KATI PORKKA

Differentially Expressed Genes in Prostate Cancer

ACADEMIC DISSERTATION

To be presented, with the permission of
the Faculty of Medicine of the University of Tampere,
for public discussion in the auditorium of Finn-Medi 1,
Lenkkeilijänkatu 6, Tampere, on October 24th, 2003, at 12 o'clock.

Acta Universitatis Tamperensis 959
University of Tampere
Tampere 2003

ACADEMIC DISSERTATION
University of Tampere, Institute of Medical Technology
Finland

Supervised by
Professor Tapio Visakorpi
University of Tampere

Reviewed by
Docent Anne Kallioniemi
University of Tampere
Docent Jarmo Wahlfors
University of Kuopio

Distribution

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

Tel. +358 3 215 6055
Fax +358 3 215 7685
taju@uta.fi
http://granum.uta.fi

Cover design by
Juha Siro

Printed dissertation
Acta Universitatis Tamperensis 959
ISBN 951-44-5771-4
ISSN 1455-1616

Electronic dissertation
Acta Electronica Universitatis Tamperensis 283
ISBN 951-44-5772-2
ISSN 1456-954X
http://acta.uta.fi

Tampereen yliopistopaino Oy Juvenes Print
Tampere 2003

CONTENTS

LIST OF ORIGINAL COMMUNICATIONS **5**

ABBREVIATIONS **6**

ABSTRACT **7**

INTRODUCTION **9**

REVIEW OF THE LITERATURE **10**
1. Natural history of prostate cancer 10
2. Molecular mechanisms of prostate cancer 11
 2.1 Chromosomal alterations in prostate cancer 13
  2.1.1 Losses of genetic material in prostate cancer 13
  2.1.2 Gains of genetic material in prostate cancer 16
  2.1.3 Epigenetic changes in prostate cancer 19
 2.2 Tumor suppressor genes in prostate cancer 20
  2.2.1 PTEN 21
  2.2.2 TP53 23
  2.2.3 NKX3.1 24
  2.2.4 GSTP1 24
  2.2.5 CDH1 25
  2.2.6 Other tumor suppressor genes 25
 2.3 Oncogenes in prostate cancer 27
  2.3.1 MYC 27
  2.3.2 ERBB2 28
  2.3.3 BCL-2 29
3. Methods for studying differential gene expression 29
 3.1 Subtractive hybridization 30
 3.2 Differential display 32
 3.3 Serial analysis of gene expression (SAGE) 33
 3.4 DNA microarray 34

AIMS OF THE STUDY **36**

MATERIALS AND METHODS **37**
1. Cancer cell lines and xenografts 37
2. Clinical tumor samples 37
3. Suppression subtractive hybridization (SSH) (Studies I and II) 39
4. DNA microarray (Studies I, II and III) 39
 4.1 Filter arrays (Study I) 39
 4.2 Custom-made cDNA microarrays (Study II) 40
 4.3 Commercial microarrays (Study III) 41
5. Real-time quantitative RT-PCR (Studies II-V) 41
6. FISH (Studies II, III and V) 42
7. Northern hybridization (Studies I, II, IV and V) 43
8. Southern hybridization (Study IV) 43
9. Cloning of the full-length STEAP2 cDNA (Study IV) 44
10. Sequencing (Studies I, II, IV and V) 44

11. Androgen stimulation of the LNCaP cells (Study IV) 44
12. Methylation analysis (Study IV).. 45
13. Subcellular localization of STEAP2 with GFP fusion proteins (Study IV) 45
14. mRNA in situ hybridization (Study IV) 45
15. Mutational analyses (Studies IV and V).. 46
16. Statistical analyses (Studies II, IV and V)... 46

RESULTS 47
1.Detection of differentially expressed genes by combining SSH and cDNA library array (Studies I and II) 47
1.1 Subtraction efficiency (Study I) 47
1.2 Sensitivity and linearity of cDNA filter array hybridization (Study I)................. 47
1.3 Genes showing higher expression in BPH than in PC-3 (Study I)....................... 48
1.4 Genes showing overexpression in PC-3 (Study II)... 49
2. Detection of differentially expressed genes using commercial microarray slides (Study III) 49
3. Overexpression and amplification of RAD21, KIAA0196 and elongin C in prostate cancer (Studies II and III) 50
3.1 RAD21 and KIAA0196 (Study II) 51
3.2 Elongin C (Study III).. 52
4. Cloning and characterization of STEAP2 (Study IV) 53
4.1 STEAP2 gene and protein 53
4.2 Expression of STEAP2... 55
5. pHyde (Study V).. 56
5.1 Mutation and copy number analyses 56
5.2 Expression analysis.. 57

DISCUSSION 59
1. Detection of differentially expressed genes by combining SSH and cDNA library array 59
1.1 Combination of SSH and array hybridization (Studies I and II) 59
1.2 Validation of the SSH and the cDNA array hybridization methods: enrichment, sensitivity and linearity (Study I).. 60
1. 3 Differentially expressed genes.. 62
1.3.1 Genes showing higher expression in BPH than in PC-3 (Study I)................ 62
1.3.2 Genes overexpressed in PC-3 (Study II) 63
2. RAD21, KIAA0196 and elongin C as putative target genes for 8q gain 65
2.1 RAD21 and KIAA0196 (Study II) 65
2.2 Elongin C (Study III).. 66
3. STEAP2 (Study IV).. 69
4. pHyde (Study V).. 71

SUMMARY AND CONCLUSIONS 75

ACKNOWLEDGEMENTS 77

REFERENCES 79

ORIGINAL COMMUNICATIONS 97

LIST OF ORIGINAL COMMUNICATIONS

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

I. Porkka K and Visakorpi T (2001): Detection of differentially expressed genes in prostate cancer by combining suppression subtractive hybridization and cDNA library array. J Pathol 193:73-79.

II. Porkka K, Tammela T, Vessella R, Visakorpi T (2003): RAD21 and KIAA0196 are amplified and overexpressed in prostate cancer. Genes Chromosomes Cancer, in press.

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

IV. Porkka K, Helenius M and Visakorpi T (2002): Cloning and characterization of a novel six-transmembrane protein STEAP2, expressed in normal and malignant prostate. Lab Invest 82:1573-1582.

V. Porkka K, Nupponen N, Tammela T, Vessella R and Visakorpi T (2003): Human pHyde is not a classical tumor suppressor gene in prostate cancer. Int J Cancer 106:729-735.

ABBREVIATIONS

AR	androgen receptor
BCL2	B-cell CLL/lymphoma 2
BPH	benign prostatic hyperplasia
BRCA2	breast cancer susceptibility gene 2
CAV1	caveolin 1
CD44	CD44 antigen (homing function and Indian blood group system)
CDH1	cadherin 1 (E-cadherin)
CGH	comparative genomic hybridization
DCC	deleted in colon cancer
DD	differential display
DHPLC	denaturing high-performance liquid chromatography
DHT	dihydrotestosterone
EDNRB	endothelin receptor B gene
EIF3S3	eukaryotic translation initiation factor 3, subunit 40 (eIF3-p40)
ERBB2	avian erythroblastic leukemia viral oncogene homolog 2
EST	expressed sequence tag
FISH	fluorescence *in situ* hybridization
GFP	green fluorescent protein
GSTP1	glutathione S-transferase pi-class
KAI1	“kang ai” prostate cancer antimetastasis gene
KLF6	kruppel-like factor 6
LOH	loss of heterozygosity
MET	met proto-oncongene, hepatocyte growth factor receptor
MXI1	Max interactor 1
MYC	avian myelocutomatosis viral oncogene homolog
NKX3.1	NK homeobox (Drosophila) family 3A homolog
PIN	prostatic intraepithelial neoplasia
PCR	polymerase chain reaction
PSA	prostate specific antigen
PSCA	prostate stem cell antigen
PTEN	phosphatase and tensin homolog deleted on chromosome 10
RB1	retinoblastoma 1
SAGE	serial analysis of gene expression
SSH	suppression subtractive hybridization
STEAP	six-transmembrane epithelial antigen of the prostate
STEAP2	six-transmembrane epithelial antigen of the prostate 2
TIARP	TNFα-induced adipose-related protein
TP53	tumor protein p53
VHL	von Hippel-Lindau gene

ABSTRACT

To identify genes that could be involved in the development of prostate cancer, a combination of suppression subtractive (SSH) and cDNA microarray hybridizations was used for the detection of differentially expressed genes. Both methods were first validated by studying the efficiency of the subtraction as well as the sensitivity and the linearity of the array hybridization. Two subtracted cDNA libraries were constructed using a prostate cancer cell line PC-3 as a model for cancer and benign prostatic hyperplasia (BPH) tissue as a non-cancerous model, and subsequently screened for differentially expressed genes by cDNA microarray hybridization.

Using SSH and cDNA microarray hybridization, RAD21 and KIAA0196 genes were found to be overexpressed in PC-3. In the real-time quantitative RT-PCR analysis, RAD21 and KIAA0196 also showed elevated expression in clinical prostate carcinomas. Using fluorescence *in situ* hybridization analysis (FISH), amplifications of the RAD21 and the KIAA0196 genes were detected in 30-40% of hormone-refractory prostate cancers. Overexpression of elongin C gene, located in 8q21, was detected by utilizing commercial cDNA microarray slides and the PC-3 cell line containing amplification of the 8q region. In FISH analysis, amplifications of the elongin C gene were detected in about 20% of hormone-refractory tumors. Expression of elongin C was also increased in hormone-refractory tumors according to the real-time quantitative RT-PCR analysis. In conclusion, KIAA0196, RAD21, and elongin C are putative target genes for the common amplification of 8q in prostate cancer.

An anonymous EST, expressed in BPH but not in PC-3, was detected using a combination of the SSH and array hybridization methods. Full-length cloning of this EST revealed a novel gene, designated as STEAP2, which encodes for a putative six-transmembrane protein. Using a green fluorescent protein (GFP) fusion construct, it was demonstrated that STEAP2 protein is located mainly in the plasma membrane, as well as in vesicle-like structures in the cytoplasm. STEAP2 was shown to be predominantly expressed in prostate epithelial cells, and the expression is increased in prostate carcinoma cells. The biological function of STEAP2 is still unknown, but as a cell-surface antigen, STEAP2 is a potential diagnostic or therapeutic target in prostate cancer.

One of the closest homologs of the STEAP2 protein is a rat protein pHyde. Rat pHyde has been reported to display tumor suppressive activities in human prostate cancer cells. To study whether human pHyde is a classical tumor suppressor gene in prostate cancer, xenograft and clinical prostate carcinoma samples were screened for pHyde mutations using DHPLC analysis as well as sequencing. In addition, gene copy numbers of pHyde were analyzed by FISH. Of the 68 samples analyzed, only two (3%) contained mutations in the pHyde gene suggesting that biallelic inactivation of pHyde is a rare event and that pHyde is not a classical tumor suppressor in prostate cancer.

INTRODUCTION

Cancer is a disease involving dynamic genetic changes. Inactivating mutations in tumor suppressor genes and activating mutations in oncogenes gradually lead to transformation of a benign cell to a malign derivative. There is a lot of evidence suggesting that tumorigenesis is a multistep process in which mutations accumulate in the genome in succession to clonal selection and expansion of the most aggressive phenotype, “the fittest”, in terms of Darwinian evolution (Kinzler and Vogelstein, 1996; Nowell, 1976; Hanahan and Weinberg, 2000). The multiple genetic changes are believed to provide the cancer cells with novel capabilities giving them growth advantage over normal cells, ultimately leading to invasion throughout the whole body. Hanahan and Weinberg have suggested that there are six essential alterations in the cell physiology that are the hallmarks of malignant growth: 1) self-sufficiency in growth signals, 2) insensitivity to antigrowth signals, 3) escape from programmed cell death (apoptosis), 4) unlimited replicating potential, 5) angiogenesis and 6) tissue invasion and metastasis. These characteristics are believed to be common to all types of human cancers (Hanahan and Weinberg, 2000).

The molecular basis of the tumorigenic process of prostate is poorly understood. The present study was set up to find genes that could be involved in the initiation and progression of prostate cancer. Using a combination of two methods, suppression subtractive hybridization and cDNA array hybridization, genes were detected that are expressed differentially in prostate cancer compared to benign prostate. The differentially expressed genes were characterized, and their clinical significance in prostate carcinomas was analyzed.

REVIEW OF THE LITERATURE

1. Natural history of prostate cancer

Prostate cancer is now the most common malignancy among men in Finland (Finnish Cancer Registry, 2000), as well as in many other Western industrialized countries. Prostate cancer arises from glandular epithelium, most often in the peripheral zone of the prostate. Prostatic intraepithelial neoplasia (PIN) is also often found in the peripheral zone, and is believed to be a premalignant stage of prostate carcinoma. However, as many early prostate carcinomas do not contain lesions of PIN, it is not considered to be a prerequisite for cancer (reviewed by DeMarzo et al., 2003). Prostate cancer progression is a multistep process, in which an organ-confined tumor eventually invades through the capsule of the prostate into its surroundings and metastasizes, first to local lymph nodes and finally to distant organs, mainly bones. Localized, organ-confined prostate cancer is curable by either radical prostatectomy or radiotherapy. For advanced prostate cancer, hormonal therapy is the standard treatment. As almost all prostate cancers originally require androgens in order to grow, androgen withdrawal leads to regression of the tumor. During the treatment, however, an androgen-independent cancer cell population arises, after which a hormone-refractory cancer develops (reviewed by Arnold and Isaacs, 2002). There is no effective treatment for hormone-refractory prostate cancer.

The latent form of prostate cancer is very common. Microscopic lesions of cancer have been found in autopsies from more than 50% of 70-80 year old men (Sheldon et al., 1980). A vast majority of these histological cancers would most probably never have developed into a clinical cancer. The critical mechanism that triggers the progression of some of the small histological cancers to an aggressive disease is unknown. It has also been suggested that the microscopic prostate carcinomas already represent two different forms of prostate cancer: those that will remain latent, and those that will gradually develop into clinical disease (reviewed by Selman, 2000). The molecular basis of this fundamental difference is not known.

2. Molecular mechanisms of prostate cancer

In 1996, Kinzler and Vogelstein proposed a model for a multistep genetic progression of colorectal cancer, based on the finding that specific mutations are associated with certain steps of colorectal tumorigenesis (Kinzler and Vogelstein, 1996). The model has also been applied to prostate cancer progression, as it has been found that certain chromosomal changes are commonly associated with certain stage of prostate cancer (Isaacs et al., 1994; Visakorpi et al., 1995b). Unlike in colorectal cancer, however, in prostate cancer the individual target genes of these chromosomal changes are still mostly unknown. In the following chapters, chromosomal aberrations typical for prostate cancer are described, and the role of the potential target genes is discussed. The significance of some well-established tumor suppressor genes and oncogenes in prostate cancer is considered. Genetic alterations detected at different stages of prostate cancer are illustrated in Figure 1.

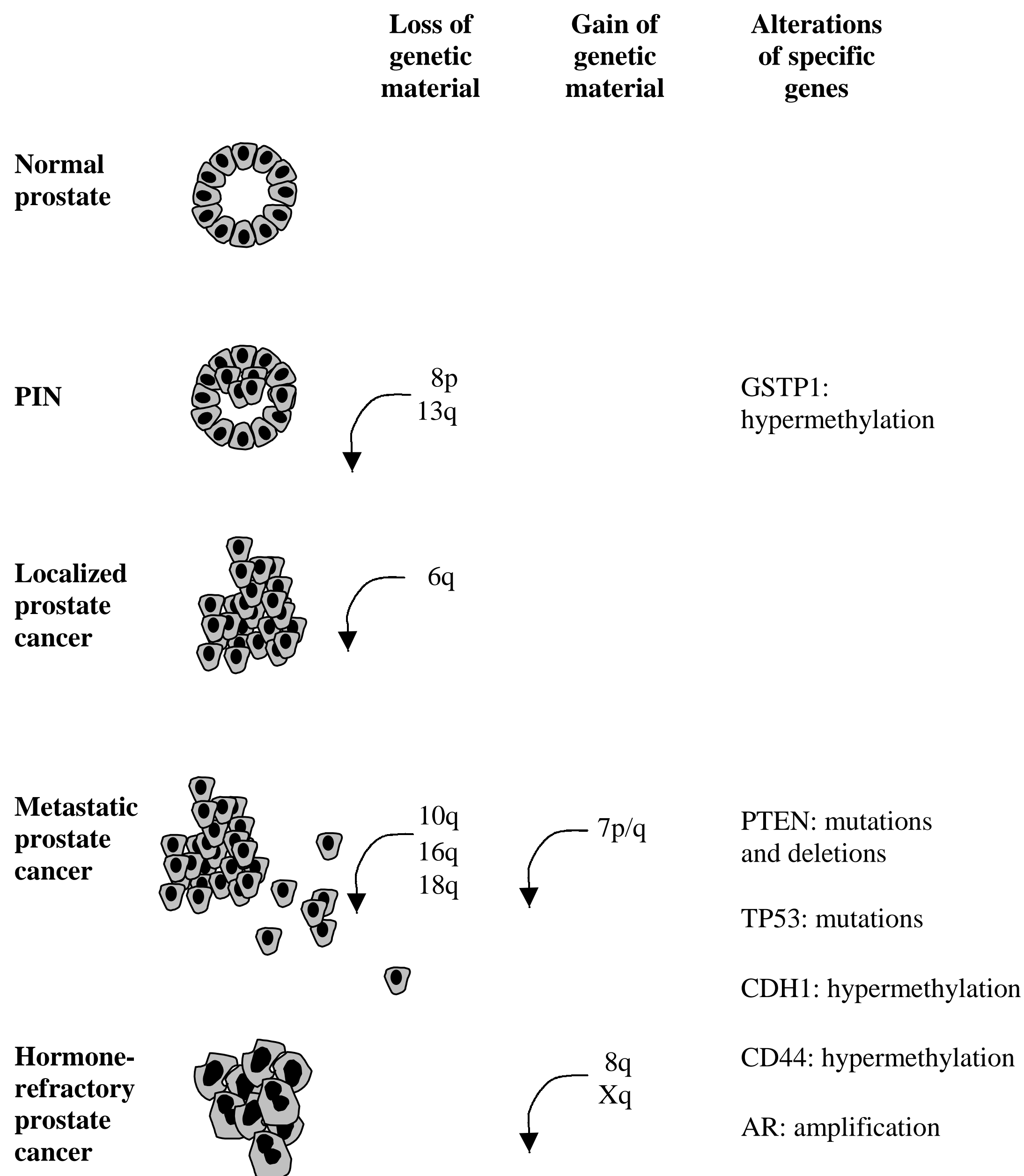

Figure 1. Genetic alterations detected at different stages of prostate cancer. In the early stages of prostate cancer, losses of genetic material are more common than gains or amplifications, whereas in metastatic and hormone-refractory tumors, gains are also frequently detected. The alterations of specific genes have been found almost exclusively in advanced tumors, meaning that the genes involved in the initiation of prostate cancer are still mostly unidentified.

2.1 Chromosomal alterations in prostate cancer

Modern molecular genetic methods, such as the analysis of loss of heterozygosity (LOH) using microsatellite markers and comparative genomic hybridization (CGH) have enabled the rapid characterization of genomic aberrations that are typical for various cancers, and also for the different stages of each cancer. These studies have revealed two main features characteristic of prostate cancer. First, losses of genetic material are much more common than gains or amplifications (Visakorpi et al., 1995b; Fu et al., 2000; Alers et al., 2001), indicating that tumor suppressor genes, which are believed to harbor the frequently deleted regions, probably play an important role in the tumorigenesis of the prostate. Secondly, many of the chromosomal losses can already be detected at the early stages of prostate cancer, whereas gains and amplifications are mostly seen in advanced tumors, suggesting that oncogenes become activated in the advanced stage of the disease (Visakorpi et al., 1995b; Cher et al., 1996; Alers et al., 2001; El Gedaily et al., 2001).

2.1.1 Losses of genetic material in prostate cancer

The chromosomal regions most commonly showing losses in prostate cancer are 6q, 8p, 10q, 13q, 16q, and 18q (reviewed by Elo and Visakorpi, 2001; DeMarzo et al., 2003). These regions are therefore believed to harbor tumor suppressor genes that are involved in the tumorigenesis of the prostate.

One of the most common chromosomal alterations in prostate cancer is loss of the 8p region (reviewed by Elo and Visakorpi, 2001; Dong, 2001). In LOH, FISH and CGH analyses, loss of 8p has also been detected in high-grade PIN lesions (Dong, 2001), which are considered to be premalignant stages of prostate cancer. About 40% of prostate carcinomas involve the loss of 8p according to CGH studies (Dong, 2001), and in metastatic and hormone-refractory tumors it has been detected in up to 70-80% of the tumor samples (Cher et al., 1996; Nupponen et al., 1998b). At least two minimally deleted regions, 8p21 and 8p22, have been identified, suggesting that several tumor suppressor genes may be located at 8p. The most promising target gene for the loss is a homeobox gene NKX3.1 at 8p21 (He et al., 1997), the significance of which in prostate

cancer is discussed in more detail in Section 2.2.3. Other candidate target genes studied include N33, FEZ1 and PRTLS (MacGrogan et al., 1996; Ishii et al., 1999; Fujiwara et al., 1995), all located at the 8p22 region. Although FEZ1 has been demonstrated to suppress cancer cell growth (Ishii et al., 2001), no mutations or other evidence of inactivation of this gene or the two others in prostate carcinomas have been reported. Quite recently, an association between germline mutations in the macrophage scavenger receptor 1 gene (MSR1), located at 8p22, and prostate cancer risk was discovered (Xu et al., 2002). However, mutations in the MSR1 gene in sporadic prostate cancer have not so far been reported.

The second most frequently deleted chromosomal region in prostate cancer is 13q. Although the loss of 13q is seen already in PIN lesions, it has been shown to be associated with clinical aggressiveness of prostate cancer (reviewed by Dong, 2001). At least three distinct regions of allelic loss, 13q14, 13q21-22 and 13q33, have been detected in prostate cancer (Hyytinen et al., 1999). The strongest target tumor suppresssor gene for the 13q loss has been the retinoblastoma gene (RB1) at 13q14 (Friend et al., 1986). Although loss of RB1 expression in prostate cancer has been detected in many studies (Phillips et al., 1994; Tricoli et al., 1996; Cooney et al., 1996b; Mack et al., 1998), there has been no correlation between the decreased expression and LOH at the RB1 locus (Cooney et al., 1996b; Li et al., 1998; Latil et al., 1999). In addition, mutations in the RB1 gene seem to be rare in prostate cancer (Tricoli et al., 1996; Li et al., 1998). As the minimal region of deletion at 13q14 associated with prostate cancer is actually also located outside the RB1 locus (Yin et al., 1999), RB1 is not likely to be the target gene of the 13q loss. Other target genes suggested are, for example, BRCA2 at 13q12 and endothelin receptor B gene (EDNRB) at 13q21. The BRCA2 gene has not been found to be altered in prostate cancer (Latil et al., 1996), whereas EDNRB has been reported to be hypermethylated and downregulated in prostate cancer (Nelson et al., 1997). More recent studies have shown, however, that EDNRB is not located at the minimal region of deletion (Dong et al., 2000).

Deletions at 10q have been detected in about 27% of prostate cancer samples studied by CGH, and in 30-60% of samples in LOH analyses (reviewed by Dong, 2001). According to CGH studies, the minimal regions of deletion are at 10cen-q21 and at 10q26 (Nupponen et al., 1998b), whereas the highest rate of LOH has been reported at

the region 10q23-q24 (Gray et al., 1995; Lacombe et al., 1996), where the well-known tumor suppressor gene PTEN is located (Li et al., 1997; Steck et al., 1997; Li and Sun, 1997). PTEN is likely to be one of the target genes for 10q loss, as evidenced by deletions and mutations as well as downregulation of the gene observed in a high proportion of prostate carcinoma samples (Cairns et al., 1997; Gray et al., 1998; McMenamin et al., 1999). The role of PTEN in prostate cancer is more thoroughly discussed in Section 2.2.1. However, LOH at 10q23-q24 has been detected much more frequently than biallelic inactivation of the PTEN gene, suggesting that there may be another, as yet unidentified target gene located close to PTEN. Another candidate target gene for the 10q loss has been the MXI1 gene, located at 10q25. MXI1 has an interesting function as an antagonist of MYC, and it has been shown to suppress prostate cancer cell proliferation *in vitro* (Taj et al., 2001). Although there is one study reporting a high rate of MXI1 mutations in prostate cancer (Eagle et al., 1995), in most other studies mutations in the MXI1 gene have been found to be rare (Gray et al., 1995; Kawamata et al., 1996; Kuczyk et al., 1998). In addition, as the MXI1 locus is not included in the minimal regions of deletion detected by neither CGH nor LOH analyses, the MXI1 gene is not likely to be one of the target genes of the 10q loss.

Loss of 16q has frequently been detected in prostate cancer by both CGH and LOH analyses (reviewed by Dong, 2001). LOH at 16q has been most commonly observed in advanced tumors and is associated with poor prognosis (Li et al., 1999; Elo et al., 1997; Elo et al., 1999). Perhaps the most intensively studied putative target gene for the 16q loss is E-cadherin (CDH1). However, according to various LOH studies, the common minimally deleted region appears to be at 16q23-q24, excluding the CDH1 locus at 16q22 (Dong, 2001). Even if CDH1 was not the target gene for 16q loss, it may still be implicated in the tumorigenesis of the prostate, as further discussed in Section 2.2.5.

Deletion of 18q is relatively common in prostate cancer according to CGH and LOH studies (Dong, 2001), and it has been detected mainly in advanced stages of the disease (Ueda et al., 1997; Padalecki et al., 2000). Three target genes, SMAD2, SMAD4 and DCC, have been suggested for 18q loss. Although downregulation of DCC expression has been reported in one study (Gao et al., 1993), no mutations have been found either in the DCC gene or in the SMAD2 and the SMAD4 genes (Ueda et al., 1997; Yin et al.,

2001). The DCC and the SMAD4 genes also seem to be located outside the minimal region of deletion (Yin et al., 2001).

