SARI LAITINEN

Prostate Cancer

From molecular genetics to prognostic markers

■

ACADEMIC DISSERTATION
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To my Beloved Family
and Toffo & Rollo

YHTEENVETO

Tämän väitöskirjan tavoitteena oli määrittää eturauhassyövän geneettisiä muutoksia ja eturauhassyövän etenemiseen liittyviä mekanismeja ksenografti (vierassiirrännäis)-mallien ja potilasmateriaalin avulla sekä löytää uusia keinoja eturauhassyöpäpotilaiden ennusteen määrittämiseksi täydellisen eturauhasenpoistoleikkauksen jälkeen.

Tutkittujen ksenografti-mallien kromosomimuutokset muistuttivat ihmisen eturauhassyöpää. Ksenografti-mallien ja etäpesäkkeiden kromosomimuutokset olivat yleisesti ottaen samankaltaisia aikaisemmin julkaistujen tulosten kanssa viitaten kromosomi alueisiin, joissa tärkeimmät syöpägeenit ja kasvurajoitegeenit sijaitsevat. Eri etäpesäkkeiden geneettinen samankaltaisuus viittaa siihen, että tappavien syöpäsolupesäkkeiden valinta tapahtuu jo siinä vaiheessa kun syöpä alkaa levitä ja etäpesäkkeen kasvupaikka ei välttämättä tarjoa kasvuetua kyseiselle pesäkkeelle. Mieshormonitoiminnan estäminen vaikuttaa vähentävän eturauhasen solujen lisääntymisaktiivisuutta useiden kuukausien ajan, mutta suurimmassa osassa eturauhassyövistä solut jatkavat lisääntymistä mikäli mieshormonitoimintaa estetään vaiheittaisesti. Vaikuttaa siltä, että kasvaimen biologinen aktiivisuus on määritelty jo diagnoosivaiheessa. Ki-67, EZH2 ja MCM7 immunovärjäyksien avulla, sekä kahden viimeisen yhdistelmällä, voidaan löytää potilaat joilla on korkea uusiutumisriski täydellisen eturauhasenpoistoleikkauksen jälkeen ja niiden avulla voidaan valita potilaita liitännäishoitokokeiluihin. Matala Ki-67 -värjäys vaikuttaa tunnistavan potilasryhmän, jolla on matala syövän uusiutumisen riski. Näitä potilaista voitaisiin hoitaa mahdollisesti ennemmin aktiivisella seurannalla kuin välittömällä täydellisellä eturauhasenpoistoleikkauksella.

Tämä väitöskirja tuotti tärkeää tietoa eturauhassyövän geneettisistä muutoksista, käyttäytymisestä ja kehityksestä sekä menetelmistä, joilla voidaan tarkemmin määritellä eturauhassyövän ennustetta täydellisen eturauhasenpoistoleikkauksen jälkeen.

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LIST OF ORGINAL COMMUNICATIONS

This thesis consists of the following publications referred to in the text by their Roman numerals. In addition, unpublished data is included:

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

II. Laitinen S, Martikainen PM, Tammela TL, Visakorpi T (2007): Cellular changes in prostate cancer cells induced by intermittent androgen suppression. Eur Urol 52:725-32.

III. Laitinen S, Martikainen PM, Tolonen T, Isola J, Tammela TLJ, Visakorpi T (2008): EZH2 and MCM7 are independent prognostic markers in prostatectomy treated patients. Int J Cancer 122:595-602.

ABBREVIATIONS

ADP	adenosine diphosphate
AKT	protein kinase B, PKB
AMARC	α-methylacyl-CoA racemase
AR	androgen receptor
BAC	bacterial artificial chromosome
BCL-2	b-cell CLL/lymphoma 2
BRCA	breast cancer 2, early onset
CAB	complete androgen blocade
CABP	cytosolic calcium binding protein
CDH1	cadherin 1, E-cadherin
cDNA	complementary DNA
aCGH	array comparative genomic hybridisation
cCGH	chromosomal comparative genomic hybridisation
COX-2	cyclooxygenase -2
DCC	deleted in colon cancer
DNA	deoxyribonucleic acid
dUTP	deoxyribonucleic acid
EIF3S3	a subunit of a translation factor eIF3
ELAC2	elaC homolog 2 (E. coli)
ERG	v-ets erythroblastosis virus E26 oncogene like, p55
ETS	avian erythroblastosis virus E26 homolog
ETV	ets variant gene
EZH2	polycomb group protein, enhancer of zeste homolog 2
FISH	fluorescence in situ hybridisation
GSTP	pi-class glutathione S-transferase gene
HGPIN	high -grade prostatic intraepithelial neoplasia
HIF-1	hypoxia inducible factor homolog
HPC	hereditary prostate cancer
IAS	intermittent androgen suppression
Ki-67	cell proliferation-associated nuclear antigen
LOH	loss of heterozygosity
LZTS1	leucine zipper, putative tumor suppressor 1
LHRH	luteinising-hormone releasing hormone
MAB	maximum androgen blocade
MCM	minichromosome maintenance protein
MYC	myelocytomatosis viral oncogene homolog
MSR1	macrophage scavenger receptor 1
MX11	MAX interactor 1, isoform b
bNED	biochemical no-evidence of disease
NH2	amino-terminal
NKX3.1	NK homeobox (Drosophila) family 3A homolog
PAC	P1-derived artificial chromosome
PCNA	proliferating cell nuclear antigen
PIA	proliferative inflammatory atrophy

PIN	prostatic intraepithelial neoplasia
PSA	prostate specific antigen
PTEN	phosphatase and tensin homolog (mutated in multiple advanced cancers 1)
RB1	retinoblastoma 1
RNA	ribonucleic acid
shRNA	small hairpin RNA
siRNA	small interfering RNA
RNASEL	2'-5'-oligoadenylate-dependent ribonuclease L
SAM	significance analysis of microarrays
SKY	spectral karyotyping
SMAD	mothers against decapentaplegic (Drosophila)
SNP	single nucleotide polymorphism
TMPRSS2	transmembrane protease, serine 2
TP53	tumor protein 53, p53
TUNEL	TdT- mediated dUTP-biotin nick end labelling
TURP	transurethral resection of the prostate
VEGF	vascular endothelial growth factor

ABSTRACT

The objective of this thesis was to explore the genetic changes and the genetic nature of prostate cancer progression using xenograft *in vivo* models and patient material and to seek out novel means to determine prognosis more accurately in prostate cancer patients after radical prostatectomy.

The xenografts studied resembled clinical prostate tumors in their chromosomal alterations. Chromosomal aberrations found in the xenografts and also in the metastases were generally similar compared to previously published chromosomal comparative genomic hybridization findings indicating the chromosomal regions harboring the most important oncogenes and tumor suppressor genes for prostate cancer. The strong clonal relationship between the metastases suggests that the selection of the lethal tumor clone already takes place at the time of cancer dissemination and the metastatic environment does not exert selection pressure on the metastatic clone. Androgen withdrawal seems to reduce the cell proliferation activity of prostate cancer cells for several months, but the majority of tumors emerging during the first withhold of castration are resistant to the subsequent rounds of castration in terms of cell proliferation activity. The biological aggressiveness of the tumor seems already to be defined at the time of diagnosis. Ki-67, EZH2 and MCM7 immunostainings and the combination of the latter two identified patients with a very high risk of recurrence after radical prostatectomy and could be useful in identifying patients for adjuvant therapy trials. Low Ki-67 immunostaining identified a subgroup of patients with a very low risk of disease progression, suggesting that such patients could be treated with active surveillance instead of immediate prostatectomy.

This thesis generated valuable information on the genetic changes and the genetic nature of prostate cancer progression and found novel approaches for more accurate determination of prostate cancer prognosis after radical prostatectomy.

INTRODUCTION

The prostate is situated in the pelvic cavity. It is a firm, partly glandular and partly muscular organ around the urethra and can be felt for the most part through rectum. The normal prostate diameter is about 2-3 cm and shape is somewhat conical. The normal volume in a young adult is approximately 20 g.

This male organ has become an extremely important target for cancer research since prostate cancer has become the most common malignancy among men in western countries. According to the Finnish Cancer Registry, in 2005 (http://www.cancerregistry.fi; 7.10.2007), 5,327 men were diagnosed with prostate cancer, compared to the second most common male cancer, lung cancer with 1,589 new cases. Prostate cancer accounted for 37.7% of all male malignancies. The highest mortality was from lung cancer, 1,416 cases (26%), prostate cancer being the second most common cause of male cancer mortality with 774 deaths (14%). The most significant risk factors for prostate cancer identified so far are age, family history and ethnicity (Gann, 2002). For example, the incidence of prostate cancer is approximately 60% higher in African-American than in European-American men and the mortality rate from the disease is more than twice as high in the United States (Powell, 2007).

The environmental risk factors are poorly understood. Dietary fat intake has been proposed to be one of the risk factors but has not been confirmed (Park *et al.*, 2007). Both high and low levels of blood vitamin D are associated with a higher prostate cancer risk (Tuohimaa *et al.*, 2004) and low vitamin D level related to metabolic syndrome is associated with higher prostate cancer risk (Tuohimaa *et al.*, 2007). Hence, the association between vitamin D levels and prostate cancer is not straightforward and more studies are required. In a Finnish cancer prevention study (SETTI) patients with higher circulating concentrations of the major vitamin E fractions, alpha-tocopherol and gamma-tocopherol had lower prostate cancer risk (Weinstein *et al.*, 2005). In addition, it was found that long-term supplementation with alpha-tocopherol substantially reduced prostate cancer incidence and mortality in male smokers (Heinonen *et al.*, 1998).

Prostate cancer affects more and more men due to the generalized diagnostics and the increasing life expectancy. Its negative effect on quality of life is, without dispute, prominent. Overall, prostate cancer seems to be quite a complex disease as far as genetic manifestation before and after treatment is concerned. Therefore it is extremely important to define the genetic aberrations underlying prostate cancer development and progression and to seek out novel approaches for monitoring and treating prostate cancer.

REVIEW OF THE LITERATURE

1. Natural history of prostate cancer

Prostate carcinoma originates in the glandular epithelium of the prostate. In histological studies, prostatic intraepithelial neoplasia (PIN) and more advanced high-grade prostatic intraepithelial neoplasia (HGPIN) can be found together with cancer (Bostwick and Brawer, 1987; Bostwick, 1998). However, many early prostate carcinomas do not contain PIN lesions, nor is this considered to be a prerequisite for cancer (DeMarzo *et al.*, 2003). It has been reported that the incidence of PIN increases with advancing age and HGPIN especially may represent a marker for biologically significant prostate cancer and may be a precursor of prostatic carcinoma (Bostwick *et al.*, 2004b). Recent hypothesis is that exposure to environmental agents such as infections and dietary carcinogens, and hormonal imbalances could lead to injury of the prostate and to the development of chronic inflammation (De Marzo *et al*., 2007a & 2007b). This could lead to cancerous lesions designated as proliferative inflammatory atrophy (PIA), a precursor of PIN (DeMarzo *et al.*, 1999; DeMarzo *et al*.,2007a&2007b). However, the evidence that prostate inflammation contributes to prostatic carcinogenesis is not particularly strong (Nelson *et al.*, 2004).

Most prostate cancers grow slowly through the capsule and metastasize first to local lymph nodes and finally to distant organs such as the spine, liver, brain, lungs, distant lymph nodes etc. Depending on whether the prostate cancer is localized, locally advanced or metastasized, treatment options are active surveillance, surgical or chemical castration, radiation therapy, chemotherapy or different combination therapies. Localized intracapsular prostate cancer can be cured, although 20-40% of the cases will relapse (Bill-Axelson *et al.*, 2005; Carver *et al.*, 2006). Once the tumor has invaded the capsule, the rate of relapses increases significantly. Because the growth of prostate cancer is androgen dependent, androgen withdrawal leads to regression of the tumor. However, during treatment the androgen independent cancer cell population arises and a hormone-refractory cancer develops (Arnold and Isaacs, 2002). At this point there is no curable treatment and the expected survival time is only about 17 months (Petrylak *et al.*, 2004).

2. Treatment of prostate cancer

Since the clinical use of prostate specific antigen (PSA) and more convenient prostate needle biopsies became generally available in the 1990´s, most of the prostate cancers found these days are localized (Isola *et al.*, 2001; Epstein and Herawi, 2006). Thus, curative treatment is a viable option.

2.1 Localized prostate cancer

If the tumor is localized (T1b-2N0M0), prostate cancer can be treated with radical prostatectomy. Those patients have a high chance of remaining disease-free (Manoharan *et al.*, 2003; Bill-Axelson *et al.*, 2005). However, a significant proportion of operated patients experience disease progression. The risk of developing metastases after rising PSA following radical prostatectomy is high, 44 % with a medium time of 7.5 years after the recurrence (Pound *et al.*, 1999). It has been demonstrated that prostatectomized patients with lymph-node metastases treated with early androgen withdrawal have better survival prespects than patients treated with deferred hormonal therapy (Messing *et al.*, 2006). In addition, prostatectomy-treated patients with locally advanced but lymph node negative disease benefit from early hormonal therapy (Wirth *et al.*, 2004). These findings suggest that adjuvant hormonal therapy in conjunction with prostatectomy could be beneficial. The critical question is the selection of patients for adjuvant treatment. It would be important to be able to identify patients with a high risk of recurrence. It is known that even short neoadjuvant or adjuvant hormonal treatment can cause permanent metabolic alterations, this emphasizes the importance of the patient selection.

Radical external radiation therapy is a treatment option for radical prostatectomy in T1-2N0M0 tumors and can be used as an option for hormonal treatment or as adjuvant therapy in T3 tumors. The local control of the disease is similar to radical prostatectomy and according to retrospective studies local recurrence in 10 to 15 year follow up has been about 5% in T1, 12-26% in T2 and 19-43% in T3 stage tumors (Duncan *et al.*, 1993; Basgshaw *et al.*, 1994; Eastham *et al.*, 1997). It is a good option for patients who are not willing or not suitable for surgical treatment. Immediate external irradiation after radical prostatectomy has been shown to improve biochemical progression-free survival and local control in patients with positive surgical margins or pT3 prostate cancer who are at high risk of progression (Bolla *et al.*, 2005). Another widely used radiation therapy option is brachytherapy, where radioactive seeds are implanted in the prostate in transrectal ultrasound guidance in various positions (Holm *et al.*, 1983). This form of therapy is most appropriate for well differentiated T1-2 tumors. Overall survival rate at 5 years has been as good as 96.7% and 10 to 12 year survival rates range from 87% to 93% bNED (biochemical no-evidence of disease) survival (Lawton *et al.*, 2007; Potters *et al.*, 2005). It has been suggested that adjuvant hormonal treatment for these patients could increase the rate of cure in radiotherapy treated patients (D'Amico *et al.*, 2004; Denham *et al.*, 2005).

Active surveillance is also a treatment option in localized prostate cancer for patients with low-grade disease (T1-2N0M0, Gr 1, Gleason score 2-6) (Chodak *et al.*, 1994; Albertsen *et al.*, 1998; Meng *et al.*, 2003; Wu *et al.*, 2004; Klotz, 2005; Hardie *et al.*, 2005).

If the tumor has grown outside the capsule of the prostate, but is still localized, radical radiation therapy (for T1-3N0M0) and/or bicalutamide

androgen deprivation treatment (for T1-3NXM0) are options. In locally advanced prostate cancer (T3N0-1M0) micrometastases may have already been sent and early androgen deprivation using castration and/or antiandrogens is a recommended treatment (Crawford, 2003). Studies of combining prior androgen withdrawal with radiotherapy have been controversial, though it has been shown to lengthen survival (Anderson *et al.*, 2003; Bolla *et al.*, 2003). The outcome of those with locally advanced (T3N0-1M0) prostate cancer depends largely on how far the cancer has actually spread and what treatment option is used. A rough estimate of overall survival at five years could be approximately 70-80 percent, a third of the patients would experience recurrence in 10 years and progress to hormone-refractory state (Medical Research Council Prostate Cancer Working Party Investigators Group, 1997; Bolla *et al.*, 1997; Ward *et al.*, 2005; Kawakami *et al.*, 2006; Akaza *et al.*, 2006).

2.2. Advanced prostate cancer

A treatment option for advanced, metastasized prostate cancer (T3-4N1M1a-c) is primary androgen deprivation (Huggins and Hodges, 1941; Tammela, 2004) using surgical (orchiectomy) or chemical castration (GnRH agonists and antagonists), estrogen treatment (oestrogen agonists like estramustine), antiandrogen treatment (non-steroidal bicalutamide and flutamide or steroidal cyproterone acetate) in combinations (maximum/complete androgen blockade MAB/CAB), chemotherapy (docetaxel, mitoxantrone, prednisone) or radiation therapy as palliative therapy.

Intermittent androgen suppression (IAS) is, for the moment, an experimental form of treatment, where chemical castration (alone or combined with antiandrogens) is given periodically to achive remission or allowing the disease to progress (Akakura *et al.*, 1993). Phases are monitored using PSA measurement. Cyclic administration could prolong the development of androgen-independent tumor clone and delay the emergence of the hormone-refractory state (Kyprianou *et al.*, 1991; Akakura *et al.*, 1993). In several studies IAS has been shown to decrease morbidity, but no effect on survival has so far been shown (Klotz *et al.*, 1986; Goldenberg *et al.*, 1995; Grossfeld *et al.*, 1998; Horwich *et al.*, 1998; Lane *et al.*, 2004).

Advanced prostate cancer can be treated with the previously described options to remission, but more than 50% of patients with metastatic disease will relapse within 2 years from the beginning of castration (Denis *et al.*, 1993; Crawford *et al.*, 1989). When prostate cancer does not respond to treatment and androgen-independent hormone-refractory state is reached, the treatment options are few. All earlier mentioned therapies can be used. In addition, chemotherapy with e.g. docetaxel and estramustine has proved to be beneficial (Petrylak *et al.*, 2004; Tannock *et al.*, 2004). According to some reports MAB has also increased overall survival by 3-6 months compared to castration alone (Caubet *et al.*, 1997; Denis *et al.*, 1998; Bennett *et al.*, 1999).

3. Prognostic markers of prostate cancer

Although prostate cancer has been studied extensively, there are still only a few clinically used prognostic markers. As more therapies become available, it will be important to identify the right patients for the right treatment. Today, the most commonly used prognostic markers in clinical practice evaluating the efficacy of the treatment of prostate cancer are prostate specific antigen (PSA), Gleason score and T-stage (T) (Partin *et al.*, 1994&1997; Stamey *et al.*, 1999; Kattan *et al.*, 1999; Nelson *et al.*, 2002; Sivridis *et al.*, 2002; Freedland *et al.*, 2005). The TNM classification has evolved under the influence of changed diagnoses and treatment and helps the clinician in the planning of treatment, in the evaluation of the results of treatment, and makes the exchange of information easier, but it can give only some indication of prognosis (Sobin, 2003). Indeed, pT-stage has been proven to be have prognostic value in T1-2 prostate cancers and after radical prostatectomy (Frazier *et al.*, 1993; Noordzij *et al.*, 1997; Ravery *et al.*, 2000; Partin *et al.*, 2001; Li *et al.*, 2005; Yokomizo *et al.*, 2006).

3.1 Prostate specific antigen (PSA)

Prostate-specific antigen (PSA) is a protein produced by normal prostate epithelial cells. This enzyme participates in the dissolution of the seminal fluid coagulum and the largest amounts of PSA are found in the seminal fluid (Albin *et al.*, 1970; Wang *et al.*, 1979). Some PSA escapes from the prostate and can be found in the serum. Diseases such as infection, inflammation, and cancer in the prostate may produce a breakdown in the basement membrane of the glands, the prostatic stroma, and the capillary endothelial cell, allowing more PSA to enter the circulation. In 1980, Papsidero *et al.* (1980) developed a serological test allowing PSA to be measured in the serum. In 1987, Stamey *et al.* (1987) at Stanford University published the first definitive clinical study investigating the utility of PSA in prostate cancer. Since then, extensive investigation and various uses for this protein have been reported.

