundschuh M, Sybrecht GW, Meese E: Translation initiation factor eIF-4gamma is encoded by an amplified gene and induces an immune response in squamous cell lung carcinoma. Hum Mol Genet 1997, 6:33–39
20. Scott PA, Smith K, Poulsom R, De Benedetti A, Bicknell R, Harris AL: Differential expression of vascular endothelial growth factor mRNA vs protein isoform expression in human breast cancer and relationship to eIF-4E. Br J Cancer 1998, 77:2120–2128
21. Miyazaki S, Imatani A, Ballard L, Marchetti A, Buttitta F, Albertsen H, Nevanlinna HA, Gallahan D, Callahan R: The chromosome location of the human homolog of the mouse mammary tumor-associated gene INT6 and its status in human breast carcinomas. Genomics 1997, 46:155–158
22. Jenkins ZA, Haag PG, Johansson HE: Human eIF5A2 on chromosome 3q25–q27 is a phylogenetically conserved vertebrate variant of eukaryotic translation initiation factor 5A with tissue-specific expression. Genomics 2001, 71:101–109
23. Guan X-Y, Sham JST, Tang TC-M, Fang Y, Huoi K-K, Yang J-M: Isolation of a novel candidate oncogene within a frequently amplified region at 3q26 in ovarian cancer. Cancer Res 2001, 61:3806–3809
24. Kononen J, Bubendorf L, Kallioniemi A, Bärlund M, Schraml P, Leighton S, Torhorst J, Mihatsch MJ, Sauter G, Kallioniemi O-P: Tissue microarrays for high-throughput molecular profiling of tumor specimens. Nat Med 1998, 4:844–847
25. Dixon WJ: BMDP Statistical Software. Los Angeles, University of California Press, 1981
26. Visakorpi T, Hyytinen E, Koivisto P, Tanner M, Keinänen R, Palmberg C, Palotie A, Tammela T, Isola J, Kallioniemi O-P: In vivo amplification of the androgen receptor gene and progression of human prostate cancer. Nat Genet 1995, 9:401–406
27. Reiter RE, Gu Z, Watabe T, Thomas G, Szigeti K, Davis E, Wahl M, Nisitani S, Yamashiro J, Le Beau MM, Loda M, Witte ON: Prostate stem cell antigen: a cell surface marker overexpressed in prostate cancer. Proc Natl Acad Sci USA 1998, 95:1735–1740
28. Chang GT, Steenbeek M, Schippers E, Blok LJ, van Weerden WM, van Alewijk DC, Eussen BH, van Steenbrugge GJ, Brinkmann AO: Characterization of a zinc-finger protein and its association with apoptosis in prostate cancer cells. J Natl Cancer Inst 2000, 92:1414–1421
29. Sato K, Qian J, Slezak JM, Lieber MM, Bostwick DG, Bergstralh EJ, Jenkins RB: Clinical significance of alterations of chromosome 8 in high-grade, advanced, nonmetastatic prostate carcinoma. J Natl Cancer Inst 1999, 91:1574–1580