Deletions at 6q, especially at the 6q15-q22 region, have been found in prostate cancer in several LOH and CGH studies (reviewed by Dong, 2001). Although mapping studies have revealed at least two putative tumor suppressor loci at 6q (Cooney et al., 1996a), no promising target genes for the 6q loss in prostate cancer have so far been suggested.

2.1.2 Gains of genetic material in prostate cancer

High-level amplifications of chromosomal regions are rare in prostate cancer, especially in primary prostate cancer, suggesting a minor role for oncogenes in the tumorigenesis of prostate (Kallioniemi and Visakorpi, 1996). In hormone-refractory and metastatic tumors, 7p/q, 8q and Xq are the chromosomal regions that have most commonly shown gains in CGH studies (reviewed by Elo and Visakorpi, 2001).

Gain of 8q is the most common chromosomal alteration detected in hormone-refractory and metastatic prostate carcinomas by CGH (Nupponen et al., 1998b), with almost 90% of advanced tumors showing gain of 8q, compared to only 5% of primary tumors (Visakorpi et al., 1995b; Cher et al., 1996; Nupponen et al., 1998b). Gain of 8q has been shown to be associated with an aggressive phenotype of prostate cancer (Alers et al., 2000). Although the gain usually covers the entire long arm of the chromosome 8, two independently amplified subregions, 8q21 and 8q23-q24, have been identified (Cher et al., 1996; Nupponen et al., 1998b). Probably the most intensively studied putative target gene for 8q gain is MYC, located at 8q24. MYC is a well-known oncogene that is activated in many human cancers (reviewed by Pelengaris et al., 2002). MYC has been shown to be amplified and overexpressed in prostate carcinomas (Jenkins et al., 1997; Nupponen et al., 1999), and its increased copy number seems to be associated with poor prognosis of prostate cancer (Sato et al., 1999). The involvement of MYC in prostate cancer is discussed in more detail in Section 2.3.1. Even though many researchers suggest that MYC is a target gene of the 8q23-q24 gain, it may not be the only one. This view is supported by a finding that in breast cancer, in which gain of 8q is also very common, overexpression of MYC is rarely due to an increased copy number of the

MYC gene (Bieche et al., 1999). Another suggested candidate for the gain at 8q23-q24 is EIF3S3, a subunit of a translation inititation factor eIF3. The EIF3S3 gene, located at 8q23, has been shown to be amplified in 30% of hormone-refractory prostate cancers and in 50% of metastases (Nupponen et al., 1999; Saramäki et al., 2001). Amplification of EIF3S3 has been shown also to be associated with high Gleason score (Saramäki et al., 2001). The EIF3S3 gene is most often amplified together with the MYC gene, but in some tumors, the copy number of EIF3S3 is higher than that of MYC (Nupponen et al., 1999). A third putative target gene for the 8q23-q24 gain is a prostate stem cell antigen gene (PSCA), located at 8q24 (Reiter et al., 1998). PSCA protein expression has been demonstrated to correlate with prostate tumor stage, grade and androgen independence (Gu et al., 2000), but studies on the mRNA levels of PSCA have not confirmed this correlation (Ross et al., 2002). Even though co-amplification of PSCA and MYC has been detected in a subset of advanced prostate carcinomas, MYC often seems to be independently amplified without amplification of PSCA, suggesting that PSCA may not be the primary target of the 8q amplicon (Reiter et al., 2000; Tsuchiya et al., 2002). Fine mapping of the minimal region of the 8q23-q24 amplicon has revealed tens of other genes residing in this region (Nupponen et al., 2000, Tsuchiya et al., 2002). No further evidence of the potential involvement of any of these genes in clinical prostate cancer has so far been reported. For the gain of the 8q21 region, no promising target genes have been identified.

Gain at both arms of the chromosome seven has frequently been detected in prostate cancer in CGH analyses (Visakorpi et al., 1995b; Alers et al., 2001). In FISH analysis, at least one extra copy of the entire chromosome seven is often observed in prostate carcinoma samples (Alcaraz et al., 1994; Visakorpi et al., 1994; Bandyk et al., 1994; Wang et al., 1996; Cui et al., 1998), and aneusomy of chromosome 7 has been shown to be associated with advanced tumor stage and poor prognosis of prostate cancer (Bandyk et al., 1994; Alcaraz et al., 1994; Cui et al., 1998; Alers et al., 2000). In CGH studies, the minimal regions of gain have been narrowed down to 7p21-p15, 7q21 and 7q31 (Nupponen et al., 1998b). One of the target genes suggested for the 7p/q gain is the well-known oncogene MET. The MET gene encodes for a receptor tyrosine kinase that binds a hepatocyte growth factor/scatter factor (HGF/SF). Overexpression of the MET protein and mRNA has been detected in PIN lesions, as well as in higher grade tumors and metastases (Pisters et al., 1995; Humphrey et al., 1995; Watanabe et al., 1999;

Knudsen et al., 2002), the expression being significantly higher in high-grade tumors compared to PIN lesions and in metastases compared to primary tumors (Pisters et al., 1995; Humphrey et al., 1995; Knudsen et al., 2002). Another putative target gene for the gain of chromosome seven is the caveolin gene (CAV1). Elevated expression of CAV1 has been detected in prostate cancer (Yang et al., 1998), with correlation between increased expression and progression of the disease (Yang et al., 1999). Suppression of CAV1 expression has been shown to convert androgen-insensitive prostate cancer cells to the androgen-sensitive phenotype, which is then again reversed by the induction of the CAV1 expression (Nasu et al., 1998). Both the CAV1 and the MET genes are located at one of the minimal regions of gain, at 7q31. Paradoxically, deletions and allelic losses at the region 7q31 have also frequently been detected in prostate cancer (Zenklusen et al., 1994; Takahashi et al., 1995; Cui et al., 1998; Jenkins et al., 1998b; Zenklusen et al., 2000), suggesting that a tumor suppresor gene, instead of an oncogene, may be located at 7q31. In fact, many researchers have reported results indicating that CAV1 actually displays tumor suppressive activities (Galbiati et al., 1998; Zhang et al., 2000b), and that the expression of CAV1 is decreased in many human cancers (Wiechen et al., 2001b, Wiechen et al., 2001a). The 7q arm, especially the region 7q31, seems to be genetically unstable, probably due to a common fragile site at 7q31 (FRA7G) (Jenkins et al., 1998a). This may partly explain the controversial results observed.

Gain of chromosome X has been detected by CGH in over 50% of hormone-refractory prostate cancers, whereas in primary tumors it is not seen (Visakorpi et al., 1995b). At the region Xq12-q13, even high-level amplifications can be detected (Visakorpi et al., 1995b). The gene for the androgen receptor (AR) is located at this region, and in further studies it has been shown that the AR gene is amplified in 30% of hormone-refractory prostate carcinomas (Visakorpi et al., 1995a). The increased copy number of the AR gene is associated with its overexpression, although high expression of AR is frequently also detected in hormone-refractory tumors without the AR amplification (Linja et al., 2001). No amplifications of AR have been found in tumors prior to hormone therapy, suggesting that AR amplification is selected during androgen deprivation and may be a possible mechanism for the failure of the therapy (Visakorpi et al., 1995a; Koivisto et al., 1997). Another possible mechanism could be point mutation that changes the ligand binding or transactivation capacity of AR. The rate of AR mutations in prostate cancer has been widely studied with somewhat contradictory results. Although in one study,

AR mutations were reported to be quite frequent in tumors already prior to hormone-therapy (Tilley et al., 1996), in most of the other studies mutations in samples from untreated patients have been reported to be rare (Newmark et al., 1992; Elo et al., 1995; Evans et al., 1996; Segawa et al., 2002). Several research groups have detected AR mutations in patients with androgen-independent prostate cancer, but the reported mutation frequencies vary widely (reviewed by Balk, 2002). The highest rates of AR mutations have been found in hormone-refractory tumors or metastatic lesions after anti-androgen therapy (Taplin et al., 1999; Haapala et al., 2001). Specific mutations have been found to be associated with the type of hormone therapy used (Taplin et al., 1999; Haapala et al., 2001; Hyytinen et al., 2002), suggesting that the mutations occur in response to selective pressure of the treatment in a way similar to the selection for AR amplification.

2.1.3 Epigenetic changes in prostate cancer

In addition to chromosomal changes that alter gene copy numbers, epigenetic changes, such as DNA hypo- or hypermethylation and histone acetylation or deacetylation, have also been studied quite extensively during the last decade. It has been discovered that in many cancer types, the genome as a whole is hypomethylated, whereas some specific regulatory regions in the genome frequently show hypermethylation (reviewed by Rennie and Nelson, 1999; Jones and Baylin, 2002). DNA hypomethylation has been suggested to promote chromosomal instability, as evidenced by its association with chromosomal alterations (Schulz et al., 2002; Jones and Baylin, 2002). Hypermethylation of promoter regions of specific genes, on the other hand, can lead to the silencing of tumor suppressor genes, being functionally equivalent to inactivating mutations in the genes. Several tumor suppressor genes have been reported to be frequently hypermethylated in prostate cancer, e.g. glutathione-S-transferase π-class gene (GSTP1), E-cadherin (CDH1) and CD44, (Lee et al., 1997; Li et al., 2001; Lou et al., 1999). These genes, as well as their roles in prostate cancer, are more closely described in Chapter 2.2. Together with DNA methylation, histone acetylation and deacetylation are believed to control gene expression (Rennie and Nelson, 1999). There are a few studies suggesting that histone acetylation/deacetylation could be one mechanism for altered expression of some genes in prostate cancer (e.g. p21/WAF/Cip1

and hDAB2IP) (Sowa et al., 1997; Chen et al., 2003b), but so far there is very little direct evidence of this.

2.2 Tumor suppressor genes in prostate cancer

Tumor suppressor genes are genes that, when functioning normally, prevent cells from becoming malignant by controlling, for example, the cell cycle, proliferation, apoptosis and cell adhesion mechanisms. Inactivation of a tumor suppressor gene by mutation, deletion or downregulation of the expression, for example, by hypermethylation of the promoter leads to loss of the tumor suppressing function, thus rendering the cell susceptible to transforming stimuli. When the concept of a tumor suppressor gene came into being, it was suggested that both alleles of the gene must be inactivated for its activity to be lost (known as the "two-hit" theory) (Knudson, 1971; reviewed by Knudson, 2001). The mechanisms for inactivation of a tumor suppressor gene have been considered to be mutation, deletion or chromosomal loss (reviewed by Knudson, 2001; Devilee et al., 2001), but recently it has been suggested that other mechanisms, such as hypermethylation, could also mediate tumor suppressor inactivation (Esteller et al., 2000; Grady et al., 2000).

The number of tumor suppressor genes found to fulfill the criteria of the "two-hit" theory (i.e. showing inactivation of both alleles) has been quite low. Therefore, it has been suggested that for some tumor suppressor genes, loss of only one allele could result in insufficient amount of the gene product (haploinsufficiency), leading to cancer formation (Fero et al., 1998; Song et al., 1999; Cook and McCaw, 2000; Kwabi-Addo et al., 2001).

In prostate cancer, most of the classical tumor suppressors known to be crucial in many other cancers (e.g. APC, RB1, and VHL) do not seem to have a significant role. In the following sections, tumor suppressor genes that have been shown to be involved in the development of prostate cancer are described. The most significant of these are also presented in Table 1.

Table 1. Genes shown to be altered in prostate cancer.

Gene name	Chromos. location	Identified function	Alteration in prostate cancer	References
PTEN	10q23	Lipid/ tyrosine phosphatase	Mutations and deletions in advanced tumors	Cairns et al., 1997; Gray et al., 1998; Suzuki et al., 1998
TP53	17p13	Transcription factor, cell cycle regulator	Mutations in advanced tumors	Bookstein et al., 1993; Navone et al., 1993; Heidenberg et al., 1995
NKX3.1	8p21	Transcription factor	LOH, loss of expression in advanced tumors	Bowen et al., 2000
GSTP1	11q13	Detoxifying enzyme	Hypermethylation in PIN lesions and in carcinomas	Lee et al., 1994; Lee et al., 1997; Brooks et al., 1998
CDH1	16q22	Cell adhesion	Hypermethylation	Graff et al., 1995; Li et al., 2001
KAI1	11p11	Metastasis suppressor	Decreased expression	Dong et al., 1996; Ueda et al., 1996
CD44	11p11	Cell adhesion	Hypermethylation	Lou et al., 1999; Verkaik et al., 1999; Noordzij et al., 1999; Verkaik et al., 2000
AR	Xq12-q13	Androgen receptor	Amplifications in hormone-refractory tumors	Visakorpi et al., 1995a
MYC	8q24	Transcription factor, regulates e.g. proliferation, differentiation, apoptosis	Amplifications in primary and in advanced tumors	Jenkins et al., 1997; Nupponen et al., 1998b; Bubendorf et al., 1999; Reiter et al., 2000

2.2.1 PTEN

The PTEN tumor suppressor gene (Li et al., 1997), also known as MMAC1 (Steck et al., 1997) or TEP1 (Li and Sun, 1997), is located at 10q23, a region that often shows loss of genetic material in prostate cancer by both LOH and CGH studies (reviewed by Dong, 2001). The PTEN gene encodes for a dual specificity phosphatase that regulates crucial signal transduction pathways. PTEN functions mainly as a lipid phosphatase, its main target molecule *in vivo* being a signaling lipid, phosphatidylinositol 3,4,5-

trisphosphate (PIP-3) (Maehama and Dixon, 1998; Myers et al., 1998). By dephosphorylating PIP-3, PTEN downregulates the Akt/PKB signaling pathway that promotes cell survival and inhibits apoptosis. In addition, PTEN displays weak tyrosine phosphatase activity. The cellular substrates of PTEN tyrosine phosphatase activity are focal adhesion kinase (FAK) and an adapter protein Shc (Tamura et al., 1998; Gu et al., 1999), which regulate cell adhesion and migration. However, the *in vivo* tyrosine phosphatase actitivity of PTEN, compared to its phospholipase activity, is controversial (reviewed by Yamada and Araki, 2001). In addition, functional studies have shown that it is the lipid phosphatase activity of PTEN that is required for the tumor suppressive effects (Myers et al., 1998).

Deletions and other mutations of PTEN have frequently been detected in a wide variety of cancers, especially in glioblastomas and in endometrial carcinomas, but also in renal, ovarian, breast and prostate cancers (Steck et al., 1997; Li et al., 1997; Cairns et al., 1997). Although deletions and mutations of PTEN gene have also been detected in primary prostate carcinomas, (Cairns et al., 1997; Gray et al., 1998), alterations of PTEN have more often been reported to be infrequent in primary tumors (Wang et al., 1998; Feilotter et al., 1998; Pesche et al., 1998; Dong et al., 1998). Instead, inactivation of PTEN has been shown to be commonly associated with advanced stages of prostate cancer, as evidenced by the high rate of mutations and deletions detected in cell lines and xenografts as well as in metastatic lesions of prostate cancer (Steck et al., 1997; Li et al., 1997; Vlietstra et al., 1998; Suzuki et al., 1998). Since the frequency of LOH at the PTEN locus has been reported to be higher (about 40%, on average) than the rate of PTEN mutations in prostate cancer, (Fernandez and Eng, 2002), alternative mechanisms for inactivation of the remaining allele have been studied. One potential explanation for the low rate of biallelic alteration might be that inactivation of only one allele of PTEN would be sufficient to promote tumor growth (haploinsufficiency). This hypothesis is supported by the heterozygous $Pten^{+/-}$ TRAMP mouse model, in which the loss of one allele of PTEN results in an increased rate of prostate cancer progression (Kwabi-Addo et al., 2001).

2.2.2 TP53

The tumor suppressor gene TP53 is the most commonly mutated gene in human cancer. TP53 gene encodes for the tumor protein p53, which is a key regulator of the cell cycle, controlling the transition from the G1 phase to the S phase. Under conditions conducive to DNA damage, TP53 may either induce apoptosis or arrest the cell cycle for DNA repair (reviewed by Morris, 2002). Mutated TP53 protein has a prolonged half-life, leading to nuclear accumulation of the abnormal protein (reviewed by MacGrogan and Bookstein, 1997). Because of the nuclear accumulation, the presence of the TP53 mutation can be indirectly detected by immunohistochemistry. The frequency of TP53 mutations in prostate cancer has been studied using both the immunohistochemical detection of the protein and direct sequencing of the gene. Although there is some discrepancy in the reports concerning the actual mutation frequencies, most prostate cancer researchers agree that mutations in the TP53 gene are more common in advanced (poorly differentiated, metastatic and/or hormone-refractory) prostate carcinomas than in early, localized prostate cancer (Bookstein et al., 1993; Navone et al., 1993; Heidenberg et al., 1995).

Nuclear accumulation of the TP53 protein has also been shown to be associated with many clinical parameters, such as short progression interval and poor prognosis, transition from androgen-dependent to androgen-independent growth, incidence of distant metastasis and overall survival (Visakorpi et al., 1992; Navone et al., 1993; Grignon et al., 1997). However, in some other studies, no correlation between TP53 immunoreactivity and progression interval or survival has been found (Brooks et al., 1996). There are several studies reporting heterogeneity of TP53 mutation between tumors in multifocal prostate cancer and also within the same tumor (Mirchandani et al., 1995; Gumerlock et al., 1997; Navone et al., 1999). This may partly explain the controversial results obtained in different studies on the frequency and the prognostic value of TP53 mutation in prostate cancer.

2.2.3 NKX3.1

The human homeobox gene NKX3.1, located at 8p21 (He et al., 1997), is the strongest candidate for the commonly observed 8p deletion in prostate cancer. Several different mouse models with NKX3.1 deficiency have been demonstrated to have abnormal ductal morphogenesis, hyperplasias and PIN-like lesions in the prostate (Bhatia-Gaur et al., 1999; Abdulkadir et al., 2002; Kim et al., 2002a). In addition, it has been shown that loss of NKX3.1 function and PTEN function together leads to synergistic activation of the Akt/PKB signaling pathway (Kim et al. 2002b), which promotes cell survival and inhibits apoptosis.

The functional activities of NKX3.1 as well as its location at the commonly deleted chromosomal region strongly support the hypothesis that NKX3.1 is the one of target genes for the 8p deletion. However, although loss of the NKX3.1 locus is frequently detected in prostate cancer, no mutations in the remaining allele have been found (Voeller et al., 1997; Xu et al., 2000b; Ornstein et al., 2001). Loss of NKX3.1 expression has been shown to be associated with hormone-refractory disease and advanced tumor stage (Bowen et al., 2000), but overexpression of NKX3.1 in prostate carcinomas has also been reported (Xu et al., 2000b). The lack of mutations in the remaining allele of NKX3.1 has raised speculation of haploinsufficiency as the mechanism to abolish the tumor suppressive activity of NKX3.1. NKX3.1 mouse models support this hypothesis, since heterozygous mutant mice lacking only one allele of NKX3.1 gene also develop hyperplasias and PIN-like lesions in their prostate (Bhatia-Gaur et al., 1999; Abdulkadir et al., 2002).

2.2.4 GSTP1

Glutathione S-tranferase π–class gene (GSTP1) is the most commonly altered gene in prostate cancer detected so far (reviewed by Elo and Visakorpi, 2001). Silencing of the GSTP1 expression by hypermethylation of the promoter region has been detected in 90-100% of prostate carcinoma samples (Lee et al., 1994; Lee et al., 1997; Brooks et al., 1998), and also in a high proportion of PIN samples (Brooks et al., 1998), suggesting that it is an early event in prostate tumorigenesis. Glutathione S-transferases are

detoxifying enxymes that catalyze the conjunction of glutathione with harmful, electrophilic molecules either endogenously or exogenously produced, thereby protecting cells from carcinogenic factors. GSTP1 is usually not hypermethylated in BPH tissue (Lee et al., 1994; Goessl et al., 2000). Therefore, it has been suggested that detection of the hypermethylated GSTP1 could be used as a diagnostic marker for prostate cancer. This has already been shown to be feasible by studies in which hypermethylation of GSTP1 has been detected by methylation-specific PCR from body fluids (Goessl et al., 2000; Cairns et al., 2001) and needle biopsies (Harden et al., 2003).

2.2.5 CDH1

Cadherins are transmembrane glycoproteins that mediate calcium-dependent cell-cell adhesion. The E-cadherin gene (CDH1), a member of the cadherin family, is located at chromosomal region 16q22. As loss of the 16q arm is frequently observed in advanced prostate cancer by CGH as well as by LOH studies (reviewed by Dong, 2001), CDH1 has been the most promising target gene for the 16q loss. However, no somatic mutations in the CDH1 have been found (Suzuki et al., 1996; Li et al., 1999), and the more recent studies have shown that the most commonly deleted regions at 16q are actually outside the CDH1 locus (Li et al., 1999). Instead, decreased expression of the E-cadherin protein in prostate cancer has been reported (Umbas et al., 1992; Li et al., 1999), and the decreased expression has been shown to correlate with histopathological grade, tumor stage, tumor progression and overall survival of prostate cancer patients (Umbas et al., 1992; Cheng et al., 1996; Richmond et al., 1997). On the other hand, in some studies, no correlation between expression of E-cadherin and tumor stage has been found (Rubin et al., 2001). Decreased expression in prostate cancer cell lines and in clinical tumors has been shown to be accompanied by hypermethylation of the CDH1 promoter (Graff et al., 1995; Li et al., 2001).

2.2.6 Other tumor suppressor genes

KAI1 was first identified as a metastasis suppressor gene by transfecting it into rat prostate cancer cells (Dong et al., 1995). Expression of KAI1 has been reported to be

downregulated both in primary prostate tumors and metastatic lesions (Dong et al., 1996; Ueda et al., 1996), and an inverse correlation between KAI1-positive cells and the Gleason score of the tumor has been found (Ueda et al., 1996). However, since no somatic mutations or allelic loss at the KAI1 locus at 11p11 or hypermethylation of the promoter region have been detected (Dong et al., 1996; Sekita et al., 2001), the mechanism of the downregulation of KAI1 expression remains uknown.

CD44 gene is located at the same chromosomal region as the KAI1 gene (11p11), and it encodes for a transmembrane glycoprotein that participates in specific cell-cell and cell-extracellular matrix interactions. As with KAI1, CD44 has also been suggested to be a metastasis suppressor gene (Gao et al., 1997). Downregulation of CD44 expression, as well as hypermethylation of the CD44 promoter, has been detected in prostate cancer (Verkaik et al., 1999; Lou et al., 1999; Noordzij et al., 1999; Verkaik et al., 2000). Both decreased expression and hypermethylation have been shown to be associated with the progression and metastasis of prostate cancer (Noordzij et al., 1999; Kito et al., 2001).

Quite recently, allelic loss of the Kruppel-like factor 6 (KLF6), a transcription factor with an unknown function, was detected in 77% of primary prostate carcinoma samples. In the same study, mutations in the KLF6 gene were found in 55% of the samples (Narla et al., 2001). Transfected wild-type KLF6 was shown to reduce cell proliferation and to upregulate p21 expression in a p53-dependent manner, whereas mutated KLF6 proteins did not show this activity (Narla et al., 2001). In another study soon after, the rate of KLF6 alterations in prostate cancer was, however, reported to be much lower, with LOH occurring in 19% of cell lines and xenografts and in 28% of tumors (Chen et al., 2003a). Missense mutations of KLF6 were detected in only 9% of the samples, and no truncation mutations were reported. In addition, genetic alterations of KLF6 were mainly detected in high-grade tumors and metastases, not in early tumors. As the results of these two studies differ so markedly from each other, further studies are needed to fully ascertain the frequency and the signification of KLF6 alterations in prostate cancer.

2.3 Oncogenes in prostate cancer

Oncogenes are genes whose activation leads to the transformation of a benign cell to a malignant phenotype. The activation mechanism may, for example, be gene amplification leading to overexpression of the gene, translocation of the gene into a transcriptionally active domain in the genome or activating point mutation in the gene. The knowledge of oncogenes in prostate cancer is very limited, and none of the traditional oncogenes is known to be significant in the tumorigenesis of the prostate. In the following sections, the oncogenes that have been most widely studied regarding to their role in prostate cancer are introduced.

2.3.1 MYC

The oncogene MYC encodes for a transcription factor implicated in various cellular processes, such as cell growth, proliferation, loss of differentiation and apoptosis. Elevated or deregulated expression of MYC has been detected in many human cancers, and it is often associated with aggressive and poorly differentiated tumors (reviewed by Pelengaris et al., 2002). As the MYC gene is located at chromosomal region 8q24, which is one of the most frequently amplified regions in advanced prostate cancer (Elo and Visakorpi, 2001), it has been a strong target gene for the 8q23-q24 amplification. The MYC gene has been found to be amplified in 8% of primary prostate carcinomas and in 11-35% of advanced tumors (Jenkins et al., 1997; Nupponen et al., 1998b; Bubendorf et al., 1999; Reiter et al., 2000). Amplification of MYC has in some studies been shown to correlate with overexpression of its protein product as well as with tumor grade and poor prognosis (Jenkins et al., 1997; Sato et al., 1999). However, in some other studies, no correlation between MYC amplification and clinical outcome has been found (Kaltz-Wittmer et al., 2000).

The putative oncogenic function of MYC in prostate cancer has been studied both *in vitro* and *in vivo*: a MYC antisense oligonucleotide has been demonstrated to have antiproliferative effects on prostate cancer cell lines (Balaji et al., 1997), and a retroviral construct of an antisense MYC reduced tumor size when injected into established tumors in nude mice (Steiner et al., 1998). Transgenic mice with chronic overexpression

of MYC in their prostate have been reported to develop epithelial abnormalities similar to low-grade PIN lesions in humans, but these abnormalities have not been found to progress to carcinomas (Zhang et al., 2000a). In conclusion, the role of MYC in prostate cancer remains unclear.