The standard PSA reference range is 0-4 ng/ml. The detailed reference range is determined age specific, because PSA levels tend to increase with age. The PSA level rise in men with benign prostatic hyperplasia (BPH) and is a good marker for prostate volume (Collins *et al.*, 1993; Bosch *et al.*, 1995&2004; Hochberg *et al.*, 2000; Mochtar *et al.*, 2003; Pinsky *et al.*, 2006). PSA is useful in helping to identify men from whom a prostate biopsy should be taken. For clinical purposes, PSA is considered prostate organ specific but not prostate cancer specific. A major limitation of PSA as a prostate cancer marker is the overlap in values between BPH and prostate cancer. (McNaughton *et al.*, 1997; Carter *et al.*, 1997; Catalona *et al.*, 1998; Thompson *et al.*, 2005). In order to differentiate BPH and cancer, free to total PSA ratio has been suggested to be useful in cases with total PSA between 2-11 ng/ml. A ratio over 15% suggests BPH and in proximately half of the cancer patients the ratio is under 15%.

Studies on using PSA for screening large populations of men for prostate cancer are still underway to determine if PSA screening reduces mortality. At least, more local prostate cancers than advanced are found through PSA-screening (Chadwick *et al.*, 1991; Smith *et al.*, 1996; Isola *et al.*, 2001; Gosselaar *et al.*, 2006; Aus *et al.*, 2006; Ilic *et al.*, 2006). PSA is widely used in monitoring the treatment of prostate cancer. PSA can be used to track the response to therapy in men with prostate cancer. It is used to detect recurrence following radical prostatectomy, with or without radiation therapy. Some 2-3 weeks after radical prostatectomy PSA should be undetectable, if not, it is an indication of residual cancer (Pound *et al.*, 1999; D'Amico *et al.*, 1998&2000; Rogers *et al.*, 2004; Dotan *et al.*, 2005). When hormonal therapy is used, PSA reacts quite rapidly in the course of the natural progression of the disease and is the most used marker monitoring the effect of hormonal or combition treatments (Bolla *et al.*, 1997&2002; Palmberg *et al.*, 1999; Benaim *et al.*, 2002b; D'Amico *et al.*, 2004; Stewart *et al.*, 2005; Bianco *et al.*, 2005; Pilepich *et al.*, 2005; Rodrigues *et al.*, 2006) and it is also considered to be a strong independent prognostic factor after adrogen deprivation in metastatic prostate cancer (Hussain *et al.*, 2006). While PSA is still a good prognostic factor for lethal prostate cancer, recent studies have proved that PSA is a poor predictor of lethal prostate cancer among patients with localized prostate cancer who are managed by watchful waiting (Fall *et al.*, 2007). There is also evidence that finasteride consumption improves the sentitivity of PSA (Thompson *et al.*, 2006). Despite the fact that PSA has been demonstrated to have prognostic value, clearly more accurate means are needed.

3.2 Histological grading of prostate cancer

In 1966 Donald F. Gleason created a grading system for prostatic carcinoma based on the architectural pattern of the tumor (Gleason, 1966; Bailar *et al.*, 1966; Mellinger *et al.*, 1967). In his system the grade was defined as the sum of the two most common grade patterns and reported as the Gleason score. Gleason first intended to classify carcinomas into four patterns, but he found a small group of distinctive tumors of a clear cell pattern and these were placed in a separate fifth category. He further improved the classification in 1974 and 1977 (Gleason, 1974&1977). Since the treatment of prostate cancer has changed over time the scoring system was updated in 2004 by the World Health Organization Classification of Tumors (Epstein *et al.*, 2004) and in 2005 for prostate cancer by the International Society of Urological Pathology (ISUP) Consensus Conference on Gleason Grading of Prostatic Carcinoma (Epstain *et al.*, 2005) (Fig 1.). Comparable classification to Gleason grading is the WHO/Mostofi grading system, where nuclear grade along with glandular differentiation is taken into account (Mostofi, 1974). Gleason grading is more widely used as its has been shown in many studies to be a good prognostic tool (Allsbrook et al., 2001a&2001b; Lilleby *et al.*, 2001; Benaim *et al.*, 2002a; Mitchell *et al.*, 2007) and also constantly elaborated (Epstein *et al.*, 2005).

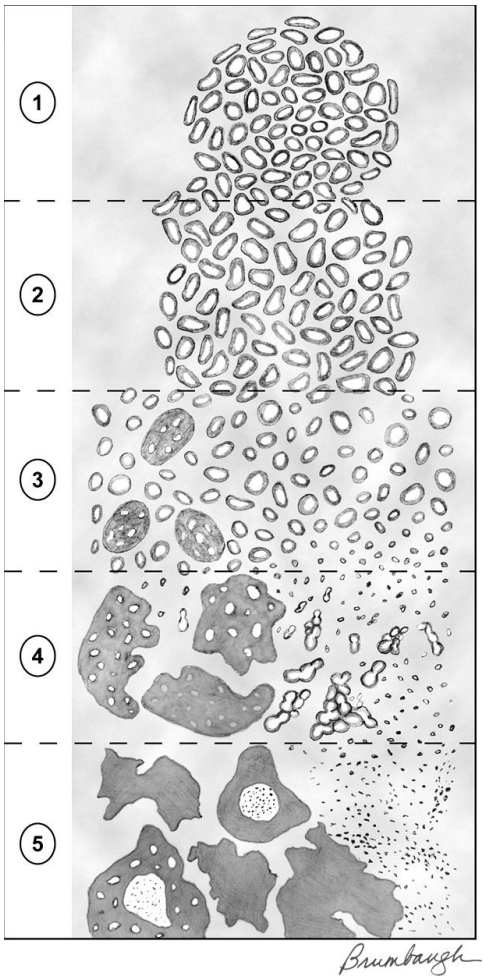

Fig 1. Schematic diagram of modified Gleason grading system.
From: Epstein JI (2005): Am J Surg Pathol 29:1228-1242.

3.3 Other prognostic markers

One approach to evaluate the behavior of prostate cancer is to construct a nomogram. Nomogram is a graphical calculating device with a two-dimensional diagram designed to allow the approximate graphical computation of a function. Nomograms are usually designed to perform a specific calculation with tables of values built in to the construction of the scales. Currently there are more than 40 published prostate cancer nomograms to help with multiple decisions, from the risk of prostate cancer to survival after the development of metastatic, castration-independent disease (Stephenson *et al.*, 2006). The Kattan-nomogram, for example, has been recalibrated to identify substantial groups of prostate cancers that are likely indolent and the nomogram could be used to detect patients for active surveillance (Roemeling *et al.*, 2007). Another approach to obtain additional information about the aggressiveness of prostate cancer is to use various widely studied markers. The most important immunohistochemical

markers proven to have prognostic value in prostate cancer are presented in Table 1. The prognostic markers used in this study are more profoundly discussed in the following chapters 3.3.1 to 3.3.4.

3.3.1 Proliferative activity

Ki-67 antigen identifies cells in proliferative phases of the cell cycle (G1, S, G2 and M), but not at rest (GO phase) or in the early G1 phase (Gerdes *et al.*, 1984). Many studies have shown that the proliferative fraction of primary prostate tumors is associated with grade and/or stage and can even predict survival (Koivisto *et al.*, 1997a; Keshgegian *et al.*, 1998; Uzoaru *et al.*, 1998; Masuda *et al.*, 1998; Bubendorf *et al.*, 1998). The expression of Ki-67 has been shown to be higher in HGPIN and prostate cancer than in normal tissue in radical prostatectomy specimens and in the biopsy specimens (Mucci *et al.*, 2000) and to predict outcome in tissue microarray models (Rubin *et al.*, 2002). After radiotherapy Ki-67 immunostaining has been proven to be a strong independent predictor of failure using biochemical criteria (Cowen *et al.*, 2002).

Another way to measure tumor progression defined by proliferation activity is the definition of Proliferating Cell Nuclear Antigen (PCNA). PCNA activity has been shown to be an independent predictor of progression after prostatectomy, especially in patients with a low risk of progression, compared to Ki-67 (Taftachi *et al.*, 2005). In some studies PCNA associates with tumor grade and patients with lower PCNA score have survived significantly longer than those with higher PCNA scores (Harper *et al.*, 1992; Kallakury *et al.*, 1999). Proliferative activity can also be defined by flow cytometry and e.g. S+G2/M phase has been shown to be an independent predictor of poor survival in patients with non-metastatic prostatic carcinoma (Visakorpi, 1992a; Shockley *et al*., 1996; Borre *et al.*, 1998).

3.3.2 Apoptosis

Apoptosis is the physiologically relevant mode of cell death that counterbalances cell proliferation (Slee *et al.*, 1999). Tissue homeostasis in the normal prostate gland is maintained by the quantitative relationship between the rate of cell proliferation and the rate of apoptotic cell death (Woo *et al.*, 1998). Various apoptosis regulator proteins, e.g. p53 and bcl-2, have been shown to have independent prognostic value in local prostate cancer (Bubendorf *et al.*, 1996; Bauer *et al.*, 1996; Stapleton *et al.*, 1998; Bruckheimer *et al.*, 2000). Apoptosis is apparently an initial event in prostate cancer progression. The prognostic value of apoptosis is controversal, because it seems that prostate cancer progression is due to proliferation rather than apoptosis (Matsushima *et al.*, 1999; Miayata *et al.*, 2005; Ohlson *et al.*, 2005).

Caspases are members of the cysteine protease family. They are synthesized as inactive proenzymes selectively cleaved after an aspartate residue to produce the active enzyme (Alnemri *et al.*, 1996; Cohen, 1997). Caspases are the key mediators of apoptosis and the activation starts a cascade within the caspase

family hierarchy. There are two families of caspases based on the lengths of their NH2-terminal prodomains. Caspase-1, -2, -4, -5, -8, -9, and -10 have long prodomains and function in targeting and regulating apoptosis. Caspase-3, -6, and -7 have short prodomains and are responsible for the execution of apoptosis by operating at the downstream end of the DNA repair enzyme poly(ADP-ribose) polymerase, whose cleavage is essential for apoptosis induction (Whyte, 1996; Slee *et al.*, 1999). Caspase -3 is one of the primary executioners of apoptosis, necessary for the cleavage of a large number of proteins, chromatin margination, DNA fragmentation, chromatin condensation and nuclear collapse during apoptosis (Woo *et al.*, 1998; Slee *et al.*, 2001). It has been shown that the loss of caspase-1 protein is a potential step in the loss of apoptotic control during prostate tumorigenesis and that the pattern of caspase-1 and -3 expression in prostatic tumors may have prognostic significance in disease progression (Winter *et al.*, 2001).

Another method to measure apoptosis is TdT- mediated dUTP-biotin nick end labeling (TUNEL) (Gavrieli *et al.*, 1992). Labeling detects DNA fragmentation, *in situ* visualization, at nuclear level. Cytokeratin fragmentation has also been proven to be a marker of apoptosis. Cytokeratins are cleaved by caspases during apoptosis and released from tumor cells by unknown mechanism. They can be measured e.g. as serum markers for evaluating the clinical progression of cancer in patients with epithelial malignancies (Kramer *et al.* 2004). In the study by Duan *et al.* (2003) immunohistochemistry for activated caspase-3 and cleaved cytokeratin 18 was compared with the TUNEL method and the result was that caspase-3 immunostaining was reliable and showed good correlation with cytokeratin 18 and TUNEL.

3.3.3 EZH2

Varambally *et al.* (2002) found that the expression of *EZH* (polycomb group protein, enhancer of zeste homolog 2), is increased in prostate cancer metastases and also in localized tumors with poor prognosis. Subsequently, Rhodes *et al.*, (2003) showed that increased expression of *EZH2* combined with decreased expression of *CDH1* is associated with brief progression-free survival. Although the detailed function of *EZH2* is incompletely known, it is believed to be the catalytically active component of polycomb repressive complexes 2, 3 and 4 (PRC2/3/4) (Kuzmichev *et al.*, 2005). *EZH2* is essential in early embryonic development, as shown by 100% embryonic lethality in homozygous knockout mice (O´Carroll *et al.*, 2001). In addition, the inhibition of *EZH2* expression by transfection with small interfering RNA (siRNA) or by small hairpin RNA (shRNA) has been shown to lead to cell cycle arrest in G1, G2, and G2/M (Varambally *et al.*, 2002; Bracken *et al.*, 2003; Tang *et al.*, 2004; Croonquist *et al.*, 2005). It has also been shown that *EZH2* expression is strongly associated with cell proliferation activity in many malignancies, including prostate cancer (Kleer *et al.*, 2003; Bachmann *et al.*, 2006). Overexpression of *EZH2* has also been shown to promote neoplastic transformation of breast epithelial cells, and to

be associated with the aggressiveness of breast cancer (Kleer *et al.*, 2003). Saramäki *et al.* (2006) have also shown that *EZH2* gene is amplified in about 20% of hormone-refractory prostate carcinomas.

3.3.4 MCM7

Minichromosome maintenance protein 7 (MCM7) has been suggested to be a molecular marker of prostate cancer aggressiveness. MCM proteins are part of the replication system complex that licenses DNA replication and found to be markers of cell proliferation (Maiorano *et al.*, 2000; Meng *et al.*, 2001; Padmanabhan *et al.*, 2004). Both MCM2 (Meng *et al.*, 2001) and MCM7 (Padmanabhan *et al.*, 2004) have been shown to be proliferation markers.

*MCM7 (*minichromosome maintenance 7) gene is located at 7q21.3. Recently Ren *et al.*, (2006) found that *MCM7* amplification and protein expression have been associated with prostate cancer relapse, 76.5% (52/68) patients with *MCM7* amplification relapsed compared to only 12.3% (7/57) of patients without *MCM7* amplification. In terms of MCM7 protein expression, 76.3% patients with MCM7 expression experienced a recurrence within 5 years after radical prostatectomy compared with only 26.5% patients with weak MCM7 expression. The cancers with high *MCM7* amplification were also considered to be more aggressive.

Table1. Prognostic markers studied by immunohistochemistry. Studies including multivariate analysis are included to evaluate the independent value of the marker. (N.O.S= not significant, SE= standard error)

Prognostic marker	Study material	Multivariate analysis		Publication
		RR (95%CI)	*p*-value	
Ki-67	125 TURP samples,; pT0-pT4, M0-Mx	2.51 (1.39-4.53)	0.0023	Stattin P *et al.* JUrol 157:219-222, 1997.
	156 TURP samples; pT1-pT4, M0-Mx	2.57 (1.57-4.21)	0.03	Dunsmuir WD *et al.* BJU Int 86:869-78, 2000.
	91 needle biopsies (a) , radical prostatectomy samples; pT2-3 (b)	3.6 (1.40-8.90) (b)	0.006	Rubio J *et al.* Eur Urol 48:745-51, 2005.
EZH2	259 radical prostatectomy samples; pT2-pT4 (EZH2+ECAD)	3.19 (1.50-6.77)	0.003	Rhodes DR *et al.* J Natl Cancer Inst 95:661-8,2003.
	104 radical prostatectomy samples	3.4 (1.2-9.5)	0.037	Bachmann IM *et al.* J Clin Oncol 24:268-73, 2006.
	147 patients; biopsy (n=1), radical prostatectomy (n=29), TURP (n=117)	1.9290 (1.2-3.2)	0.0103	Foster CS *et al.* Oncogene 23:5871-9, 2004.
E-cadherin	76 radical prostatectomy samples; pT2-pT3a, N0-1	0.001 (SE) (-0.023-0.002)	0.023	De Marzo AM *et al.* Urology 53:707-13, 1999.
	259 radical prostatectomy samples; pT2-pT4 (EZH2+ECAD)	3.19 (1.50-6.77)	0.003	Rhodes DR *et al.* J Natl Cancer Inst 95:661-8, 2003.
	70 radical prostatectomy samples; pT2	---	0.005	Wu TT *et al.* J Urol 170:78-81, 2003.
Cox-2	60 radical prostatectomy samples; pT1c-pT2b, N0-1	16.442 (4.656-58.067)	0.0001	Cohen BL *et al.* Int J Cancer 119:1082-7, 2006.
p53	175 radical prostatectomy samples; pT1-pT3	---	0.0009	Bauer JJ *et al.* J Urol 156:1511-6, 1996.
	76 preoperative biopsies (a), radical prostatectomy sample; pT1a-pT2c	---	0.02 (a)	Brewster SF *et al.* J Urol 161:1238-43, 1999.
	(b)		0.01 (b)	
	221 prostate cancers; watchful waiting; pT1a-pT2, pT3-4, M0-M1	2.68 (1..35-5 .33)	0.002	Borre M *et al.* J Urol 164:716-21, 2000.
Bcl-2	175 radical prostatectomy samples; pT1-pT3	---	0.007	Bauer JJ *et al.* J Urol 156:1511-6, 1996.
	76 preoperative biopsies (a), prostatectomy samples; pT1a-pT2c (b)	---	0.01 (b)	Brewster SF *et al.* J Urol 161:1238-43, 1999.
	79; 40 radical prostatectomy samples (a), 39 HRPC patients before	N.O.S	N.O.S (a)	Yoshino T *et al*. Clin Cancer Res 12:6116-24.
	taxane based chemotherapy (b)	9.188 (2.288-36.894)(b)	<0.01 (b)	
p27	86 radical prostatetomy samples, pT2a-pT3b	4.99	0.0081	Yang RM *et al.* J Urol 159:941-5, 1998.
	92 radical prostatetomy samples	3.26	0.045	Vis A *et al.* J Urol 164:2156-61, 2000.
	161 radical prostatetomy samples; pT2 (neg. surgical margins)(a) ,	5.15 (1.41-18.83) (a)	0.013 (a)	Freedland SJ *et al.* Urology 61:1187-92, 2003.
	pT2-4(pos. surgical margins) (b)	N.O.S (b)	N.O.S (b)	
AMACR	204 radical prostatetomy samples (a), 188 watchful waiting; pT1a-pT1b	2.12 (1.04-4.32) (a)	0.0006 (a)	Rubin MA *et al.* Cancer Epidemiol Biolmarkers Prev
	(b)	4.06 (1.82-9.06) (b)	0.039 (b)	14:1424-32, 2005.

4. Molecular mechanisms of prostate cancer

The development and progression of prostate cancer involves both germ-line and somatic genetic aberrations inactivating tumor suppressor genes and activating oncogenes. Today, the chromosomal changes associated with prostate cancer are quite well known (see Chapter 4.1), whereas the precise genes are less well known.

Epidemiological studies have shown that hereditary factors are important in prostate cancer development (Grönberg *et al.*, 2003). It has been suggested that about 40% of prostate cancer risk could be heritable (Lichtenstein *et al.*, 2000). Linkage analysis have revealed several high-penetrance susceptibility loci and genes, such as *CABP, HPC1, PCAP*, HPC20, HPCX, *HPC2/ELAC2, RNASEL, MSR1* and 8q24 (Gibbs *et al.*, 1999; Smith *et al.*, 1996; Berthon *et al.*, 1998; Berry *et al.*, 2000; Xu *et al.*, 1998&2001; Tavtigian *et al.*, 2001; Carpten *et al.*, 2002; Freedman *et al.*, 2006; Yeager *et al.*, 2007). Further studies of these genes and loci have suggested that they explain only a small fraction of inheritance of prostate cancer risk. It has been estimated that in Finnish population hereditary prostate cancer (i.e. the man has inherited a high-risk allele) covers approximately 5-10% of all prostate cancers and the major locus for disequilibrium of a haplotype is HPCX region (Baffoe-Bonnie *et al.*, 2005).

