Genetic Analysis of Prostate Cancer

Dirk van Alewijk

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Cover: A ceiling view of "La Grande Arche" - La Défense in Paris, illustrating a different point of view regarding the inactivating mechanisms of tumour suppressor genes in prostate cancer. During my traineeship in Paris, I appreciated the elegance and splendour of how artwork can be embedded into architectural designs, which initiated my fascination for detail photography. I have tried to use this to give varying insights to each of the different chapter covers.

Genetic Analysis of Prostate Cancer

Genetische analyse van prostaatkanker

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Voor "mijn vrouwen"

INDEX

List of abbreviations

AR	Androgen Receptor
BPH	Benign Prostate Hyperplasia
CGH	Comparative Genome Hybridization
DRE	Digital Rectal Examination
EST	Expressed Sequence Tag
FISH	Fluorescence *in situ* Hybridization
FITC	Fluoresceine Isothiocyanate
HD	Homozygous Deletion
HPC	Hereditary prostate cancer
JPS	Juvenile Polyposis Syndrome
LOH	Loss of Heterozygosity
LUTS	Lower Urinal Tract Symptoms
MMR	Mismatch Repair
PCR	Polymerase Chain Reaction
PIN	Prostatic Intraepithelial Neoplasia
PrCa	prostate cancer
PSA	prostate specific antigen
PSP94	Prostatic Secretory Protein 94
RCC	Renal Cell Carcinoma
RDA	Representational Difference Analysis
RFLP	Restriction Fragment Length Polymorphism
RT	Reverse Transcriptase
SCID	Severe Combined Immunodeficient
SKY	Multicolour Spectral Karyotyping
SNP	Single Nucleotide Polymorphisms
SSCP	Single Stranded Conformation Polymorphism
STS	Sequence Tagged Site
TNM	Tumour, Nodes and Metastasis
TSG	Tumour Suppressor Gene
TRAMP	Transgenic Adenocarcinoma of Mouse Prostate
TRUS	Transrectal Ultrasound
TUR	Transurethral resection
UGM	Urogenital sinus Mesenchyme
WS	Werner Syndrome

List of gene abbreviations

1D12A	pre-T/NK cell associated protein
AK057533	
AKT1	v-akt murine thymoma viral oncogene homologue 1 (*RAC* / *PKB*)
AKT3	v-akt murine thymoma viral oncogene homologue 3
ANXA7	Annexin A7 (*ANX7*)
APC	Adenomatosis Polyposis Coli
AR	Androgen Receptor
BAD	BCL2-Antagonist of cell Death
BCL2	B-cell CLL/Lymphoma 2
BC-PC	Brain Cancer and Prostate Cancer
BRCA2	Breast Cancer 2
CASP9	caspase 9, apoptosis-related cysteine protease
CD44	CD44 antigen
CDC42	Cell Division Cycle 42
CDH1	Cadherin 1 (E-cadherin)
CDKN1A	Cyclin-Dependent Kinase inhibitor 1A (*WAF1* / *P21* / *CIP1*)
CDKIN1B	Cyclin-Dependent Kinase inhibitor 1B (*P27* / *KIP1*)
CDKN2A	Cyclin-Dependent Kinase inhibitor 2A (*P16* / *MTS1*)
CLU	Clusterin
COPEB	Core Promoter Element Binding protein (*KLF6*)
CREB	cAMP Responsive Element Binding protein
CUGBP2	CUG triplet repeat, RNA Binding Protein 2
CYP17A1	Cytochrome P450, family 17, subfamily A, polypeptide 1 (*CYP17*)
CYP3A4	Cytochrome P450, family 3, subfamily A, polypeptide 4
DCC	Deleted in Colon Cancer
DMBT1	Deleted in Malignant Brain Tumours 1
EIF3S3	Eukaryotic translation Initiation Factor 3, Subunit 3 gamma, 40kDa
ELAC2	elaC homolog 2
eNOS	Endothelial Nitric Oxide Synthase
EPHX2	Epoxide Hydrolase 2
FLJ11218	hypothetical protein FLJ11218
FLJ30656	hypothetical protein FLJ30656
FOXO3A	Forkhead box O3A (*FKHRL1*)
FRAP1	FK506 binding protein 12-Rapamycin Associated Protein 1 (*mTOR*)
GATA3	GATA binding protein 3
GNRH1	Gonadotropin-Releasing Hormone 1 (*LHRH*)
GSK3	Glycogen Synthase Kinase 3 alpha (beta)
GSTM1	Glutathione S-Transferase M1
GTF2E2	General Transcription Factor IIE, polypeptide 2, beta 34kDa
HRAS	v-Ha-ras Harvey Rat Sarcoma viral oncogene homologue
HSD3B1	Hydroxy-delta-5-Steroid Dehydrogenase, 3 Beta- and steroid delta-isomerase 1
HSD3B2	Hydroxy-delta-5-Steroid Dehydrogenase, 3 Beta- and steroid delta-isomerase 2
HPC1	Hereditary Prostate Cancer 1
HPC2	Hereditary Prostate Cancer 2
HPCX	Hereditary Prostate Cancer X
IKK	IkappaB Kinase
KRAS	v-Ki-ras2 Kirsten Rat Sarcoma 2 viral oncogene homologue
LAPSER1	
LZTS1	Leucine Zipper, putative Tumour Suppressor 1 (*FEZ1*)
MADH4	Mothers Against Decapentaplegic Homologue 4 (*SMAD4* / *DPC4*)
MDM2	Mouse Double Minute 2 homologue
MINPP1	Multiple Inositol Polyphosphate histidine Phosphatase, 1
MSMB	Microseminoprotein, Beta- (*PSP-94*)