2.3.2 ERBB2

ERBB2 (also known as HER-2/neu) is a receptor tyrosine kinase belonging to the epidermal growth factor receptor family. The role of ERBB2 is important in breast cancer, in which it is amplified and overexpressed in 20-30% of cases and is also a prognostic marker (reviewed by Hayes and Thor, 2002). Since ERBB2 is capable of activating the androgen receptor signaling pathway at low levels of androgens (Craft et al., 1999; Yeh et al., 1999), it has been suggested that ERBB2 might be implicated in the development of androgen-independent prostate cancer. Over the past decade, expression of the ERBB2 protein in prostate cancer has been widely studied. Although some researchers have reported that ERBB2 is overexpressed in prostate cancer (Myers et al., 1994; Gu et al., 1996; Signoretti et al., 2000), others have not detected overexpression of ERBB2 (Visakorpi et al., 1992; Reese et al., 2001; Savinainen et al., 2002). It has been reported that the level of ERBB2 expression is as low in prostate tumors as it is in breast tumors without the gene amplification (Savinainen et al., 2002).

Amplifications of the ERBB2 gene in prostate cancer have been reported by only one research group (Ross et al., 1997a; Ross et al., 1997b), whereas other investigators have found ERBB2 amplifications to be very rare in prostate cancer (Bubendorf et al., 1999; Signoretti et al., 2000; Reese et al., 2001; Savinainen et al., 2002; Lara et al., 2002). After the introduction of the new anti-ERBB2 antibody, trastuzumab (Herceptin), which is used in the treatment of advanced, ERBB2 positive breast cancer, trials with Herceptin in prostate cancer have been initiated. Even though growth inhibition after treatment with Herceptin was observed with prostate cancer xenograft and cell line models (Agus et al., 1999), in clinical trials no positive effects on prostate cancer patients have been seen (Morris et al., 2002). In summary, ERBB2 does not seem to be as significant in prostate cancer as it is e.g. in breast cancer.

2.3.3 BCL-2

BCL-2 is an anti-apoptotic factor that has been found to be involved in many human cancers. The oncogenic function of BCL-2 in prostate cancer cells has been widely studied. It has been demonstrated that the expression of BCL-2 protects prostate cancer cells from apoptotic stimuli and increases their tumorigenic potential (Raffo et al., 1995). Inhibition of BCL-2 expression has been reported to induce apoptosis in prostate cancer cell lines (Dorai et al., 1999) and to delay the progression of hormone-refractory tumors after castration in a mouse model (Miyake et al., 1999). In addition, prostate xenograft tumors overexpressing BCL-2 have been shown to display increased angiogenetic potential (Frenandez et al., 2001). The role of BCL-2 in clinical prostate carcinomas has also been studied, however with somewhat contradictory results. In normal prostate, only basal cells express the BCL-2 protein (McDonnell et al., 1992). Elevated expression of BCL-2 has been detected in prostate cancer especially after androgen withdrawal and in hormone-refractory tumors (McDonnell et al., 1992; Stattin et al., 1996; McDonnell et al., 1997; Furya et al., 1996), suggesting that BCL-2 could be involved in the progression of androgen-independent prostate cancer. On the other hand, some researchers have reported high expression of BCL-2 in PIN lesions and in primary tumors, but not in metastases (Stattin et al., 1996), whereas others have found BCL-2 to be expressed in high-grade PIN but not in clinical carcinomas (Johnson et al., 1998). According to some studies, BCL-2 overexpression predicts progression of prostate cancer and is associated with high grade tumors (Bubendorf et al., 1996; Krajewska et al., 1996), but others have found no correlation between BCL-2 expression and tumor stage or prognosis (Stattin et al., 1996; McDonnell et al., 1997). No high-level amplifications of the BCL-2 gene have been detected in prostate carcinomas (Nupponen et al., 1998b). To summarize, the mechanisms of the oncogenic function of BCL-2 in cancer cells are quite well characterized, but there is no decisive proof of the significance of BCL-2 in prostate cancer.

3. Methods for studying differential gene expression

Positional cloning is one strategy to search for genes involved in cancer. This has been carried out either by linkage analysis or by first screening genetic alterations at the

chromosomal level, and subsequently revealing the target genes underlying the chromosomal changes. Another way of detecting genes potentially involved in tumorigenesis is to monitor differences in gene expression. Several powerful techniques for studying differential expression have been developed, the most widely used being subtractive hybridization, differential display, serial analysis of gene expression and cDNA microarray hybridization. These four techniques, with their advantages and disadvantages, are described below. The various characteristics of the techniques are compared in Table 2.

Table 2. Characteristics of the subtractive hybridization, differential display, SAGE and DNA microarray methods.

Method	Qualitative/ Quantitative	Expression comparison	Detection of novel genes	High throughput of samples	Global expression profiling	Costs
Subtractive hybridization	qualitative	one-way	yes	no	no	low
Differential display	qualitative	two-way	yes	no	no	low to medium
SAGE	quantitative	two-way	yes	no	yes	medium
DNA microarray	quantitative	two-way	usually no	yes	yes	high

3.1 Subtractive hybridization

Subtractive hybridization is used to isolate nucleic acids present in one sample but not in another. The two samples compared are called “tester” and “driver”. The tester is the sample from which differentially represented sequences are to be isolated, and the driver is the reference sample. Subtraction is based on hybridization of sequences present in both the tester and the driver samples, and subsequent separation of the driver and the tester-driver hybrids from unhybridized tester DNA. To achieve as complete subtraction as possible, an excess amount of driver DNA is used in hybridization. The first protocols of subtractive hybridization used methods like hydroxyapatite columns, biotinylation or immobilization of the driver or enzymatic hybrid removal to separate the tester-driver hybrids from the single-stranded tester sample (reviewed by Sagerström et al., 1997). The enrichment of the differentially represented sequences was inefficient,

and so large amounts of starting material were needed, as well as several rounds of subtractive hybridization.

In a new modification of subtractive hybridization, called representational difference analysis (RDA), a PCR based, positive selection of the tester-DNA was used instead of physical separation of double-stranded and single-stranded DNA, allowing subtractive hybridization with much smaller amounts of starting material (Lisitsyn et al., 1993; Hubank and Schatz, 1994). However, the subtraction with the RDA protocol was still biased with high abundant sequences, and rare sequences were missed.

The latest and the best developed version of subtractive hybridization, called suppression subtractive hybridization (SSH), combines normalization with subtraction in the same step (Diatchenko et al., 1996). As a result, concentrations of the high and the low abundance cDNA species become equalized, the subtraction is effective with all sequences, and the probability of detecting rare, differentially expressed genes is increased. In selection, a phenomenon called suppression PCR is utilized to selectively suppress amplification of the non-target DNA molecules, whereas the target molecules are exponentially amplified in the same reaction. SSH is a powerful tool to enrich differentially expressed genes, but since the subtraction can never be complete, a secondary screening process is necessary. The subtracted cDNA population can be used to construct a cDNA library followed by screening with another method, or it can be used as a complex probe to screen other libraries, for example. Only one-way comparisons (up or downregulation) between the two cell populations can be made in one subtraction experiment, and only two samples can be compared at a time. SSH is also suitable for detecting novel sequences. The disadvantage of the method is that since the protocol includes a restriction enzyme digestion, the resulting cDNAs are typically 500-600-bp fragments. Therefore, some additional effort is often required to obtain the full-length sequences of the transcripts. However, several novel, differentially expressed, cancer-related genes, such as ING1 in neuroblastoma and breast cancer (Garkatsev et al., 1996) as well as STEAP, EIF3S3 and Trp-p8 in prostate cancer (Hubert et al., 1999; Nupponen et al., 1999; Tsavaler et al., 2001) have been identified using the SSH method.

3.2 Differential display

Differential display (DD) was first described in 1992 (Liang and Pardee, 1992) as an effective method to separate and clone individual, differentially expressed transcripts. In DD, two or more mRNA populations are first reverse transcribed into cDNA using a poly-A anchoring primer. Subsequently, the poly-A anchoring primer is used in combination with a short, arbitrary primer to randomly amplify the cDNA species by PCR. Finally, the PCR reactions are run in parallel in a high-resolution gel to visualize the expression patterns. Differentially expressed genes are identified by extracting and cloning the PCR products from the gel. The advantage of DD lies in its simplicity. By combining three frequently used molecular biology methods; reverse transcription, PCR and polyacrylamide gel electrophoresis, DD allows a rapid screening of differentially expressed genes in two or more samples at a time. The bottleneck of the method, however, is the cloning of the individual genes. PCR products from several different mRNA species may lie in one band, and so the PCR products usually have to be cloned before sequencing. The major disadvantage of DD is the high percentage (up to more than 50%) of false positive clones. In addition, the high redundancy of genes may be detected as a result of the capability of the arbitrary primers to anneal to several target sequences in the same transcript. Meanwhile, some transcripts are not amplified at all. Analysis of complex mRNA populations would require about 240 primer combinations to achieve 95% coverage of the transcriptome (Liang and Pardee, 1992). Thus, DD is not suitable for global expression profiling.

DD can be used to detect both over and underrepresented genes at the same time and in principle, even very low-abundant transcripts can be detected by DD, if the primer combination used is capable of annealing to the sequence (Wan et al., 1996). However, differences in high-abundant transcripts may not be detected by DD, because saturation of the PCR reactions finally leads to equalization of the amounts of the products (Matz and Lukyanov, 1998). One of the advantages of DD is that only a small amount (<5 µg of total RNA) of starting material is needed, although the rate of false positive clones is likely to be higher when very small amounts of RNA (50 ng) are used (Matz and Lukyanov, 1998). Novel genes can also be discovered by DD; for example, several prostate cancer associated genes, such as PTI-1 (Shen et al., 1995), GC79 (Chang et al.,

1997), caveolin (Yang et al., 1998), DD3 (Bussemakers et al., 1999) and UROC28 (An et al., 2000) have been identified using DD.

3.3 Serial analysis of gene expression (SAGE)

The SAGE method was introduced in 1995 by Velculescu et al. Gene expression analysis by SAGE is based on two principles: first, that a short (9-14 bp) fragment of a transcript called “tag”, is sufficient to identify the corresponding transcript, and secondly, that concatenation of the tags allows sequence-based identification of a large number of transcripts in one experiment. In contrast to the other two methods for genes expression analysis described above (subtractive hybridization and differential display), SAGE also provides quantitative information of the gene expression, because the relative number of each individual tag among other tags reflects the abundance of the corresponding transcript in the cell. Since the protocol includes PCR amplification of only short DNA fragments (“ditags”) with common adaptor primers, no artifacts in gene expression results can be produced by selective amplification of some sequences over others. SAGE can be applied for both global expression profiling and detecting differential expression of individual genes. In principle, novel genes can also be found using SAGE. However, since the expression analysis is based on 9 to 14-bp cDNA fragments, full-length cloning of the novel sequence requires extra work, such as cDNA library screening or rapid amplification of cDNA ends (RACE). One of the advantages of SAGE is that global expression profiling is possible without any previous sequence information. SAGE data is also easily portable, meaning that data produced by different laboratories can be combined. As a part of the National Institutes of Health Cancer Genome Anatomy Project (CGAP) of the USA, a SAGE database currently containing over five million tags from more than a hundred cell types (Boon et al., 2002) is maintained and freely accessible at the website of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/SAGE).

Performing SAGE analysis is basically simple, requiring only a high-throughput sequencing system. However, the more complete expression profile is desired, the more tags need to be sequenced. Typically, more than 200 000 tags are sequenced in one SAGE experiment, which is both time-consuming and expensive. Therefore, SAGE is

not suitable for the analysis of a large number of samples at a time. Even though novel genes, such as the prostate-specific, androgen-responsive gene PMEPA1 (Xu et al., 2000a), have been detected using SAGE, it has mainly been applied for comparing the expression profiles of two different cell types. For example, gene expression in colorectal, pancreatic, breast and prostate cancer cells (Saha et al., 2001; Zhang et al., 1997; Nacht et al., 1999; Waghray et al., 2001), and also in tumor endothelial cells of colon cancer (St. Croix et al., 2000) has been studied using SAGE.

3.4 DNA microarray

DNA microarray, introduced by Schena et al. in 1995, is a high-throughput method for monitoring gene expression. The microarrays are prepared by robotically printing cDNA or oligonucleotide fragments onto glass slides, or by synthesizing oligonucleotide probes *in situ* on silicon-based chips (reviewed by Holloway et al., 2002). RNA from the samples to be studied is reverse-transcribed into cDNA in the presence of fluorescently labeled nucleotides. Usually, the two samples that are being compared are labeled with two different fluorochromes and hybridized together to the probes on the slide, although in some applications (e.g. Affymetrix GeneChip arrays), each sample is hybridized to a separate chip. The fluorescent signals from each spot are then measured using a special reader, and signal ratios are calculated. The choice between cDNA and oligonucleotide probes may not be simple, as they both have their advantages and disadvantages: cDNA clones are easier and cheaper to propagate, but their use is limited by the availability of representative cDNA libraries. Oligonucleotide probes, in principle, can be obtained for any gene, but they must be very carefully designed, as the base composition can easily affect the hybridization results (Holloway et al., 2002).

One of the disadvantages of the microarray method is the costs: the manufacture of the microarray slides is expensive, and special equipment is needed both for printing the slides and for detecting the hybridization signals. In addition, the expression analysis made by microarray is always limited to those genes that have been spotted onto the slides. Although it is possible to study the expression of unidentified genes using microarray hybridization by spotting unknown genes, such as anonymous ESTs or

randomly picked clones from a cDNA library, most of the commercially available microarrays contain only known genes. It has been demonstrated that even very subtle changes, such as 10-20% differences in expression, can be detected by microarray hybridization (Lockhart et al., 1996). However, the drawback is that conventional hybridization protocols usually require a substantial amount of starting material (typically 20-75 µg of total-RNA) (Holloway et al., 2002), which can be problematic with certain types of samples.

The microarray method allows monitoring and comparing of the gene expression of up to tens of thousands of genes simultaneously (Lockhart et al., 1996). The hybridization protocol is simple enough to permit studies with several different samples in one experiment, making it feasible for expression profiling of groups of samples. Consequently, microarray experiments often produce a vast amount of data, which is why data analysis has become the major challenge of the method. In order to extract meaningful information from tens of thousands of data points, datamining software programs are used to discover patterns and rules from large data sets (reviewed by Holloway et al., 2002). Although datamining tools are extremely useful in pattern recognition or clustering of genes and/or samples, they cannot answer fundamental questions like "what kind of data is interesting" or "what is a significant difference". Finding a piece of data that has some biological meaning is the real challenge of a microarray experiment.

In spite of the limitations described, microarray hybridization has become a popular and a widespread method in studying differential expression. It has been used for identifying individual, differentially expressed genes (Lin et al., 1999; Liau et al., 2000; Finlin et al., 2001), but more often for global gene expression profiling and subsequent clustering of genes and/or samples on the basis of the similarities in their expression profiles (Eisen et al., 1998; Iyer et al., 1999). With clustering analysis, it has been possible to even classify certain subtypes of B-cell lymphomas and breast cancers according to their expression profiles (Alizadeh et al., 2000; Perou et al., 2000). With the aid of constantly improving datamining tools, the microarray method is at present perhaps the most powerful for studying gene expression.

AIMS OF THE STUDY

The general aim of the study was to identify genes that might be involved in the tumorigenesis of the prostate. The specific aims were:

1. To evaluate the utility of a combination of suppression subtractive hybridization (SSH) and cDNA microarray hybridization to detect genes that are differentially expressed in prostate cancer.

2. To identify the differentially expressed genes by combining SSH and the microarray hybridization or by utilizing commercial microarrays.

3. To evaluate the clinical significance of the differentially expressed genes in prostate cancer.

4. To clone and characterize the differentially expressed, anonymous EST detected by using SSH and the microarray hybridization.

5. To study whether pHyde is a classical tumor suppressor gene in prostate cancer.

MATERIALS AND METHODS

1. Cancer cell lines and xenografts

Prostate cancer cell lines PC-3 (ATCC number CRL-1435), DU145 (ATCC number HTB-81), LNCaP (ATCC number CRL-1740), 22Rv1 (ATCC number CRL-2505) and NCI-H660 (ATCC number CRL-5813), breast cancer cell lines T47D (ATCC number HTB-133), MCF-7 (ATCC number HTB-22), MDA436 (ATCC number HTB-130), SK-Br-3 (ATCC number HTB-30), MDA415 (ATCC number HTB-128) and ZR75-1 (ATCC number CRL-1500), as well as a cell line originating from fibrocystic disease of the mammary gland, MCF10A (ATCC number CRL-10317), were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). A breast cancer cell line EFM19 was purchased from DSMZ GmbH (Braunschweig, Germany) and a normal prostate epithelial cell line, PrEC, from Clonetics (Walkersville, MD, USA). The cells were cultured under the recommended conditions.

Ten prostate cancer xenografts (LuCaP 23.1, 23.8, 23.12, 35, 41, 49, 58, 69, 70 and 73) were made available by Robert L. Vessella (Department of Urology, University of Washington, USA). The xenografts have been established from human prostate carcinomas and propagated in intact male nude mice (BALB/C *nu/nu*).

2. Clinical tumor samples

The BPH tissue material used in Study I was obtained from Tampere University Hospital. The clinical prostate tumor material obtained from Tampere University Hospital was used for the real-time RT-PCR, FISH and mutational analyses in Studies II, IV and V. The clinico-pathological characteristics of the sample materials are described in Table 3. The BPH samples were obtained from prostatectomy specimens from patients with prostate cancer. However, the samples were histologically verified not to contain any cancer cells. The samples used for the real-time quantitative RT-PCR and the FISH analyses (Studies II, IV and V) were freshly frozen. For the mutational analysis in Study V, fifty-three freshly frozen and three formalin-fixed, paraffin-

embedded prostate carcinoma samples were utilized. The sample materials used in the real-time RT-PCR and the mutational analyses were mostly but not completely overlapping. The use of the clinical tumor samples was approved by the ethical committee of Tampere University Hospital.

Table 3. Clinicopathological characteristics of the clinical prostate tumor material used in Studies II, IV and V.

Characteristic	Samples used for the mutation analysis (Study V)	Samples used for the FISH and the quantitative RT-PCR analyses (Studies II, IV and V)
BPH:		
Total no. of cases	**0**	**9**
Untreated carcinomas:		
Total no. of cases	**38**	**30**
TNM-stage:		
T2N0M0	20	15
T2N1M0	1	1
T3N0M0	13	12
T3NXM0	1	0
T3NXM1	1	0
T4NXM0	1	0
T4NXM1	0	1
TXN0M0	1	1
Histological grade (WHO system):		
I	10	10
II	22	15
III	6	5*
Hormone-refractory carcinomas:		
Total no. of cases	**18**	**12***
Time of the hormonal therapy prior to the TURP (months)	7-80	15-60

Abbreviations: BPH, benign prostate hyperplasia; TNM, tumor, lymph node, metastasis; TURP, transurethral resection of prostate
* = Samples used for the FISH analyses in Studies II and V

In Study III, tissue microarrays (TMA) consisting of formalin-fixed, paraffin-embedded tumor tissues obtained from Tampere University Hospital were utilized in FISH analysis. The TMA contained samples from 35 untreated, prostate cancer specimens and from 35 locally-recurrent, hormone-refractory prostate carcinomas.

3. Suppression subtractive hybridization (SSH) (Studies I and II)

SSH was carried out using the PCR-Select™ cDNA Subtraction Kit (Clontech, Palo Alto, CA, USA) according to the manufacturer's instructions. Total-RNA from the prostate cancer cell line PC-3 and from BPH tissue was extracted using the TRIzol reagent (Invitrogen, Leek, The Netherlands), and poly $(A)^+$ RNA was isolated from the total RNA preparations using Oligotex resin (Qiagen GmbH, Hilden, Germany). In the first subtraction (Study I), BPH was used as a tester and PC-3 as a driver. The second subtraction (Study II) was performed with PC-3 as a tester and BPH as a driver. After the subtractions, BPH and PC-3 cDNA libraries were constructed by cloning the subtracted cDNA fragments into pCR®II-TOPO TA cloning vector (Invitrogen). For a subtraction efficiency test, a cDNA library from unsubtracted cDNA from the same BPH sample used for the subtraction was constructed in the same way.

4. DNA microarray (Studies I, II and III)

4.1 Filter arrays (Study I)

In Study I, 432 clones from the subtracted BPH library and 432 clones from the unsubtracted BPH were randomly picked and grown in liquid 96-well cultures. For internal standards, IMAGE clones of common housekeeping genes ubiquitin (IMAGE: 628344), G3PDH (IMAGE: 645081) and 40S ribosomal protein (IMAGE: 362274) were obtained from GenomeSystems (St. Louis, MO, USA.) The inserts of the cDNA clones from the subtracted libraries and from the IMAGE clones were PCR-amplified using adaptor-specific primers (Clontech) or vector-specific primers respectively. The PCR-amplified inserts from the subtracted and the unsubtracted BPH libraries, as well as from the IMAGE clones were arrayed onto nylon membranes (GeneScreen Plus,

NEN™ Life Science Products Inc., Boston, MA, USA) using a 96-pin Multi-blot Replicator tool (V&P Scientific Inc., San Diego, CA, USA). The DNA was denatured after gridding by soaking the membranes in 1.5 M NaOH, after which the membranes were neutralized in 1.5 M NaCl/ 0.5M Tris, pH 7.2/ 0.001M EDTA.

Total RNA from BPH tissue and from the cell lines was extracted using TRIzol reagent (Invitrogen), and poly(A)$^+$ RNA from the total RNA using Oligotex resin (Qiagen). ^{32}P-dCTP labeled probes were synthesized from 500 ng of poly(A)$^+$ RNA using Oligo-dT primer (Invitrogen) and SuperScript II RNase H- Reverse Transcriptase (Invitrogen) according to the manufacturer's instructions. For a sensitivity and linearity test, various amounts of *in vitro* transcribed poly(A)$^+$ RNA (Riboprobe® *in vitro* Transcription Systems, Promega, Madison, WI, USA) coding for prostate specific antigen (PSA) were spiked to the labeling reactions. The labeled probes were purified with NucTrap Probe Purification Columns (Stratagene, La Jolla, CA, USA), denatured and hybridized to the membranes. After washes, the membranes were exposed to Phosphoimager screens (Molecular Dynamics Inc., Sunnyvale, CA, USA,) and the hybridization signals were detected and analyzed using the Phosphoimager scanner and ImageQuaNT software program (Molecular Dynamics). Background subtracted hybridization signals within the same hybridization were normalized using ubiquitin and 40S ribosomal protein as references. Genes showing greater than two-fold difference in signal intensity between separate hybridizations were classified as differentially expressed.

4.2 Custom-made cDNA microarrays (Study II)

In Study II, 960 cDNA clones from the subtracted PC-3 library were picked, cultured and PCR-amplified as described in Section 4.1. The PCR products were purified by ethanol precipitation and arrayed onto glass slides at the Finnish DNA Microarray Centre (Turku Centre for Biotechnology, Turku, Finland). For cDNA labeling, total RNA was extracted from the PC-3 (test sample) and the LNCaP (reference sample) cells using RNeasy Midi Kit (Qiagen). Cy3 and Cy5 labeled probes were synthesized from 50 µg of total RNA with 40 U of AMV reverse transcriptase (Finnzymes, Espoo, Finland) and 2 µg of Oligo dT Primer (Invitrogen). After alkaline hydrolysis of the RNA template, the probes were purified and concentrated using the Microcon YM-30

columns (Millipore Corporation, Bedford, MA, USA). Hybridization was carried out at +65°C for 16 h. After washes, fluorescent intensities of Cy3 and Cy5 were measured using the ScanArray 4000 laser confocal scanner (GSI Lumonics, Billerica, MA, USA) and quantitated with the QuantArray software program (Packard Bioscience, Billerica, MA, USA). Local background was subtracted from the signal volume of each spot, and the mean value of the housekeeping genes ubiquitin, G3PDH and 40S ribosomal protein was used for normalization of the Cy3/Cy5 signals.

4.3 Commercial microarrays (Study III)

In Study III, Atlas Glass Human 1.0 Microarray slides containing oligos representing 1081 genes were purchased from Clontech. Total RNA from cell lines was extracted using the TRIzol reagent (Invitrogen), and poly(A)$^+$ RNA from the total RNA using the Oligotex resin (Qiagen). Normal prostate poly(A)$^+$ RNA from Clontech was used as the reference sample. Cy3 and Cy5 labeled probes were synthesized from 2 µg of poly(A)$^+$ RNA using the Micromax Direct cDNA Microarray system (NEN Life Science Products Inc., Boston, MA, USA) except for the cDNA synthesis primers, which were replaced by the CDS Primer Mix from Clontech. The synthesized probes were purified with Microcon YM-30 columns (Millipore Corporation) and added to the GlassHyb Hybridization Solution (Clontech). After hybridization and washes, Cy3 and Cy5 signals were measured and quantitated. Local background was subtracted from the signal volume of each spot, and the mean signal value of eight housekeeping genes was used for the normalization of the Cy3/ Cy5 signals.

5. Real-time quantitative RT-PCR (Studies II-V)

The tumor samples used in the real-time quantitative RT-PCR were histologically examined using hematoxylin-eosin stained sections to ensure the presence of more than 60% of carcinoma or hyperplasic tissue in the samples. Subsequently, small amounts of frozen tissue were scraped for total RNA extraction with the RNeasy Mini Kit (Qiagen). From the cell lines, total RNA was isolated with either the TRIzol reagent or with the

RNeasy Midi Kit. RNA samples from normal human tissues were purchased from Clontech.