Studies on somatic genetic alterations have revealed several genes involved in prostate cancer progression, such as *ERG, GSTP1, TP53, PTEN, NKX3.1, CDH1, EIF3S3* and *AR* (Umbas *et al.*, 1992; Visakorpi *et al.*, 1995a; Bowen *et al.*, 2000; Kwabi-Addo *et al.*, 2001; Linja *et al.*, 2001; Varambally *et al.*, 2002; Nakayama *et al.*, 2003; Tomlins *et al.*, 2005). The most recent important discovery has been the fusion gene, in which the untranslated region of *TMPRSS2* (21q22.3) fuses with the ETS family transcription factor, either with *ERG* (21q22.2), *ETV1* (7p21.2) or *ETV4* (17p21) gene (Chinnaiyan, 2005; Tomlins *et al.*, 2005). *TMPRSS2:ERG* fusion is found in approximately 30-70% of prostate cancers mainly through genomic deletion between *ERG* and *TMPRSS2* (Perner *et al.*, 2006; Saramäki *et al.*, 2007). The high frequency of the range of detection is probably due to methodological and material differences, most likely rearrangement is present in about one third of prostate cancers (Saramäki *et al.*, 2007). *TMPRSS2:ERG* fusion has been shown to be associated with downregulation of cell death pathways (Iljin *et al.*, 2006; Wang *et al.*, 2006). The fusion seems to identify a distinct subgroup of tumors with a favorable prognosis and has been shown not to be associated with classical prognostic factors like Gleason score, pT-stage or PSA nor with cell proliferation activity in prostatectomy specimens (Saramäki *et al.*, 2007).

The silencing of *GSTP* (pi-class glutathione S-transferase gene), which encodes a detoxifying enzyme, results from aberrant methylation at the CpG island in promoter-5' and occurs in the vast majority of cases of HGPIN and in 90% of prostate cancers (Lee *et al.*, 1994; Bastian *et al.*, 2004). Promoter hypermethylation has been shown to be an independent prognostic factor for relapse in patients with prostate cancer following radical prostatectomy (Rosenbaum *et al.*, 2005). Detecting DNA methylation of *GSTP1* has been

proposed to be a novel biomarker in the diagnosis of prostate cancer and is thus the most common genetic alteration described in prostate cancer (Hopkins *et al.*, 2007).

The *TP53* (alias *p53*) tumor suppressor gene plays a role in cell cycle control, DNA repair, and apoptosis (Levine *et al.*, 1991; Vogelstein *et al.*, 1992). Mutations in the *TP53* gene are common events in a wide variety of human malignancies. However, in prostate cancer the mutation seems to be rare in early cancer, but found in later stages of the disease (Hall *et al.*, 1995; Brooks *et al.*, 1996; Mottaz *et al.*, 1997; Navone *et al.*, 2000). *TP53* overexpression has been shown to be strongly associated with *TP53* mutations (Navone *et al.*, 1993; Bookstein *et al.*, 1993) There is evidence that *AR* and *TP53* expression is balanced during the androgen-dependent growth of prostate cancer, which is lost during further progression of the disease (Cronauer *et al.*, 2004). *TP53* overexpression has been found to be associated with advanced stage and poor survival (Visakorpi *et al.*, 1992b; Navone *et al.*, 1993; Bookstein *et al.*, 1993; Thomas *et al.*, 1993; Myers *et al.*, 1994; Bauer *et al.*, 1995).

PTEN (Phosphatase and tensin homolog (mutated in multiple advanced cancers 1)) has been shown to be an inhibitor of PI3K pathway through its downstream molecule AKT in regulating various cell functions including cell proliferation, cell transformation, cell apoptosis, tumor growth and angiogenesis. *PTEN* loss or mutation is common in human prostate cancer. *PTEN* is shown to inhibit angiogenesis by regulating the expression of *HIF-1* and *VEGF* expression through AKT activation in PC-3 cells (Fang *et al.*, 2007). It seems that *PTEN* inactivation generally occurs relatively late in prostate cancer progression and it has been shown that *PTEN* expression is reduced in a large subset of advanced prostate cancers (Halvorsen *et al.*, 2003; Dreher *et al.*, 2004). High frequency of LOH compared to frequency of mutations in *PTEN* suggested that it is targeted by haploinsufficiency and that the loss of the second allele is actively selected during disease progression (Kwabi-Addo *et al.*, 2001; Hill *et al.*, 2005). Further, homozygous deletions, rather than mutations or epigenetic silencing, have been found to be the major mechanism of gene inactivation at this locus (Cairns *et al.*, 1997; Dong *et al.*, 1998; Feilotter *et al.*, 1998). This hypothesis has recently been strengthened by the recurrent finding of homozygous deletions encompassing the *PTEN* region in several prostate cancer cell lines and xenografts (Vlietstra *et al.*, 1998; Hermans *et al.*, 2004), as well as in primary tumors (Verhagen *et al.*, 2006). Pourmand *et al.* (2007) showed that patients with *PTEN* mutation had a significantly higher Gleason score, poorer prognosis, and higher rate of metastasis, but could not show that this mutation predicts the prognosis.

The prostate-specific homeodomain protein *NKX3.1* is a tumor suppressor gene that is commonly down-regulated in human prostate cancer. *NKX3.1* protein is reduced in focal atrophy and PIN but has not been shown to be related to 8p allelic loss. It seems that *NKX3.1* reduces protein levels early in human prostate carcinogenesis, which may facilitate both proliferation and DNA damage in atrophic and PIN cells. Deletions on chromosome 8p are associated with more advanced invasive and aggressive disease (Bethel *et al.*, 2006). In

mice, loss of even one *NKX3.1* allele causes prostatic epithelial hyperplasia and eventual PIN formation (Magee *et al.*, 2003). It is believed that haploinsufficiency is enough to cause altered phenotype or e.g. hypermethylation of promoter regions could have initiative role in prostate cancer (Chaib *et al.*, 2003; Santarosa *et al.*, 2004; Li *et al.*, 2005).

The loss of *CDH1*(alias *E-cadherin*) expression has been shown to be more common in advanced prostate cancer and has been proposed, but not demonstrated, to be a metastasis suppressor gene (Umbas *et al.*, 1992). Bonilla *et al.* (2006) showed recently that *CDH1* is likely a low-penetrant prostate cancer susceptibility gene. In another study by Yegnasubramanian *et al.* (2004) *CDH1* was not abnormally hypermethylated in prostate cancer. Clearly more information is needed to determine the significance of this gene is prostate cancer progression.

As noted the growth of prostate cancer is heavily dependent on androgens, whose action is mediated by the nuclear androgen receptor *(AR)*. *AR* activates the expression of the target gene network and mediates the transcription pathway. *AR* has been proven to play a crucial role in malignant prostate, thus all untreated and the majority of hormone-refractory prostate carcinomas express *AR* and the expression is increased in the hormone-refractory state of the disease (Ruizeweld de Winter *et al.*, 1994; Visakorpi *et al.*, 1995a; Hobisch *et al.*, 1995; Koivisto *et al.* 1997b; Latil *et al.*, 2001; Linja *et al.*, 2001; Gelmann, 2002; Chen *et al.*, 2004). It has been suggested that at the time of diagnosis prostate cancer may already consist of both androgen dependent and independent tumor cells, and that the the selection pressure of the androgen ablation induces the clonal selection and growth of hormone independent cells from a heterogenous cancer cell population (Craft *et al.*, 1999; Taplin *et al.*, 1999). Alternatively, androgen dependent tumor cells may adapt to the low levels of serum androgens during the castration due to polysomy of X-chromosome and the additional copies of the *AR* (Röpke *et al.*, 2004). It has been shown that hormone-refractory tumors emerging during castration re-activate the *AR* signaling pathway by mechanisms such as *AR* gene amplification and overexpression (Visakorpi *et al.*, 1995a; Linja *et al.*, 2001; Linja and Visakorpi, 2004). It has also been suggested that during the androgen withdrawal *AR* could be activated by adrenal androgens (Stanbrough *et al.*, 2006; Tan *et al.*, 1997) or by other ligands (Metzger *et al.*, 2003). *AR* mediated pathways assist the tumor to survive even under strict androgen removal (Baldi *et al.*, 2003; Heinlein *et al.*, 2004; Mulholland *et al.*, 2006). In addition, it has recently been shown by Chen *et al.* (2004) that increased expression of *AR* in prostate cancer xenografts is necessary and sufficient to convert androgen-sensitive growth into hormone-refractory growth. They also proved that hormone-refractory growth is ligand-dependent and requires the nuclear action of *AR*.

Mutations of *AR* gene are rare in early-stage, untreated tumors and become more common in late-stage hormone independent prostate tumors. Instead, the highest frequency of mutations seems to be in prostate cancers treated with antiandrogen, flutamide or bicalutamide. Mutation frequencies of 10-30% have been reported in such cases (Taplin *et al.*, 1999&2003; Haapala *et al.*, 2001;

Linja and Visakorpi, 2004). Han *et al*. (2005) have recently shown that mutated *AR* causes oncogenic transformation of prostate leading to development of PIN, which progressed to invasive and metastatic disease in 100% of transgenic mice examined. *AR* mutations are most often missense point mutations (threonine at position 877 is substituted to alanine) and are located in the coding regions of the *AR* gene. Mutations in non-coding regions have been much less studied. Waltering *et al.* (2006) recently reported that no recurrent mutations were identified in noncoding regulatory regions of *AR* and mutations do not explain the overexpression of *AR* in hormone-refractory prostate cancer.

4.1 Chromosomal alterations of prostate cancer

There are several techniques to study chromosomal alterations in solid tumors. Chromosomal comparative genomic hybridization (cCGH) to detect DNA copy number aberrations was first described in 1992 by Kallioniemi *et al.* In cCGH diversely labeled tumor and normal DNA are hydridized to normal peripheral blood lymphocytes and the ratios of the hydridized signals are analyzed with computer software. This invention revolutionized molecular cytogenetics. Since then it has been possible to generate genome-wide information about copy number alterations from an individual solid tumor from a single hydridization. Because good quality metaphases are difficult to obtain from clinical tumor samples, the method offers advantages compared to other classical cytogenetic analyses that are reliant on cancer cell metaphases like multiplex fluorescence *in situ* hybridization (M-FISH) or spectral karyotyping (SKY) (Speicher *et al.*, 1996; Schröck *et al.*, 1996; Macville et al., 1997). Otherwise M-FISH and SKY are convenient methods for studying cancer, because simultaneous identification of copy number changes and translocations from single hybridization can be obtained. Another method, loss of heterozygosity (LOH) analyses, provides information on allelic balance utilizing polymorphic markers (de Nooij-van Dalen *et al.*, 1998; Varella-Garcia *et al.*, 1998; White *et al.*, 1998). However, LOH data cannot always be interpreted as physical copy number losses, because the remaining allele may be duplicated after the loss of the first allele.

Variety of DNA microarray technologies provide more precise genome-wide profiling of gene expression and gene copy number changes even to the level of single nucleotide polymorphisms (SNPs) (Schena *et al.*, 1995&1996). In microarray technologies the sample is hybridized with the normal genetic information presented as cDNA, BACs, PACs (Solinas-Toldo *et al.*, 1997; Pinkel *et al.*, 1998) or oligonucleotides (Lockhart *et al.*, 1996, Wang *et al.*, 1998; Lucito *et al.*, 2003; Snijders *et al.*, 2001; Barrett *et al.*, 2004).

It has been shown that the frequency of chromosomal changes is associated with the stage of prostate cancer. In the early stages of prostate cancer, losses of the genetic material are more common than gains or amplification, whereas in metastatic and hormone-refractory tumors, gains are more frequently detected. This indicates that the aberrations in tumor suppressor genes may be important in

the initiation of the prostate cancer and the oncogenes are activated later (Visakorpi *et al.*, 1995b; Elo and Visakorpi, 2001; Porkka and Visakorpi, 2004).

4.1.1 Losses of genetic material

The chromosomal regions mainly showing losses in prostate cancer by cCGH are 6q, 8p, 10q, 13q, 16q, 18q (Visakorpi *et al.*, 1995b; Cher *et al.*, 1996; Nupponen *et al.*, 1998a; Alers *et al.*, 2000 & 2001; Fu *et al.*, 2000; Chu *et al.*, 2003; Teixeira *et al.*, 2004; Ribeiro *et al.*, 2006). However, only very few target tumor suppressor genes have been identified. Deletions at 6q15-q22 region have been reported in many studies by LOH and cCGH (Dong, 2001). According to cCGH studies, 23% of the primary tumors and about 40% in recurrent tumors of prostate have a loss in this region (Visakorpi *et al.*, 1995a; Alers *et al.*, 2001). Although, there have been suggestions of putative suppressor loci at 6q (Cooney *et al.*, 1996), no probable target genes for that region have been identified.

The most common chromosomal deletion is the loss of 8p, which has already been found in the HGPIN stage of prostate cancer (Visakorpi *et al.*, 1995b; Zitzelsberger *et al.*, 2001) and has been proposed to be involved in early genetic events in prostate cancer progression according to cCGH studies (Ribeiro *et al.*, 2006). Three different regions of loss have been identified (Jenkins *et al.*, 1998; Macoska *et al.*, 1995; Paris *et al.*, 2004), and so far putative genes for these regions are *NKX3-1* (8p21.2), *LZTS1* (8p22), and *MSR1* (8p22) (He *et al.*, 1997; Ishii *et al.*, 1999; Hawkins *et al.*, 2002). According to cCGH studies, about 30-40% of prostate carcinomas involve the loss of 8p (Dong, 2001; Ribeiro *et al.*, 2006) and in metastatic and hormone-refractory tumors it has been noted in 70-80% of samples (Cher *et al.*, 1996; Nupponen *et al.*, 1998b).

Deletions at 10q have been detected in 27% of the prostate cancer samples studied by cCGH, and in 30-60% of those studied by LOH (Dong, 2001). Two minimal regions of loss have been found, one proximally around centromere (10cen-q21 region) and the other distally (10q-23-ter) (Nupponen *et al.*, 1998a; Hermans *et al.*, 2004). At the last-mentioned region a well-known tumor suppressor gene *PTEN* at 10q23.3 is located and another suggested candidate gene *MXI1* (MAX interactor 1, isoform b) at 10q25.2 (Zervos *et al.*, 1993; Prochownik *et al.*, 1998; Suzuki *et al.*, 1998).

The second most frequently deleted chromosomal region is 13q, and is detected in approximately 30-40% of primary tumors (Visakorpi *et al.*, 1995b; Alers *et al.*, 2001; Teixeira *et al.*, 2004) and about in 50-75% of metastatic tumors (Cher *et al.*, 1996; Nupponen *et al.*, 1998b). Three separate regions of loss have been detected, 13q14, 13q21-22, 13q33 (Hyytinen *et al.*, 1999) and although two well-known genes *BRCA* (breast cancer 2, early onset) and *RB1* (retinoblastoma 1 gene) are situated at 13q13.1, no evidence of significance of these has been reported in sporadic prostate cancer (Tricoli *et al.*, 1996; Li *et al.*, 1998; Latil *et al.*, 1999). In any case, recent regression analyses of cCGH studies have revealed that 13q loss is an early event and a predictor of locally invasive prostate cancer (Ribeiro *et al.*, 2006).

Several regions of loss at 16q have been shown in LOH and cCGH and reported to be associated with advanced tumors with poor prognosis (Suzuki *et al.*, 1996; Latil *et al.*, 1997; Elo *et al.*, 1997&1999; Li *et al.*, 1999; Teixeira *et al.*, 2004; Härkönen *et al.*, 2005). In local tumors studied by cCGH it was seen in 20-30 % (Visakorpi *et al.*, 1995b; Alers *et al.*, 2001) and in advanced stage in 40-55% of prostate cancer tumors (Cher *et al.*, 1996; Nupponen *et al.*, 1998b; Teixeira *et al.*, 2004). One promising target gene in this region is *CDH1* at 16q22.1. Ribeiro *et al.* (2006) showed in their regression analysis of cCGH studies that 16q loss is an intermediate event in progression of prostate cancer and the loss of 8p is often followed by 16q loss.

According to cCGH studies, deletion of 18q is found in 13-19% of local tumors (Visakorpi *et al.*, 1995b; Fu *et al.*, 2000; Alers *et al.*, 2001) and in 28-42% of advanced prostate tumors (Nupponen *et al.*, 1998b; Ribeiro *et al.*, 2006). There have been several candidate genes for 18q loss (*SMAD2, SMAD4, DCC*), but none of them has been proved to explain the aberration (Ueda *et al.*, 1997; Yin *et al.*, 2001). In cCGH based regression analysis 18q loss has proven to be an intermediate event in the progression of prostate cancer and 13q loss might be followed by 6q and 18q losses (Ribeiro *et al.*, 2006).

4.1.2 Gains of genetic material

The most common gained regions in prostate cancer are 7p/q, 8q and Xq. (Visakorpi *et al.*, 1995b; Cher *et al.*, 1996; Nupponen *et al.*, 1998a; Alers *et al.*, 2000&2001; Fu *et al.*, 2000; Skacel *et al.*, 2001; El Gedaily *et al.*, 2001; Chu *et al.*, 2003; Teixeira *et al.*, 2004; Ribeiro *et al.*, 2006).

7q is quite frequently a gained region in prostate cancer. In cCGH studies about 10-20% of local tumors and 56% in recurrent tumors have been shown gain in this region (Visakorpi *et al.*, 1995b; Chu *et al.*, 2003). It is associated with late events in prostate cancer evolution and with poor prognosis (Alers *et al.*, 2000; Ribeiro *et al.*, 2006). *MCM7* gene is located at the 7q21.3.

The most common gain is 8q, and it is prevalently detected in metastatic and hormone-refractory prostate carcinomas (Visakorpi *et al.*, 1995b; Alers *et al.*, 2000; van Dekken *et al.*, 2003). As the highest, almost 90% of the advanced tumors carry 8q gain, compared to primary tumors with the lowest incidence of 6% (Visakorpi *et al.*, 1995b; Cher *et al.*, 1996; Nupponen *et al.*, 1998b). Two minimal regions of gain have been detected by cCGH in hormone-refractory tumors: 8q21 and 8q23-24 (Visakorpi *et al.*, 1995b; Cher *et al.*, 1996; Nupponen *et al.*, 1998b). A well-known oncogene *MYC* is located in 8q24 and over expressed in many cancers, but it has not been shown to be overexpressed in prostate carcinomas (Savinainen *et al.*, 2004) and it seems to be associated with poor prognosis (Sato *et al.*, 1999; Alers *et al.*, 2000). Another interesting gene in the 8q23-24 region is the earlier disscussed *EIF3S3*gene. *EIF3S3* (a subunit of a translation factor eIF3) gene has been shown to be amplified in 9% of local tumors, in 50% of the metastases and 30% of the hormone-refractory tumors studied by cCGH (Nupponen *et al.*, 1999; Saramäki *et al.*, 2001). *MYC*

(myelocytomatosis viral oncogene homolog) and *EIF3S3* have been shown to be coamplified in recurrent prostate tumors, but the *EIF3S3* gene has been proposed to be the candidate gene for the 8q amplification (Nupponen *et al.*, 1999; Saramäki *et al.*, 2001; Savinainen *et al.*, 2004). The gain of *EIF3S3* is also associated with poor prostate cancer specific survival (Saramäki *et al.*, 2001). cCGH based regression study Ribeiro *et al.* (2006) showed that 8p is followed by 16q loss and 8q gain and therefore it is proposed that 8q gain is an intermediate event in prostate cancer evolution leading to invasive or metastatic disease.

By cCGH, gain of Xq has been detected in over 50% of hormone-refractory prostate carcinomas, whereas in primary tumors it is not seen (Visakorpi *et al.*, 1995b). At the region Xq12-q13, where *AR* gene is located, high-level amplifications have been found in 30% of hormone-refractory prostate carcinomas (Visakorpi *et al.*, 1995a). The role of *AR* in prostate cancer is discussed in Chapter 4.

AIMS OF THE STUDY

The overall aim of this dissertation was to determine the genetic changes and the nature of prostate cancer progression in models and in patient material and to seek out novel approaches to more accurately determine prostate cancer prognosis after radical prostatectomy.

The more specific objectives were:

1. To determine the usefulness of xenografts in studying prostate cancer
2. To define the genetic changes and the clonality of metastatic prostate cancer
3. To evaluate the cellular effects of intermittent androgen deprivation
4. To evaluate the prognostic significance of immunohistochemical markers Ki-67, EZH2**,** MCM7 and *EIF3S3* in radical prostatectomy material

MATERIALS AND METHODS

The materials and methods used in this study are listed below. More detailed descriptions of the materials and methods will be found in the original publications I-III.