MSR1	Macrophage Scavenger Receptor 1
MXI1	MAX Interacting protein 1
MYC	v-myc avian Myelocytomatosis viral oncogene homologue
N33	Putative prostate cancer tumor suppressor
NEFL	Neurofilament, Light polypeptide 68kDa
NKX3-1	NK3 transcription factor related, locus 1
NRAS	Neuroblastoma RAS viral (v-ras) oncogene homologue
PAP	Prostatic Acid Phosphatase
PAPSS2	3'-Phosphoadenosine 5'-Phosphosulfate Synthase 2
PDGF	Platelet Derived Growth Factor alpha (beta)
PDGFRL	Platelet Derived Growth Factor Receptor-Like (*PRLTS*)
PG1	Putative prostate cancer
PI3K	Phosphoinositide-3-Kinase, class 2, alpha polypeptide (beta)
POLB	DNA Polymerase Beta
PTEN	Phosphatase and Tensin homologue (*MMAC1* / *TEP1*)
PTK2	Protein Tyrosine Kinase 2 (*FAK2*)
RB1	Retinoblastoma 1
RBPMS	RNA Binding Protein with Multiple Splicing
RNASEL	Ribonuclease L (2',5'-oligoisoadenylate synthetase-dependent)
SHC	(SRC Homology 2 domain Containing) transforming protein 1
SKP2	S-phase Kinase-associated Protein 2
SRD5A2	Steroid-5-alpha-Reductase, Alpha polypeptide 2
TGFB1	Transforming Growth Factor, Beta 1
TNFRSF6	Tumour Necrosis Factor Receptor Superfamily, member 6
TP53	Tumour Protein p53 (*P53*)
TP73	Tumour protein p73 (*P73*)
UNC5D	Unc-5 homologue D
VDR	Vitamin D (1,25- dihydroxyvitamin D3) Receptor
WNT	Wingless-type MMTV integration site family
WRN	Werner Syndrome

CHAPTER 1

GENERAL INTRODUCTION

1.1   PROSTATE CANCER

1.1.1 Morphology, function, and development of the prostate

The human prostate has the size of a chestnut and envelops the urethra as it exits the bladder, below the bladder neck. It is the largest of the male accessory sex glands, which also include the seminal vesicles, and bulbourethral gland. The prostate is composed of glandular structures, which are tightly fused within a common capsule. The prostate can be subdivided in a transitional, a central, and a peripheral zone (Figure 1.1) (McNeal 1997). Histologically, the glandular structures are complex arrays of luminal structures. Except for the larger ducts near the urethra, the ductal-acinar system is lined by highly differentiated secretory columnar epithelial cells layered on undifferentiated nonsecretory basal epithelial cells, thus forming a continuous layer adjacent to the basement membrane (Figure 1.2). The glandular structures are supported by stroma. The stromal compartment encompasses all cellular and extra-cellular elements outside the epithelial basement membrane and includes smooth muscle cells, blood vessels, lymphatic tissues, nerves, and fibroblasts embedded in a loose collagenous matrix (reviewed by Cunha et al. 1987)(McNeal 1997).
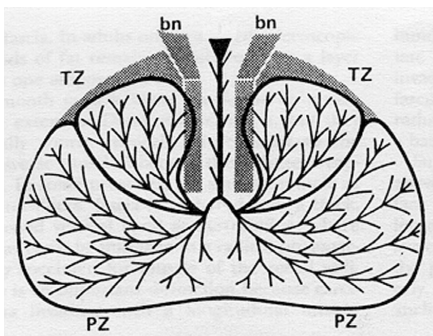
Figure 1.1.   Anatomy of the human prostate that shows the location of the peripheral zone (PZ), transition zone (TZ), and bladder neck (bn) (McNeal 1997).
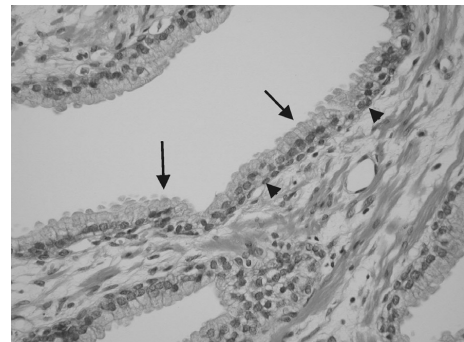
Figure 1.2.   Hematoxylin-eosin stained tissue section of a normal prostate. Columnar secretory epithelial cells (arrows) are layered on nonsecretory basal epithelial cells (arrow heads), which are scattered along the basement membrane.