The total RNA samples were first reverse transcribed with Oligo dT Primer (Invitrogen) and SuperScript II RNase H^- Reverse Transcriptase (Invitrogen). Subsequently, the cDNA was used as the template in the real-time quantitative PCR, using the LightCycler apparatus (Roche Applied Science, Basel, Switzerland) with either SYBR Green I or hybridization probe detection method (FastStart DNA Master SYBR Green I or FastStart DNA Master Hybridization Probes, Roche Applied Science). Thermal cycling was performed as previously described (Helenius et al., 2001; Linja et al., 2001). For a standard curve, cDNA from normal human tissue (prostate or liver) or from prostate cancer cell lines was used to make serial dilutions. PCR primers were designed to anneal to separate exons in order to avoid amplification from any contaminating genomic DNA. To ensure the specificity of the PCR product, a melting curve analysis was performed after the cycling in the SYBR Green application, and all the PCR products were also analyzed in an agarose gel. For normalization, expression of TATA-box binding protein (TBP) was measured from each sample. The normalized expression values were calculated by dividing the expression value of the gene of interest by the expression value of TBP.

6. FISH (Studies II, III and V)

For a gene copy number analysis by FISH, human genomic BAC clones containing the genes of interest were obtained from ResGen™ Invitrogen Corporation (Huntsville, AL, USA). The BAC probes were labeled with digoxigenin-dUTP (Roche Applied Science, Basel, Switzerland) using nick translation. FITC-labeled, chromosome 8 centromeric probe (pJM128) was used as a reference in Studies II and III, and SpectrumOrange-labeled, chromosome 2 centromeric probe (Vysis Inc., Downers Grove, IL, USA) in Study V. Metaphase chromosome preparations from normal blood lymphocytes and from cell lines were prepared using routine techniques. Tissue sections from freshly frozen xenograft and carcinoma samples, as well as from paraffin-embedded tumor samples were pretreated as previously described (Helenius et al., 2001; Saramäki et al., 2001). The dual-color FISH was performed for all the samples according to the

previously published guidelines (Hyytinen et al., 1994). After washes, the samples were stained with either anti-digoxigenin-FITC or anti-digoxigenin-Rhodamine (Roche Applied Science) and counterstained with 0.1 M 4,6-diamino 2-phenylindole in Vectashield antifading solution (Vector Laboratories Inc., Burlingame, CA, USA). The fluorescent signals were scored from non-overlapping epithelial cells using Olympus BX50 epifluorescence microscope (Tokyo, Japan). According to the gene copy numbers, the tumors were classified into three different categories: normal (two copies of the gene and the centromere signals per cell), low-level gain (three or four copies of the gene per cell) and high-level amplification (five or more copies of the gene per cell or signal clusters).

7. Northern hybridization (Studies I, II, IV and V)

Twenty micrograms of total RNA was electrophoresed in an agarose-formaldehyde gel, blotted and hybridized using standard protocols (Sambrook and Russell, 2001). In addition, commercial MTN blots containing poly $(A)^+$ RNA from normal human tissues were purchased from Clontech and hybridized according to the manufacturer's protocol. The cDNA fragments used as probes in the hybridizations were labeled with ^{32}P-dCTP using the Rediprime II labeling system (Amersham Biosciences, Piscataway, NJ, USA). The hybridization signals were detected using the Storm PhosphorImager (Molecular Dynamics).

8. Southern hybridization (Study IV)

Thirty micrograms of genomic DNA from cell lines and from normal human blood lymphocytes was digested with *XbaI* and separated in agarose gel electrophoresis. Blotting and hybridization were performed using standard protocols (Sambrook and Russell, 2001). A cDNA probe containing the full-length mRNA sequence of the six-transmembrane epithelial antigen of the prostate 2 (STEAP2) was labeled using the Rediprime II labeling kit (Amersham Biosciences). The hybridization signals were detected using Storm PhosphorImager (Molecular Dynamics).

9. Cloning of the full-length STEAP2 cDNA (Study IV)

The first 600 bp of the STEAP2 cDNA sequence were revealed from the clone from the subtracted BPH library. Next, a contig of matching sequences was assembled using EST sequences retrieved from public databases. In addition, the genomic sequence of STEAP2 was utilized to make exon predictions using the Grail and Genscan programs. The exons were then cloned by RT-PCR (Titan One Tube RT-PCR System, Roche Applied Science) from normal human prostate poly $(A)^+$ RNA (Clontech) using primers designed for the predicted exons. The final 5' and 3' untranslated sequences were cloned from normal human prostate poly $(A)^+$ RNA (Clontech) using the SMART RACE protocol (Clontech). A sequence similarity search was carried out using the BLAST program with blastn and blastx algorithms. Predictions for secondary structures and subcellular localization were made using the PSORT II and the SOSUI programs.

10. Sequencing (Studies I, II, IV and V)

Sequencing was performed using the ABI PRISM ®BigDye™ Terminator Cycle Sequencing Ready Reaction Kit with the ABI 310 and ABI3100 sequencers (PerkinElmer Life Science Inc., Boston, MA, USA).

11. Androgen stimulation of the LNCaP cells (Study IV)

The LNCaP cells were cultured in phenol-red free RPMI medium with 10% charcoal stripped serum for 24 h before androgen stimulation. The medium was then replaced with fresh medium containing dihydrotestosterone (DHT) (Sigma-Aldrich, St. Louis, MO, USA) at final concentrations of 0 M; 10^{-10} M; 10^{-9} M; 10^{-8} M and 10^{-7} M. The cells were cultured in the presence of DHT for 24 h and harvested. Total RNA was extracted from the stimulated cells using the RNeasy Mini Kit (Qiagen).

12. Methylation analysis (Study IV)

The DU145 and the PC-3 cells showing very low expression of STEAP2 were cultured to 50% confluence, after which a demethylating agent, 5-aza-2'-deoxycytidine (Sigma-Aldrich) was added to the medium at final concentrations of 0 µM; 0.5 µM and 0.75 µM. Fresh medium with the demethylating agent was replaced after every 48 h, and after five days the cells were harvested. Total RNA was extracted from the cells with TRIzol reagent (Invitrogen). STEAP2 expression in the treated cells was studied using both Northern hybridization and the real-time quantitative RT-PCR as described above.

13. Subcellular localization of STEAP2 with GFP fusion proteins (Study IV)

The coding region of STEAP2 was subcloned into pEGFP-N3 and pEGFP-C1 expression vectors (Clontech) to produce constructs for N-terminal and C-terminal GFP fusion proteins respectively. The DU145 and the LNCaP cells were cultured to 80% confluence and transfected with 6-8 µg of the purified STEAP2-pEGFP plasmids using the lipofectamine method (Lipofectamine PLUS reagent, Invitrogen). After transfection the cells were cultured for two days and then fixed with 4% paraformaldehyde/ 5% saccharose/ 1 x PBS for 30 min, counterstained with 0.1 M 4,6-diamino 2-phenylindole in Vectashield antifading solution (Vector Laboratories Inc.) and visualized using a confocal laser scanning microscope (UltraView, PerkinElmer Life Science Inc.)

14. mRNA in situ hybridization (Study IV)

A 600-bp fragment of STEAP2 cDNA was subcloned into pBluescript vector, which was then linearized to produce ^{33}P-rUTP labeled antisense and sense riboprobes using *in vitro* transcription method (Riboprobe® *in vitro* Transcription Systems, Promega). Hybridization to formalin-fixed, paraffin-embedded prostate tissue sections was performed as previously described (Nupponen et al., 1999). The sections were counterstained with hematoxylin and visualized using a Nikon Microphot-SA

fluorescence microscope with an epipolarization filter enabling simultaneous imaging of tissue morphology and hybridization signal.

15. Mutational analyses (Studies IV and V)

Genomic DNA was isolated from prostate cancer cell lines and tumor tissues using proteinase K/ phenol extraction method (Sambrook and Russell, 2001). In Study IV, the four coding exons and the exon-intron boundaries of the STEAP2 gene were PCR-amplified and directly sequenced from the cell line and the xenograft samples. In Study V, the four coding exons as well as exon-intron boundaries of the pHyde gene were PCR amplified from the cell line, xenograft and clinical tumor material as previously described (Xu et al., 2001). Mutational analyses of the cell line and the xenograft samples were performed using direct sequencing. For the clinical tumor material, heteroduplex analysis was performed according to the previously published guidelines (Xu et al., 2001) using an automated denaturing high-performance liquid chromatography (DHPLC) instrument equipped with an analytical 2.1 x 75 mm Eclipse dsDNA experimental column (Agilent Technologies). All putative heteroduplex samples were reanalyzed by sequencing.

16. Statistical analyses (Studies II, IV and V)

The association of the gene expression levels with tumor type and histological grade was calculated using non-parametric tests: Kruskall-Wallis, with Dunn’s multiple comparison test (Studies II, IV and V), and Mann-Whitney U-test (Studies II and V). Pearson’s chi-square test was used to study the association of regions of chromosomal gains detected by CGH and localization of genes found to be overexpressed by cDNA microarrays (Study II).

RESULTS

1.Detection of differentially expressed genes by combining SSH and cDNA library array (Studies I and II)

In Studies I and II, two subtracted cDNA libraries were constructed: one with BPH as a tester and PC-3 as a driver (Study I), and the other with PC-3 as a tester and BPH as a driver (Study II). Both cDNA libraries were screened for differentially expressed genes using array hybridization: filter array hybridization in Study I and glass array hybridization in Study II.

1.1 Subtraction efficiency (Study I)

Prior to the construction of the subtracted BPH cDNA library, a subtraction efficiency test was performed according to the manufacturer's protocol (Clontech) to ensure that the subtraction had been successful. To study the degree of enrichment more closely, an unsubtracted cDNA library was prepared from the same BPH tissue sample with essentially the same protocol omitting the subtraction step. Subsequently, 432 clones were randomly picked from both cDNA libraries, arrayed onto nylon membranes and hybridized with a cDNA probe complementary to prostate specific antigen (PSA), a gene that was known to be expressed in BPH but not in PC-3. Of all the clones that according to PCR analysis contained inserts (412 clones in the unsubtracted and 386 clones in the subtracted cDNA library), one clone (0.24%) in the unsubtracted library and 15 clones (3.86%) in the subtracted library represented PSA, corresponding to 16-fold enrichment of PSA cDNA in the subtracted library.

1.2 Sensitivity and linearity of cDNA filter array hybridization (Study I)

To study the sensitivity and linearity of the cDNA filter array hybridization method, seven identical cDNA array membranes were prepared. The membranes were hybridized with ^{32}P-dCTP labeled probes synthesized from 500 ng of poly(A)$^{+}$ RNA

from the PSA negative DU145 cells, spiked with 0, 10, 25, 50, 100, 500 and 1000 pg of *in vitro* transcribed PSA poly(A)$^+$ RNA. The smallest amount of PSA poly(A)$^+$ RNA giving a hybridization signal that was reliably distinguished from the background was 50 pg, corresponding to 0.01% of the total amount of poly(A)$^+$ RNA used for the labeling. Linearity curve showed that the linear range of the hybridization signal was from 50 pg to 1000 pg. The maximum variation of the normalized signal intensities between the separate hybridizations measured from the non-PSA clones was two-fold.

1.3 Genes showing higher expression in BPH than in PC-3 (Study I)

Next, the subtracted BPH cDNA arrays were hybridized with ^{32}P-dCTP labeled probes synthesized from poly(A)$^+$ RNA from BPH as well as from the cell lines PC-3, DU145, LNCaP, PrEC, MFC10-A and MCF-7. Of the 386 clones that contained inserts according to the PCR analysis, 76 (20%) showed no hybridization signal with any of the probes. Altogether, 111/310 (=36%) clones showing hybridization signals were more highly expressed in BPH than in PC-3. Of these 111 clones, 54 were expressed only in BPH and in none of the cell lines studied, whereas 57 clones were also expressed in at least one of the cell lines. Seven clones (2%) out of the 386 clones showed higher hybridization signal in PC-3 than in BPH.

Of the clones showing higher hybridization signal in BPH than in PC-3, 42 clones were sequenced, revealing 21 different genes: 16 known genes, two anonymous ESTs and three mitochondrial sequences. Thus, the redundancy of the library was approximately two-fold. The expression of 17 of the clones (HLA class clones were excluded) was reanalyzed by Northern hybridization, confirming the differential expression of eight genes (myosin light polypeptide kinase, lumican, α-tropomyosin, PSA, phosphatidic acid phosphatase 2a, EST AI689722, glandular kallikrein 2 and IGFBP7). Twelve clones showing a hybridization signal only in BPH in the cDNA array hybridization were also sequenced. One of these clones represented a gene called hevin. Northern hybridization confirmed that hevin was moderately expressed in BPH, but not in any of the cell lines studied.

1.4 Genes showing overexpression in PC-3 (Study II)

The subtracted PC-3 cDNA library was arrayed onto glass slides and used for microarray hybridization with fluorescently labeled PC-3 (sample) and LNCaP (reference) probes. Clones showing a median signal ratio >3 were classified to be overexpressed in PC-3 and sequenced. The sequencing revealed 68 different genes (Table 4) with both known and unknown functions. When the chromosomal locations of these genes were retrieved from public databases and combined with data from previously published CGH studies (Nupponen et al., 1998a), it was found that the overexpressed genes clustered in regions that showed gains in PC-3. According to the CGH studies, chromosomal regions showing gain of the genetic material cover about 837 Mb (~27%) of the total of 3133 Mb (excluding chromosome Y) of the PC-3 genome. As 36 (54%) of the 67 overexpressed genes (the chromosomal location of one of the 68 genes was unknown) were localized in the gain regions, the association between overexpression and localization in the regions of gain was strong ($p<0.001$). The association was even stronger when the same analysis was performed with only regions showing high-level amplifications (CGH ratio >1.5). In PC-3, high-level amplifications of three regions, 8q13-qter, 10cen-q23 and 14q22-q24, have been detected in CGH studies covering about 174 Mb (~6%) of the genome. Of the 67 genes overexpressed in PC-3, 14 (21%) were localized in such regions.

2. Detection of differentially expressed genes using commercial microarray slides (Study III)

In Study III, commercial microarray slides containing 1081 oligonucleotide targets were utilized to study differential expression in prostate cancer cell lines PC-3, DU145 and LNCaP compared to normal prostate cells. The ten most highly overexpressed genes in each cell line were selected for further study. The selected 22 different genes could be classified into the following functional categories: cell cycle regulators (cell division cycle 2, cyclin-dependent kinase 5, cyclin B1 and cyclin 2A), ribosomal proteins (ribosomal protein S21 and ribosomal protein 3a), DNA polymerases and replication-associated proteins (topoisomerase II α and replication protein A3), proteases (urokinase type plasminogen activator and calpain 2), nuclear receptors/ transcription

factors (orphan hormone nuclear receptor and c-myc), apoptosis/ cell proliferation – associated proteins (cellular apoptosis susceptibility protein and apoptosis inhibitor 4) and miscellaneous (nucleoside diphosphate kinase A, 90-kd heat shock protein A, elongin C, caveolin 2, calmodulin, KIAA0175, prostate-specific membrane antigen and G-protein activated inward rectifier potassium channel 2).

The chromosomal locations of these genes were retrieved from public databases, and expression profiles were constructed by plotting the expression ratios of the genes against the physical maps of the chromosomes containing the overexpressed genes. Subsequently, the expression profiles were compared with previously published CGH profiles of the cell lines (Nupponen et al., 1998a). Prostate cancer cell lines show only few regions of high-level amplification: PC-3 contains a high-level amplification of 8q13-qter, 10cen-q23 and 14q21-q24, and DU145 contains a gain of 14q including amplification at 14cen-q21, whereas LNCaP does not show any amplification. By comparing the expression profiles with the CGH profiles, two genes showing consistent overexpression with amplification of the corresponding chromosomal region were detected. These genes were urokinase type plasminogen activator (uPA) at 10q22 and elongin C at 8q21. The expression ratio of uPA was 7.1 in PC-3 and 1.6 in DU145, whereas in LNCaP the expression was below the detection limit. The expression ratio of elongin C was 5.5 in PC-3, 1.0 in DU145 and 2.1 in LNCaP.

3. Overexpression and amplification of RAD21, KIAA0196 and elongin C in prostate cancer (Studies II and III)

By combining the SSH and the array hybridization methods, as well as by using the commercial microarray slides, a number of differentially expressed genes in prostate cancer was detected in Studies I, II and III. Further study of a subset of the differentially expressed genes led to the detection of overexpression and amplification of three genes, RAD21, KIAA1096 and elongin C, in clinical prostate carcinomas (Studies II and III).

3.1 RAD21 and KIAA0196 (Study II)

In Study II, 68 different genes were classified to be overexpressed in PC-3 according to the microarray results (ratio >3). To validate the results of the microarray hybridization, and also to study the expression of selected genes in three other prostate cancer cell lines (DU145, LNCaP and 22Rv1) as well as in nine prostate cancer xenografts (LuCaP 23.1, 23.8, 23.12, 35, 49, 58, 69, 70 and 73), real-time quantitative RT-PCR was used. Of the 68 genes, we selected 23 genes that were clustered in chromosomal regions that in CGH studies have shown gains in PC-3. These were genes at 8q (eight genes), 10p/q (six genes), 14q (four genes) and 17q (five genes). In addition, we selected six genes on the basis of high overexpression, indicated by a high ratio and a high clone frequency in the SSH library (more than three clones with the hybridization signal ratio >3). The real-time quantitative RT-PCR analysis confirmed the overexpression of all 29 genes in PC-3. When the expression data was compared with the previously published CGH profiles of the cell lines and the xenografts (Nupponen at al., 1998a; Laitinen at al., 2002), it was found that seven genes (KIAA0196, RAD21, CHRAC1, BM-009, EXT1, FBXO32 and PRG1) were overexpressed mainly in samples containing amplification at the chromosomal regions harboring the genes.

Subsequently the expression of these seven genes was studied in clinical prostate tumor material. According to the real-time quantitative RT-PCR analysis, expression of RAD21 was significantly higher in carcinoma samples than in BPH samples (p=0.0255, Kruskall-Wallis test). In addition, KIAA0196 showed significantly higher expression in hormone-refractory tumors than in BPH samples (p=0.0252, Mann-Whitney U-test). A tendency to increased expression in carcinoma samples compared to BPH samples was also detected with CHRAC1, although the differences were not statistically significant (p=0.0908, Kruskall-Wallis test). The rest of the genes did not show increased expression in carcinoma samples.

The copy numbers of RAD21, KIAA0196 and CHRAC1 genes were studied by FISH analysis. Since all three genes were located at the chromosomal region 8q24, the prostate cancer cell line PC-3 and a breast cancer cell line SK-Br-3, both containing amplification at 8q23-24, were first studied. As a control, the prostate cancer cell line LNCaP showing no alterations at 8q was used. In PC-3, RAD21, KIAA0196 and

CHRAC1 were all amplified with 6-12 copies of the gene and 2-4 copies of chromosome eight centromere (ratio ~3). In SK-Br-3, RAD21 and KIAA0196 showed high-level amplifications with 13-15 copies of RAD21 and 10-12 copies of KIAA0196, compared to one copy of the chromosome eight centromere. Only two copies of CHRAC1 were detected in SK-Br-3. In LNCaP cells, four copies of RAD21, KIAA0196 and CHRAC1 as well as of the chromosome eight centromere were detected. Since amplification of the region 8q23-24 is frequently detected in advanced prostate cancer, the copy numbers of RAD21, KIAA1096 and CHRAC1 were also studied in ten prostate cancer xenografts as well as in twelve hormone-refractory prostate tumors using FISH analysis. RAD21 was amplified in 7 (32%), KIAA0196 in 8 (36%) and CHARC1 in 4 (18%) of the samples studied. The samples showing high-level amplification of KIAA0196 also had a higher expression value in the real-time quantitative RT-PCR than samples with low-level gain or normal copy number (p=0.0051, Mann-Whitney U-test). RAD21 and CHRAC1 did not show any statistically significant association between the gene copy number and the expression.

Finally the results of the cDNA microarray hybridization and the real-time quantitative RT-PCR of RAD21 and KIAA0196 were verified by Northern hybridization. Of the prostate cancer cell lines, the highest expression of both RAD21 and KIAA0196 was detected in PC-3. The results confirmed the microarray and quantitative RT-PCR results.

3.2 Elongin C (Study III)

In Study III the results of the microarray hybridization were verified by measuring the expression of uPA, elongin C and TOP2A in the prostate cancer cell lines PC-3, DU145 and LNCaP using real-time quantitative RT-PCR analysis. Differences in gene expression between the cell lines were similar with both methods. The expression of TOP2A was highest in DU145 and the expression of uPA in PC-3. The expression of elongin C was over five times higher in PC-3 than in DU145 and in LNCaP. The expression of elongin C was also studied in seven breast cancer cell lines (T47D, MDA436, SK-Br-3, MDA415, ZR75-1, MCF-7 and EFM19) and in one cell line

originating from fibrocystic disease of the mammary gland (MCF10A). The highest expression of elongin C was detected in SK-Br-3.

According to the public databases, elongin C is located at chromosomal region 8q21. This was also confirmed by the FISH analysis using a gene-specific probe. In FISH analysis, elongin C showed amplification in PC-3 with 6-12 copies of the gene compared to 2-4 copies of the chromosome eight centromere (ratio ~3). High-level amplification was also detected in SK-Br-3, with about 12 copies of elongin C compared to only one chromosome eight centromere (ratio ~12). DU145 contained three and LNCaP four copies of both the gene and the centromere (ratio=1). In order to study the frequency of elongin C amplification in clinical prostate cancer, the copy number of elongin C was studied by FISH in 35 untreated prostate carcinomas and in 35 hormone-refractory tumors. In the untreated carcinomas, no amplifications of elongin C were detected, but 12 (34%) of the samples contained a low-level gain of the gene. Of the hormone-refractory tumors, eight (23%) showed high-level amplification and 19 (54%) low-level gain of elongin C.

4. Cloning and characterization of STEAP2 (Study IV)

In Study I, one of the clones that showed a hybridization signal in BPH but not in PC-3 was an anonymous EST. In Study IV the cloning and characterization of this EST (STEAP2) are described.

4.1 STEAP2 gene and protein

In BLAST search, the anonymous EST from the subtracted BPH cDNA library matched to a genomic sequence from chromosomal region 7q21.13 (GenBank accession no. AC002064) and to several EST sequences (e.g. AI023663, AI051210, R09227). Full-length cloning of the cDNA revealed four different splicing forms, three of which resulted from differential splicing of the last exon. According to the Northern hybridization with probes specific to the three different splicing forms of the last exon, the longest version turned out to be present in the major transcript, which was about 7.5

kb long. The fourth splicing form contained an extra 113-bp exon upstream of the first putative coding exon, introducing a new translation starting codon to the transcript. However, translation from this starting codon would have produced a polypeptide of only 14 amino acids in length, due to a stop codon in the following exon in this reading frame. Therefore it is not likely that this exon is present in the major transcript of the gene.

Southern hybridization did not reveal any major rearrangement of the gene in PC-3, DU145 and LNCaP cell lines. Nor were any nonsense or frameshift mutations of the gene found when the cell lines PC-3, DU145, LNCaP and 22Rv1 were sequenced. However, four sequence variations were detected by sequencing. One of them was a neutral change at codon 272 (CTC to CTT) with no amino acid change, but three of them were substitutions that changed the amino acid: Phe17Cys (TTT to TGT), Arg456Gln (CGA to CAA) and Met475Ile (ATG to ATT). All of these variants were also detected in normal DNA samples (n=10), indicating that they are normal polymorphisms, not mutations. The allele frequencies in normal DNA samples were 45% for T (Phe) and 55% for G (Cys) at codon 17, 45% for C and 55% for T (both coding for leucine) at codon 272, 40% for G (Arg) and 60% for A (Gln) at codon 456 and 10% for G (Met) and 90% for T (Ile) at codon 475.

The major transcript of the gene encodes for a putative polypeptide of 490 amino acids, with the predicted molecular mass of 56 kDa. According to the secondary structure predictions made by PSORT II and the SOSUI software programs, the polypeptide is a membrane protein with six putative membrane-spanning domains. The protein was predicted to be located either at the endoplasmic reticulum (probablility 39.1%) or at the plasma membrane (probability 34.8%). Sequence similarity search with the blastn algorithm did not reveal any significant similarities at the DNA level. However, search at the amino acid level with the blastx algorithm resulted in three highly homologous proteins, with 50% identity and 70% similarity on average. These homologs were a human six-transmembrane epithelial antigen of the prostate (STEAP) (Hubert et al., 1999), a rat tumor suppressor pHyde (Rinaldy and Steiner, 1999) and a TNF-α-induced adipose-related protein (TIARP) (Moldes et al., 2001) from mouse. Since the novel gene shared so many similarities with the STEAP gene, both at the amino acid level and at the expression level (described below), it was named STEAP2. Soon after the

completion of the cloning and characterization of the STEAP2 gene, Korkmaz et al. (2002) reported the cloning of the same gene under the name STAMP1 (six-transmembrane protein of the prostate 1). The cDNA sequence of STAMP1 (GenBank accession no. AY008445) is almost identical to the STEAP2 cDNA sequence, differing in only a few bases and containing a slightly different 5' untranslated region.

The subcellular localization of the STEAP2 protein was experimentally studied by transfecting the DU145 and the LNCaP cells with a fusion protein construct of green fluorescent protein (GFP) and STEAP2. The green fluorescent signal was seen in the plasma membrane, and also in vesicle-like structures all over the cytoplasm. No signal was detected in the nucleus.

4.2 Expression of STEAP2

In normal human tissues, STEAP2 was most highly expressed in prostate, according to both the Northern hybridization and the real-time quantitative RT-PCR analyses. The expression of STEAP2 was also detectable by Northern hybridization in ovary, and by the RT-PCR in all the tissues studied (skeletal muscle, mammary gland, testis, uterus, heart, kidney, lung, trachea, brain and liver), but at levels ten-fold lower than in the prostate. *In situ* hybridization with prostate tissue showed that epithelial cells and carcinoma cells were the major source of the STEAP2 expression in the prostate. Subsequently the real-time quantitative RT-PCR was used to study the expression of STEAP2 in clinical prostate tumor material. The expression of STEAP2 was three-fold higher in the carcinoma samples than in the BPH samples ($p=0.002$, Kruskall-Wallis test). No difference in the expression was detected between untreated and hormone-refractory carcinomas, and there was no association between the histological grade and the expression of STEAP2.