List of materials used:

- Xenografts: LuCaP 23.8, 23.12, 35, 41, 49, 58, 69, 70, 73 and LAPC-4AD, LAPC-4AI, LAPC-9AD, LAPC-9AI LuCaPs from Dr.Robert L.Vessella (vessella@u.washington.edu) and LAPCs from: Dr. Charles L. Sawyers (martinb@mskcc.org) (Pretlow *et al*.,1993; Reiter and Sawyers, 2001) — Study I
- Prostate cancer cell line 22Rv1 (ATCC ,Rockville, MO, USA) — Study I
- 29 prostate cancer patients in following T- stages: T1c (n=1), T2 (n=1), T3 (n=15), T4 (n=12), Nx (n=29), M0 (n=15) and M+ (n=14) (Tampere University Hospital, Tampere, Finland) — Study II
- 226 radical prostatectomy samples: pT2-3 (Tampere University Hospital, Tampere, Finland) — Study III

List of used methods:

- Comparative genomic hybridization (cCGH) — Study I
- Fluoresence in situ hydridization (FISH) — Study III
- multi-color fluorescence in situ hybridization (armFISH) — Study I
- Immunohistochemistry — Study II, III

In study II and III the immunohistochemical stainings Ki-67, EZH2, MCM7 and caspase-3 were evaluated as percentige of positive stained cells. Cut-off value of semiquantitative groupings were determined according to case distribution.

All studies with clinical samples were approved by the local Ethical Committees. Studies II-III were approved by Tampere University Ethical Committee and the National Authority for Medicolegal Affairs.

The unpublished material to define the genetic changes and the clonality of metastatic prostate cancer can be seen in Table 2. Altogether 85 metastatic samples from various metastatic sites were obtained from 29 men who died of prostate cancer and underwent autopsy as part of a rapid autopsy program at the Johns Hopkins Hospital. Patients had been treated by androgen-deprivation therapy. Tissues were snap-frozen and stored at -80°C as described previously (Suzuki *et al.,* 1998). The method used was chromosomal comparative genomic hybridization (cCGH). More detailed descriptions of the cCGH method will be found in Study I. The cCGH results were analyzed first by SAM (Significance Analysis of Microarrays) to calculate an estimate of the median false discovery

rate (FDR). SAM uses repeated permutations of the data to determine if the expression of any gene is significantly related to the response. The cutoff for significance is determined by a tuning parameter delta, chosen by the user based on the false positive rate. The Cluster/TreeView was used for clustering. The hierarchical clustering was applied to identify potentially clonally related metastases within and among the study subjects. In hierarchical clustering the Spearman Rank Correlation, uncentered correlation, centered correlation, absolute uncentered correlation and Kendall's Tau correlation was used. TreeView was used to visualize the results.

Table 2. Distribution and number of distinct metastatic (n=85) lesions studied in 29 patients.

Case No.	Metastatic site					Total No.
	LN	B	L	SuD	O.S.	
A1				1		1
A2	1	1	1			3
A3	2			1		3
A4			1			1
A5	1	1				2
A7				2		2
A8	1		1			2
A9	1					1
A10	3				1	4
A11	1					1
A12	3					3
A13		2				2
A14	1		1			2
A16	1			1	2	4
A17	5	1		2		8
A18	2					2
A19	1	1				2
A21		1	3		1	5
A22	1	1			2	4
A23			2			2
A24	1	2			1	4
A26					2	2
A27	1					1
A28	2				2	4
A29	1				1	2
A30		2	2			4
A31	1			1	2	4
A32	2	2			1	5
A33	2			1	2	5
=	34	14	11	9	17	**85**

LN= lymph node metastasis, (e.g. para-aortic, mediastinal, inguinal, perigastric, axillary, iliac), **B**= bone metastasis, (e.g. sternum, vertebrae, humerus), **L**=liver metastasis, **SuD**= subdural metastasis, **O.S.=** other sites, (e.g. , adrenal).

RESULTS AND DISCUSSION

1. Genetic alterations in prostate cancer xenografts by cCGH

On average 13 (range 5-28) alterations per xenograft, 5 gains (1-13) and 8 losses (1-15) were found. Most often gained chromosome arms were 7q (43% of the cases), 8q (64%), and Xq (50%). High-level sub-arm amplifications were found at 2p21-pter (LuCaP 70), 3q26-qter (LuCaP 41), 7p14-q11 (LuCaP 70), 7q32-qter (LuCaP 41), 8cen-q21 (LuCaP 23.8), 8q21-q22 (LuCaP 35), 8q22-qter (LuCaP 69), 8q23-qter (LuCaP 58, LuCaP 70), 8q24-qter (LuCaP 23.8, LAPC-4AD, LAPC-4AI), 9q34-qter (LAPC-4AD), 16cen- p12 (LuCaP 70) and Xcen-q13 (LuCaP 69). Most often lost chromosome arms were 2q (71%), 5q (50%), 6q (79%), 8p (64%), 13q (50%), 18q (57%). The minimal common regions of deletions were 2q21-q22, 5q13-q21, 6cen-q22, 8p21-pter, 13q22-qter, and 18q21-qter. 22Rv1, which is an *in vitro* growing cell line from CWR22R androgen independent xenograft, (Nagabhushan *et al.*, 1996; Sramkoski *et al.*, 1999) revealed very similar cCGH findings to those previously published for the original androgen-dependent CWR22 (Bubendorf *et al.*, 1999). In our study, by CGH, gains of 1q, 7p15-qter, 8p12-p22, and 12 and losses of 2q13-31 were found. The cell line was also analyzed with armFISH, which revealed three related clones. Karyotypes were 51, XY, +i(1)(q10), der(2)t(2;4)(p13;q35)del(2)(q?), +3, der(4)t(2;4)(p13;q35), t(6;14)(q15;q32), +7, +8, +12[7]/ 49, XY, idem, +der(1)t(1;8)(q11;q12?), -3, -8 [11] /49, XY, idem, +der(1)t(1;8)(q11;q12?), -3, -8, t(7;19). Altogether three balanced translocations were found. These were t(2;4)(p13;q35), t(6;14)(q15;q32), and t(7;19).

These genetic aberrations have all been previously found by the cCGH analyses of clinical prostate tumors (Visakorpi *et al.*, 1995b; Joos *et al.*, 1995; Cher *et al.*, 1996; Nupponen *et al.*, 1998b; Alers *et al.*, 2000). Minimal regions of deletion in prostate cancer at 8p21 corresponded well, e.g. *NKX3.1* gene is located there. Nine out of the 14 cases showed gain of 8q and most of them showed high-level amplification of 8q23-q24, which is the most common minimal amplified region of 8q (Cher *et al.*,1996; Nupponen *et al.*, 1998b). LuCaP 35 showed high-level amplification of 8q21-q22, representing the other minimal commonly amplified region. Therefore, the xenografts are also likely to be valuable in the identification of the target genes for the 8q gain. LuCaP 69 showed high-level amplification at Xq12-q13 by cCGH. It has previously been shown that this xenograft contains a high-level amplification of *AR* gene in the region (Linja *et al.*, 2001). Another xenograft containing *AR* gene amplification is LuCaP 35 (Linja *et al.*, 2001), which showed a gain of Xpter-q13. Saramäki *et al.* (2006) recently published microarray CGH (aCGH) data of same LuCaP xenografts. In comparison of aCGH and cCGH, the results were strikingly similar (90% agreement) and aCGH found only a few additional regions of gains or losses compared with cCGH. aCGH was presumably more capable of detecting smaller alterations in pericentromeric regions.

The cCGH findings of the xenografts, LuCaP 23.8 and LuCaP 23.12 deriving from lymph node and liver metastases from a single autopsy (Ellis *et al.*, 1996), were very similar. A previously published cCGH analysis of a third xenograft, LuCaP 23.1 established from the same autopsy also indicated the same alterations as well as gains at 5q, 6q, 12q (Williams *et al.*, 1997). The findings suggest that a single clone was the source of metastases to different sites, and that the subsequent metastatic lesions underwent undetectable, if any, additional genomic alterations. The finding in this one case is similar to results in the analysis of multiple metastatic lesions from several autopsy cases in unpublished material.

Comparison both androgen-dependent (AD) and androgen-independent (AI) sublines of LAPC-4 and -9 showed that the androgen-dependent and androgen - independent xenografts have nearly identical genetic alterations. This may be due to the fact that the xenograft cell population has already gone through several selections, and therefore the cells are likely to be genetically more homogenous. It is not yet known whether there is a difference between the xenografts and tumors in man in the mechanisms of progression from androgen dependence to androgen independence.

Most of the xenografts resembled prostate tumors in their chromosomal alterations, which indicates that these are very useful in studying prostate cancer. The genetic composition of each xenograft allows one to choose the best model for studying a particular question or for the identification of genes involved in the development and progression of prostate cancer. Additionally, according to this study, the transition of the growth from androgen dependence to independence does not involve major chromosomal aberrations, amplification of the AR gene being a possible exception.

2. Genetic alterations and clonality of metastatic prostate cancer by cCGH

A total of 85 prostate cancer metastatic lesions from 29 patients who had died of prostate cancer were analyzed by cCGH. The most frequently lost chromosome arms (lost in more than one-third of the cases) were: 13 q (83 %), 8p (83%), 16q (59 %), 4q (45%), 6q (41%), 4p (38%). The minimal commonly lost regions in these chromosome arms were: 4cen-p14, 4cen-q28, 6cen-q23, 8cen-p21, 13q14-q31, 16q22-qter, whereas the most frequently gained chromosome arms were: 8q (86%), 16p (66%), Xq (59%) and 7q (38%). The minimal commonly gained regions were: 7q31-qter, 8q23-qter, 16p12-pter Xq12-q13.

The large number of genetic alterations found emphasizes the importance of genetic instability in the progression of prostate cancer. Compared to previous cCGH analyses on untreated primary prostate carcinomas, these metastatic lesions contained over 5 times more alterations and over 14 times more loci containing gain than the primary tumors (Visakorpi *et al.*, 1995b). Altered regions were mostly the same, as has previously been suggested in either locally-recurrent hormone-refractory or hormone-naive metastatic lesions of prostate cancer (Visakorpi *et al.*, 1995b; Cher *et al.*, 1996; Nupponen *et al.*, 1998b; Fu *et al.*, 2000; Alers *et al.*, 2000&2001; Teixeira *et al.*, 2004; Ribeiro *et al.*, 2006)

demonstrating that no large regional genomic alteration is specific to the metastatic prostate cancer phenotype. Additionally, this data is similar to the earlier cCGH findings of xenografts in Study I. The xenografts were also established from tumors progressing during androgen withdrawal from hormone-refractory tumors. Commonly aberrated lost chromosomal regions in clinical samples and xenografts were 6q, 8p and 13q and gained regions were 7q, 8q, Xq. Chromosomal alterations in xenografts closely resemble the clinical samples of recurrent prostate cancer.

To study the clonal relationship of the metastatic lesions, unsupervised clustering analysis was utililized (Fig 2.). In 24 cases more than one metastatic lesion was available. SAM was used to filter the data to indicate 220 significant markers. Out of 220 markers, 95 contained mostly gains and 125 contained mostly losses. These 220 markers were further clustered with Cluster program and visualized with Treeview program. The clustering analysis separated samples into "correct" clusters in 14 out of 24 cases (58.3%) indicating a strong clonal relationship. 3/24 (12.5%) of all metastases in individual cases were clustered to completely different groups indicating a weak clonal relationship. In 2 cases 80% and in another 2 cases 75% of the metastases were clustered into the same group, whereas in 1 case each, 67%, 50%, and 40% of the metastases clustered into same group.

In the clustering analysis, a strong genetic relationship within cases was found. The finding of a close clonal relationship of the multiple metastatic lesions is somewhat surprising, since it has been shown that primary tumors often contain several genetically only distantly related clones. For example, studies on whole-mount prostatectomy specimens have shown that the prostate gland may contain several carcinoma foci, containing different genetic changes (Sakr *et al.*, 1994; Jenkins *et al.*, 1997; Cheng *et al.*, 1998; Macintosh *et al.*, 1998). It has also been shown that metastatic lesions do not necessarily resemble the primary tumor or that metastases originate from a minor tumor focus (Jenkins *et al.*, 1997). In addition, we earlier analyzed prostate tumor specimens by cCGH from patients before hormonal treatment and during the treatment at the time of local progression. Only half of such primary-recurrent tumor pairs showed a close genetic relationship as evidenced by the large number of shared genetic alterations in the primary and recurrent tumors (Nupponen *et al.*, 1998b). In consequence, the results here suggest that there are two major types of metastatic prostate cancer at the level of the individual patient: uniclonal metastatic prostate cancer, whose origins can be traced to a single genomically aberrant prostate cell, and multiclonal metastatic prostate cancer, which originates from two or more genomically aberrant prostate cells. Note that in Study I, xenografts LuCaP 23.8 and LuCaP 23.12 (derived from metastases from a single autopsy) the genetic aberrations found were very similar, suggesting the same clonal mechanism. The fact that metastatic lesions share nearly identical macrogenomic alterations strongly suggests that the key mechanisms of the metastatic event in different lesions are the same.

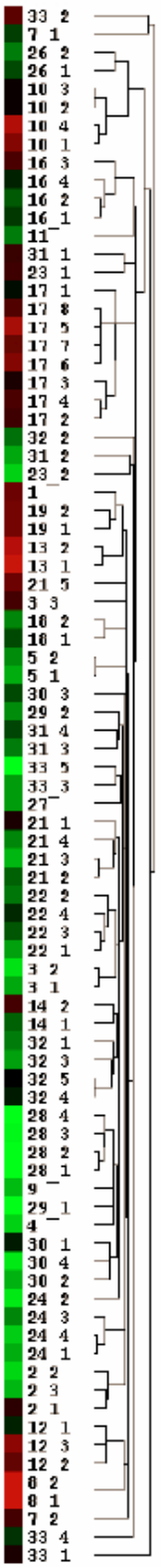

Fig 2. Treeview visualization of the unsupervised hierarchical cluster-analysis of 220 significant markers filtered with SAM program. Case numbers with the metastasis site code (see Table 2.) are shown on the right.

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In conclusion, when prostate cancer cells metatasize, they appear to have all the necessary genomic information needed to survive outside the prostate. The primary tumors being genetically more heterogeneous, but the metastatic lesions more homogeneous, it is also likely that the primary tumors do not function as a deposit of cancer cells that spread cells over a long period of time. Instead, the dissemination possibly takes place in a relatively short period of time, and the metastatic clones continue to live independently outside the prostate gland without interference and competition of other tumor clones from the prostate gland. According to our results, it seems that the metastatic environment (bone, visceral, etc.) does not select the metastatic clone, and the different metastases do not evolve much at the site of the metastases. Understanding and predicting therapeutic success in an individual will depend to some degree on clonality as well as genomic alteration pattern for metastatic lesions in a given patient, and the uniclonal and multiclonal forms of metastatic prostate cancer have potentially important implications for the treatment of metastatic prostate cancer. Further studies are needed to determine whether the macrogenomic uniclonality identified in the majority of metastatic prostate cancer patients studied here extends to the microgenomic (individual base pair) level.

3. Cellular changes in prostate cancer treated by intermittent androgen deprivation

The effect of primary androgen withdrawal was evaluated using the whole material (n=29) consisting patients in IAS (n=23) and in continuous withdrawal group (n=6). There was no statistically significant difference in the Gleason score before and after castration (p=0.813). Although cases with Gleason score 6-8 at the time of diagnosis were evaluated, 5/10 cases showed increased Gleason score after 6 months of castration. The proliferation activity determined by Ki-67 immunostaining was significantly (p= 0.002) reduced 6 months after the beginning of androgen withdrawal. Altogether 9/15 cases showed reduction in the Ki-67 staining upon the first castration. The rate of apoptosis as determined by cleaved-caspase 3 showed no changes after 6 months of castration.

The main finding was the significant reduction in the cell proliferation rate induced by castration after 6 months of treatment. The reduction was already apparent 3 months after the beginning of castration, but due to the small number of samples at 3 months, the reduction was not statistically significant at that time. The finding is similar to those of earlier studies showing a decreased cell proliferation rate already 7 days after castration and up to 8 months after the treatment (Westin *et al.*, 1995; Paterson *et al.*, 1999; Matsushima *et al.*, 1999; Miayata *et al.*, 2005; Ohlson *et al.*, 2005).

There was no effect on apoptosis after castration. It is possible that castration increases apoptosis only immediately after androgen withdrawal, whereas the long-term inhibitory effect of castration on cancer growth is due to a decreased

proliferation rate. One possible mechanism for lack of increased apoptosis after 3 or 6 months of castration could be failure to achieve castration levels of testosterone in LHRH analogue treated patients. In addition, it seems that both moderately and poorly differentiated tumors respond similarly to castration in terms of apoptosis, because there was no association between Gleason score at the time of diagnosis and apoptosis rate after castration.

Contrary to expectation, no statistically significant castration-induced changes in the Gleason score were found. However, it should be noted that a large fraction of the cases already had a Gleason score of 8-10 at the time of diagnosis. Thus, the number of tumors that could theoretically show an increase in Gleason score was quite low. Half of the cases with a Gleason score of 8 or less actually showed an increase after 6 months of castration, but it is possible that tumors with lower Gleason score might respond differently to the IAS. One explanation could be that androgen withdrawal, either by orchiectomy or LHRH analogue, as well as combined androgen blockade, possibly leads to morphological changes resulting in an increased Gleason score (Bostwick, 1994&2004; Vaillaincourt *et al.*, 1996). It has also been shown that although a 3-month treatment with LHRH analogue affected the histology of tumors, it did not significantly affect the Gleason score, a finding which concurs with this data (Rubin *et al.*, 2005).

Only the samples from the IAS arm (n=23) were used in determining the changes in the Gleason score, Ki-67, and cleaved caspase-3 staining during the subsequent cycles of androgen withdrawal. There were no significant changes in the Gleason scores in association with androgen withdrawal in any treatment cycles and no evidence of up-shift in the Gleason score during the course of the disease. Ki-67 was reduced during the first androgen withdrawal, but showed no statistically significant changes in subsequent rounds of withdrawal. At the first progression, the level of Ki-67 staining was about the same as at the time of diagnosis and remained about the same during subsequent cycles of remission and progression. Nor were there any significant changes in cleaved caspase-3 staining during the IAS cycles either. In addition, androgen withdrawal led to a significant reduction in PSA in every cycle and there was no sign of an increase in PSA at any point of the treatment. All available samples (n=113) showed strong nuclear staining with anti-AR antibody, no cytoplasmic staining was found in any of the samples.

The fact that subsequent rounds of androgen withdrawal had no statistically significant effect on the proliferation rate suggests that mechanisms regulating cell growth are less related to serum levels of androgens than at the time of diagnosis. The tumors may have acquired the mechanisms for growing in the presence of low levels of androgens already prior to the second cycle of androgen withdrawal. These findings concur with data from a preclinical study by Buhler *et al.* (2000), who used a human prostate cancer xenograft model, LuCaP 23.12, to investigate the effect of IAS. No cycling in tumor volume during different cycles of IAS was found in this xenograft.

It has been shown that hormone-refractory tumors emerging during castration re-activate the *AR* signaling pathway by mechanisms such as *AR* gene amplification and overexpression (Linja and Visakorpi, 2004). The

immunohistochemistry of AR showed strong nuclear staining in all samples irrespective of the treatment cycle (castration or no castration), consistent with earlier findings of nuclear AR staining during IAS in a human prostate xenograft model (Bladou *et al.*, 1996). It is known that after activation AR binds to androgen and is translocated into the nucleus. In untreated prostate cancer as well as in hormone-refractory prostate cancer, AR is predominantly seen in the nuclei. The findings here indicate that AR is activated even during the androgen withdrawal, possibly by the remaining adrenal androgens (Tan *et al.*, 1997; Stanbrough *et al.*, 2006) or by other ligands (Metzger *et al.*, 2003).