The function of the prostate is to add components to the ejaculate. Prostatic secretions contain many different proteins. A major component is prostate specific antigen (PSA), which belongs to the family of kallikrein-like proteases. Two other major secreted proteins are prostatic acid phosphatase (PAP), and microseminoprotein beta (MSMB / PSP-94; Lilja and Abrahamsson 1988). PSA is known to be involved in semen liquefaction (Lilja 1985; Lundwall and Lilja 1987), but the physiological functions of PAP and MSMB are still unknown. Other products of the prostate are kallikreins, plasminogenactivator, pepsinogen II, metalloproteases, caseinolytic and gelatinolytic activities, and Zn-α2-Glycoprotein (reviewed by Wilson 1995).

The development of the prostate occurs via mesenchymal-epithelial interactions in which the urogenital sinus mesenchyme (UGM) is thought to induce ductal morphogenesis, and epithelial growth. This prostatic development is induced by androgens. In early development, androgenic effects on epithelial development are mainly elicited via androgen receptors (AR) in the mesenchymal cells. The stromal-epithelial interactions in the prostate continue into adulthood (reviewed by Cunha 1994; Marker et al. 2003).

1.1.2 The epidemiology of prostate cancer

Prostate cancer (PrCa) is the most frequently diagnosed cancer in men, and the second leading cause of male cancer death in Western countries (Greenlee et al. 2001). Out of all cancers in men, the incidence of PrCa increases most rapidly with age (Carter and Coffey 1990; Jacobsen et al. 1995; Potosky et al. 1995; Merrill et al. 1997; Post et al. 1998). From autopsy studies and from studies of radical cystoprostatectomy specimens removed for bladder cancer, it is known that approximately 10% of men in their fifties to 70% of men in their eighties, harbour microscopic foci of well or moderately differentiated adenocarcinoma in the prostate, although there is no clinical evidence of PrCa (latent PrCa; reviewed by Gittes 1991; Scardino et al. 1992; Matzkin et al. 1994; Ohori and Scardino 1994). Extrapolated to 50-year-old American men, it is estimated that about one in four latent PrCas becomes clinically manifest (Carter and Coffey 1990; Coffey 1993), whereas about one of three patients with clinical PrCa eventually die of the disease (Scardino et al. 1992).

Although the incidence of latent PrCa might be comparable worldwide, there are large variations in clinical incidence and mortality among different geographical and ethnic populations (Carter and Coffey 1990). The highest incidence rate was found in African-American men in the United States, followed by Caucasian men in Northern America, Western Europe and Australia. The lowest rates were found among Chinese and Japanese men (Coffey 1993). Some of these population differences may be attributed to life style and dietary factors (Hanley et al. 1995; Uchida et al. 1995).

1.1.3 Clinicopathological aspects of prostate cancer

1.1.3.1   Histological typing of prostate cancer

Most PrCa are adenocarcinomas that arise in the peripheral zone of the prostate. It is generally accepted that PrCa originates from stem cells or progenitor cells of the luminal epithelial cells (De Marzo et al. 1998). The clinical stages that are distinguished in PrCa are pre-malignant, locally confined, infiltrating, and metastatic disease (Figure 1.3). The pre-malignant stage is characterized by severe cytological aberrations in the luminal epithelial cells with preservation of the glandular architecture. These changes are also known as prostatic intraepithelial neoplasia (PIN), and are subdivided into a low- and high-grade form. High-grade PIN is considered to be the immediate precursor of locally confined PrCa (Bostwick et al. 1997). Local prostate cancer is characterized by invasive tumour growth without penetration through the prostatic capsule. In locally advanced cancer, the tumour infiltrates the tissues surrounding the prostate after penetration of the capsule. When PrCa metastasizes, secondary deposits are found in lymphatic and bone tissue. In late stages, PrCa metastases can be found in skin, lung, and liver tissues. Two commonly used clinical staging systems for PrCa are the Whitemore-Jewlett classification (Catalona and Avioli 1987; Gittes 1991), and the TNM (tumour, nodes and metastasis) staging system (Schroder et al. 1992).