Of the prostate cancer cell lines (PC-3, DU145, LNCaP and 22Rv1), the LNCaP cells showed about 30-fold higher expression of STEAP2 than the others. Since LNCaP is the only androgen-sensitive prostate cancer cell line, whether the expression of STEAP2 is regulated by androgens was studied by culturing the LNCaP cells with the presence of various concentrations of dihydrotestosterone (DHT). No differences in the

expression of STEAP2 were detected after the stimulations, whereas the expression of the positive control gene, PSA, correlated with the concentration of DHT. To study if the expression of STEAP2 is silenced in the other cell lines by hypermethylation, the PC-3 and the DU145 cells were cultured in the presence of a demethylating agent, 5-aza-2'-deoxycytidine. The treatment did not restore the expression of STEAP2, whereas the expression of the positive control gene, estrogen receptor alpha, was increased in DU145.

5. pHyde (Study V)

One of the closest homologs of the STEAP2 protein that was cloned and characterized in Study IV is a rat tumor suppressor pHyde (Rinaldy and Steiner, 1999). In Study V, mutation and copy number analyses of the human pHyde gene were performed in order to find out whether pHyde is a classical tumor suppressor gene in prostate cancer. In addition, expression of pHyde was studied in normal human tissues as well as in prostate carcinoma samples.

5.1 Mutation and copy number analyses

The sequence of the human pHyde gene was retrieved from the public databases by BLAST search. Both the cDNA (GenBank Acc.no. AK00196) and the genomic sequences (GenBank Acc.no. AC016673 and AC016736) were found, enabling us to construct the exon-intron organization of the human pHyde gene. Four human prostate cancer cell lines (PC-3, DU145, LNCaP and 22Rv1) as well as eight prostate cancer xenografts (LuCaP 23.1, 35, 41, 49, 58, 69, 70 and 73) were screened for pHyde mutations by sequencing the four coding exons and the exon-intron boundaries. No sequence alterations were detected in the cell lines. In xenografts, two missense mutations were found: a homozygous alteration, Ile305Thr (ATC to ACC), in LuCaP 69, and a heterozygous alteration, Ala184Thr (GCC to ACC), in LuCaP 58. These two alterations were not detected in DNA samples from healthy blood donors (n=50), indicating that they are real mutations, not polymorphisms. In addition, three silent substitutions were detected in the xenografts: GGC to GGT at codon 32 in LuCaP 73

and CTG to CTA at codon 379 as well as TTC to TTT at codon 391, both in LuCaP 58. Fifty-six clinical prostate carcinoma samples were also screened for pHyde mutations using a DHPLC analysis. No truncating or missense mutations were detected, but the same silent substitution that was found in the xenograft LuCaP 58 (TTC to TTT at codon 391) was also detected in four clinical tumor samples. The same alteration was found in normal DNA samples as well, indicating that it is a polymorphic variant of the gene.

The cell lines and xenografts were also analyzed by FISH for copy number changes. The cell lines PC-3, DU145 and LNCaP contained equal numbers of the pHyde locus and the chromosome two centromere, but the cell line 22Rv1 had only one copy of pHyde compared with two centromeric signals. A deletion at the pHyde locus was also detected in two xenografts, LuCaP 69 and 70. Of the grade III untreated primary prostate carcinoma samples (n=5), one contained deletion of the pHyde gene, with two copies of pHyde and four copies of the chromosome two centromere.

5.2 Expression analysis

In normal human tissues, expression of pHyde was detected in colon, small intestine, ovary, prostate, placenta, liver and heart by Northern hybridization. With the real-time quantitative RT-PCR, the expression was detected in skeletal muscle, mammary gland, lung, trachea and kidney. The expression was weak but also detectable in testis, uterus and brain tissues. According to the quantitative RT-PCR measurements, the expression of pHyde was highest in the prostate.

Northern hybridization and quantitative RT-PCR analyses were utilized to study the expression of pHyde in prostate cancer cell lines, xenografts and in clinical prostate tumors. In the cell lines, pHyde was expressed at levels about two times higher in PC-3 and DU145 than in LNCaP and 22Rv1. All the xenografts (LuCaP 23.1, 35, 41, 49, 58, 69, 70 and 73) expressed pHyde, showing no clear differences between the samples. No statistically significant differences ($p=0.602$) were found in the expression of pHyde in BPH, untreated prostate carcinomas and hormone-refractory tumors. However, there was an association between the expression of pHyde and histological grade in the

untreated carcinoma samples, with poorly differentiatied (grade III) tumors showing lower expression than well and moderately differentiated (grades I and II) tumors ($p=0.007$).

DISCUSSION

1. Detection of differentially expressed genes by combining SSH and cDNA library array

1.1 Combination of SSH and array hybridization (Studies I and II)

In Studies I and II, the SSH method was used to enrich genes that are differentially expressed in prostate cancer. However, as the subtraction can never be complete, another method, such as Northern analysis or RT-PCR, is always needed to confirm the differential expression of the genes. Only a limited number of genes can be analyzed in one Northern hybridization, and designing and testing primers for hundreds of different genes for RT-PCR is also both laborious and expensive. cDNA array hybridization is therefore an optimal method for secondary screening of the subtracted cDNA population, as expression of a large number of genes can be simultaneously studied in one array hybridization.

Nowadays, there are also a wide variety of commercial cDNA arrays available for studying differential expression. However, the commercial arrays mainly contain known genes, thus representing only a fraction of the human genes expressed. As expression analysis by cDNA microarray is restricted to what has been printed on the slide or filter, it is also not applicable for detection of novel genes, unless custom-made arrays are used. For these reasons, subtracted cDNA libraries were constructed and screened for truly differentially expressed genes using the array techniques in Studies I and II. Since the cDNA was subtracted before the library construction, differentially expressed genes were enriched, and at the same time, the number of common housekeeping genes was decreased. This reduced the number of clones to be picked for a representative collection of potentially interesting genes. On the other hand, using the array techniques, it was feasible to considerably increase the number of clones screened for differential expression.

One of the disadvantages of using the subtracted cDNA libraries for the detection of differentially expressed genes was that transcripts from only one sample (BPH tissue sample or PC-3 cell line) were represented in each of the two libraries. Using pooled RNA preparations from several tissue samples may have made the subtracted cDNA libraries more representative. However, as prostate carcinoma tumors are very heterogeneous, pooling could also have resulted in dilution of expression differences present in one individual sample but not in the others, thus reducing the possibility of capturing all differentially expressed genes.

Combining the SSH and the cDNA array hybridization methods led to the identification of two genes (RAD21 and KIAA0196) that were found to be overexpressed and amplified in prostate cancer, and also to the cloning and characterization of one novel gene (STEAP2). Although the strategy includes steps that, without automatization, require a lot of manual effort (e.g. picking of the clones, PCR amplification and purification of the PCR products, sequencing of the clones etc.), it is still a powerful tool for the rapid identification of putative new target genes of the chromosomal alterations in prostate cancer, especially when applied together with data on chromosomal alterations

1.2 Validation of the SSH and the cDNA array hybridization methods: enrichment, sensitivity and linearity (Study I)

To study the degree of enrichment after subtraction, the number of clones representing PSA was studied in unsubtracted and in subtracted cDNA libraries. It was found that the number of PSA clones was 16-fold in the subtracted cDNA library compared to the unsubtracted library. Further enrichment of differentially expressed genes could have been achieved by performing additional rounds of subtractive hybridization or by adding more cycles to the suppression PCR. However, a higher degree of enrichment is also accompanied by reduced complexity, thus decreasing the likelihood of detecting all differentially expressed genes. As secondary screening of the subtracted cDNA library was included in the process of detecting truly differentially expressed genes, maximal enrichment was not so crucial. Instead, it was more important to keep the complexity of

the cDNA library as high as possible, so that more differentially expressed genes could be detected.

The detection limit in cDNA array hybridization was found to be 50 pg, corresponding to 0.01% of the 500 ng of poly(A)$^+$ RNA used for the labeling. The transcripts that are present in a cell at a time can be divided into three classes on the basis of their abundance: the rare, the intermediate and the abundant class. It has been estimated that if there are 15 000 different genes expressed in a cell at a time, about 14 200 of them belong to the rare class, about 840 to the intermediate class and about 10 to the abundant class (Hastie and Bishop, 1976). Together, the rare class transcripts have been estimated to comprise about 37% of the total amount of transcripts in a cell (Hastie and Bishop, 1976). From this, it can be calculated that each of the rare class mRNA species would thus represent about 0.003% of the total amount of transcripts in a cell (37% divided by 14 200). Correspondingly, the mRNA species belonging to the intermediate class were estimated to comprise about 41% of all the transcripts in a cell (Hastie and Bishop, 1976), each individual mRNA species thus representing about 0.05% of the total amount of transcripts (41% divided by 840). The detection limit of 0.01% observed in the array hybridization in Study I would thus be between the rare (0.003%) and the intermediate (0.05%) classes.

Since the vast majority of the mRNA species in a cell belongs to the rare abundance class, improving the sensitivity of the array hybridization would be extremely beneficial. Higher sensitivity could be achieved by increasing the starting material used for the probe labeling, or by prolonging the hybridization time. There is, however, a risk of thereby saturating the signals of the more abundant transcripts. According to the results of the linearity test, the linear range of the array hybridization signals was from 50 to 1000 pg, corresponding to 0.01%-0.2% of the poly(A)$^+$ RNA used for the labeling. This indicates that using this protocol it was possible to reliably study differential expression of transcripts including those of greater abundance.

1. 3 Differentially expressed genes

1.3.1 Genes showing higher expression in BPH than in PC-3 (Study I)

In Study I, genes expressed in BPH and not in PC-3 were detected. Fifty-four (49%) of all the differentially expressed clones were found to be expressed only in BPH and in none of the cancer cell lines studied. This was an expected result, as the subtracted cDNA library used in the study was constructed from BPH. In addition to epithelial cells, the BPH tissue also contains other types of cells, such as stromal cells and blood cells, whereas the cancer cell lines consist of cells of epithelial origin only. Indeed, sequencing of the differentially expressed clones revealed that many of them represented genes known to be expressed in stromal cells (e.g. decorin) (Pulkkinen et al., 1992). However, a subset of the clones was also likely to represent genes that are expressed in the epithelium of normal prostate but not in cancer cell lines. An example of such a gene is hevin, which in previous studies has been shown to be expressed in normal prostatic epithelial cells and to be downregulated in prostate cancer cell lines and in metastatic prostate carcinomas (Nelson et al., 1998).

Fifty-seven (51%) of all the differentially expressed clones were expressed, in addition to BPH, in at least some of the cell lines studied, indicating that they represented genes expressed in epithelial cells. Forty-two of them were sequenced and studied using Northern hybridization, which confirmed the differential expression of eight different genes (myosin light chain polypeptide kinase, lumican, α-tropomyosin, PSA, PAP2a, glandular kallikrein 2, IGFBP7 and an anonymous EST). Of these, PSA, PAP2a and glandular kallikrein 2 were expected findings, as they all are androgen-regulated genes, thus known to be downregulated in the AR-negative cell line PC-3. In addition to PC-3, downregulation of PAP2a has been detected at least in colon tumors (Leung et al., 1998).

IGFBP7 (also known as IGFBP-Pr1/mac25) belongs to the multigenic family of insulin-like growth factor binding proteins. IGFBP7 has been suggested to possess tumor suppressive potential in prostate cancer, as transfection of IGFBP7 has been shown to delay doubling time, decrease colony formation and increase apoptotic response in a

prostate cancer cell line (Sprenger et al., 1999). At the mRNA level, expression of IGFBP7 has been detected in both stromal and epithelial cells of normal prostate (Hwa et al., 1998), but at the protein level, the expression has not been detected in normal prostate tissue (Degeorges et al., 1999).

Alpha-tropomyosin (TPM1) is a cell-motility protein that binds to the actin filaments. Intrestingly, the 3' untranslated region of the α-tropomyosin gene has been reported to show tumor suppressor activity in myogenic cells (Rastinejad et al., 1993). TPM1 gene is located at 15q22, a region that shows deletion in PC-3, suggesting that at least one copy of the TPM1 gene may be missing in this cell line.

Lumican is a member of a leucine-rich proteoglycan family involved in cell migration. In breast, cervical and in colorectal tissues, it has been shown to be expressed in fibroblast-like cells adjacent to both normal and cancerous epithelium (Leygue et al., 1998; Leygue et al., 2000; Naito et al., 2002; Lu et al., 2002). Since lumican was identified here as a gene expressed in BPH, it is entirely possible that the main source of the lumican expression is the stroma.

Of the last two differentially expressed genes, the myosin light chain polypeptide kinase has not been reported to be implicated in cancer by other investigators. The gene represented by the anonymous EST (STEAP2) was cloned and characterized, and is discussed in Chapter 3.

1.3.2 Genes overexpressed in PC-3 (Study II)

In Study II, genes overexpressed in PC-3 were detected. The subtracted cDNA library constructed using PC-3 as a tester and BPH as a driver was screened using cDNA microarray hybridization. In the microarray hybridization, the prostate cancer cell line LNCaP was used as the reference to PC-3. PC-3 is an androgen-independent cell line, which contains chromosomal alterations typical for late stage prostate cancer (e.g. gains of 8q, 7p/q and 10q), whereas LNCaP is an androgen-sensitive cell line resembling the early stages of prostate cancer with respect to chromosomal alterations (for example, no gains or amplifications detected by CGH) (Nupponen et al., 1998a). With this strategy,

the aim was to maximize the likelihood of detecting genes that might be overexpressed due to a gene amplification, which is believed to be one mechanism for the activation of oncogenes in the late stages of prostate cancer.

All in all, 68 different genes overexpressed in PC-3 (ratio>3) were identified. When the chromosomal locations of these genes were retrieved and compared to the chromosomal alterations of PC-3, it was found that more than half of the genes (54%) were located at regions showing gains in PC-3 (Nupponen et al., 1998a). The percentage is high, even though it should be noted that not every single gene located at a region of gain is necessarily amplified. Interestingly, there were a few regions showing high clustering of the overexpressed genes, such as 8q24, 10p11 and 17q21, suggesting that a gain of a chromosomal region can lead to overexpression of several genes harboring the amplified locus.

Quantitative RT-PCR confirmed the overexpression of all the 29 genes selected for further study. In order to identify those genes that were likely also to be overexpressed in clinical prostate cancer and not only in the cell lines, prostate cancer xenograft samples were utilized in the RT-PCR analyses. Xenografts resemble clinical prostate tumors more closely than the cell lines, in terms of their behavior. In addition, they also contain chromosomal changes typical for clinical cancers, whereas of the cell lines only PC-3 shows such alterations (Laitinen et al., 2002; Nupponen et al., 1998a). Many of the genes showing high overexpression in PC-3, such as ITGB1, NRP1, DKK1, ANXA7, C14orf31 and IGFBP4, were found to be expressed only in the cell lines, and in none of the xenografts. These genes are likely to be those whose expression is altered because of the *in vitro* culturing of the cells. Some of the genes, on the other hand, such as COTL1 and ANXA2, seemed to be overexpressed in all samples, suggesting that the expression of these genes was probably diminished in LNCaP, the reference, rather than overexpressed in the other samples. By utilizing the xenografts along with the cell lines, the number of genes to be analyzed in the clinical sample material was reduced from twenty-nine to seven, thus making the analysis of the clinical material more feasible.

2. RAD21, KIAA0196 and elongin C as putative target genes for 8q gain

Gain of the long arm of chromosome eight is one of the most common chromosomal alterations in late-stage prostate carcinomas (Cher et al., 1996; Nupponen et al., 1998b), and it has been shown to be associated with aggressive phenotype of prostate cancer (Alers et al., 2000). Although the gain usually covers the entire 8q arm, two independently amplified subregions, 8q21 and 8q23-q24, have been detected (Cher et al., 1996; Nupponen et al., 1998b). In Studies II and III, three overexpressed and amplified genes located at these minimal regions of amplification were detected, namely RAD21 and KIAA0196 at 8q24 and elongin C at 8q21.

2.1 RAD21 and KIAA0196 (Study II)

Several putative target genes have been suggested for the commonly amplified region 8q23-q24, such as MYC, EIF3S3 and PSCA (Jenkins et al., 1997; Nupponen et al., 1999; Reiter et al., 1998). In Study II, clustering of the genes overexpressed in PC-3 was found to be particularly high at the region 8q24, from which six different genes were identified: FBOX32, RAD21, KIAA0196, BM-009, CHRAC1 and EXT1. Of these, FBOX32, BM-009 and EXT1 did not show elevated expression in hormone-refractory prostate carcinomas, indicating that they are not likely to be involved in prostate cancer progression. Instead, expression of RAD21 and KIAA0196 was significantly inreased in prostate carcinoma samples, and CHRAC1 also showed a tendency of increasing expression in carcinomas. The RAD21 and the KIAA0196 genes were also found to be amplified in 30-40% of hormone-refractory prostate carcinoma and xenograft samples, whereas the CHRAC1 gene was not as frequently amplified. The breast cancer cell line SK-Br-3 also showed high-level amplifications of both RAD21 and KIAA0196, but not of CHRAC1. High expression of KIAA0196 was found to be associated with amplification of the gene in hormone-refractory carcinomas. These results suggest that KIAA0196, and possibly also RAD21, should be considered as putative target genes for the 8q23-q24 amplification in prostate cancer.

The function of KIAA0196 is unknown, and it shows no homologies to known genes. Instead, RAD21 (also known as hr21/ Scc1/ Mcd1/ NXP1/ KIAA0078) is a human

ortholog of the *S.pombe* RAD21, and has been quite intensively studied in both yeast and humans. The RAD21 protein is a component of the cohesin complex that holds sister chromatids together during mitosis (Nasmyth et al., 2000; Hirano, 2000). At the onset of anaphase, proteolytic cleavage of the RAD21 protein triggers the separation of the sister chromatids into daughter cells. The presence of an uncleavable mutant of the RAD21 protein has been shown to be associated with aneuploidy in interphase and formation diplochromosomes in mitosis (Hauf et al., 2001). It has also been reported that a caspase-mediated cleavage of the RAD21 protein occurs during apoptosis, causing amplification of the proapoptotic death signal (Chen et al., 2002; Pati et al., 2002). All these functional activities can be linked to cancer progression, thus making RAD21 a promising candidate for a target gene for the 8q23-q24 amplification. Whether the possible overproduction of the RAD21 protein is involved in cell malignancy remains to be studied.

2.2 Elongin C (Study III)

No promising target genes have been identified for 8q21 amplification. In Study III, elongin C, located at 8q21, was detected to be overexpressed in PC-3 compared to DU145, LNCaP and to normal prostate by microarray hybridization as well as by quantitative RT-PCR. When the expression profiles obtained by microarray hybridization were compared with the previously published CGH profiles (Nupponen et al., 1998a), it was found that overexpression of elongin C was associated with gain of the chromosomal region harboring the gene. The elongin C gene was found to be amplified in the prostate cancer cell line PC-3 as well as in the breast cancer cell line SK-Br-3, both containing the gain at the 8q21 region. These cell lines also showed much higher expression of elongin C than the cell lines without amplification of the gene. In addition, about 20% of hormone-refractory prostate carcinomas were found to contain amplification of elongin C. No amplifications of elongin C were detected in primary, untreated prostate carcinomas, indicating that the amplification is associated with late stage of prostate cancer, as 8q gain in general. As the expression on elongin C was also elevated in hormone-refractory prostate carcinomas (p=0.0184, Kruskall-Wallis test) (Porkka et al., unpublished data), elongin C is a putative target gene for the 8q21 amplification.

Elongin C (TCEB1) is a subunit of the elongin complex (SIII) that activates transcription performed by RNA polymerase II. The elongin complex is composed of three subunits: A, B, and C. Elongin A is the transcriptionally active subunit, whereas elongins B and C are regulatory units that activate and stabilize the complex. RNA polymerase II has a tendency to pause at several positions after the transcription initiation site. Binding of elongin A to the subunits B and C reactivates the RNA polymerase, leading to high transcription level of the gene (Aso et al., 1995).

Binding to the elongin complex is not the only functional activity of elongin C; it has been demonstrated that the protein product of the von Hippel-Lindau gene (VHL) can also bind to the elongin C subunit (Duan et al., 1995; Kibel et al., 1995). Mutations in the VHL gene are associated with the inherited von Hippel-Lindau cancer syndrome and also with the majority of haemangioblastomas and renal carcinomas (reviewed by Kaelin, 2002). The tumorigenic mutations in the VHL gene have been reported to occur frequently in the domain responsible for elongin C binding (Stebbins et al., 1999). First, it was suggested that VHL negatively regulates transcription elongation by binding to elongin C, thus preventing the formation of the elongin complex. More recently, however, it has been found that VHL binds to a multiprotein complex including elongin B, elongin C, Cul2 and Rbx1 proteins (Pause et al., 1997; Lonergan et al., 1998; Kamura et al., 1999). This complex targets other proteins for ubiquitinylation-mediated degradation. It has been shown that hypoxia-induced proteins HIF1-α and HIF2- α are targets, and the current view is that mutations in the VHL gene lead to accumulation of HIF1-α and HIF2- α proteins. This in turn results in increased expression of their target-genes, such as the vascular endothelial growth factor (VEGF), platelet-derived growth factor B chain (PDGFβ), and transforming growth factor α (TGFα), thus promoting tumor growth (reviewed by Kaelin, 2002). The complex network of elongin C and VHL interactions is illustrated in Figure 2.

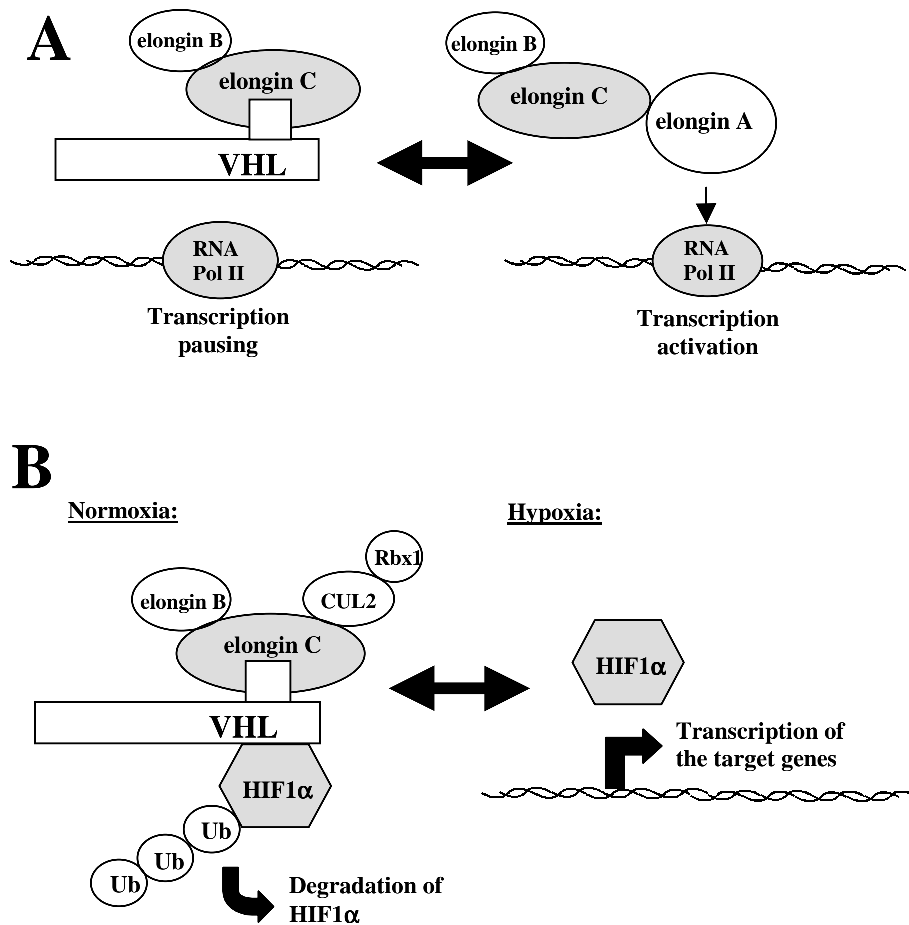

Figure 2. Interactions of the elongin C and the VHL proteins. **A)** Binding of elongin A to elongins B and C reactivates RNA polymerase II after pausing. Binding of elongin C to VHL prevents the formation of the elongin complex, and RNA polymerase stops at the pausing site. **B)** Binding of elongins C and B, CUL2 and Rbx1 proteins to the VHL protein directs HIF1α to the ubiquitin-mediated degradation in normoxia. In the absence of the multiprotein complex during hypoxia, free HIF1α induces transcription of its target genes.

The role of elongin C in the interaction with VHL seems to be protective against tumorigenesis. Overexpression of elongin C could in that case promote tumor growth only if the protein were inactivated by a mutation. No mutations were, however, detected in the cell lines PC-3 and SK-Br-3 showing amplification and overexpression of elongin C (Rauhala et al., unpublished data). One possible mechanism by which a cancer cell might achieve growth advantage by the elevated expression of elongin C is the overall inreased transcriptional activity that the formation of the elongin complex

may induce. However, since it has been shown that the elongin C protein is capable of forming complexes with several proteins, it is possible that is has also other, unidentified functions, some of which may better explain the amplification and overexpression of the elongin C gene in prostate cancer.