The finding that, except in a few individual cases, both the Gleason score and the proliferation rate remained about the same during the progression of the disease and during each step of the IAS cycles suggests that the histological differentiation as well as the proliferation activity of a particular tumor are defined already at an early stage of tumorigenesis. There is no additional dedifferentiation during the progression of the disease, suggesting that the biological aggressiveness does not shift during progression. As noted in unpublished material, the primary tumors are likely to be genetically heterogeneous, but the metastatic lesions are homogeneous and the metastatic clones continue to live independently outside the prostate gland without interference and/or competition from other tumor clones from the prostate gland. These findnings support the conception that the capability to metatasize may be defined already in early tumorgenesis. However, since this material was enriched by tumors with a high Gleason score it is entirely possible that tumors with a low Gleason score could show dedifferentiation during the progression of the disease. This should be further studied.

4. Significance of prognostic markers Ki-67, EZH2, MCM7 and *EIF3S3* in localized prostate cancer treated with radical prostatectomy

For the evaluation of the prognostic value of Ki-67, and novel molecular markers EZH2, MCM7 and *EIF3S3*, a population-based radical prostatectomy material of 249 patients was used. The analyses were successful in 229 cases for Ki-67, in 213 for EZH2, in 221 for MCM7 and in 195 for *EIF3S3*. The markers were compared to the parameters routinely used in the clinic (pT-stage, Gleason score, PSA). The end point was biochemical failure.

The best discriminatory cut-off values were selected for evaluation of the Kaplan-Meier curves utilizing Mantel-Cox test for the diagnostic PSA, Ki-67, EZH2, and MCM7 immunostainings. PSA and Ki-67 gave the best discrimination when the material was divided into three groups. In the material as whole both PSA and Ki-67 were strongly associated with a short progression-free time (p=0.0037, and p=0.0010 respectively). For the EZH2 and MCM7, dichotomous grouping seemed to have the best prognostic value (p=0.0001 both)**.** Both markers identified a small group of patients with a very poor prognosis. The combination of these two markers identified a patient group of about 12% of all patients (n=24), whose median progression-free time was about

2.2 years compared to about 9.2 years in the rest of the patients. Of the 24 patients with high EZH2 or MCM7 cell fraction, 19 (73%) experienced disease progression during follow-up. In the material as whole, pT-stage and Gleason score ($p<0.0001$ respectively) were associated both with progression free survival, and increased immunostainings of EZH2, MCM7, and Ki-67 were significantly associated with a high Gleason score and short progression free survival.

The independent value of the prognostic markers was estimated next. The association of each marker with clinicopathological variables showed that immunostaining of Ki-67 ($p=0.0107$), EZH2 ($p=0.0012$) and MCM7 ($p=0.0021$) was associated with Gleason score. Ki-67 ($p=0.0265$) and MCM7 ($p=0.0004$) stainings also correlated with pT-stage, whereas EZH2 did not. There was no significant association between any of the markers and diagnostic PSA value. Patient´s age was associated with Ki-67 staining but not with other markers. In the analysis of the association of the markers with each other, Ki-67 staining was associated with EZH2 ($p=0.0001$), MCM7 ($p=0.0068$) staining and *EIF3S3* gene copy number ($p=0.0389$). EZH2 and MCM7 stainings were not associated with each other.

In multivariate analysis (Cox-regression model) pT-stage, MCM7 and Ki-67 were independent prognostic markers, which e.g. Gleason score was not. The relative risk value (RR) of pT-stage, MCM7 and Ki-67 were 1.97 (95%Cl 1.23-3.15), 2.65 (95%Cl 1.22-5.70), and 1.85 (95%Cl 1.14-3.01) respectively. EZH2 showed almost significant independent prognostic value ($p=0.0561$). Combination of EZH2 and MCM7 increased the reliability and the relative risk (RR) for the combined variable was 2.92 (95%Cl 1.66-5.15) in the multivariate analysis, ensuring the prognostic value of combination of EZH2 and MCM7. Amplification of the *EIF3S3* gene or gain of chromosome 8 had no prognostic value ($p=0.3382$). When current clinically used prognostic markers, pT- stage (3 versus 2), Gleason score (<7, versus 7, versus >7), and PSA were forced into the Cox regession model, Ki-67 ($p=0.004$) and EZH2 ($p=0.011$) improved in prognostic fit significantly and MCM7 almost significantly ($p=0.053$).

In truly radical cases (n=226), *i.e.* pN0, and serum PSA value below the detection limit after surgery, independent prognostic markers were: EZH2, 3.14 (1.38-7.16), MCM7, 2.70 (1.16-6.30), and PSA 1.51 (1.03-2.20), whereas Ki-67 and pT-stage had almost significant prognostic value. Gleason score failed to achieve independent prognostic value in this patient group.

In addition, low immunostaining of Ki-67 identified a subgroup of patients with very low risk of disease progression. There were 15 patients with Ki-67 staining ≤1% and only 1 (7%) experienced progression. In the group of patients with Ki-67 >1% (n=71) 26 (37%) experienced disease progression ($p=0.0302$, Fisher´s exact test). The relative risk (RR) of disease progression in a patient with low Ki-67 was 0.09 (95%-Cl 0.01-0.69). The Gleason under 7 patient group analyzed with Ki-67 staining showed a significant association with progression-free survival ($p=0.0049$). Diagnostic PSA ($p=0.0658$) and also EZH2 ($p=0.0878$) were not significantly associated with prognosis, nor were pT-stage, MCM7 or *EIF3S3*.

There is an increasing interest in testing the effect of adjuvant treatment in conjunction with the radical prostatectomies in patients with a high risk of disease progression. The critical question is the selection of patients for adjuvant treatment. How to identify patients with a high risk of recurrence? This study suggests that EZH2 and MCM7 immunostainings, separately or combined, seem to independently identify patients with a very high risk of recurrence after radical prostatectomy and could be useful in identifying patients for adjuvant therapy trials. The risk of developing metastases after a rising PSA following radical prostatectomy is high, 44%, with an average time of 7.5 years to recurrence (Pound *et al.*, 1999). It has also been shown that prostatectomized patients with lymph-node metastases treated with early androgen withdrawal have better survival prospects than patients treated with deferred hormonal therapy (Messing *et al.*, 2006) and prostatectomy-treated patients with locally advanced, but lymph node negative disease benefit from early hormonal therapy (Wirth *et al.*, 2004). These findings suggest that adjuvant hormonal therapy in conjunction with prostatectomy could be beneficial. However, the benefit of such adjuvant therapies in conjunction with prostatectomy has not been shown. The results of two large studies on hormone-refractory cancer (HRPC) indicated that docetaxel is effective in the treatment of symptomatic HRPC (Petrylak *et al.*, 2004; Tannock *et al.*, 2004). Cytotoxic treatment may also be effective as adjuvant therapy. In this study the end point was biochemical failure, which does not correlate with prostate cancer specific mortality for a certainty. Nevertheless progression –free survival is a convenient way to study how long the patient stays in remission and the disease does not progress.

Another obvious finding in this study was that low Ki-67 immunostaining seemed to identify a subgroup of patients with a very low risk of disease progression, suggesting that such patients could be treated with active surveillance instead of immediate prostatectomy. It is well known that a large portion of prostatectomy treated patients are actually overtreated and active surveillance can be a good treatment option instead of immediate prostatectomy for patients with good prognosis. This finding must be confirmed in needle biopsies.

Interestingly, Gleason score as a current prognostic marker was associated with progression-free survival, but failed to show independent prognostic value in any of the multivariate analyze calculated. It is proposed that there has been a stage and grade shift of Gleason score in currently diagnosed prostate cancer and the prognostic power of the Gleason score system has been diminished. Instead, in this study as a current prognostic marker pT-score had independent prognostic value. Clearly more prognostic markers are needed to incorporate with the current markers to evaluate the prognosis after prostatectomy more reliably.

CONCLUSIONS

The main findings and conclusions in this thesis were:

1. Most of the xenografts studied by cCGH resembled prostate tumors in their chromosomal alterations. This indicates that they are very useful in studying prostate cancer. According to the genetic composition of each xenograft reported in the study, one may choose the best model for studying a particular question or for the identification of genes involved in the development and progression of prostate cancer.

2. Chromosomal aberrations found in hormonally treated metastatic prostate cancer are similar to the earlier cCGH findings of untreated metastases and hormone-refractory prostate cancer and it seems that no large regional genomic alteration is specific to the metastatic prostate cancer phenotype. It is likely that the primary tumors do not function as a deposit of cancer cells that spread cells over a long period of time; instead the cancer cell dissemination may take place in a relatively short period of time, and the metastatic clones continue to live independently outside the prostate gland without interference and competition from other tumor clones from the prostate gland. Understanding and predicting therapeutic success in an individual will depend to some degree on clonality as well as on genomic alteration pattern for metastatic lesions in a given patient. The uniclonal and multiclonal forms of metastatic prostate cancer have potentially important implications for the treatment of metastatic prostate cancer.

3. Androgen withdrawal reduces the cell proliferation activity of prostate cancer cells for several months, but the majority of tumors emerging during the first withhold of castration are resistant to the subsequent rounds of castration in terms of cell proliferation activity. The biological aggressiveness of the tumor seems already to be defined at the time of diagnosis. The reduction of PSA levels at each castration as well as the constant nuclear expression of AR indicate continuous activation of *AR* signaling despite the androgen withdrawal.

4. Ki-67, EZH2 and MCM7 immunostainings, separately or the last two combined, identify patients with a very high risk of recurrence after radical prostatectomy and they could be useful in selecting patients for adjuvant therapy trials. Low Ki-67 immunostaining seemed to identify a subgroup of patients with a very low risk of disease progression, suggesting that such patients could be treated with active surveillance instead of immediate prostatectomy.

In conclusion, the results here suggest that the histological differentiation, proliferation activity and the biological aggressiveness of a particular tumor seem to be defined already at an early stage of tumorigenesis. According to our results, there seems to be no additional dedifferentiation during the progression of the disease, suggesting that the biological aggressiveness does not shift during the progression of prostate cancer.

In the evolution of metastasis the selection of a tumor clone which metastasize takes place at the time when a clone disseminates from the prostate gland and the metastatic environment does not seem to exert selection pressure on the metastatic clone. The metastatic clones continue to live independently outside the prostate gland without interference and competition of other tumor clones from the prostate gland. As primary tumors are genetically heterogeneous, but metastatic lesions are homogeneous, it is also possible that the primary tumors do not function as a deposit of cancer cells that spread cells over a long period of time. Because the metastatic lesions share the genetic alterations, this strongly suggests that that no large regional genomic alteration is specific to the metastatic prostate cancer phenotype. This may indicate that once novel therapies targeted against the genetically altered mechanisms are developed, all metastases will respond to such treatments.

The long-term inhibitory effect of castration on prostate cancer growth is due to a decreased proliferation rate and apoptosis is increased only immediately after the androgen withdrawal. Additionally, the transition of the growth from androgen dependence to independence does not seem to involve major chromosomal aberrations.

With Ki-67, EZH2 and MCM7 immunostainings, separately or the last two combined, patients with a very high risk of recurrence after radical prostatectomy can be identified. This could be useful in identifying patients for adjuvant therapy trials. Low Ki-67 immunostaining seems to identify a subgroup of patients with a very low risk of disease progression, suggesting that such patients could be treated with active surveillance instead of immediate prostatectomy.

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ORIGINAL COMMUNICATIONS

Chromosomal Aberrations in Prostate Cancer Xenografts Detected by Comparative Genomic Hybridization

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A major problem in studying prostate cancer has been the lack of model systems because of the difficulties in growing prostate cancer cells in vitro. Recently, however, several human prostate cancer xenografts, grown in immune-deficient mice, have been established. Here, we characterized 13 such xenografts (LuCaP 23.8, 23.12, 35, 41, 49, 58, 69, 70, 73, LAPC-4AD, LAPC-4AI, LAPC-9AD, and LAPC-9AI) as well as one prostate cancer cell line (22Rv1) derived from a xenograft for chromosomal alterations by comparative genomic hybridization and a modification of multicolor fluorescence in situ hybridization. On average, the xenografts contained 13 (range 5–28) aberrations, 5 (1–13) gains, and 8 (1–15) losses, per case. The chromosome arms that most often contained losses were 2q, 5q, 6q, 8p, 13q, and 18q, and gains were 7q, 8q, and Xq. The same regions were previously shown to be often altered in advanced prostate carcinomas in patients. The androgen-dependent and corresponding androgen-independent sublines of LAPC-4 and LAPC-9 shared all genetic alterations, suggesting that the transition of the growth from androgen dependency to independence does not involve major chromosomal aberrations in these two models. In conclusion, the identified genetic aberrations lay the groundwork for further detailed genetic analyses of these xenografts.

INTRODUCTION

Prostate cancer has become the most common malignancy of men in many Western industrialized countries, including Finland (Finnish Cancer Registry, 2000). During the last decade, a major effort has been in research of the mechanisms of prostate cancer, and finding new therapeutic strategies to combat the disease. One major problem in studying prostate cancer has been the shortage of model systems. However, it has been almost impossible to establish permanent in vitro growing prostate cancer cell lines. Therefore, the vast majority of the in vitro studies has been performed with three commercially available cell lines, PC-3, DU145, and LNCaP (Reiter and Sawyers, 2001). A few additional commonly available cell lines also exist. However, the origin of these other cell lines has recently been questioned. For example, PPC-1 and ALVA31 have actually been shown to be identical to PC-3 (Chen, 1993; Pan et al., 2001). It has also been shown that cell lines TSU-Pr1 and JCA-1 most likely originate from bladder instead of prostate cancer, further diminishing the number of cell lines usable for prostate cancer research (van Bokhoven et al., 2001). Of the cell lines, LNCaP is the only androgen-responsive one, which, however, contains a mutation in the androgen receptor (*AR*) gene (Reiter and Sawyers, 2001). Thus, there has not been a single commonly available cell line modeling androgen-dependent prostate cancer.

During the last few years, a number of prostate cancer xenografts, grown in immune-deficient mice, have successfully been established (Pretlow et al., 1993; Ellis et al., 1996; van Weerden et al., 1996; Klein et al., 1997; Craft et al., 1999; Pinthus et al., 2000). They represent both androgen-dependent and -independent forms of the disease. In a short period of time, these xenografts have become a major resource for prostate cancer research. They have already been utilized, for example, for gene discovery and functional analysis of signaling pathways (Reiter and Sawyers, 2001).

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The genetic composition of each model system forms a basis for utilizing each model. The first step in the genetic characterization of cancers is the identification of chromosomal abnormalities. Because the xenografts usually do not grow well in vitro, it has often not been possible to karyotype them with, for example, classical G-banding technique. Comparative genomic hybridization (CGH) provides an alternative method for screening samples for DNA sequence copy number changes throughout the entire genome (Kallioniemi et al., 1992). Because no metaphase chromosomes from the samples are required, CGH is well suited for studying the xenografts. However, only a few of the xenografts have been analyzed by CGH or other similar tools (Williams et al., 1997; Bubendorf et al., 1999). Here, we analyzed 13 human prostate cancer xenografts as well as one cell line (22Rv1) originating from xenografts by using CGH. In addition, the 22Rv1 cell line was analyzed with a modification of multicolor fluorescence in situ hybridization (armFISH).

MATERIALS AND METHODS

Samples

The material consisted of 13 human prostate cancer xenografts (LuCaP 23.8, 23.12, 35, 41, 49, 58, 69, 70, 73, LAPC-4AD, LAPC-4AI, LAPC-9AD, and LAPC-9AI) grown in mice as well as one prostate cancer cell line (22Rv1) obtained from ATCC (Rockville, MD). The LuCaP and LAPC xenografts can be requested from Dr. Robert L. Vessella (vessella@u.washington.edu) and Dr. Charles L. Sawyers, respectively (CSawyers@mednet.ucla.edu). DNA was isolated from the xenografts and cell line using routine techniques. 22Rv1 was cultured under recommended conditions, and metaphase spreads were obtained using standard techniques.

CGH

CGH was carried out as described before, with minor modifications (Visakorpi et al., 1995b). Briefly, DNAs from xenografts and the cell line were labeled with FITC-dUTP (DuPont, Boston, MA) and normal reference male DNA with Texas Red-dUTP (DuPont) using nick-translation. Labeled DNAs together with unlabeled Cot-1 DNA (Life Technologies, Grand Island, NY) were hybridized to normal lymphocyte metaphase slides up to 72 hr. After washes, the slides were counterstained with an antifade solution (Vectashield; Vector Laboratories, Burlingame, CA) containing DAPI (4,6-diamine-2-phenylindole). Five high-quality metaphase cells from each hybridization were captured using a Photometrics ImagePoint CCD camera (Photometrics, Tuscon, AZ) mounted on an Olympus BX50 epifluorescence microscope (Tokyo, Japan) and IPLab Spectrum software program (Scananalytics, Fairfax, VA). Relative DNA sequence copy number changes were detected by analyzing the fluorescence intensities of green (tumor) and red (normal) signals along the length of all chromosomes in the metaphase spreads using the Quips CGH analysis program (Vysis, Downers Grove, IL). Hybridizations of FITC-labeled normal male DNA against Texas Red–labeled normal female DNA, in each hybridization batch, were used as negative controls. The mean green-to-red ratio and corresponding standard deviation (SD) for all autosomes remained between 0.85 and 1.15 in these control hybridizations. Thus, chromosomal regions with a mean ratio of 0.85 or less were considered lost and those with a ratio 1.15 or more gained, in the prostate tumors. High-level amplifications were defined as small regions with a ratio > 1.4. Because of the large heterochromatic regions, the Y chromosome was excluded from CGH analysis.

armFISH

armFISH, a modification of mFISH, was essentially done as previously described (Karhu et al., 2001). Briefly, a commercially available mFISH reagent kit (24XCyte; MetaSystems GmbH, Altslussheim, Germany) was supplemented with a set of chromosome arm–specific painting probes (Guan et al., 1996). The armFISH hybridization cocktail contained 5 μl of mFISH-kit probe reagent and 0.5 μl of digoxigenin-labeled chromosome arm–specific painting probes composed of either p or q arms of all human chromosomes. After hybridization and washes, the analysis was done in two steps. First, mFISH images were captured by a Zeiss Axioplan II epifluorescence microscope (Carl Zeiss Jena GmbH, Jena, Germany) equipped with filters for DAPI, DEAC, FITC, Cy3, Texas Red, and Cy5 (Chroma Technology, Brattleboro, VT). Second, the arm-specific probes were detected by horseradish peroxidase–conjugated anti-digoxigenin (Boehringer Mannheim GmbH, Mannheim, Germany) followed by a signal amplification step with biotinyl tyramide (NEN™) and visualization with LaserPro™ IR790 fluorochrome (Molecular Probes, Eugene, OR) with Cy7 filter (Chroma Technology). ISIS 3.2.0 software (MetaSystems, GmbH) was used for mFISH and armFISH analysis. First, the chromosomes were classified and translocations

were detected according to normal mFISH analysis, followed by identification of the chromosome arms involved in the translocations.

RESULTS

Figure 1 summarizes the DNA sequence copy number alterations in the xenografts and 22Rv1 cell line found by CGH. All samples showed chromosomal aberrations. On average, there were 13 (range 5–28) alterations per sample, 5 gains (1–13), and 8 losses (1–15). The chromosome arms that most often contained gains were 7q (43% of the cases), 8q (64%), and Xq (50%). High-level amplifications were found at 2p21–pter (LuCaP 70), 3q26–qter (LuCaP 41), 7p14–q11 (LuCaP 70), 7q32–qter (LuCaP 41), 8cen–q21 (LuCaP 23.8), 8q21–q22 (LuCaP 35), 8q22–qter (LuCaP 69), 8q23–qter (LuCaP 58, LuCaP 70), 8q24–qter (LuCaP 23.8, LAPC-4AD, LAPC-4AI), 9q34–qter (LAPC-4AD), 16cen-p12 (LuCaP 70), and Xcen-q13 (LuCaP 69). Chromosome arms that most often contained losses were 2q (71%), 5q (50%), 6q (79%), 8p (64%), 13q (50%), and 18q (57%). The minimal common regions of deletions were 2q21–q22, 5q13–q21, 6cen–q22, 8p21–pter, 13q22–qter, and 18q21–qter.