3. STEAP2 (Study IV)

In Study I, one of the differentially expressed genes detected by combining the SSH and the array hybridization methods was an anonymous EST. In Study IV, the full-length cDNA of this novel gene, named STEAP2 (six-transmembrane epithelial antigen of the prostate 2), was cloned and characterized. Cloning and characterization of this same gene was independently also reported by Korkmaz et al. (2002) under the name STAMP1 (six-transmembrane protein 1). Korkmaz et al. presented very similar findings, the only exception being the size of the transcript. Korkmaz et al. reported that in Northern analysis, a major band of approximately 6.5 kb and minor bands of 2.2, 4.0 and 4.5 kb were detected, whereas in Study IV, only one major band of approximately 7.5 kb was seen. There may be several explanations for this. First, in Northern analysis, the ribosomal RNA subunits 18S and 28S often show some non-specific background at 2.2 and 4.5 kb positions of the lane, which is why it is difficult to interpret bands of these sizes. Secondly, the cDNA sequence cloned by Korkmaz et al. had a slightly different 5' UTR than that reported in Study IV, indicating that there may be several different forms of the UTR regions for STEAP2. In study IV, it was shown that also differential splicing of some of the exons occurs. Therefore, using different probes, different forms of the transcript can be seen. The major transcript detected in these two studies, however, is probably the same, the differences merely reflecting the difficulties in estimating the exact size of a transcript as long as 6.5-7.5 kb in Northern analysis. In all cases, the putative coding region reported in these two studies is essentially identical, with variations in only few bases.

STEAP2 was first identified as a gene expressed in normal prostate tissue and BPH as well as in the LNCaP cell line, but in none of the other prostate cancer cell lines. Since LNCaP is the only androgen-sensitive cell line, it was hypothesized that the expression of STEAP2 could be regulated by androgens. However, stimulation of the LNCaP cells

with DHT did not induce the expression of STEAP2. In addition, Korkmaz et al. did not detect any alterations in STEAP2 expression after castration in nude mice (Korkmaz et al., 2002). These results indicate that STEAP2 is not an androgen-regulated gene. No mutations, major rearrangements or hypermethylation of the STEAP2 gene were detected in the cell lines either, leaving the mechanism of the loss of expression of STEAP2 unsolved. It is known, however, that the *in vitro* culturing of the cells leads to the loss of the expression of many genes that are expressed in prostate tumors, the androgen receptor being a good example (Linja et al., 2001). Therefore, it is important also to analyse tumor samples in addition to the cell lines.

In clinical prostate tumor samples, expression of STEAP2 was significantly higher in carcinoma samples than in BPH samples. Korkmaz et al. also showed by mRNA *in situ* hybridization that STEAP2 expression was increased in prostate cancer cells (Korkmaz et al., 2002). The STEAP2 gene is located at 7q, which is one of the most frequently gained chromosomal arms in prostate cancer (Visakorpi et al., 1995b), and has been shown to be associated with aggressive phenotype (Alcaraz et al., 1994). Whether the high expression of STEAP2 is associated with the gain of 7q, remains to be studied. The STEAP2 gene was found to be polymorphic, with three single nucleotide polymorphisms (SNP) that also change the amino acid. Studies elucidating the possible associations of these SNPs with prostate cancer are ongoing.

No functional domains in the STEAP2 protein were recognized by any prediction program. Thus, predictions about the functional activites of STEAP2 can only be made based on the sequence similarity of the STEAP2 protein with other proteins. STEAP2 belongs to a family of six-transmembrane proteins together with the six-transmembrane epithelial antigen of the prostate (STEAP), (Hubert et al., 1999), a putative tumor suppressor pHyde (Rinaldy and Steiner, 1999), and the TNFα-induced adipose-related protein (TIARP) (Moldes et al., 2000). These proteins are about 50% identical and 70% similar to each other. Interestingly, all the human genes except for pHyde are located at 7q21, probably due to ancient duplications of the ancestor gene.

STEAP was originally cloned from a prostate cancer xenograft modeling metastatic prostate cancer (Hubert et al., 1999). The expression pattern of STEAP is very similar to that of STEAP2, as they both are highly prostate-specific and show higher expression in

LNCaP than in the other prostate cancer cell lines. Neither of them is induced by androgens, however (Hubert et al., 1999). Nevertheless, the expression patterns of STEAP and STEAP2 are not identical, so even though they are both located at 7q21 very close to each other, they are not likely to share a common regulatory region. The TIARP gene was cloned from mouse 3T3-L1 cells, and it was shown to be induced by TNFα, but also to be spontaneously expressed in differentiating mouse adipocyte cells (Moldes et al., 2001). Both the TIARP and the STEAP proteins were shown to be located in the plasma membrane, at cell-cell junctions (Moldes et al., 2001; Hubert et al., 1999). Since the six-transmembrane proteins often function as ion channels or water channels (Dolly and Parcej, 1996; Reizer et al., 1993), the authors suggested that TIARP and STEAP could be transporter proteins. According to the GFP fusion protein, STEAP2 was also localized mostly in the plasma membrane, suggesting a role in cell-to-cell trafficking for STEAP2.

The fourth member of this six-transmembrane protein family is a putative tumor suppressor pHyde, which was first cloned from a rat prostate cancer cell line (Rinaldy and Steiner, 1999). In functional studies, the rat pHyde has shown tumor suppressive activities (Steiner et al., 2000), and it has been demonstrated to promote apoptosis in prostate cancer cells (Rinaldy et al., 2000). STEAP2, in contrast, is not likely to possess a tumor suppressive function, as the expression of STEAP2 was significantly increased in prostate carcinoma samples. To fully clarify the function of STEAP2, further functional studied are now in progress. Whatever function STEAP2 may possess in the prostate, however, as a cell surface antigen it is a potential diagnostic or therapeutic target in prostate cancer.

4. pHyde (Study V)

One of the closest homologs of the novel gene, STEAP2, cloned in Study IV is the rat tumor suppressor pHyde (Rinaldy and Steiner, 1999). The rat pHyde has been shown to inhibit proliferation of human prostate cancer cell lines and to reduce tumor growth in nude mice (Steiner et al., 2000). In addition, it has been demonstrated that rat pHyde can induce apoptosis in prostate cancer cells through a caspase-3 dependent pathway (Rinaldy et al., 2000; Zhang et al., 2001). The functional activities of rat pHyde make it

a promising candidate for a novel prostate cancer tumor suppressor gene. However, since no data about the pHyde gene or its expression in human has previously been reported, the significance of pHyde as a tumor suppressor gene is unclear.

In Study V, 68 prostate carcinoma samples consisting of cell lines, xenografts and clinical prostate tumors were analyzed for mutations in the pHyde gene. Missense mutations were detected in two samples (3%), both of which were xenografts. No truncating mutations were found. Mutational analysis of clinical tumor samples by direct sequencing is often problematic due to a substantial number of contaminating normal cells that may mask the mutations in the cancer cells. Therefore, direct sequencing was applied here only for the analysis of the cell lines and the xenografts representing pure malignant cell populations (xenografts containing only a minute number of normal mouse cells). Mutational analysis of the clinical tumors was performed using the DHPLC method, which is sensitive enough to detect mutations that are present in only a small fraction of the sample (Xiao and Oefner, 2001). Since no mutations in the pHyde gene were detected in the clinical samples by this method, it can be concluded that they truly are rare.

The pHyde gene was originally identified as a gene whose expression was lost in a high-metastatic rat prostate cancer cell subline when compared to a low-metastatic subline (Rinaldy and Steiner, 1999). This might suggest that the possible inactivation of the pHyde gene could be a late event in prostate cancer progression, in which case mutations could only be detected in advanced tumor samples. However, the sample material used in Study V also contained metastatic and hormone-refractory tumors as well as cell lines and xenografts, most of which have been established from metastatic lesions of prostate cancer. Therefore, if mutation of pHyde is a late event in prostate cancer progression, more than just two mutations should have been detected in this analysis. Thus, it is concluded that mutation of pHyde is not a frequent event in prostate cancer progression.

To study copy number alterations of the pHyde gene, prostate cancer cell lines PC-3, DU145, LNCaP and 22Rv1 as well as eight prostate cancer xenografts (LuCaP 23.1, 35, 41, 49, 58, 69, 70 and 73) were analyzed by FISH. In addition, the poorly differentiated (grade III) prostate carcinoma samples (n=5) were also analyzed, because they showed

significantly lower expression of pHyde than the well and moderately differentiated (grades I-II) carcinomas. Deletions of the pHyde gene were detected in one cell line (22Rv1), two xenograft samples (LuCaP 69 and 70) and in one grade III carcinoma sample. All these samples have in previous CGH analyses been shown to sustain losses at the pHyde locus at 2q14 (Laitinen et al., 2002, Visakorpi et al., 1995b). One of these samples, the xenograft LuCaP 69, had been shown in this study to have mutation in the pHyde gene. Of all the samples studied here, this was the only one showing alterations in both alleles of the gene. According to the "two-hit" theory , both alleles of a classical tumor suppressor gene are inactivated in cancer, typically through a mutation of one allele and deletion of the other (reviewed by Knudson, 2001; Devilee et al., 2001). The mutation in the remaining allele of pHyde in LuCaP69 should therefore be inactivating to fulfill the criteria of a classical tumor suppressor gene. According to the secondary structure predictions, the mutation is not located in any of the six putative membrane-spanning domains of the pHyde protein. Since no other functional domains in the pHyde protein can be recognized using prediction programmes, the functional significance of the mutation remains unclear. However, since this was the only case in which both alleles of the pHyde gene were altered, the results do not support the idea that pHyde is a classical tumor suppressor gene in prostate cancer.

Increasing evidence suggests that mechanisms other than mutation and deletion, such as promoter hypermethylation, can also mediate inactivation of tumor suppressor genes. Hypermethylation has in some reports even been shown to be the second inactivating event in addition to loss of one allele (Esteller et al., 2000; Grady et al., 2000). Many of the tumor suppressor genes that are believed to be implicated in prostate cancer, such as GSTP1, NKX3.1, CDH1, KAI1 and CD44, have shown no biallelic inactivation in prostate carcinomas. Instead, decreased expression of these genes, either by hypermethylation or by an unknown mechanism, has been reported to be associated with tumorigenesis of prostate (Lee et al., 1994; Bowen et al., 2000; Umbas et al., 1992; Dong et al., 1996; Noordzij et al., 1999).

The methylation status of the pHyde gene was not analyzed in Study V. Instead, expression of the pHyde gene was studied in prostate cancer cell lines, xenografts and tumor samples as well as in normal human tissues. The pHyde gene showed ubiquitous expression in normal human tissues, suggesting that the biological function of pHyde is

not restricted to prostate. In the cell lines, the expression of pHyde was highest in the androgen-independent cell lines DU145 and PC-3, but in the xenografts no clear differences between androgen-independent and androgen-dependent xenografts were seen, indicating that pHyde expression is not related to androgen-dependence.

The lowest expression of pHyde was detected in the cell line 22Rv1 containing the deletion of the pHyde gene. In the xenograft LuCaP 70 that showed deletion of one copy and an intact remaining copy of the pHyde gene, the expression of pHyde was likewise very low. Of the untreated, primary prostate carcinomas studied, the poorly differentiated tumors (grade III, n=5) showed significantly lower expression of pHyde than the well or moderately differentiated tumors (grades I-II, n=25). However, only one of the grade III tumors exhibited deletion of the pHyde gene, indicating that deletion of one allele of the gene is not the main reason for decreased expression of pHyde. Whether this is an epigenetic event, such as hypermethylation, remains to be studied. There was no statistically significant difference in the expression of pHyde between the BPH, untreated primary carcinoma or hormone-refractory tumors. Therefore, it is concluded that expression of pHyde is not associated with prostate cancer progression.

The first functional studies demonstrating the tumor suppressive potential of pHyde were carried out using rat pHyde cDNA. Quite recently, Passer et al. (2003) reported the cloning of the human pHyde gene (called TSAP6 by the authors). Passer et al. showed that the pHyde promoter contains a functional p53-responsive element and that the expression of pHyde is induced by p53 (Passer et al., 2003). In addition, they demonstrated that the blocking of pHyde with an antisense-RNA impairs p53-mediated apoptosis, and overexpression of pHyde sensitizes cells to apoptosis (Passer et al., 2003). Even though the human pHyde gene seems to have growth inhibiting potential *in vitro*, the data obtained from the clinical material in Study V does not support the idea that pHyde is involved in the development of prostate cancer.

SUMMARY AND CONCLUSIONS

The present study was set up to identify genes that might be involved in tumorigenesis of the prostate.

Suppression subtractive hybridization and cDNA microarray hybridization were used in combination to detect genes that are expressed differentially in prostate cancer. The methods were first validated by evaluating the subtraction efficiency as well as the sensitivity and linearity of the array hybridization. In conclusion, the combination of SSH and microarray hybridization was found to be suitable for the detection of differentially expressed genes in prostate cancer.

By combining the SSH and the microarray hybridization methods, two overexpressed genes from the chromosomal region 8q24, RAD21 and KIAA0196, were identified. These genes showed increased expression in clinical prostate carcinomas, and were also found to be amplified in 30-40% of hormone-refractory tumors. The results suggest that KIAA0196 and RAD21 are putative target genes for the common amplification of the 8q23-q24 region in prostate cancer.

By utilizing commercial microarray slides, overexpression of elongin C gene was detected. The elongin C gene, located in chromosomal region 8q21, was found to be amplified in about 20% of hormone-refractory tumors. As the hormone-refractory tumors also showed elevated expression of elongin C, it can be considered a putative target gene for the amplification of region 8q21.

Using SSH and cDNA microarray methods, a differentially expressed, anonymous EST was detected. Full-length cloning of the EST revealed a novel gene, named STEAP2, that encodes for a 490 amino acid long polypeptide with six putative transmembrane domains. A green fluorescent protein fusion construct indicated that the STEAP2 protein is localized in the plasma membrane, as well as in vesicle-like structures in the cytoplasm. STEAP2 is predominantly expressed in prostate epithelial cells, and its expression is elevated in prostate cancer. Although the androgen-sensitive prostate cancer cell line LNCaP is the only cell line expressing STEAP2, the expression of STEAP2 is not regulated by androgens. The biological function of STEAP2 has not yet

been solved. However, as a cell surface antigen STEAP2 is a potential diagnostic or therapeutic target in prostate cancer.

One of the closest homologs of the STEAP2 protein is rat protein pHyde. Rat pHyde had been shown to have tumor suppressive potential in human prostate cancer cells. In order to study whether pHyde is a classical tumor suppressor gene, mutational as well as copy number analysis of the pHyde gene in prostate carcinoma samples was performed. Out of the 68 samples studied, only two (3%) showed mutations in the pHyde gene, indicating that mutations of pHyde are rare in prostate cancer. Only one of the samples contained both mutation and deletion of the pHyde gene (“two hits”), suggesting that pHyde is not a classical tumor suppressor in prostate cancer. In addition, no differences in the expression of pHyde in benign and malign prostate tissues were detected, indicating that pHyde is not likely to be involved in the development of prostate cancer.

ACKNOWLEDGEMENTS

This study was carried out in the Laboratory of Cancer Genetics, Institute of Medical Technology, University of Tampere and Tampere University Hospital, during the years 1998-2003.

I want to express my deepest gratitude to my supervisor, Professor Tapio Visakorpi, M.D., Ph.D., for admitting me to his research group and for giving me the most fascinating and challenging project to work on. His wide expertise in the fields of both molecular biology and medicine has been invaluable in the completion of this thesis. As a supervisor he has been patient and supportive, never too busy to help and always so well organized. It has been a great pleasure to work under his supervision.

I express sincere thanks to Professor Olli Silvennoinen, M.D., Ph.D., the Head of the Institute of Medical Tchnology, University of Tampere, for providing the research facilitites for this study. I also want to express my gratitude to Professor Howy Jacobs, Ph.D., and to Professor Kalle Saksela, M.D., Ph.D., the former and the present Heads of Tampere Graduate School in Biomedicine and Biotechnology, for providing me the graduate school position to carry out this work. Howy is acknowledged also for organizing such high-quality and stimulating courses for the graduate school students.

I am very grateful to Docent Anne Kallioniemi, M.D. Ph.D., University of Tampere, and Docent Jarmo Wahlfors, Ph.D., University of Kuopio, for their valuable comments on this thesis.

I wish to thank Ms. Virginia Mattila, M.A., for carefully reviewing the language of this manuscript.

I want to thank all my co-authors, Professor Teuvo Tammela, M.D., Ph.D., Professor Robert L. Vessella, Ph.D., Docent Minna Tanner, M.D., Ph.D., Docent Nina Nupponen, Ph.D., Merja Helenius, PhD., and Outi Saramäki, M.Sc, for their important contribution to this work.

Thanks are also due to each and everyone in "Tapsalab" for all the good times shared both at work and leisure. I am also grateful to all the people in the old "SGY", where I first started my work, for being so helpful and friendly to a newcomer.

My special thanks go to my dearest friends, Marika Linja, M.Sc., and Päivikki Kauraniemi, M.Sc., for sharing both the good and the bad days of research and life.

My warmest thanks to my parents, Tuula and Pekka, for their love and support, or "Love and Substral", as we used to call it!

Finally, with all my heart I want to thank my husband, Jukka, for his love, patience and understanding during these years. His unfailingly positive attitude towards life has helped me through the hard times over and over again. Thank you!

This study was financially supported by the Finnish Cancer Society, the Pirkanmaa Cancer Society, the Pirkanmaa Regional Fund of the Finnish Cultural Foundation and the Scientific Foundation of the city of Tampere.

Tampere, September 2003

Kati Porkka

REFERENCES

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

Agus DB, Scher HI, Higgins B, Fox WD, Heller G, Fazzari M, Cordon-Cardo C and Golde DW (1999): Response of prostate cancer to anti-Her-2/neu antibody in androgen-dependent and -independent human xenograft models. Cancer Res 59:4761-4764.

Alcaraz A, Takahashi S, Brown JA, Herath JF, Bergstralh EJ, Larson-Keller JJ, Lieber MM and Jenkins RB (1994): Aneuploidy and aneusomy of chromosome 7 detected by fluorescence in situ hybridization are markers of poor prognosis in prostate cancer. Cancer Res 54:3998-4002.

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

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

Alizadeh AA, Eisen MB, Davis RE, Ma C, Lossos IS, Rosenwald A, Boldrick JC, Sabet H, Tran T, Yu X, Powell JI, Yang L, Marti GE, Moore T, Hudson J Jr, Lu L, Lewis DB, Tibshirani R, Sherlock G, Chan WC, Greiner TC, Weisenburger DD, Armitage JO, Warnke R, Staudt LM, et al. (2000): Distinct types of diffuse large B-cell lymphoma identified by gene expression profiling. Nature 403:503-511.

An G, Ng AY, Meka CS, Luo G, Bright SP, Cazares L, Wright GL Jr and Veltri RW (2000): Cloning and characterization of UROC28, a novel gene overexpressed in prostate, breast, and bladder cancers. Cancer Res 60:7014-7020.

Arnold JT and Isaacs JT (2002): Mechanisms involved in the progression of androgen-independent prostate cancers: it is not only the cancer cell's fault. Endocr Relat Cancer 9:61-73.

Aso T, Lane WS, Conaway JW and Conaway RC (1995): Elongin (SIII): a multisubunit regulator of elongation by RNA polymerase II. Science 269:1439-1443.

Balaji KC, Koul H, Mitra S, Maramag C, Reddy P, Menon M, Malhotra RK and Laxmanan S (1997): Antiproliferative effects of c-myc antisense oligonucleotide in prostate cancer cells: a novel therapy in prostate cancer. Urology 50:1007-1015.

Balk SP (2002): Androgen receptor as a target in androgen-independent prostate cancer. Urology 60:132-138.

Bandyk MG, Zhao L, Troncoso P, Pisters LL, Palmer JL, von Eschenbach AC, Chung LW and Liang JC (1994): Trisomy 7: a potential cytogenetic marker of human prostate cancer progression. Genes Chromosomes Cancer 9:19-27.

Bhatia-Gaur R, Donjacour AA, Sciavolino PJ, Kim M, Desai N, Young P, Norton CR, Gridley T, Cardiff RD, Cunha GR, Abate-Shen C and Shen MM (1999): Roles for Nkx3.1 in prostate development and cancer. Genes Dev 13:966-977.

Bieche I, Laurendeau I, Tozlu S, Olivi M, Vidaud D, Lidereau R and Vidaud M (1999): Quantitation of MYC gene expression in sporadic breast tumors with a real-time reverse transcription-PCR assay. Cancer Res 59:2759-2765.

Bookstein R, MacGrogan D, Hilsenbeck SG, Sharkey F and Allred DC (1993): p53 is mutated in a subset of advanced-stage prostate cancers. Cancer Res 53:3369-3373.

Boon K, Osorio EC, Greenhut SF, Schaefer CF, Shoemaker J, Polyak K, Morin PJ, Buetow KH, Strausberg RL, De Souza SJ and Riggins GJ (2002): An anatomy of normal and malignant gene expression. Proc Natl Acad Sci U S A 99:11287-11292.

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

Brooks JD, Bova GS, Ewing CM, Piantadosi S, Carter BS, Robinson JC, Epstein JI and Isaacs WB (1996): An uncertain role for p53 gene alterations in human prostate cancers. Cancer Res 56:3814-3122.

Brooks JD, Weinstein M, Lin X, Sun Y, Pin SS, Bova GS, Epstein JI, Isaacs WB and Nelson WG (1998): CG island methylation changes near the GSTP1 gene in prostatic intraepithelial neoplasia. Cancer Epidemiol Biomarkers Prev 7:531-536.

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

Bubendorf L, Sauter G, Moch H, Jordan P, Blochlinger A, Gasser TC and Mihatsch MJ (1996): Prognostic significance of Bcl-2 in clinically localized prostate cancer. Am J Pathol 148:1557-1565.

Bussemakers MJ, van Bokhoven A, Verhaegh GW, Smit FP, Karthaus HF, Schalken JA, Debruyne FM, Ru N and Isaacs WB (1999): DD3: a new prostate-specific gene, highly overexpressed in prostate cancer. Cancer Res 59:5975-5979.

Cairns P, Okami K, Halachmi S, Halachmi N, Esteller M, Herman JG, Jen J, Isaacs WB, Bova GS and Sidransky D (1997): Frequent inactivation of PTEN/MMAC1 in primary prostate cancer. Cancer Res 57:4997-5000.

Cairns P, Esteller M, Herman JG, Schoenberg M, Jeronimo C, Sanchez-Cespedes M, Chow NH, Grasso M, Wu L, Westra WB and Sidransky D (2001): Molecular detection of prostate cancer in urine by GSTP1 hypermethylation. Clin Cancer Res 7:2727-2730.

Chang GT, Blok LJ, Steenbeek M, Veldscholte J, van Weerden WM, van Steenbrugge GJ and Brinkmann AO (1997): Differentially expressed genes in androgen-dependent and -independent prostate carcinomas. Cancer Res 57:4075-4081.

Chen C, Hyytinen ER, Sun X, Helin HJ, Koivisto PA, Frierson HF Jr, Vessella RL and Dong JT (2003a): Deletion, Mutation, and Loss of Expression of KLF6 in Human Prostate Cancer. Am J Pathol 162:1349-1354.

Chen F, Kamradt M, Mulcahy M, Byun Y, Xu H, McKay MJ and Cryns VL (2002): Caspase proteolysis of the cohesin component RAD21 promotes apoptosis. J Biol Chem 277:16775-16781.

Chen H, Toyooka S, Gazdar AF and Hsieh JT (2003b): Epigenetic regulation of a novel tumor suppressor gene (hDAB2IP) in prostate cancer cell lines. J Biol Chem 278:3121-3130.

Cheng L, Nagabhushan M, Pretlow TP, Amini SB and Pretlow TG (1996): Expression of E-cadherin in primary and metastatic prostate cancer. Am J Pathol 148:1375-1380.
Cher ML, Bova GS, Moore DH, Small EJ, Carroll PR, Pin SS, Epstein JI, Isaacs WB and Jensen RH (1996): Genetic alterations in untreated metastases and androgen-independent prostate cancer detected by comparative genomic hybridization and allelotyping. Cancer Res 56:3091-3102.
Cook WD and McCaw BJ (2000): Accommodating haploinsufficient tumor suppressor genes in Knudson's model. Oncogene 19:3434-3438.
Cooney KA, Wetzel JC, Consolino CM and Wojno KJ (1996a): Identification and characterization of proximal 6q deletions in prostate cancer. Cancer Res 56:4150-4153.
Cooney KA, Wetzel JC, Merajver SD, Macoska JA, Singleton TP and Wojno KJ (1996b): Distinct regions of allelic loss on 13q in prostate cancer. Cancer Res 56:1142-1145.
Craft N, Shostak Y, Carey M and Sawyers CL (1999): A mechanism for hormone-independent prostate cancer through modulation of androgen receptor signaling by the HER-2/neu tyrosine kinase. Nat Med 5:280-285.
Cui J, Deubler DA, Rohr LR, Zhu XL, Maxwell TM, Changus JE and Brothman AR (1998): Chromosome 7 abnormalities in prostate cancer detected by dual-color fluorescence in situ hybridization. Cancer Genet Cytogenet 107:51-60.
Degeorges A, Wang F, Frierson HF Jr, Seth A, Chung LW and Sikes RA (1999): Human prostate cancer expresses the low affinity insulin-like growth factor binding protein IGFBP-rP1. Cancer Res 59:2787-2790.
DeMarzo AM, Nelson WG, Isaacs WB and Epstein JI (2003): Pathological and molecular aspects of prostate cancer. Lancet 361:955-964.
Devilee P, Cleton-Jansen AM and Cornelisse CJ (2001): Ever since Knudson. Trends Genet 17:569-573.
Diatchenko L, Lau YF, Campbell AP, Chenchik A, Moqadam F, Huang B, Lukyanov S, Lukyanov K, Gurskaya N, Sverdlov ED and Siebert PD (1996): Suppression subtractive hybridization: a method for generating differentially regulated or tissue-specific cDNA probes and libraries. Proc Natl Acad Sci U S A 93:6025-6030.
Dolly JO and Parcej DN (1996): Molecular properties of voltage-gated K+ channels. J Bioenerg Biomembr 28:231-253.
Dong JT (2001): Chromosomal deletions and tumor suppressor genes in prostate cancer. Cancer Metastasis Rev 20:173-193.
Dong JT, Chen C, Stultz BG, Isaacs JT and Frierson HF Jr (2000): Deletion at 13q21 is associated with aggressive prostate cancers. Cancer Res 60:3880-3883.
Dong JT, Lamb PW, Rinker-Schaeffer CW, Vukanovic J, Ichikawa T, Isaacs JT and Barrett JC (1995): KAI1, a metastasis suppressor gene for prostate cancer on human chromosome 11p11.2. Science 268:884-886.
Dong JT, Sipe TW, Hyytinen ER, Li CL, Heise C, McClintock DE, Grant CD, Chung LW and Frierson HF Jr (1998): PTEN/MMAC1 is infrequently mutated in pT2 and pT3 carcinomas of the prostate. Oncogene 17:1979-1982.
Dong JT, Suzuki H, Pin SS, Bova GS, Schalken JA, Isaacs WB, Barrett JC and Isaacs JT (1996): Down-regulation of the KAI1 metastasis suppressor gene during the progression of human prostatic cancer infrequently involves gene mutation or allelic loss. Cancer Res 56:4387-4390.

Dorai T, Perlman H, Walsh K, Shabsigh A, Goluboff ET, Olsson CA and Buttyan R (1999): A recombinant defective adenoviral agent expressing anti-bcl-2 ribozyme promotes apoptosis of bcl-2-expressing human prostate cancer cells. Int J Cancer 82:846-852.

Duan DR, Pause A, Burgess WH, Aso T, Chen DY, Garrett KP, Conaway RC, Conaway JW, Linehan WM and Klausner RD (1995): Inhibition of transcription elongation by the VHL tumor suppressor protein. Science 269:1402-1406.

Eagle LR, Yin X, Brothman AR, Williams BJ, Atkin NB and Prochownik EV (1995): Mutation of the MXI1 gene in prostate cancer. Nat Genet 9:249-255.

Eisen MB, Spellman PT, Brown PO and Botstein D (1998): Cluster analysis and display of genome-wide expression patterns. Proc Natl Acad Sci U S A 95:14863-14868.

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

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

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

Elo JP, Kvist L, Leinonen K, Isomaa V, Henttu P, Lukkarinen O and Vihko P (1995): Mutated human androgen receptor gene detected in a prostatic cancer patient is also activated by estradiol. J Clin Endocrinol Metab 80:3494-3500.

Elo JP and Visakorpi T (2001): Molecular genetics of prostate cancer. Ann Med 33:130-141.

Esteller M, Silva JM, Dominguez G, Bonilla F, Matias-Guiu X, Lerma E, Bussaglia E, Prat J, Harkes IC, Repasky EA, Gabrielson E, Schutte M, Baylin SB and Herman JG (2000): Promoter hypermethylation and BRCA1 inactivation in sporadic breast and ovarian tumors. J Natl Cancer Inst 92:564-569.

Evans BA, Harper ME, Daniells CE, Watts CE, Matenhelia S, Green J and Griffiths K (1996): Low incidence of androgen receptor gene mutations in human prostatic tumors using single strand conformation polymorphism analysis. Prostate 28:162-171.

Feilotter HE, Nagai MA, Boag AH, Eng C and Mulligan LM (1998): Analysis of PTEN and the 10q23 region in primary prostate carcinomas. Oncogene 16:1743-1748.

Fernandez M and Eng C (2002): The expanding role of PTEN in neoplasia: a molecule for all seasons? Clin Cancer Res 8:1695-1698.

Fernandez A, Udagawa T, Schwesinger C, Beecken W, Achilles-Gerte E, McDonnell T and D'Amato R (2001): Angiogenic potential of prostate carcinoma cells overexpressing bcl-2. J Natl Cancer Inst 93:208-213.

Fero ML, Randel E, Gurley KE, Roberts JM and Kemp CJ (1998): The murine gene p27Kip1 is haplo-insufficient for tumour suppression. Nature 396:177-180.

Finlin BS, Gau CL, Murphy GA, Shao H, Kimel T, Seitz RS, Chiu YF, Botstein D, Brown PO, Der CJ, Tamanoi F, Andres DA and Perou CM (2001): RERG is a

novel ras-related, estrogen-regulated and growth-inhibitory gene in breast cancer. J Biol Chem 276:42259-42267.

Fujiwara Y, Ohata H, Kuroki T, Koyama K, Tsuchiya E, Monden M and Nakamura Y (1995): Isolation of a candidate tumor suppressor gene on chromosome 8p21.3-p22 that is homologous to an extracellular domain of the PDGF receptor beta gene. Oncogene 10:891-895.

Friend SH, Bernards R, Rogelj S, Weinberg RA, Rapaport JM, Albert DM and Dryja TP (1986): A human DNA segment with properties of the gene that predisposes to retinoblastoma and osteosarcoma. Nature 323:643-646.

Fu W, Bubendorf L, Willi N, Moch H, Mihatsch MJ, Sauter G and Gasser TC (2000): Genetic changes in clinically organ-confined prostate cancer by comparative genomic hybridization. Urology 56:880-885.

Furuya Y, Krajewski S, Epstein JI, Reed JC and Isaacs JT (1996): Expression of bcl-2 and the progression of human and rodent prostatic cancers. Clin Cancer Res 2:389-398.

Galbiati F, Volonte D, Engelman JA, Watanabe G, Burk R, Pestell RG and Lisanti MP (1998): Targeted downregulation of caveolin-1 is sufficient to drive cell transformation and hyperactivate the p42/44 MAP kinase cascade. EMBO J 17:6633-6648.

Gao X, Honn KV, Grignon D, Sakr W and Chen YQ (1993): Frequent loss of expression and loss of heterozygosity of the putative tumor suppressor gene DCC in prostatic carcinomas. Cancer Res 53:2723-2727.

Gao AC, Lou W, Dong JT and Isaacs JT (1997): CD44 is a metastasis suppressor gene for prostatic cancer located on human chromosome 11p13. Cancer Res 57:846-849.

Garkavtsev I, Kazarov A, Gudkov A and Riabowol K (1996): Suppression of the novel growth inhibitor p33ING1 promotes neoplastic transformation. Nat Genet 14:415-420.

Goessl C, Krause H, Muller M, Heicappell R, Schrader M, Sachsinger J and Miller K (2000): Fluorescent methylation-specific polymerase chain reaction for DNA-based detection of prostate cancer in bodily fluids. Cancer Res 60:5941-5945.

Grady WM, Willis J, Guilford PJ, Dunbier AK, Toro TT, Lynch H, Wiesner G, Ferguson K, Eng C, Park JG, Kim SJ and Markowitz S (2000): Methylation of the CDH1 promoter as the second genetic hit in hereditary diffuse gastric cancer. Nat Genet 26:16-17.

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

Gray IC, Phillips SM, Lee SJ, Neoptolemos JP, Weissenbach J and Spurr NK (1995): Loss of the chromosomal region 10q23-25 in prostate cancer. Cancer Res 55:4800-4803.

Gray IC, Stewart LM, Phillips SM, Hamilton JA, Gray NE, Watson GJ, Spurr NK and Snary D (1998): Mutation and expression analysis of the putative prostate tumour-suppressor gene PTEN. Br J Cancer 78:1296-1300.

Grignon DJ, Caplan R, Sarkar FH, Lawton CA, Hammond EH, Pilepich MV, Forman JD, Mesic J, Fu KK, Abrams RA, Pajak TF, Shipley WU and Cox JD (1997): p53 status and prognosis of locally advanced prostatic adenocarcinoma: a study based on RTOG 8610. J Natl Cancer Inst 89:158-165.

Gu K, Mes-Masson AM, Gauthier J and Saad F (1996): Overexpression of her-2/neu in human prostate cancer and benign hyperplasia. Cancer Lett 99:185-189.
Gu J, Tamura M, Pankov R, Danen EH, Takino T, Matsumoto K and Yamada KM (1999): Shc and FAK differentially regulate cell motility and directionality modulated by PTEN. J Cell Biol 146:389-403.
Gu Z, Thomas G, Yamashiro J, Shintaku IP, Dorey F, Raitano A, Witte ON, Said JW, Loda M and Reiter RE (2000): Prostate stem cell antigen (PSCA) expression increases with high gleason score, advanced stage and bone metastasis in prostate cancer. Oncogene 19:1288-1296.
Gumerlock PH, Chi SG, Shi XB, Voeller HJ, Jacobson JW, Gelmann EP and deVere White RW (1997): p53 abnormalities in primary prostate cancer: single-strand conformation polymorphism analysis of complementary DNA in comparison with genomic DNA. The Cooperative Prostate Network. J Natl Cancer Inst 89:66-71.
Haapala K, Hyytinen ER, Roiha M, Laurila M, Rantala I, Helin HJ and Koivisto PA (2001): Androgen receptor alterations in prostate cancer relapsed during a combined androgen blockade by orchiectomy and bicalutamide. Lab Invest 81:1647-1651.
Hanahan D and Weinberg RA (2000): The hallmarks of cancer. Cell 100:57-70.
Harden SV, Guo Z, Epstein JI and Sidransky D (2003): Quantitative GSTP1 methylation clearly distinguishes benign prostatic tissue and limited prostate adenocarcinoma. J Urol 169:1138-1142.
Hastie ND and Bishop JO (1976): The expression of three abundance classes of messenger RNA in mouse tissues. Cell 9:761-774.
Hauf S, Waizenegger IC and Peters JM (2001): Cohesin cleavage by separase required for anaphase and cytokinesis in human cells. Science 293:1320-1323.
Hayes DF and Thor AD (2002): c-erbB-2 in breast cancer: development of a clinically useful marker. Semin Oncol 29:231-245.
He WW, Sciavolino PJ, Wing J, Augustus M, Hudson P, Meissner PS, Curtis RT, Shell BK, Bostwick DG, Tindall DJ, Gelmann EP, Abate-Shen C and Carter KC (1997): A novel human prostate-specific, androgen-regulated homeobox gene (NKX3.1) that maps to 8p21, a region frequently deleted in prostate cancer. Genomics 43:69-77.
Heidenberg HB, Sesterhenn IA, Gaddipati JP, Weghorst CM, Buzard GS, Moul JW and Srivastava S (1995): Alteration of the tumor suppressor gene p53 in a high fraction of hormone refractory prostate cancer. J Urol 154:414-421.
Helenius MA, Saramäki OR, Linja MJ, Tammela TL and Visakorpi T (2001): Amplification of urokinase gene in prostate cancer. Cancer Res 61:5340-5344.
Hirano T (2000): Chromosome cohesion, condensation, and separation. Annu Rev Biochem 69:115-144.
Holloway AJ, van Laar RK, Tothill RW and Bowtell DD (2002): Options available--from start to finish--for obtaining data from DNA microarrays II. Nat Genet 32 Suppl:481-489.
Hubank M and Schatz DG (1994): Identifying differences in mRNA expression by representational difference analysis of cDNA. Nucleic Acids Res 22:5640-5648.
Hubert R S, Vivanco I, Chen E, Rastegar S, Leong K, Mitchell SC, Madraswala R, Zhou Y, Kuo J, Raitano AB, Jakobovits A, Saffran DC and Afar DEH (1999): STEAP: A prostate-specific cell-surface antigen highly expressed in human prostate tumors. Proc Natl Acad Sci USA 96:14523-14528.

Humphrey PA, Zhu X, Zarnegar R, Swanson PE, Ratliff TL, Vollmer RT and Day ML (1995): Hepatocyte growth factor and its receptor (c-MET) in prostatic carcinoma. Am J Pathol 147:386-396.

Hwa V, Tomasini-Sprenger C, Bermejo AL, Rosenfeld RG and Plymate SR (1998): Characterization of insulin-like growth factor-binding protein-related protein-1 in prostate cells. J Clin Endocrinol Metab 83:4355-4362.

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

Hyytinen ER, Haapala K, Thompson J, Lappalainen I, Roiha M, Rantala I, Helin HJ, Jänne OA, Vihinen M, Palvimo JJ and Koivisto PA (2002): Pattern of somatic androgen receptor gene mutations in patients with hormone-refractory prostate cancer. Lab Invest 82:1591-1598.

Hyytinen E, Visakorpi T, Kallioniemi A, Kallioniemi OP and Isola JJ (1994): Improved technique for analysis of formalin-fixed, paraffin-embedded tumors by fluorescence *in situ* hybridization. Cytometry 16:93-99.

Isaacs WB, Bova GS, Morton RA, Bussemakers MJ, Brooks JD and Ewing CM (1994): Genetic alterations in prostate cancer. Cold Spring Harb Symp Quant Biol 59:653-659.

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

Ishii H, Vecchione A, Murakumo Y, Baldassarre G, Numata S, Trapasso F, Alder H, Baffa R and Croce CM (2001): FEZ1/LZTS1 gene at 8p22 suppresses cancer cell growth and regulates mitosis. Proc Natl Acad Sci U S A 98:10374-10379.

Iyer VR, Eisen MB, Ross DT, Schuler G, Moore T, Lee JC, Trent JM, Staudt LM, Hudson J Jr, Boguski MS, Lashkari D, Shalon D, Botstein D and Brown PO (1999): The transcriptional program in the response of human fibroblasts to serum. Science 283:83-87.

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

Jenkins RB, Qian J, Lee HK, Huang H, Hirasawa K, Bostwick DG, Proffitt J, Wilber K, Lieber MM, Liu W and Smith DI (1998a): A molecular cytogenetic analysis of 7q31 in prostate cancer. Cancer Res 58:759-766.

Jenkins R, Takahashi S, DeLacey K, Bergstralh E and Lieber M (1998b): Prognostic significance of allelic imbalance of chromosome arms 7q, 8p, 16q, and 18q in stage T3N0M0 prostate cancer. Genes Chromosomes Cancer 21:131-143.

Johnson MI, Robinson MC, Marsh C, Robson CN, Neal DE and Hamdy FC (1998): Expression of Bcl-2, Bax, and p53 in high-grade prostatic intraepithelial neoplasia and localized prostate cancer: relationship with apoptosis and proliferation. Prostate 37:223-229.

Jones PA and Baylin SB (2002): The fundamental role of epigenetic events in cancer. Nat Rev Genet 3:415-428.

Kaelin WG Jr (2002): Molecular basis of the VHL hereditary cancer syndrome. Nat Rev Cancer 2:673-682.

Kallioniemi OP and Visakorpi T (1996): Genetic basis and clonal evolution of human prostate cancer. Adv Cancer Res 68:225-255.

Kaltz-Wittmer C, Klenk U, Glaessgen A, Aust DE, Diebold J, Lohrs U and Baretton GB (2000): FISH analysis of gene aberrations (MYC, CCND1, ERBB2, RB, and AR) in advanced prostatic carcinomas before and after androgen deprivation therapy. Lab Invest 80:1455-1464.

Kamura T, Koepp DM, Conrad MN, Skowyra D, Moreland RJ, Iliopoulos O, Lane WS, Kaelin WG Jr, Elledge SJ, Conaway RC, Harper JW and Conaway JW (1999): Rbx1, a component of the VHL tumor suppressor complex and SCF ubiquitin ligase. Science 284:657-661.

Kawamata N, Park D, Wilczynski S, Yokota J and Koeffler HP (1996): Point mutations of the Mxil gene are rare in prostate cancers. Prostate 29:191-193.

Kibel A, Iliopoulos O, DeCaprio JA and Kaelin WG Jr (1995): Binding of the von Hippel-Lindau tumor suppressor protein to Elongin B and C. Science 269:1444-1446.

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

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

Kinzler KW and Vogelstein B (1996): Lessons from hereditary colorectal cancer. Cell 87:159-170.

Kito H, Suzuki H, Ichikawa T, Sekita N, Kamiya N, Akakura K, Igarashi T, Nakayama T, Watanabe M, Harigaya K and Ito H (2001): Hypermethylation of the CD44 gene is associated with progression and metastasis of human prostate cancer. Prostate 49:110-115.

Knudsen BS, Gmyrek GA, Inra J, Scherr DS, Vaughan ED, Nanus DM, Kattan MW, Gerald WL and Vande Woude GF (2002): High expression of the Met receptor in prostate cancer metastasis to bone. Urology 60:1113-1117.

Knudson AG Jr (1971): Mutation and cancer: statistical study of retinoblastoma. Proc Natl Acad Sci U S A 68:820-823.

Knudson AG (2001): Two genetic hits (more or less) to cancer. Nat Rev Cancer 1:157-162.

Koivisto P, Kononen J, Palmberg C, Tammela T, Hyytinen E, Isola J, Trapman J, Cleutjens K, Noordzij A, Visakorpi T and Kallioniemi OP (1997): Androgen receptor gene amplification: a possible molecular mechanism for androgen deprivation therapy failure in prostate cancer. Cancer Res 57:314-319.

Korkmaz KS, Elbi C, Korkmaz CG, Loda M, Hager GL and Saatcioglu F (2002): Molecular cloning and characterization of STAMP1, a highly prostate-specific six transmembrane protein that is overexpressed in prostate cancer. J Biol Chem 277:36689-36696.

Krajewska M, Krajewski S, Epstein JI, Shabaik A, Sauvageot J, Song K, Kitada S and Reed JC (1996): Immunohistochemical analysis of bcl-2, bax, bcl-X, and mcl-1 expression in prostate cancers. Am J Pathol 148:1567-1576.

Kuczyk MA, Serth J, Bokemeyer C, Schwede J, Herrmann R, Machtens S, Grunewald V, Hofner K and Jonas U (1998): The MXI1 tumor suppressor gene is not mutated in primary prostate cancer. Oncol Rep 5:213-216.

Kwabi-Addo B, Giri D, Schmidt K, Podsypanina K, Parsons R, Greenberg N and Ittmann M (2001): Haploinsufficiency of the Pten tumor suppressor gene

promotes prostate cancer progression. Proc Natl Acad Sci U S A 98:11563-11568.
Lacombe L, Orlow I, Reuter VE, Fair WR, Dalbagni G, Zhang ZF and Cordon-Cardo C (1996): Microsatellite instability and deletion analysis of chromosome 10 in human prostate cancer. Int J Cancer 69:110-113.
Laitinen S, Karhu R, Sawyers CL, Vessella RL and Visakorpi T (2002): Chromosomal aberrations in prostate cancer xenografts detected by comparative genomic hybridization. Genes Chromosomes Cancer 35:66-73.
Lara PN Jr, Meyers FJ, Gray CR, Edwards RG, Gumerlock PH, Kauderer C, Tichauer G, Twardowski P, Doroshow JH and Gandara DR (2002): HER-2/neu is overexpressed infrequently in patients with prostate carcinoma. Results from the California Cancer Consortium Screening Trial. Cancer 94:2584-2589.
Latil A, Bieche I, Pesche S, Volant A, Valeri A, Fournier G, Cussenot O and Lidereau R (1999): Loss of heterozygosity at chromosome arm 13q and RB1 status in human prostate cancer. Hum Pathol 30:809-815.
Latil A, Cussenot O, Fournier G and Lidereau R (1996): The BRCA2 gene is not relevant to sporadic prostate tumours. Int J Cancer 66:282-283.
Lee WH, Isaacs WB, Bova GS and Nelson WG (1997): CG island methylation changes near the GSTP1 gene in prostatic carcinoma cells detected using the polymerase chain reaction: a new prostate cancer biomarker. Cancer Epidemiol Biomarkers Prev 6:443-450.
Lee WH, Morton RA, Epstein JI, Brooks JD, Campbell PA, Bova GS, Hsieh WS, Isaacs WB and Nelson WG (1994): Cytidine methylation of regulatory sequences near the pi-class glutathione S-transferase gene accompanies human prostatic carcinogenesis. Proc Natl Acad Sci U S A 91:11733-11737.
Leung DW, Tompkins CK and White T (1998): Molecular cloning of two alternatively spliced forms of human phosphatidic acid phosphatase cDNAs that are differentially expressed in normal and tumor cells. DNA Cell Biol 17:377-385.
Leygue E, Snell L, Dotzlaw H, Hole K, Hiller-Hitchcock T, Roughley PJ, Watson PH and Murphy LC (1998): Expression of lumican in human breast carcinoma. Cancer Res 58:1348-1352.
Leygue E, Snell L, Dotzlaw H, Troup S, Hiller-Hitchcock T, Murphy LC, Roughley PJ and Watson PH (2000): Lumican and decorin are differentially expressed in human breast carcinoma. J Pathol 192:313-320.
Li C, Berx G, Larsson C, Auer G, Aspenblad U, Pan Y, Sundelin B, Ekman P, Nordenskjold M, van Roy F and Bergerheim US (1999): Distinct deleted regions on chromosome segment 16q23-24 associated with metastases in prostate cancer. Genes Chromosomes Cancer 24:175-182.
Li C, Larsson C, Futreal A, Lancaster J, Phelan C, Aspenblad U, Sundelin B, Liu Y, Ekman P, Auer G and Bergerheim US (1998): Identification of two distinct deleted regions on chromosome 13 in prostate cancer. Oncogene 16:481-487.
Li DM and Sun H (1997): TEP1, encoded by a candidate tumor suppressor locus, is a novel protein tyrosine phosphatase regulated by transforming growth factor beta. Cancer Res 57:2124-2129.
Li J, Yen C, Liaw D, Podsypanina K, Bose S, Wang SI, Puc J, Miliaresis C, Rodgers L, McCombie R, Bigner SH, Giovanella BC, Ittmann M, Tycko B, Hibshoosh H, Wigler MH and Parsons R (1997): PTEN, a putative protein tyrosine phosphatase gene mutated in human brain, breast, and prostate cancer. Science 275:1943-1947.

Li LC, Zhao H, Nakajima K, Oh BR, Filho LA, Carroll P and Dahiya R (2001): Methylation of the E-cadherin gene promoter correlates with progression of prostate cancer. J Urol 166:705-709.
Liang P and Pardee AB (1992): Differential display of eukaryotic messenger RNA by means of the polymerase chain reaction. Science 257:967-971.
Liau LM, Lallone RL, Seitz RS, Buznikov A, Gregg JP, Kornblum HI, Nelson SF and Bronstein JM (2000): Identification of a human glioma-associated growth factor gene, granulin, using differential immuno-absorption. Cancer Res 60:1353-1360.
Lin B, Ferguson C, White JT, Wang S, Vessella R, True LD, Hood L and Nelson PS (1999): Prostate-localized and androgen-regulated expression of the membrane-bound serine protease TMPRSS2. Cancer Res 59:4180-4184.
Linja MJ, Savinainen KJ, Saramäki OR, Tammela TLJ, Vessella RL and Visakorpi T (2001): Amplification and overexpression of androgen receptor gene in hormone-refractory prostate cancer. Cancer Res 61:3550-3555.
Lisitsyn N, Lisitsyn N and Wigler M (1993): Cloning the differences between two complex genomes. Science 259:946-951.
Lockhart DJ, Dong H, Byrne MC, Follettie MT, Gallo MV, Chee MS, Mittmann M, Wang C, Kobayashi M, Horton H and Brown EL (1996): Expression monitoring by hybridization to high-density oligonucleotide arrays. Nat Biotechnol 14:1675-1680.
Lonergan KM, Iliopoulos O, Ohh M, Kamura T, Conaway RC, Conaway JW and Kaelin WG Jr (1998): Regulation of hypoxia-inducible mRNAs by the von Hippel-Lindau tumor suppressor protein requires binding to complexes containing elongins B/C and Cul2. Mol Cell Biol 18:732-741.
Lou W, Krill D, Dhir R, Becich MJ, Dong JT, Frierson HF Jr, Isaacs WB, Isaacs JT and Gao AC (1999): Methylation of the CD44 metastasis suppressor gene in human prostate cancer. Cancer Res 59:2329-2331.
Lu YP, Ishiwata T, Kawahara K, Watanabe M, Naito Z, Moriyama Y, Sugisaki Y and Asano G (2002): Expression of lumican in human colorectal cancer cells. Pathol Int 52:519-526.
MacGrogan D and Bookstein R (1997): Tumour suppressor genes in prostate cancer. Semin Cancer Biol 8:11-19.
MacGrogan D, Levy A, Bova GS, Isaacs WB and Bookstein R (1996): Structure and methylation-associated silencing of a gene within a homozygously deleted region of human chromosome band 8p22. Genomics 35:55-65.
McMenamin ME, Soung P, Perera S, Kaplan I, Loda M and Sellers WR (1999): Loss of PTEN expression in paraffin-embedded primary prostate cancer correlates with high Gleason score and advanced stage. Cancer Res 59:4291-4296.
Mack PC, Chi SG, Meyers FJ, Stewart SL, deVere White RW and Gumerlock PH (1998): Increased RB1 abnormalities in human primary prostate cancer following combined androgen blockade. Prostate 34:145-511.
Maehama T and Dixon JE (1998): The tumor suppressor, PTEN/MMAC1, dephosphorylates the lipid second messenger, phosphatidylinositol 3,4,5-trisphosphate. J Biol Chem 273:13375-13378.
Matz MV and Lukyanov A (1998): Different strategies of differential display: areas of application. Nucleic Acids Res 26:5537-5543.
McDonnell TJ, Navone NM, Troncoso P, Pisters LL, Conti C, von Eschenbach AC, Brisbay S and Logothetis CJ (1997): Expression of bcl-2 oncoprotein and p53 protein accumulation in bone marrow metastases of androgen independent prostate cancer. J Urol 157:569-574.

McDonnell TJ, Troncoso P, Brisbay SM, Logothetis C, Chung LW, Hsieh JT, Tu SM and Campbell ML (1992): Expression of the protooncogene bcl-2 in the prostate and its association with emergence of androgen-independent prostate cancer.Cancer Res 52:6940-6944.

Mirchandani D, Zheng J, Miller GJ, Ghosh AK, Shibata DK, Cote RJ and Roy-Burman P (1995): Heterogeneity in intratumor distribution of p53 mutations in human prostate cancer. Am J Pathol 147:92-101.

Miyake H, Tolcher A and Gleave ME (1999): Antisense Bcl-2 oligodeoxynucleotides inhibit progression to androgen-independence after castration in the Shionogi tumor model.Cancer Res 59:4030-4034.