Most of the xenografts originated from patients treated with androgen withdrawal, the only exceptions being LuCaP 49, LuCaP 58, and 22Rv1 (Table 1). These samples had, on average, fewer aberrations per case (3 gains, 5 losses; 8 total) than the rest of the xenografts (6 gains, 8 losses; 14 total). Xenografts LuCaP 23.8 and LuCaP 23.12, derived from different metastatic lesions from an autopsy (Table 1), showed the same chromosomal aberrations. From xenografts LAPC-4 and LAPC-9, both androgen-dependent (AD) and -independent (AI) forms were analyzed. In both cases, the AD and AI types demonstrated identical alterations (Figs. 1 and 2A).

The new prostate cancer cell line 22Rv1, derived from a CWR22R xenograft, showed only a few chromosomal alterations by CGH: gains of 1q, 7p15–qter, 8p12–p22, and 12, as well as a loss of 2q13–31 (Fig. 1). The cell line was also analyzed with armFISH (Fig. 2B), which revealed three related clones. Karyotypes were 51, XY, +i(1)(q10), der(2)t(2;4)(p13;q35)del(2)(q?), +3, der(4)t(2;4)(p13;q35), t(6;14)(q15;q32), +7, +8, +12[7]/49, idem, +der(1)t(1;8)(q11;q12?), −3, −8 [11]/49, idem, +der(1)t(1;8)(q11;q12?), −3, −8, t(7;19)[2]. Altogether, three balanced translocations were found: t(2;4)(p13;q35), t(6;14)(q15;q32), and t(7;19).

DISCUSSION

In addition to cell lines, prostate cancer xenografts constitute a valuable tool for studying mechanisms of the disease as well as for preclinical testing of new drugs. However, it is important that the model systems used for such studies are well characterized. The commonly used prostate cancer cell lines have already been screened for genetic alterations with various techniques, such as CGH, spectral karyotyping (SKY), and mFISH, by us and others (Nupponen et al., 1998a; Pan et al., 1999, 2001; Aurich-Costa et al., 2001; Strefford et al., 2001). Here, we describe an analysis of 13 human prostate cancer xenografts as well as a cell line derived from a xenograft. The average number of all chromosomal alterations as well as gains and losses of DNA sequences, found by CGH in the xenografts, was strikingly similar to the frequency of alterations found in hormone-refractory prostate tumors obtained directly from patients (Nupponen et al., 1998b). The majority of the xenografts studied here were established from tumors progressing during androgen withdrawal. The three xenografts established from untreated prostate cancers showed fewer aberrations than did the xenografts established from hormone-refractory tumors, in concordance with our earlier findings indicating that the number of chromosomal alterations is higher in hormone-refractory tumors (Visakorpi et al., 1995b). Thus, although we did not have the patient tumors from which the xenografts derived were available for the analyses, it is likely that the aberrations found represent the genetic alterations already present in the tumors in the patients.

The chromosome arms that most often contained losses were 2q, 5q, 6q, 8p, 13q, and 18q, which have all previously been implicated by the CGH analyses of prostate tumors (Joos et al., 1995; Visakorpi et al., 1995b; Cher et al., 1996; Nupponen et al., 1998b; Alers et al., 2000). The CGH data of the xenografts also corresponded well with the mapped minimal regions of deletion in prostate cancer. Thus, the xenografts are very useful resources for cloning putative tumor-suppressor genes likely to be located in these

Figure 1. Summary of all DNA sequence copy number changes in 13 prostate cancer xenografts and a prostate cancer cell line detected by CGH. Losses are indicated on the left side of the chromosome ideograms, gains on the right. A thick bar represents a region of high-level amplification. The samples are marked under each bar with the following numbers: 2, LuCaP 23.8; 3, LuCaP 23.12; 4, LuCaP 35; 5, LuCaP 41; 6, LuCaP 49; 7, LuCaP 58; 8, LuCaP 69; 9, LuCaP 70; 10, LuCaP 73; 11, LAPC-4AI; 12, LAPC-4AD; 13, LAPC-9AD; 14, LAPC-9AD; 15, 22Rv1. The Y chromosome was excluded from the analysis.

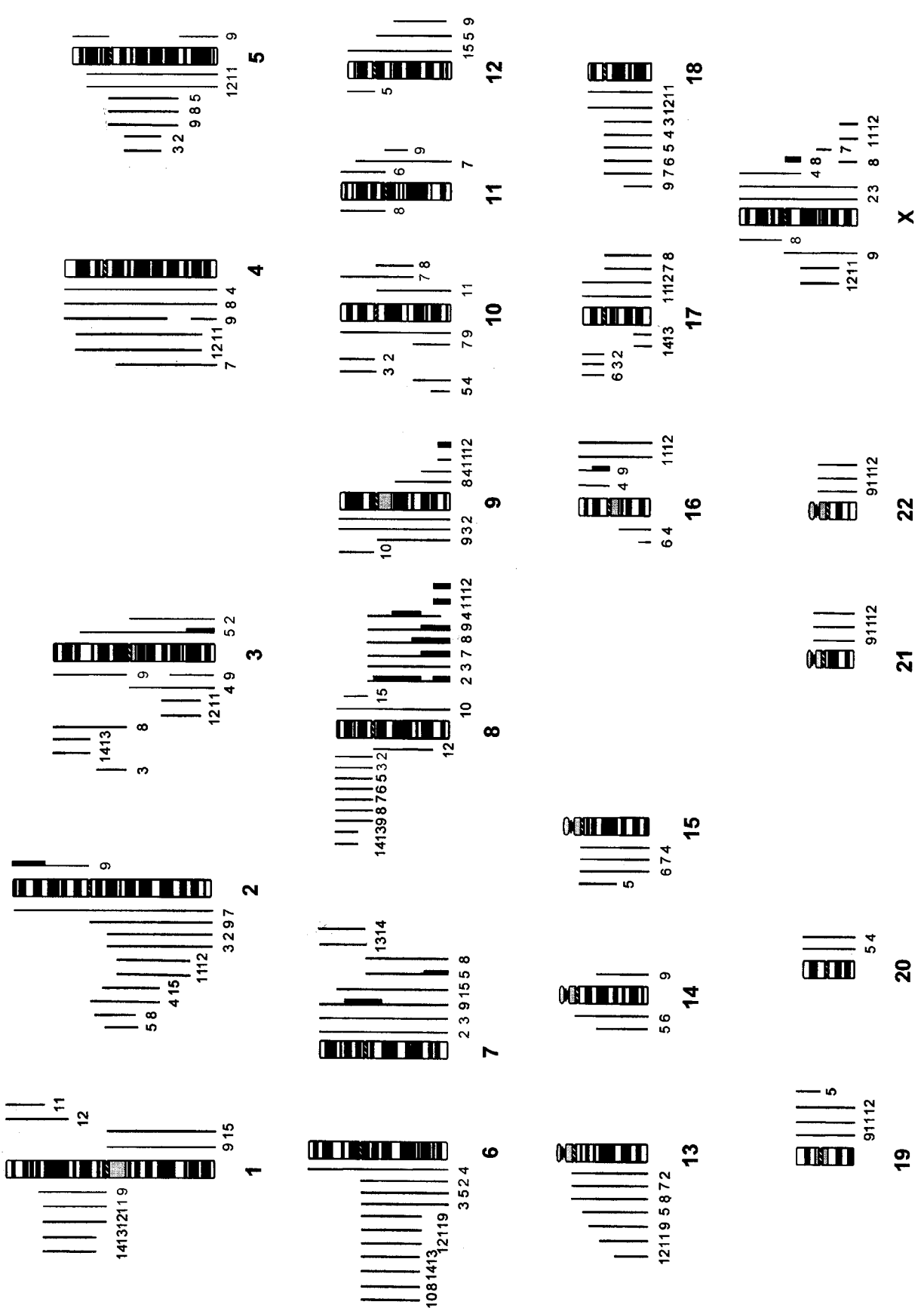

Figure 1.

TABLE 1. Description of the Xenografts and a Cell Line

Xenografts and cell line	Site originating from	Tumor state in patient	Androgen dependency in mice
LuCaP 23.8[a]	lymph node metastasis	hormone-refractory	dependent
LuCaP 23.12[a]	liver metastasis	hormone-refractory	dependent
LuCaP 35	lymph node metastasis	hormone-refractory	dependent
LuCaP 41	primary tumor	hormone-refractory	dependent
LuCaP 49[b]	omental metastasis	prior to hormonal therapy	independent
LuCaP 58	lymph node metastasis	prior to hormonal therapy	dependent
LuCaP 69	bowel metastasis	hormone-refractory	not known
LuCaP 70	liver metastasis	hormone-refractory	not known
LuCaP 73	pelvic mass	hormone-refractory	dependent
LAPC-4AD	lymph node metastasis	hormone-refractory	dependent
LAPC-4AI[c]	lymph node metastasis	hormone-refractory	independent
LAPC-9AD	femoral metastasis	hormone-refractory	dependent
LAPC-9AI[d]	femoral metastasis	hormone-refractory	independent
22Rv1	primary tumor	prior to hormonal therapy	independent

[a]The xenografts originated from different metastatic lesions from an autopsy.
[b]Small cell carcinoma of the prostate.
[c]LAPC-4A is a subline of LAPC-4.
[d]LAPC-9A is a subline of LAPC-9.

regions. It should be noted that none of the target genes for the above-mentioned deletions is known, although some candidates, such as *NKX3A* located at 8p21, have been identified (He et al., 1997; Elo and Visakorpi, 2001). Also, the most commonly gained regions, 7q, 8q, and Xq, found in the xenografts, correspond well with the CGH findings of prostate tumors from patients (Visakorpi et al., 1995b; Nupponen et al., 1998b; Alers et al., 2000). Nine out of the 14 cases showed gain of 8q, and most of them showed high-level amplification of 8q23–q24, which is the most common minimally amplified region of 8q (Cher et al., 1996; Nupponen et al., 1998b). On the other hand, LuCaP 35 showed high-level amplification of 8q21–q22, representing a second minimal commonly amplified region (Cher et al., 1996; Nupponen et al., 1998b). Therefore, the xenografts are also likely to be valuable for the identification of the target genes for 8q amplification. LuCaP 69 showed high-level amplification at Xq12–q13 by CGH. We previously showed that this xenograft contains a high-level amplification of the androgen receptor (*AR*) gene, located in the region (Linja et al., 2001). Another xenograft containing *AR* gene amplification is LuCaP 35 (Linja et al., 2001), which here showed a gain of Xpter–q13. These are the first model systems containing *AR* gene amplification, making them valuable for analysis of the significance of the amplification.

Two of the xenografts, LuCaP 23.8 and LuCaP 23.12, derived from lymph node and liver metastases (Ellis et al., 1996). The CGH findings in the two cases were similar. A previously published CGH analysis of a third xenograft (LuCaP 23.1), established from the same patient, also indicated the same alterations as well as gains at 5q, 6q, and 12q (Williams et al., 1997). The findings suggest that a single clone was the source of metastases to different sites, and that the subsequent metastatic lesions underwent undetectable, if any, additional genomic alterations. The finding in this one case is similar to what we found earlier in a direct analysis of multiple metastatic lesions from several autopsy cases (unpublished data).

Most of the xenografts have been established from hormone-refractory tumors (Table 1). Thus, it is surprising that when grown in mice, they behave in an androgen-dependent fashion. For example, castration of mice bearing one of the LuCaP 23 series of xenografts, LAPC-4 or LAPC-9, results in a decrease in tumor size (Ellis et al., 1996; Klein et al., 1997; Craft et al., 1999), consistent with features of androgen-dependent tumors. However, these xenografts will eventually progress and become androgen-independent. Here, we were able to compare both AD and AI sublines of LAPC-4 and -9. The AI sublines of the xenografts were established by implanting AD sublines into castrated mice. After 13–26 weeks, the implants started to grow to form the AI sublines (Klein et al., 1997; Craft et al., 1999). In fact, it has already been shown that androgen-dependent LAPC-9 is a mixture of AD and AI cells (Craft et al., 1999). Our data show that the AD and AI xenografts have nearly

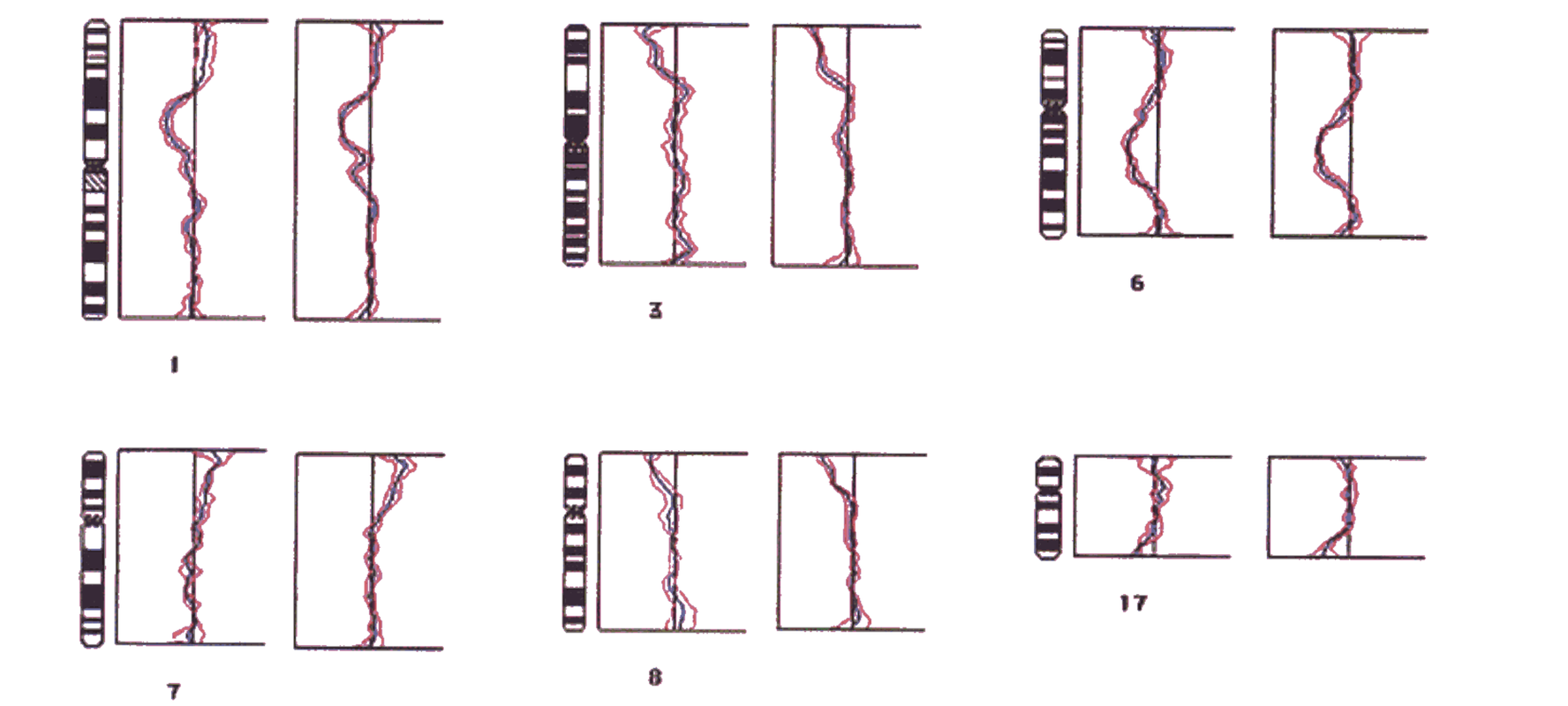

Figure 2. (**A**) Mean (blue line) ± SD (red lines) green-to-red ratio profile for altered chromosomes from pter to qter obtained from CGH analysis of xenografts LAPC-9AD (profile on the left, next to chromosome ideogram), and LAPC-9AI (profile on the right). The middle vertical line represents ratio value 1.0. Chromosomal regions with a mean ratio of 0.85 or less were considered lost and those with a ratio 1.15 or more, gained. Changes in the profiles indicate a gain at 7p and losses at 1p13–p21, 3p21–pter, 6cen–q22, 8p21–pter, and 17q23–qter in both xenografts. The remaining chromosomes did not show aberrations in either xenograft. (**B**) armFISH analysis of 22Rv1. Chromosomes presenting rearrangements are shown.

identical genetic alterations. In addition, 22Rv1, which is an in vitro growing cell line from the CWR22R AI xenograft (Nagabhushan et al., 1996; Sramkoski et al., 1999), revealed very similar CGH findings to those previously published for the original AD CWR22 (Bubendorf et al., 1999). More-

over, we previously reported that very few differences are found in the AD and AI sublines of LNCaP (Hyytinen et al., 1997). Thus, it seems that transition from androgen-dependent to androgen-independent growth in xenografts does not involve major chromosomal alterations. This finding is somewhat different from what we have found in patients.

First, we previously showed that androgen withdrawal may select amplification of the *AR* gene in vivo (Visakorpi et al., 1995a). Second, we also analyzed locally growing tumors from patients at the time of diagnosis and at the time of local progression during androgen withdrawal by CGH (Nupponen et al., 1998b). In half of the cases, the chromosomal aberrations in the hormone-refractory tumors were different from those in untreated tumors, indicating selection of a distinct clone following the androgen ablation. Cancer within the prostate gland is often multifocal and genetically heterogeneous, and thus distinct clones may be selected (Greene et al., 1991; Jenkins et al., 1997). In comparison, the xenograft cell population has already gone through several selections (castration and metastases in patients, as well as transplantation in mice), and therefore the cells are likely to be genetically more homogeneous. It is not known whether this difference between the xenografts and tumors in man will also be reflected as differences in the mechanisms of progression from androgen dependency to androgen independence.

Most of the xenografts showed typical chromosomal alterations for advanced prostate cancer (Cher et al., 1996; Nupponen et al., 1998b). Two exceptions were LuCaP 73, which showed only three aberrations, and 22Rv1, showing more gains than losses. The 22Rv1 cell line was analyzed not only by CGH but also by armFISH. The findings by armFISH were quite similar to what was previously reported by classical G-banding of the cell line as well as of the original CWR22 xenograft (Wainstein et al., 1994; Kochera et al., 1999; Sramkoski et al., 1999). Atypical for prostate cancers, the cell line contained mostly balanced translocations. It also lacked almost all common chromosomal alterations of prostate cancer, for example, losses at 6q, 8p, and 13q or gains at 7p/q and 8q. CWR22 is probably the most commonly used xenograft model for prostate cancer. However, based on the CGH and armFISH findings, one could argue that it may not be the best model, at least for genetic studies of prostate cancer.

In conclusion, the fact that most of the xenografts resembled prostate tumors by their chromosomal alterations indicates that they are very useful in studying prostate cancer. The genetic composition of each xenograft, reported here, allows one to choose the best model for studying a particular question or for the identification of genes involved in the development and progression of prostate cancer. Finally, the model systems suggested that the transition of the growth from androgen dependency to androgen independence does not involve major chromosomal aberrations, amplification of the *AR* gene being a possible exception.

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EZH2, Ki-67 and MCM7 are prognostic markers in prostatectomy treated patients

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The aim of the study was to evaluate the prognostic value of Ki-67, EZH2, MCM7 and *EIF3S3* in prostatectomy treated patients. A retrospective population-based material of 249 radical prostatectomy specimens on tissue microarrays was utilized. The median follow-up of the patients was ~5.5 years and the main end-point was biochemical progression. The expression of Ki-67, EZH2 and MCM7 was determined by immunohistochemistry and the gene copy number of *EIF3S3* was analyzed by fluorescence *in situ* hybridization (FISH). In the whole material, increased immunostainings of EZH2, MCM7 and Ki-67 were significantly associated with a high Gleason score and a short progression-free survival. In multivariate analysis, MCM7 and Ki-67 showed independent prognostic value with relative risks (RR) of 2.65 (95%-confidence interval of 1.22–5.70), and 1.85 (1.14–3.01), respectively. In subgroup analysis of patients, whose treatment was evaluated to be truly radical ($n = 226$), EZH2 (3.14, 1.38–7.16), MCM7 (2.70, 1.16–6.30) and PSA (1.5, 1.03–2.20) showed independent prognostic value. In subgroup analysis of cases with a Gleason score <7, low Ki-67 staining was associated with favorable prognosis with RR of 0.09 (0.01–0.69). In conclusion, Ki-67, EZH2 and MCM7 are potential prognostic biomarkers in prostatectomy treated patients.