Moldes M, Lasnier F, Gauthereau X, Klein C, Pairault J, Feve B and Chambaut-Guerin AM (2001): Tumor necrosis factor-alpha -induced adipose-related protein (tiarp), a cell-surface protein that is highly induced by tumor necrosis factor-alpha and adipose conversion. J Biol Chem 276:33938-33946.

Morris SM (2002): A role for p53 in the frequency and mechanism of mutation. Mutat Res 511:45-62.

Morris MJ, Reuter VE, Kelly WK, Slovin SF, Kenneson K, Verbel D, Osman I and Scher HI (2002): HER-2 profiling and targeting in prostate carcinoma. Cancer 94:980-986.

Myers MP, Pass I, Batty IH, Van der Kaay J, Stolarov JP, Hemmings BA, Wigler MH, Downes CP and Tonks NK (1998): The lipid phosphatase activity of PTEN is critical for its tumor supressor function. Proc Natl Acad Sci U S A 95:13513-13518.

Myers RB, Srivastava S, Oelschlager DK and Grizzle WE (1994): Expression of p160erbB-3 and p185erbB-2 in prostatic intraepithelial neoplasia and prostatic adenocarcinoma. J Natl Cancer Inst 86:1140-1145.

Nacht M, Ferguson AT, Zhang W, Petroziello JM, Cook BP, Gao YH, Maguire S, Riley D, Coppola G, Landes GM, Madden SL and Sukumar S (1999): Combining serial analysis of gene expression and array technologies to identify genes differentially expressed in breast cancer. Cancer Res 59:5464-5470.

Naito Z, Ishiwata T, Kurban G, Teduka K, Kawamoto Y, Kawahara K and Sugisaki Y (2002): Expression and accumulation of lumican protein in uterine cervical cancer cells at the periphery of cancer nests. Int J Oncol 20:943-948.

Narla G, Heath KE, Reeves HL, Li D, Giono LE, Kimmelman AC, Glucksman MJ, Narla J, Eng FJ, Chan AM, Ferrari AC, Martignetti JA and Friedman SL (2001): KLF6, a candidate tumor suppressor gene mutated in prostate cancer. Science 294:2563-2566.

Nasmyth K, Peters JM and Uhlmann F (2000): Splitting the chromosome: cutting the ties that bind sister chromatids. Science 288:1379-1385.

Nasu Y, Timme TL, Yang G, Bangma CH, Li L, Ren C, Park SH, DeLeon M, Wang J and Thompson TC (1998): Suppression of caveolin expression induces androgen sensitivity in metastatic androgen-insensitive mouse prostate cancer cells. Nat Med 4:1062-1064.

Navone NM, Labate ME, Troncoso P, Pisters LL, Conti CJ, von Eschenbach AC and Logothetis CJ (1999): p53 mutations in prostate cancer bone metastases suggest that selected p53 mutants in the primary site define foci with metastatic potential. J Urol 161:304-308.

Navone NM, Troncoso P, Pisters LL, Goodrow TL, Palmer JL, Nichols WW, von Eschenbach AC and Conti CJ (1993): p53 protein accumulation and gene

mutation in the progression of human prostate carcinoma. J Natl Cancer Inst 85:1657-1669.
Nelson JB, Lee WH, Nguyen SH, Jarrard DF, Brooks JD, Magnuson SR, Opgenorth TJ, Nelson WG and Bova GS (1997): Methylation of the 5' CpG island of the endothelin B receptor gene is common in human prostate cancer. Cancer Res 57:35-37.
Nelson PS, Plymate SR, Wang K, True LD, Ware JL, Gan L, Liu AY and Hood L (1998): Hevin, an antiadhesive extracellular matrix protein, is down-regulated in metastatic prostate adenocarcinoma. Cancer Res 58:232-236.
Newmark JR, Hardy DO, Tonb DC, Carter BS, Epstein JI, Isaacs WB, Brown TR and Barrack ER (1992): Androgen receptor gene mutations in human prostate cancer. Proc Natl Acad Sci U S A 89:6319-6323.
Noordzij MA, van Steenbrugge GJ, Schroder FH and Van der Kwast TH (1999): Decreased expression of CD44 in metastatic prostate cancer. Int J Cancer 84:478-483.
Nowell PC (1976): The clonal evolution of tumor cell populations. Science 194:23-28.
Nupponen NN, Hyytinen ER, Kallioniemi AH and Visakorpi T (1998a): Genetic alterations in prostate cancer cell lines detected by comparative genomic hybridization. Cancer Genet Cytogenet 101:53-57.
Nupponen NN, Isola J and Visakorpi T (2000): Mapping the amplification of EIF3S3 in breast and prostate cancer. Genes Chromosomes Cancer 28:203-210.
Nupponen NN, Kakkola L, Koivisto P and Visakorpi T (1998b): Genetic alterations in hormone-refractory recurrent prostate carcinomas. Am J Pathol 153:141-148.
Nupponen NN, Porkka K, Kakkola L, Tanner M, Persson K, Borg Å, Isola J and Visakorpi T (1999): Amplification and overexpression of p40 subunit of eukaryotic translation initiation factor 3 in breast and prostate cancer. Am J Pathol 154:1777-1783.
Ornstein DK, Cinquanta M, Weiler S, Duray PH, Emmert-Buck MR, Vocke CD, Linehan WM and Ferretti JA (2001): Expression studies and mutational analysis of the androgen regulated homeobox gene NKX3.1 in benign and malignant prostate epithelium. J Urol 165:1329-1334.
Padalecki SS, Troyer DA, Hansen MF, Saric T, Schneider BG, O'Connell P and Leach RJ (2000): Identification of two distinct regions of allelic imbalance on chromosome 18Q in metastatic prostate cancer. Int J Cancer 85:654-658.
Passer BJ, Nancy-Portebois V, Amzallag N, Prieur S, Cans C, Roborel de Climens A, Fiucci G, Bouvard V, Tuynder M, Susini L, Morchoisne S, Crible V, Lespagnol A, Dausset J, Oren M, Amson R and Telerman A (2003): The p53-inducible TSAP6 gene product regulates apoptosis and the cell cycle and interacts with Nix and the Myt1 kinase. Proc Natl Acad Sci U S A 100:2284-2289.
Pati D, Zhang N and Plon SE (2002): Linking sister chromatid cohesion and apoptosis: role of Rad21. Mol Cell Biol 22:8267-8277.
Pause A, Lee S, Worrell RA, Chen DY, Burgess WH, Linehan WM and Klausner RD (1997): The von Hippel-Lindau tumor-suppressor gene product forms a stable complex with human CUL-2, a member of the Cdc53 family of proteins. Proc Natl Acad Sci U S A 94:2156-2161.
Pelengaris S, Khan M and Evan G (2002): c-MYC: more than just a matter of life and death. Nat Rev Cancer 2:764-776.
Perou CM, Sorlie T, Eisen MB, van de Rijn M, Jeffrey SS, Rees CA, Pollack JR, Ross DT, Johnsen H, Akslen LA, Fluge O, Pergamenschikov A, Williams C, Zhu SX,

Lonning PE, Borresen-Dale AL, Brown PO and Botstein D (2000): Molecular portraits of human breast tumours. Nature 406:747-752.
Pesche S, Latil A, Muzeau F, Cussenot O, Fournier G, Longy M, Eng C and Lidereau R (1998): PTEN/MMAC1/TEP1 involvement in primary prostate cancers. Oncogene16:2879-2883.
Phillips SM, Barton CM, Lee SJ, Morton DG, Wallace DM, Lemoine NR and Neoptolemos JP (1994): Loss of the retinoblastoma susceptibility gene (RB1) is a frequent and early event in prostatic tumorigenesis. Br J Cancer 70:1252-1257.
Pisters LL, Troncoso P, Zhau HE, Li W, von Eschenbach AC and Chung LW (1995): c-met proto-oncogene expression in benign and malignant human prostate tissues. J Urol 154:293-298.
Pulkkinen L, Alitalo T, Krusius T and Peltonen L (1992): Expression of decorin in human tissues and cell lines and defined chromosomal assignment of the gene locus (DCN). Cytogenet Cell Genet 60:107-111.
Raffo AJ, Perlman H, Chen MW, Day ML, Streitman JS and Buttyan R (1995): Overexpression of bcl-2 protects prostate cancer cells from apoptosis in vitro and confers resistance to androgen depletion in vivo. Cancer Res 55:4438-4445.
Rastinejad F, Conboy MJ, Rando TA and Blau HM (1993): Tumor suppression by RNA from the 3' untranslated region of alpha-tropomyosin. Cell 75:1107-1117.
Reese DM, Small EJ, Magrane G, Waldman FM, Chew K and Sudilovsky D (2001): HER2 protein expression and gene amplification in androgen-independent prostate cancer. Am J Clin Pathol 116:234-239.
Reiter RE, Gu Z, Watabe T, Thomas G, Szigeti K, Davis E, Wahl M, Nisitani S, Yamashiro J, Le Beau MM, Loda M and Witte ON (1998): Prostate stem cell antigen: a cell surface marker overexpressed in prostate cancer. Proc Natl Acad Sci U S A 95:1735-1740.
Reiter RE, Sato I, Thomas G, Qian J, Gu Z, Watabe T, Loda M and Jenkins RB (2000): Coamplification of prostate stem cell antigen (PSCA) and MYC in locally advanced prostate cancer. Genes Chromosomes Cancer 27:95-103.
Reizer J, Reizer A and Saier MH Jr (1993): The MIP family of integral membrane channel proteins: sequence comparisons, evolutionary relationships, reconstructed pathway of evolution, and proposed functional differentiation of the two repeated halves of the proteins. Crit Rev Biochem Mol Biol 28:235-257.
Rennie PS and Nelson CC (1999): Epigenetic mechanisms for progression of prostate cancer. Cancer Metastasis Rev 17:401-409.
Richmond PJ, Karayiannakis AJ, Nagafuchi A, Kaisary AV and Pignatelli M (1997): Aberrant E-cadherin and alpha-catenin expression in prostate cancer: correlation with patient survival. Cancer Res 57:3189-3193.
Rinaldy AR, Menon RP, Lerner JL and Steiner MS (2000): Role of pHyde novel gene product as an intrinsic factor for apoptotic pathway in prostate cancer. Gan To Kagaku Ryoho 27 Suppl 2:215-222.
Rinaldy AR and Steiner MS (1999): Application of an improved cDNA competition technique to identify prostate cancer-associated gene. DNA Cell Biol 18:829-836.
Ross JS, Sheehan C, Hayner-Buchan AM, Ambros RA, Kallakury BV, Kaufman R, Fisher HA and Muraca PJ (1997a): HER-2/neu gene amplification status in prostate cancer by fluorescence in situ hybridization. Hum Pathol 28:827-833.
Ross JS, Sheehan CE, Hayner-Buchan AM, Ambros RA, Kallakury BV, Kaufman RP Jr, Fisher HA, Rifkin MD and Muraca PJ (1997b): Prognostic significance of

HER-2/neu gene amplification status by fluorescence in situ hybridization of prostate carcinoma. Cancer 79:2162-2170.

Ross S, Spencer SD, Holcomb I, Tan C, Hongo J, Devaux B, Rangell L, Keller GA, Schow P, Steeves RM, Lutz RJ, Frantz G, Hillan K, Peale F, Tobin P, Eberhard D, Rubin MA, Lasky LA and Koeppen H (2002): Prostate stem cell antigen as therapy target: tissue expression and in vivo efficacy of an immunoconjugate. Cancer Res 62:2546-2553.

Rubin MA, Mucci NR, Figurski J, Fecko A, Pienta KJ and Day ML (2001): E-cadherin expression in prostate cancer: a broad survey using high-density tissue microarray technology. Hum Pathol 32:690-697.

Sagerström CG, Sun BI and Sive HL (1997): Subtractive cloning: past, present, and future. Annu Rev Biochem 66:751-783.

Saha S, Bardelli A, Buckhaults P, Velculescu VE, Rago C, St Croix B, Romans KE, Choti MA, Lengauer C, Kinzler KW and Vogelstein B (2001): A phosphatase associated with metastasis of colorectal cancer. Science 294:1343-1346.

Sambrook J and Russell DW (2001): Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

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

Sato K, Qian J, Slezak JM, Lieber MM, Bostwick DG, Bergstralh EJ and Jenkins RB (1999): Clinical significance of alterations of chromosome 8 in high-grade, advanced, nonmetastatic prostate carcinoma. J Natl Cancer Inst 91:1574-1580.

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

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

Schulz WA, Elo JP, Florl AR, Pennanen S, Santourlidis S, Engers R, Buchardt M, Seifert HH and Visakorpi T (2002): Genomewide DNA hypomethylation is associated with alterations on chromosome 8 in prostate carcinoma. Genes Chromosomes Cancer 35:58-65.

Segawa N, Nakamura M, Shan L, Utsunomiya H, Nakamura Y, Mori I, Katsuoka Y and Kakudo K (2002): Expression and somatic mutation on androgen receptor gene in prostate cancer. Int J Urol 9:545-553.

Sekita N, Suzuki H, Ichikawa T, Kito H, Akakura K, Igarashi T, Nakayama T, Watanabe M, Shiraishi T, Toyota M, Yoshie O and Ito H (2001): Epigenetic regulation of the KAI1 metastasis suppressor gene in human prostate cancer cell lines. Jpn J Cancer Res 92:947-951.

Selman SH (2000): "Latent" carcinoma of the prostate: a medical misnomer? Urology 56:708-711.

Sheldon CA, Williams RD and Fraley EE (1980): Incidental carcinoma of the prostate: a review of the literature and critical reappraisal of classification. J Urol 124:626-631.

Shen R, Su ZZ, Olsson CA and Fisher PB (1995): Identification of the human prostatic carcinoma oncogene PTI-1 by rapid expression cloning and differential RNA display. Proc Natl Acad Sci U S A 92:6778-6782.

Signoretti S, Montironi R, Manola J, Altimari A, Tam C, Bubley G, Balk S, Thomas G, Kaplan I, Hlatky L, Hahnfeldt P, Kantoff P and Loda M (2000): Her-2-neu

expression and progression toward androgen independence in human prostate cancer. J Natl Cancer Inst 92:1918-1925.

Song WJ, Sullivan MG, Legare RD, Hutchings S, Tan X, Kufrin D, Ratajczak J, Resende IC, Haworth C, Hock R, Loh M, Felix C, Roy DC, Busque L, Kurnit D, Willman C, Gewirtz AM, Speck NA, Bushweller JH, Li FP, Gardiner K, Poncz M, Maris JM and Gilliland DG (1999): Haploinsufficiency of CBFA2 causes familial thrombocytopenia with propensity to develop acute myelogenous leukaemia. Nat Genet 23:166-175.

Sowa Y, Orita T, Minamikawa S, Nakano K, Mizuno T, Nomura H and Sakai T (1997): Histone deacetylase inhibitor activates the WAF1/Cip1 gene promoter through the Sp1 sites. Biochem Biophys Res Commun 241:142-150.

Sprenger CC, Damon SE, Hwa V, Rosenfeld RG and Plymate SR (1999): Insulin-like growth factor binding protein-related protein 1 (IGFBP-rP1) is a potential tumor suppressor protein for prostate cancer. Cancer Res 59:2370-2375.

Stattin P, Damber JE, Karlberg L, Nordgren H and Bergh A (1996): Bcl-2 immunoreactivity in prostate tumorigenesis in relation to prostatic intraepithelial neoplasia, grade, hormonal status, metastatic growth and survival. Urol Res 24:257-264.

St Croix B, Rago C, Velculescu V, Traverso G, Romans KE, Montgomery E, Lal A, Riggins GJ, Lengauer C, Vogelstein B and Kinzler KW (2000): Genes expressed in human tumor endothelium. Science 289:1197-1202.

Stebbins CE, Kaelin WG Jr and Pavletich NP (1999): Structure of the VHL-ElonginC-ElonginB complex: implications for VHL tumor suppressor function. Science 284:455-461.

Steck PA, Pershouse MA, Jasser SA, Yung WK, Lin H, Ligon AH, Langford LA, Baumgard ML, Hattier T, Davis T, Frye C, Hu R, Swedlund B, Teng DH and Tavtigian SV (1997): Identification of a candidate tumour suppressor gene, MMAC1, at chromosome 10q23.3 that is mutated in multiple advanced cancers. Nat Genet 15:356-362.

Steiner MS, Anthony CT, Lu Y and Holt JT (1998): Antisense c-myc retroviral vector suppresses established human prostate cancer. Hum Gene Ther 9:747-755.

Steiner MS, Zhang X, Wang Y and Lu Y (2000): Growth inhibition of prostate cancer by an adenovirus expressing a novel tumor suppressor gene, pHyde. Cancer Res 60:4419-4425.

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

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

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

Takahashi S, Shan AL, Ritland SR, Delacey KA, Bostwick DG, Lieber MM, Thibodeau SN and Jenkins RB (1995): Frequent loss of heterozygosity at 7q31.1 in primary prostate cancer is associated with tumor aggressiveness and progression. Cancer Res 55:4114-4119.

Tamura M, Gu J, Matsumoto K, Aota S, Parsons R and Yamada KM (1998): Inhibition of cell migration, spreading, and focal adhesions by tumor suppressor PTEN. Science 280:1614-1617.

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

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

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

Tsavaler L, Shapero MH, Morkowski S and Laus R (2001): Trp-p8, a novel prostate-specific gene, is up-regulated in prostate cancer and other malignancies and shares high homology with transient receptor potential calcium channel proteins. Cancer Res 61:3760-3769.

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

Ueda T, Ichikawa T, Tamaru J, Mikata A, Akakura K, Akimoto S, Imai T, Yoshie O, Shiraishi T, Yatani R, Ito H and Shimazaki J (1996): Expression of the KAI1 protein in benign prostatic hyperplasia and prostate cancer. Am J Pathol 149:1435-1440.

Ueda T, Komiya A, Emi M, Suzuki H, Shiraishi T, Yatani R, Masai M, Yasuda K and Ito H (1997): Allelic losses on 18q21 are associated with progression and metastasis in human prostate cancer. Genes Chromosomes Cancer 20:140-147.

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

Velculescu VE, Zhang L, Vogelstein B and Kinzler KW (1995): Serial analysis of gene expression. Science 270:484-487.

Verkaik NS, van Steenbrugge GJ, van Weerden WM, Bussemakers MJ and van der Kwast TH (2000): Silencing of CD44 expression in prostate cancer by hypermethylation of the CD44 promoter region. Lab Invest 80:1291-1298.

Verkaik NS, Trapman J, Romijn JC, Van der Kwast TH and Van Steenbrugge GJ (1999): Down-regulation of CD44 expression in human prostatic carcinoma cell lines is correlated with DNA hypermethylation. Int J Cancer 80:439-443.

Visakorpi T, Hyytinen E, Kallioniemi A, Isola J and Kallioniemi OP (1994): Sensitive detection of chromosome copy number aberrations in prostate cancer by fluorescence in situ hybridization. Am J Pathol 145:624-630.

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

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

Visakorpi T, Kallioniemi OP, Heikkinen A, Koivula T and Isola J (1992): Small subgroup of aggressive, highly proliferative prostatic carcinomas defined by p53 accumulation. J Natl Cancer Inst 84:883-887.

Vlietstra RJ, van Alewijk DC, Hermans KG, van Steenbrugge GJ and Trapman J (1998): Frequent inactivation of PTEN in prostate cancer cell lines and xenografts. Cancer Res 58:2720-2723.

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

Waghray A, Schober M, Feroze F, Yao F, Virgin J and Chen YQ (2001): Identification of differentially expressed genes by serial analysis of gene expression in human prostate cancer. Cancer Res 61:4283-4286.

Wan JS, Sharp SJ, Poirier GM, Wagaman PC, Chambers J, Pyati J, Hom YL, Galindo JE, Huvar A, Peterson PA, Jackson MR and Erlander MG (1996): Cloning differentially expressed mRNAs. Nat Biotechnol 14:1685-1691.

Wang SI, Parsons R and Ittmann M (1998): Homozygous deletion of the PTEN tumor suppressor gene in a subset of prostate adenocarcinomas. Clin Cancer Res 4:811-815.

Wang RY, Troncoso P, Palmer JL, El-Naggar AK and Liang JC (1996): Trisomy 7 by dual-color fluorescence in situ hybridization: a potential biological marker for prostate cancer progression. Clin Cancer Res 2:1553-1558.

Watanabe M, Fukutome K, Kato H, Murata M, Kawamura J, Shiraishi T and Yatani R (1999): Progression-linked overexpression of c-Met in prostatic intraepithelial neoplasia and latent as well as clinical prostate cancers. Cancer Lett 141:173-178.

Wiechen K, Diatchenko L, Agoulnik A, Scharff KM, Schober H, Arlt K, Zhumabayeva B, Siebert PD, Dietel M, Schafer R and Sers C (2001a): Caveolin-1 is down-regulated in human ovarian carcinoma and acts as a candidate tumor suppressor gene. Am J Pathol 159:1635-1643.

Wiechen K, Sers C, Agoulnik A, Arlt K, Dietel M, Schlag PM and Schneider U (2001b): Down-regulation of caveolin-1, a candidate tumor suppressor gene, in sarcomas. Am J Pathol 158:833-839.

Xiao W and Oefner PJ (2001): Denaturing high-performance liquid chromatography: A review. Hum Mutat 17:439-474.

Xu LL, Shanmugam N, Segawa T, Sesterhenn IA, McLeod DG, Moul JW and Srivastava S (2000a): A novel androgen-regulated gene, PMEPA1, located on chromosome 20q13 exhibits high level expression in prostate.Genomics 66:257-263.

Xu LL, Srikantan V, Sesterhenn IA, Augustus M, Dean R, Moul JW, Carter KC and Srivastava S (2000b): Expression profile of an androgen regulated prostate specific homeobox gene NKX3.1 in primary prostate cancer. J Urol 163:972-979.

Xu J, Zheng SL, Carpten JD, Nupponen NN, Robbins CM, Mestre J, Moses TY, Faith DA, Kelly BD, Isaacs SD, Wiley KE, Ewing CM, Bujnovszky P, Chang B, Bailey-Wilson J, Bleecker ER, Walsh PC, Trent JM, Meyers DA and Isaacs WB (2001): Evaluation of linkage and association of HPC2/ELAC2 in patients with familial or sporadic prostate cancer. Am J Hum Genet 68:901-911.

Xu J, Zheng SL, Komiya A, Mychaleckyj JC, Isaacs SD, Hu JJ, Sterling D, Lange EM, Hawkins GA, Turner A, Ewing CM, Faith DA, Johnson JR, Suzuki H, Bujnovszky P, Wiley KE, DeMarzo AM, Bova GS, Chang B, Hall MC,

McCullough DL, Partin AW, Kassabian VS, Carpten JD, Bailey-Wilson JE, Trent JM, Ohar J, Bleecker ER, Walsh PC, Isaacs WB and Meyers DA (2002): Germline mutations and sequence variants of the macrophage scavenger receptor 1 gene are associated with prostate cancer risk. Nat Genet 32:321-325.

Yamada KM and Araki M (2001): Tumor suppressor PTEN: modulator of cell signaling, growth, migration and apoptosis. J Cell Sci 114:2375-2382.

Yang G, Truong LD, Timme TL, Ren C, Wheeler TM, Park SH, Nasu Y, Bangma CH, Kattan MW, Scardino PT and Thompson TC (1998): Elevated expression of caveolin is associated with prostate and breast cancer. Clin Cancer Res 4:1873-1880.

Yang G, Truong LD, Wheeler TM and Thompson TC (1999): Caveolin-1 expression in clinically confined human prostate cancer: a novel prognostic marker. Cancer Res 59:5719-5723.

Yeh S, Lin HK, Kang HY, Thin TH, Lin MF and Chang C (1999): From HER2/Neu signal cascade to androgen receptor and its coactivators: a novel pathway by induction of androgen target genes through MAP kinase in prostate cancer cells. Proc Natl Acad Sci U S A 96:5458-5463.

Yin Z, Babaian RJ, Troncoso P, Strom SS, Spitz MR, Caudell JJ, Stein JD and Kagan J (2001): Limiting the location of putative human prostate cancer tumor suppressor genes on chromosome 18q. Oncogene 20:2273-2280.

Yin Z, Spitz MR, Babaian RJ, Strom SS, Troncoso P and Kagan J (1999): Limiting the location of a putative human prostate cancer tumor suppressor gene at chromosome 13q14.3. Oncogene 18:7576-7583.

Zenklusen JC, Hodges LC, LaCava M, Green ED and Conti CJ (2000): Definitive functional evidence for a tumor suppressor gene on human chromosome 7q31.1 neighboring the Fra7G site. Oncogene 19:1729-1733.

Zenklusen JC, Thompson JC, Troncoso P, Kagan J and Conti CJ (1994): Loss of heterozygosity in human primary prostate carcinomas: a possible tumor suppressor gene at 7q31.1. Cancer Res 54:6370-6373.

Zhang X, Lee C, Ng PY, Rubin M, Shabsigh A and Buttyan R (2000a): Prostatic neoplasia in transgenic mice with prostate-directed overexpression of the c-myc oncoprotein. Prostate 43:278-285.

Zhang W, Razani B, Altschuler Y, Bouzahzah B, Mostov KE, Pestell RG and Lisanti MP (2000b): Caveolin-1 inhibits epidermal growth factor-stimulated lamellipod extension and cell migration in metastatic mammary adenocarcinoma cells (MTLn3). Transformation suppressor effects of adenovirus-mediated gene delivery of caveolin-1. J Biol Chem 275:20717-20725.

Zhang X, Steiner MS, Rinaldy A and Lu Y (2001): Apoptosis induction in prostate cancer cells by a novel gene product, pHyde, involves caspase-3. Oncogene 20:5982-5990.

Zhang L, Zhou W, Velculescu VE, Kern SE, Hruban RH, Hamilton SR, Vogelstein B and Kinzler KW (1997): Gene expression profiles in normal and cancer cells. Science 276:1268-1272.

ORIGINAL COMMUNICATIONS