Key words: prostatic; carcinoma; neoplasia; survival; immunostaining

Prostate cancer is the most common male malignancy in many Western countries. In Finland in 2004, the age-adjusted incidence of prostate cancer was 115.3 per 100,000 men (www.cancerregistry.fi). Frequent testing for prostate specific antigen (PSA) has led to earlier detection of the cancer,[1] and more operable prostate cancers are thus found. A Scandinavian randomized clinical trial has shown that prostatectomy decreases both overall and prostate cancer specific mortality,[2] giving justification for this form of intent to cure treatment. Still, 20–40% of patients treated by radical prostatectomy experience disease recurrence.[2,3] Such patients could benefit from adjuvant therapies. Thus, it would be important to identify patients with a high-risk of recurrence at the time of surgery. There are indications that adjuvant hormonal therapy could be beneficial for patients treated with radical radiation therapy.[4,5] Less is known about the usefulness of adjuvant therapies in conjunction with prostatectomy.

The critical question is how to identify patients with a high-risk of recurrence. The currently commonly used parameters for estimating the risk of progression include preoperative PSA, Gleason score and pathological T-stage (pT).[6–8] Of these, the Gleason score is based on the evaluation of glandular differentiation by a pathologist. Significant variability in Gleason scoring between individual pathologists has been reported.[9,10] Thus, more accurate prognostic markers are needed to reliably identify the patients who are in a high-risk of prostate cancer recurrence after prostatectomy.

One of the extensively studied prognostic markers in prostate cancer is cell proliferation activity. Many studies have shown that the proliferation rate is associated with histological grade, clinical stage and survival (reviewed by Quinn *et al.*[11]). Today, cell proliferation activity is most often defined by the immunostaining of the Ki-67 antigen. However, despite the promising data, Ki-67 immunostaining has not become a routinely used assay.

The molecular mechanisms of prostate cancer progression have been intensively studied[12] in the past decade. These analyses have revealed several putative prognostic markers. For example, Varambally *et al.*[13] found that the expression of polycomb group protein, enhancer of zeste homolog 2 (EZH2), is increased in prostate cancer metastases as well as in localized tumors with a poor prognosis. Subsequently, Rhodes *et al.*[14] showed that increased expression of EZH2 combined with decreased expression of E-cadherin is associated with short progression-free survival. It has been shown that EZH2 expression is strongly associated with cell proliferation activity in many malignancies, including prostate cancer.[15,16] Although the exact function of EZH2 is incompletely known, it is believed to be the catalytically active component of the polycomb repressive complexes 2, 3 and 4 (PRC2/3/4).[17] EZH2 is essential in early embryonic development as shown by the 100% embryonic lethality in homozygous knockout mice.[18] In addition, the inhibition of EZH2 expression by transfection with small interfering RNA (siRNA) or by small hairpin RNA (shRNA) has been shown to lead to cell cycle arrest in G_1, G_2 and G_2/M.[13,19–21] Overexpression of EZH2 has also been shown to promote neoplastic transformation of breast epithelial cells and to be associated with the aggressiveness of breast cancer.[16] We have also shown that the *EZH2* gene is amplified in about 20% of hormone-refractory prostate carcinomas.[22]

Another suggested molecular marker of prostate cancer aggressiveness is minichromosome maintenance (MCM) protein 7 (MCM7). MCM proteins are part of the replication system complex that licenses DNA replication and are found to be markers of cell proliferation.[23–25] Recently, Ren *et al.*[26] showed that constitutive expression of MCM7 in prostate cancer cell line DU145 results in increased proliferation and invasion *in vitro*. It was also shown that expression of MCM7 is associated with progression-free survival in prostatectomy treated patients.

By comparative genomic hybridization (CGH), we have previously shown that one of the most common genetic aberrations in advanced prostate cancer is the gain of the long arm of chromosome 8.[27,28] And, the gain of 8q has been shown to be associated with a poor prognosis in localized prostate cancer.[29,30] We have identified a gene encoding p40 subunit of eukaryotic translation initiation factor 3 (*EIF3S3*) as a putative target gene for the gains.[31] The amplification of *EIF3S* is associated with a high Gleason score and advanced disease, as well as with poor prognosis in incidentally found prostate cancer.[32]

The aim of this study was to evaluate the prognostic value of the above mentioned markers in population-based prostatectomy

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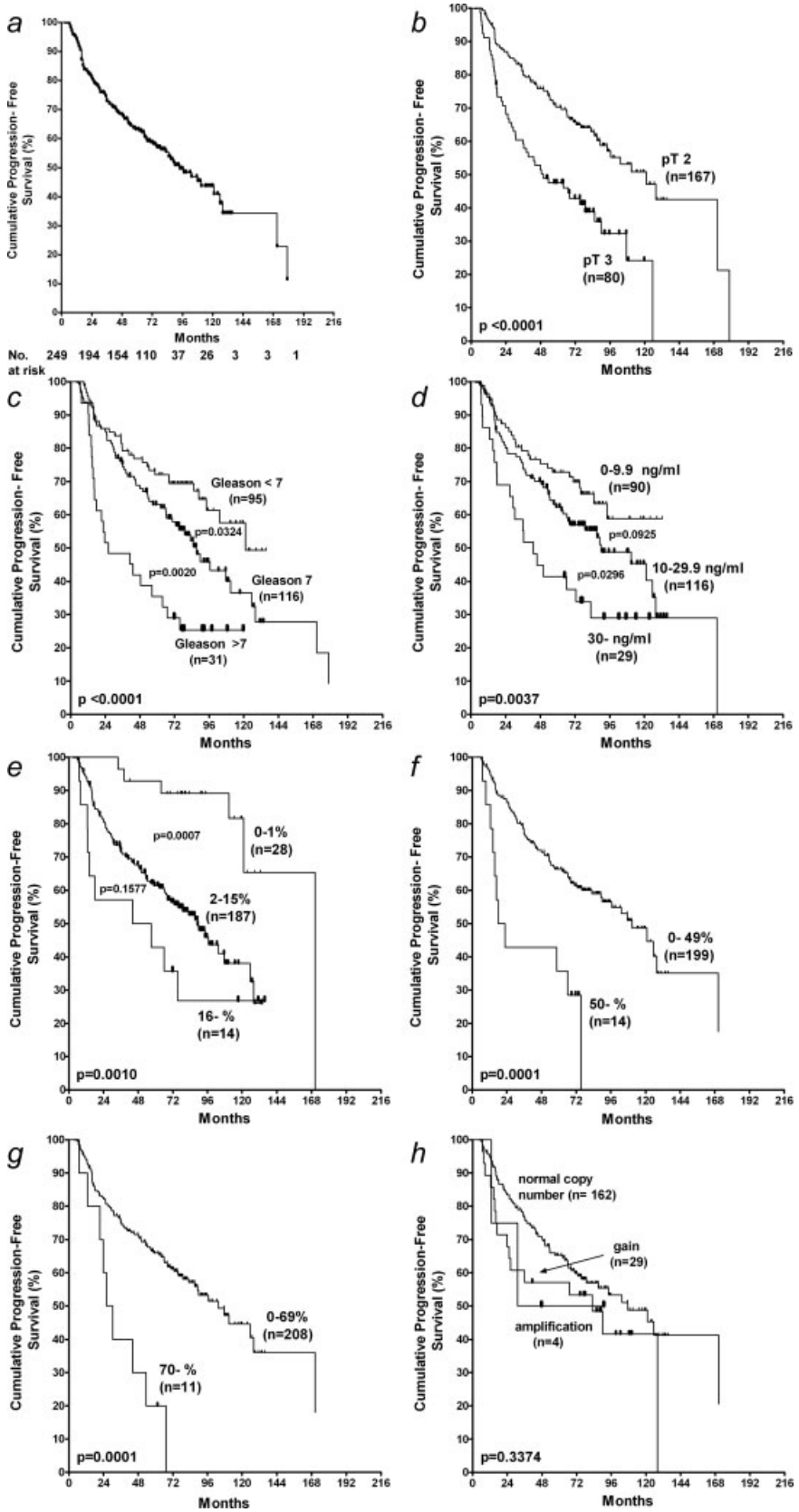

FIGURE 1 – Kaplan–Meier progression-free survival curves of the whole prostatectomy material. (*a*) overall progression-free survival, and according to (*b*) pT-stage, (*c*) Gleason score, (*d*) PSA-value, as well as immunostainings of (*e*) Ki-67, (f) EZH2, (*g*) MCM7 and (*h*) gene copy number of *EIF3S3*.

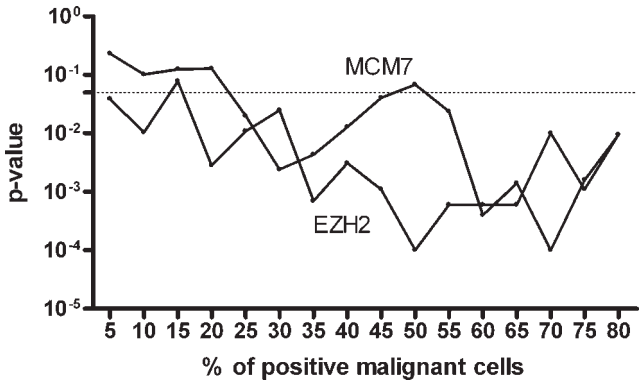

FIGURE 2 – Mantel–Cox *p*-values of Kaplan–Meier curves plotted against percentage of positive malignant cells of EZH2 and MCM7 indicate best discriminatory cut-off values of 50% and 70%, respectively.

treated patient material with long follow-up. The prognostic value of the markers was first tested separately, and subsequently in multivariate analysis was utilized to assess their value as independent prognostic markers.

Material and methods

Material

The study has been approved by the Ethical Committee of Tampere University Hospital (TAUH) and the National Authority for Medicolegal Affairs. The material consisted of consecutive prostatectomy treated patients operated at TAUH in 1982–1998. The catchment area of the TAUH district is approximately 460,000 people, and all prostatectomies in the area are performed in TAUH. During this time, 336 prostatectomies were performed. Formalin-fixed paraffin embedded samples were available from 249 (74%) cases. Three patients had received neoadjuvant and one adjuvant hormonal treatment. The tumor specimens were re-evaluated for Gleason score by a pathologist, who also selected areas for the construction of tissue microarray (TMA) blocks. Areas representing the most common and the second most common Gleason grade areas, as well as a third, if with a higher Gleason grade than in the two previous areas, were selected for the construction of TMA. The TMAs were constructed using manual tissue arrayer (Beecher Instruments, Silver Spring, MD) with 1 mm needle according to manufacturer's instructions. In the group of 249 cases, the mean age of the patients was 63 years (median 64, range 44–74). The mean follow-up time was 62 months (median 66, range 3–215 months) (Fig 1*a*). The median (lower and upper quartile) PSA value at the time of the diagnosis was 11.5 (7.3, 18.0) ng/ml. The PSA values for 14 cases were not known. The re-graded Gleason score distribution of the prostatectomy specimens was: Gleason <7; 95 (39%), Gleason $= 7$; 116 (48%) and Gleason >7; 31 (13%). Gleason score was not available from 7 cases. The tumor pT-stage[33] distribution was: pT2; 167, and pT3; 80. The pT-stage data were not available from two samples. According to the routine practice at TAUH, patients treated with prostatectomy are, 1 year from the operation, monitored in the health centers of the Hospital District, at least once a year. If there is a sign of PSA progression, patients are referred to TAUH. If not, they continue to be monitored by the health centers. Thus, serum PSA values and dates were retrieved, in addition to the patient files at TAUH, also from health centers. Detectable PSA values ($\geq$0.5 ng/ml) in two consecutive measurements or the emergence of metastases were considered as signs of progression.

Immunohistochemistry

Antibodies against Ki-67 (MM1, Novocastra™ Laboratories Ltd., Newcastle Upon Tyne, United Kingdom), EZH2 (NCL-L-EZH2, clone 6A1, Novocastra™ Laboratories Ltd., Newcastle Upon Tyne, United Kingdom) and MCM7 (sc-9966, Santa Cruz Biotechnology, Inc., CA) were used with Power Vision+™ Poly-HRP Histostaining Kit (ImmunoVision Technologies Co, Daly City, CA) according to the manufacturers' instructions. Briefly, slides were autoclaved in pretreatment buffer (5 mM Tris-HCl/1 mM EDTA, pH 9) at 121°C for 2 min, followed by incubation with the primary antibody, and diluted in pre-block solution (Ki-67 1:1,500, EZH2 1:300, MCM7 1:500) over night. After washes and blocking, the bound primary antibody was visualized with the PowerVision+™ Poly-HRP IHC Detection Kit (ImmunoVision Technologies Corporation, Brisbane, CA). The slides were counterstained with hematoxylin and mounted with Neo-Mount® (Merck, Darmstadt, Germany). Every staining batch had a negative control in which the primary antibody was omitted. Every spot was scored from nonoverlapping malignant cells using Olympus BX50 light microscope. At least 5 different randomly selected visual fields were scored in blinded fashion by one of the authors (S.L).

Fluorescence in situ *hybridization*

Dual-color FISH was performed on 5 μm sections as previously described.[34] Briefly, a locus- specific PAC probe for *EIF3S3* and a pericentromeric probe for chromosome 8 (pJM128) were labeled with digoxigenin and fluorescein isothiocyanate, respectively, by nick translation. The deparaffinized slides were treated with 1 mM NaSCN for 10 min at 80°C, followed by incubation in 60–90 mg/ml pepsin (P-7012, Sigma Chemical Co., St. Louis, MO) at 37°C for 12 min. The slides were then washed and dehydrated. The probes were applied on the slides in a hybridization mixture, co-denatured with the samples at 93°C, and hybridized for 2 days in a humid chamber at 37°C. Next, the slides were washed and the locus-specific probes were detected immunohistochemically by antidigoxigenin rhodamine. The slides were counterstained with 0.1 mM 4,6-diamidino-2-phenylindole in Vectashield antifade solution (Vector Laboratories Inc., Burlingame, CA). The FISH signals were scored from nonoverlapping malignant looking cells using an Olympus BX50 epifluorescence microscope (Tokyo, Japan). At least 5–8 different randomly selected visual fields were scored. Gain was defined as a presence of at least 3–4 signals of *EIF3S3* gene and amplification as a presence of >4 signals of *EIF3S3*.

Statistical analysis

Fisher's exact, chi-square and one-way ANOVA tests were used to evaluate the associations between the variables. Survival analysis was performed using the Kaplan–Meier method and the statistical significance of survival differences between patient groups was determined with Mantel–Cox (*i.e*. Generalized Savage) and Breslow (*i.e*. Generalized Wilcoxon) tests. The univariate and multivariate Cox-regression analyses were performed to calculate the relative risks (RR) and to estimate the independence of the prognostic markers.

Results

One to three spots from each case were present in the TMA. Thus, altogether 545 spots were scored for each variable. For the data analyses, the highest Ki-67, EZH2, MCM7 and EIF3S3 values from each case were used. Because of the fact that the number of informative spots in the TMA varied from section to section, not all 249 cases were analyzed with all markers. The analyses were successful in 229 cases for Ki-67, 213 cases for EZH2, 221 cases for MCM7 and 195 cases for *EIF3S3* copy number. The immunostainings of all cases can be viewed at http://www. webmicroscope.net/supplements/visakorpiTMA.asp using web-based virtual microscopy technique. The scoring of the nuclear staining of Ki-67 and EZH2 was straightforward due to the fact that all positive nuclei stained with about equal intensity. The MCM7 staining, however, clearly showed two different intensity levels. Thus, both strong and moderate nuclear stainings of MCM7 were separately scored. However, the moderate and strong

TABLE I – UNIVARIATE AND MULTIVARIATE ANALYSES OF THE PROGNOSTIC MARKERS[1]

Parameter[2]	Univariate		1° Multivariate		2° Multivariate	
	RR (95% Cl)	*p*-value	RR (95% Cl)	*p*-value	RR (95% Cl)	*p*-value
EZH2	3.21 (2.86–6.08)	0.002	Nonsignificant		Not included in the analysis	
Ki-67	2.23 (1.46–3.40)	0.000	1.85 (1.14–3.01)	0.013	Nonsignificant	
MCM7	3.59 (1.79–7.20)	0.002	2.38 (1.12–5.05)	0.038	Not included in the analysis	
EIFS3S	1.17 (0.94–1.24)	0.172	Not included in the analysis		Not included in the analysis	
Gleason score	1.36 (1.11–1.67)	0.002	Nonsignificant		Nonsignificant	
pT-stage	2.21 (1.52–3.21)	0.000	1.97 (1.23–3.15)	0.006	1.93 (1.21–3.06)	0.006
PSA (ng/ml)	1.59 (1.19–2.13)	0.002	Nonsignificant		Nonsignificant	
Age	1.07 (0.74–1.54)	0.715	Not included in the analysis		Not included in the analysis	
Combination of EZH2 and MCM7	3.74 (2.22–6.32)	0.000	Not included in the analysis		2.92 (1.66–5.15)	0.001

[1] $n = 181$.–[2]cut-off values, pT-score: pT3 against pT2, Gleason: >7, 7, <7, PSA: ≥30, 10–30, <10 ng/ml, Ki-67: ≥16%, 2–16%, ≤1, EZH2: ≥50%, <50%, MCM7: ≥70%, <70%, EIFS3S: amplification, gain, normal copy number, Age: ≥ 64 years, <64 years, Combination of EZH2 and MCM7: EZH2: ≥50% or MCM7 ≥70%, EZH2 <50% and MCM7 < 70%.

nuclear staining scores were finally summed up, because the combined score gave the best prognostic value. Only the sum scores are given here.

Figure 1 shows the Kaplan–Meier curves of the progression-free survival for the whole material. The overall 5- and 10-year progression free survival rates were 63% and 41%, respectively (Fig. 1*a*). The pT-stage ($p < 0.0001$, Kaplan–Meier method with Mantel–Cox test) and the Gleason score ($p < 0.0001$) were both significantly associated with progression free survival (Figs. 1*b* and 1*c*). Patients with a Gleason score above 7 had a significantly worse prognosis than patients with Gleason 7 or less. However, patients with Gleason 7 had only marginally ($p = 0.0324$) poorer prognosis than patients with a Gleason score less than 7. The difference became apparent only after a long follow-up. For the diagnostic PSA, Ki-67, EZH2 and MCM7 immunostainings, the best discriminatory cut-off values were first selected by evaluation of the Kaplan–Meier curves utilizing Mantel–Cox test. PSA and Ki-67 gave the best discrimination when the material was divided into three groups (Figs. 1*d* and 1*e*). Both PSA, with cut-off values 10 and 30 ng/ml, and Ki-67, with cut-off values 1% and 15%, were strongly associated with a short progression-free time ($p = 0.0037$, and $p = 0.0010$, respectively). For the EZH2 and MCM7, dichotomous grouping seemed to have the best prognostic value (Figs. 1*f* and 1*g*, and Fig. 2). Both markers identified a small group of patients with a very poor prognosis. Amplification of the *EIF3S3* gene or gain of chromosome 8 had no prognostic value (Fig. 1*h*). Table I shows the RR and 95%-confidence intervals (95%-CI) of markers according to the univariate Cox-regression model.

To evaluate the independent value of the prognostic markers, we first calculated the association of each marker with the clinicopathological variables as well as with each other. Table II shows the associations of the immunohistochemical and FISH analyses with clinicopathological variables. The cut-off values were the same as in the survival analyses. The immunostaining of Ki-67 ($p = 0.0107$), EZH2 ($p = 0.0012$) as well as MCM7 ($p = 0.0021$) were associated with Gleason score. Ki-67 ($p = 0.0265$) and MCM7 ($p = 0.0004$) stainings also correlated with pT-stage, whereas EZH2 did not. There was no significant association between any of the markers and diagnostic PSA value. Patients' age was associated with Ki-67 staining but not with other markers. Table III shows the association of the molecular markers with each other. Ki-67 staining was associated with EZH2 ($p = 0.0001$) and MCM7 ($p = 0.0068$) stainings, as well as with *EIF3S3* gene copy number ($p = 0.0389$). EZH2 and MCM7 stainings were not associated with each other.

Next, we used the multivariate Cox-regression model to evaluate the independent power of the prognostic makers (Table I). Ki-67, MCM7 and pT-stage showed independent prognostic value, whereas, for example, Gleason score did not ($p = 0.2050$). EZH2 showed almost significant independent prognostic value ($p = 0.0561$). Since both MCM7 and EZH2 showed strong prognostic value in univariate analysis, and they were not associated with each other (Table III), we also tested the prognostic value of combination of EZH2 and MCM7. In the multivariate analysis, the RR for the combined variable was 2.92 (1.66–5.15) (Table I). The Kaplan–Meier curve for the combined variable is shown in Figure 3. We also evaluated the added prognostic values of Ki-67, EZH2 and MCM7 by forcing the current clinically used prognostic markers, pT-stage (3 versus 2), Gleason score (>7, versus 7 versus >7) and PSA (as continuous variable) into the Cox regression model and then adding the biomarkers individually to the model. Ki-67 ($p = 0.004$) and EZH2 ($p = 0.011$) improved the fit of the model significantly, and MCM7 almost significantly ($p = 0.053$).

Next, we tested the prognostic markers (using same categorization as in Table I) in a subset of patients, whose treatment was considered to be radical, *i.e.* pN0, and the serum PSA value dropped below the detection limit after surgery. There were 226 such cases. In this group, the independent prognostic markers were: EZH2, 3.14 (1.38–7.16), MCM7, 2.70 (1.16–6.30) and PSA 1.51 (1.03–2.20).

Since prostate cancers with a Gleason score below 7 are often considered clinically insignificant, we also analyzed this patient group separately. The overall 5- and 10-year progression free survival rates were 72% and 50%, respectively (Fig. 4*a*). pT-stage, MCM7 and *EIF3S3* showed no significant prognostic value in this subgroup. Diagnostic PSA ($p = 0.0658$) and EZH2 ($p = 0.0878$) were not significantly associated with progression-free survival, whereas Ki-67 staining showed significant ($p = 0.0049$) association with progression-free survival (Figs. 4*b–d*). Low Ki-67 staining identified a subgroup of patients with a very low risk of disease progression. Out of the 15 patients with Ki-67 staining ≤1%, only 1 (7%) experienced progression, whereas 26 out of 71 (37%) of the patients with Ki-67 >1% experienced disease progression ($p = 0.0302$, Fisher's exact test). The RR of disease progression in a patient with low Ki-67 was 0.09 (95%-CI 0.01–0.69).

Discussion

The majority of prostate cancers are today diagnosed at a localized stage. Radical therapies, either surgery or radiation therapy, have thus become the primary forms of treatment. Indeed, radical prostatectomy was proven to be beneficial for patients in a randomized trial.[2] However, a significant fraction of operated patients' experiences disease progression. The risk of developing metastases after a rising PSA following radical prostatectomy is high, 44%, with an average time of 7.5 years to recurrence.[35] It has been suggested that adjuvant hormonal treatment for these patients could increase the rate of cure in radiotherapy treated patients.[4,5] However, the benefit of such adjuvant therapies in conjunction with prostatectomy has not been shown. On the other hand, it has been demonstrated that prostatectomized patients with lymph-node metastases treated with early androgen withdrawal have better survival than patients treated with deferred hormonal therapy.[36] In addition, prostatectomy-treated patients with locally

TABLE II – ASSOCIATION OF PUTATIVE PROGNOSTIC MARKERS WITH CLINICOPATHOLOGICAL VARIABLES

Variable	Ki-67, *n* (%)				EZH2, *n* (%)			MCM7, *n* (%)			EIFS3S, *n* (%)			
	0–1%	2–15%	16%	*p*-value	0–49%	50%	*p*-value	0–69%	70%	*p*-value	Normal	Gain	Amplification	*p*-value
Gleason score														
<7	15/86 (17)	70/86 (82)	1/86 (1)		76/81 (94)	5/81 (6)		76/77 (99)	1/77 (1)		63/70 (90)	6/70 (9)	1/70 (1)	
7	9/108 (8)	91/108 (84)	8/108 (8)		98/101 (97)	3/101 (3)		105/110 (96)	5/110 (4)		77/96 (80)	18/96 (19)	1/96 (1)	
>7	3/29 (10)	21/29 (73)	5/29 (17)	0.0107	20/26 (77)	6/26 (23)	0.0012	22/27 (81)	5/27 (19)	0.0021	20/25 (80)	4/25 (16)	1/25 (4)	0.3393
pT-score														
pT2	21/153 (14)	127/153 (83)	5/153 (3)		135/143 (94)	8/143 (6)		144/146 (99)	2/146 (1)		108/129 (84)	18/129 (14)	3/129 (2)	
pT3	7/74 (10)	58/74 (78)	9/74 (12)	0.0265	63/69 (91)	6/69 (9)	0.3943	63/72 (88)	9/72 (12)	0.0004	52/64 (81)	11/64 (17)	1/64 (2)	0.7987
PSA														
Mean	16.1	14.9	42.3		15.8	34.5		15.7	36.9		17.4	15.9	10.5	
SD	11.8	12.4	64.3	0.0001	12.9	65.3	0.0017	13.1	67.3	0.005	22.4	14.3	3.9	0.7754
Age														
Mean	64.7	63.3	59.9		63.3	62.2		63.2	62.8		63.4	62.9	60.3	
SD	3.9	4.7	6.2	0.0082	4.8	5.0	0.4055	4.8	5.7	0.7846	5.0	4.7	5.6	0.4255

TABLE III – ASSOCIATION OF PUTATIVE PROGNOSTIC MARKERS WITH EACH OTHER

Variable	Ki-67, *n* (%)				EZH2, *n* (%)			MCM7, *n* (%)		
	0–1%	2–15%	16%	*p*-value	0–49%	50%	*p*-value	0–69%	70%	*p*-value
EZH2										
0–49%	26/190 (14)	157/190 (82)	7/190 (4)							
50%	0/14 (0)	7/14 (50)	7/14 (50)	0.0001						
MCM7										
0–69%	22/204 (11)	172/204 (84)	10/204 (5)		174/186 (94)	12/186 (6)				
70%	0/11 (0)	8/11 (73)	3/11 (27)	0.0068	10/11 (91)	1/11 (9)	0.7319			
EIF3S3										
Normal	23/158 (14)	123/158 (78)	12/158 (8)		131/139 (94)	8/139 (6)		143/150 (95)	7/150 (5)	
Gain	0/28 (0)	28/28 (100)	0/28 (0)		28/28 (100)	0/28 (0)		27/29 (93)	2/29 (7)	
Amplification	0/4 (0)	3/4 (75)	1/4 (25)	0.0389	3/3 (100)	0/3 (0)	0.3921	2/3 (67)	1/3 (33)	0.0914

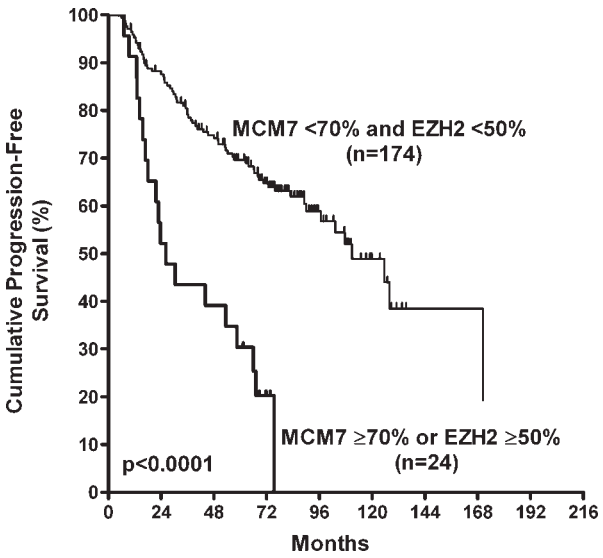

FIGURE 3 – Kaplan–Meier progression-free survival curve of the whole prostatectomy material according to combined EZH2 and MCM7 immunostainings.

advanced, but lymph node negative disease benefit of early hormonal therapy.[37] These findings suggest that adjuvant hormonal therapy in conjunction with prostatectomy could be beneficial. On the other hand, the results of two large studies on hormone-refractory cancer (HRPC) indicated that docetaxel is effective in the treatment of symptomatic HRPC.[38,39] Thus, cytotoxic treatment might also be effective as adjuvant therapy. There is an increasing interest to test this hypothesis in patients who have undergone radical prostatectomy with a high risk of disease progression. The critical question is the selection of patients for adjuvant treatment. It would be important to be able to identify patients with a high-risk of recurrence.

Here, we utilized population-based prostatectomy material to evaluate the prognostic value of several novel molecular markers. Their values were compared to the markers routinely used in the clinic. Of the molecular markers, Ki-67, EZH2 and MCM7 all showed prognostic value as previous studies have implicated,[11,13,14,24,26] whereas the gain or amplification of chromosome 8q, as defined by copy number analysis of the *EIF3S3* gene, showed little prognostic value. In multivariate analysis of the whole material Ki-67, MCM7 and pT-stage showed independent prognostic value, whereas EZH2 showed borderline ($p = 0.0561$) value. Since pT-stage Gleason score, and PSA are routinely used to assess the prognosis of the prostate cancer patients, today, we tested also whether the molecular markers have any added values.

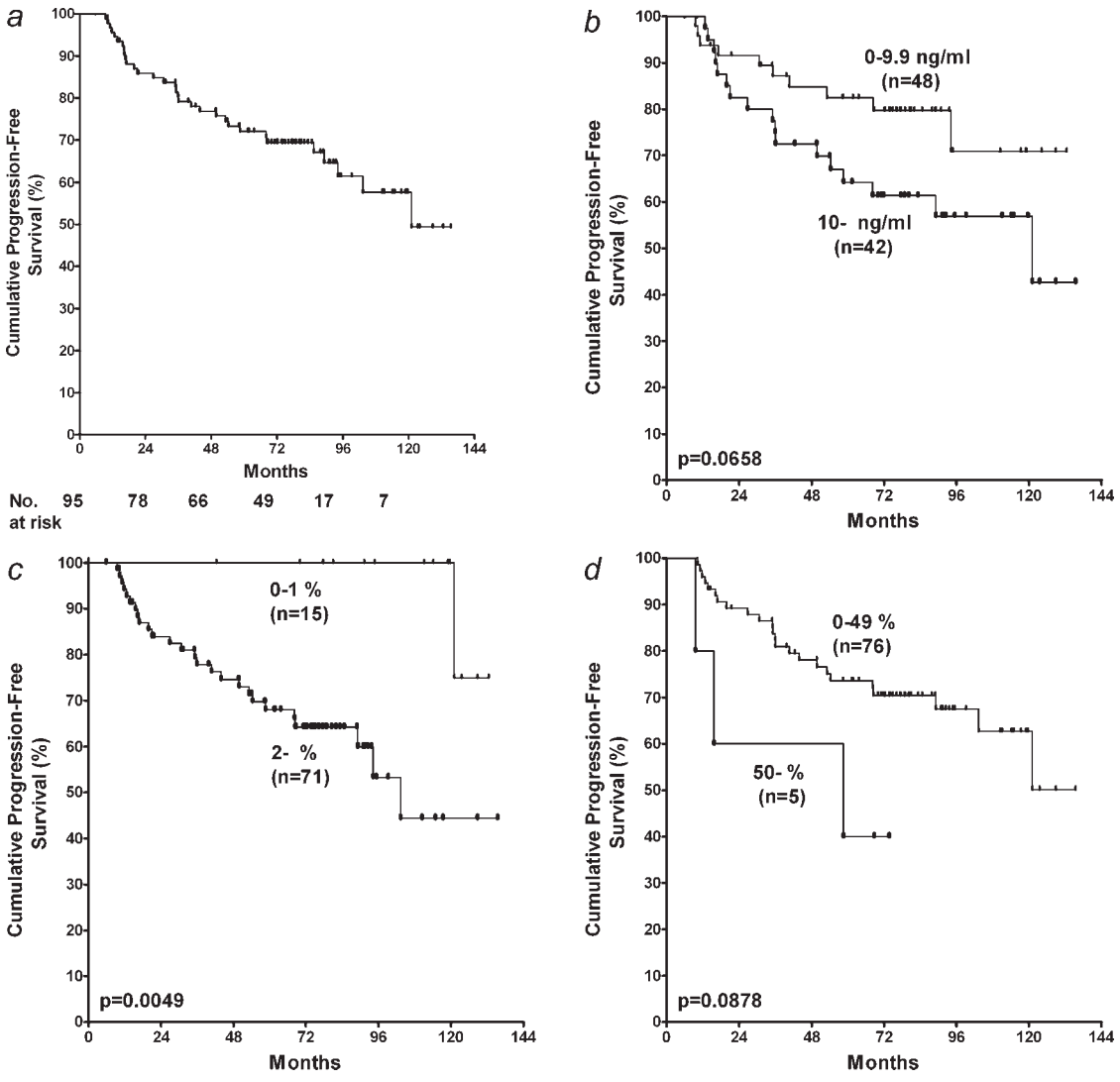

FIGURE 4 – Kaplan–Meier progression-free survival curves of the cases with Gleason score less than 7. (*a*) overall progression-free survival, and according to (*b*) PSA-value, as well as immunostainings of (*c*) Ki-67, and (*d*) EZH2.

Both Ki-67, and EZH2 improved significantly the fit of the Cox regression model, and MCM7 almost significantly. Thus, the markers have true added value as independent prognostic markers. Notably, re-graded Gleason score, which was a strong prognostic marker as itself, showed no independent prognostic value in any of the multivariate analyses. Thus, it seems that Ki-67, EZH2 and MCM7 are potentially better markers than Gleason score.

From clinical point of view, novel markers are especially needed for patients who have no lymph-node metastases and whose PSA drop to zero after surgery (*i.e.* truly radically treated patients). Therefore, we analyzed this subset of patients separately. In this group of patients EZH2, MCM7 and PSA showed independent prognostic value, whereas Ki-67 and pT-stage showed borderline, but nonsignificant prognostic value. Gleason score was not an independent prognostic marker in this patient group either.

Finally, we also analyzed the prognostic value of the markers separately in a group of patients with a Gleason score less than 7. This is a patient group that most likely includes patients with slowly growing tumors. It is well known that a large fraction of prostatectomy treated patients is actually overtreated. Active surveillance could be a more suitable form of treatment for these patients.[2,40] The key question is how to identify patients with a very good prognosis. Of the prognostic markers studied only Ki-67 was significantly associated with prognosis. The Ki-67 staining was able to identify a subgroup (17% of Gleason <7) of patients with a very good prognosis. Of the 15 patients with Ki-67 positive cell fraction $\leq 1\%$, only 1 (7%) experienced relapse about 10 years after surgery compared to a 37% recurrence rate in the rest of the patients. Thus, Ki-67 could be a clinically useful marker for the identification of patients with a very low risk of recurrence. However, the study here was done using prostatectomy specimens. In order to be useful for the identification of low-risk disease, the marker should be analyzable from needle biopsy. Therefore, the prognostic value of Ki-67 in Gleason <7 tumors should be tested in needle biopsy materials.

Varambally and co-authors identified EZH2 as a gene that is overexpressed in prostate cancer metastases.[13] They also showed that high expression determined by immunohistochemistry is associated with poor prognoses in prostatectomy treated patients. Later, the same group reported another study on the prognostic value of EZH2, in which they did not find prognostic significance for EZH2 staining alone. Instead, the combination of EZH2 and E-cadherin staining was an independent prognostic factor.[14] We have recently studied protein expression of EZH2 in the same material that was used here by using two polyclonal antibodies against EZH2.[22] The staining pattern with the polyclonal antibodies was clearly different than what we found here with a monoclonal antibody. The two polyclonal antibodies stained moderately or strongly and homogeneously about 80% of the cancer specimens. Whereas here the staining pattern resembled Ki-67 staining. The strong association between EZH2 and Ki-67 stainings, as shown here and also previously by others,[15] suggests that EZH2 is a marker of cell proliferation activity. It is likely that the polyclonal antibodies also recognize some other antigens besides EZH2. The differences in staining patterns between the different antibodies are important, since the staining with the polyclonal antibodies did not show prognostic value.[22] Recently, a positive association between EZH2 immunostaining and poor prognosis after prostatectomy was demonstrated yet with another monoclonal antibody confirming the importance of the antibodies used.[41]

Also MCM7 has previously been shown to be associated with progression-free survival in prostatectomy treated patients.[26] Here, EZH2 and MCM7 identified a small subgroup of patients with poor prognosis. Interestingly, EZH2 was strongly, and MCM7 borderline associated with high Ki-67 staining, but not with each others. Thus, the markers identified different tumors with high cell proliferation activity. The data may suggest that EZH2 and MCM7 are involved in different pathways leading to rapid proliferation. Therefore, we tested also the prognostic value of a combination of EZH2 and MCM7. The combination was able to identify a small subset of patients with a very poor prognosis. Of the 24 patients (12% of all patients) with high EZH2 and/or MCM7 cell fraction, 19 (73%) experienced disease progression during the follow-up. The median progression-free time of these patients was about 2.2 years compared to about 9.2 years in the rest of the patients.

The cut-off values used in the prognostic analyses were selected based on the best possible discriminatory effect. This approach may predispose to false positive findings. However, as Figure 2 indicates, especially, EZH2 is associated with prognosis in quite large spectrum of cut-off values. In this respect the EZH2 seems to be more robust than MCM7.

One clear finding of the study was that markers, whether molecular markers, Gleason score, or others, can identify extreme ends (high- and low-risk) of the disease behavior quite well. However, a vast majority of the patients belongs to the intermediate group by all markers. In this intermediate group of patients, there is also a significant fraction of patients who experience progression of the disease. We tested whether dividing Gleason 7 according to Gleason grades to 3 + 4 and 4 + 3 would significantly separate this intermediate group of patients. It did not (data not shown). Thus, additional markers are obviously needed to identify the high- and low-risk patients in this prognostically intermediate category.

We used population-based prostatectomy-treated patient material for the evaluation of the prognostic significance of the markers. Samples were available from about 75% of prostatectomies done in TAUH before 1999. Thus, the likelihood of biases due to patient selection is low. Also due to the fact that patients were followed up by TAUH and/or health centers from which we were able to retrieve the follow-up PSA values, practically no patients were lost in follow-up. The median follow-up time was almost 6 years, with the longest follow-up time being over 18 years. The long follow-up shows that prostate cancer progression can also take place late, 10 years after prostatectomy, suggesting that prostate cancer cells can remain dormant for long periods of time. Similar late recurrences have previously been demonstrated, for example, in breast cancer patients.[42]

In conclusion, both Ki-67, EZH2 and MCM7 (and the combination of the latter two) immunostainings seem to identify patients with a very high risk of recurrence after radical prostatectomy. Thus, they should be considered as potential markers to identify patients for adjuvant therapy trials. On the other hand, low Ki-67 immunostaining seemed to identify a subgroup of patients with a very low risk of disease progression, suggesting that such patients could be candidates for active surveillance instead of immediate prostatectomy. However, this finding should be confirmed in needle biopsy specimens. Since immunostainings of EZH2, MCM7 and Ki-67 are quantifiable and methodologically easy, it should be feasible to set up reliable assays for clinical use. Quality control studies on the reliability of the immunostainings of the biomarkers are warranted.

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