Figure 1.3.   **Morphological phases of PrCa progression.**

1.1.3.2   Diagnosis and therapy of prostate cancer

Until the late eighties in the 20th century PrCa was clinically established by digital rectal examination (DRE) in patients visiting the physician with clinical symptoms. About 15 years ago, transrectal ultrasound (TRUS) imaging and the serum prostate specific antigen (PSA) test were introduced. The final diagnosis is made on the basis of histological examination of a prostate biopsy. The serum PSA test resulted in the detection of PrCa at an earlier stage. However, it has to be realized that a positive PSA test can not only be due to PrCa, but many other explanations are possible. Before the introduction of the serum-PSA-test, a patient with PrCa would typically present in an advanced stage with micturation problems, lower urinary tract symptoms (LUTS), and / or complaints caused by metastases, for instance bone pain. The screening with the serum PSA test resulted in a dramatic increase in the incidence of diagnosed PrCa.

Treatment decisions for PrCa depend on tumour characteristics (stage, pathological grade, and serum PSA levels), the patient's life expectancy, and quality versus quantity of life trade-offs. Locally confined PrCa can be surgically removed by radical prostatectomy, or may be treated by radiation therapy. A third option is observation, also known as "watchful waiting". Infiltrative or metastatic PrCa can be treated by endocrine therapy. This treatment modality includes surgical castration, chemical castration (e.g. gonadotropin-releasing hormone 1 (GNRH1) analogues), or therapy with anti-androgens. Endocrine therapy is based on the function of androgens in prostate growth and maintenance of its structural integrity. Androgen depletion dramatically affects normal prostate development, secretory function, maintenance of structural integrity, and ultimately results in apoptosis (Cunha et al. 1987; Lee 1997; Gao and Isaacs 1998). The majority of PrCas show a partial or complete response to endocrine therapy. However, after a mean period of less than 2 years, PrCa inevitably progresses to an androgen-independent stage. Transurethral resection (TUR) can be

carried out as a palliative treatment to diminish micturation complaints and lower urinary tract symptoms. Radiation therapy may be administered for symptomatic metastases.

1.2   GENETICS OF TUMOUR DEVELOPMENT

1.2.1 Oncogenes and tumour suppressor genes

Cell growth, programmed cell death (apoptosis), and differentiation are strictly controlled by the interplay of complex molecular mechanisms (Hanahan and Weinberg 2000). Deregulation of these mechanisms can lead to aberrant cell growth, and eventually to the formation of a cancer. During the last decades, many theories regarding the molecular mechanisms of tumourigenesis have been proposed. Fundamentally, all theories postulate that cancer is a genetic disease. In a tumour cell, genes involved in the regulation of cell growth, apoptosis, differentiation and repair of DNA damage are mutated such that the encoded protein no longer functions properly. However, it is becoming more and more clear that altered gene expression also contributes to tumour growth.

Historically, genes involved in tumourigenesis have been subdivided into oncogenes and tumour suppressor genes (reviewed by Bishop 1987; Hahn and Weinberg 2002). Oncogenes are derived from cellular genes (proto-oncogenes) that have become oncogenic by activating mutations or by overexpression. If activated, (proto-)oncogenes function in cell growth stimulation or inhibition of apoptosis. Overexpression of an oncogene might be caused by amplification of the gene or by gene rearrangement (e.g. chromosomal translocation). In the latter situation, the gene is expressed under control of a different promoter. The aberrant allele of an oncogene is functionally dominant over the wild-type allele. The physiological function of many tumour suppressor genes is inhibition of cell growth or stimulation of apoptosis. In a tumour, a tumour suppressor gene might be inactivated by mutation, deletion or by down-regulation of expression by promoter methylation or other mechanisms. Classically, the wild-type allele is functionally dominant over an inactivated allele. Therefore, both alleles must be inactivated to produce an oncogenic effect. This hypothesis is generally known as Knudson's "two hit" theory (Knudson 1971). More recent insights in the malfunctioning of tumour suppressor genes provide evidence that

also loss of one functional allele (haplo-insufficiency) and epigenetics can contribute to tumourigenesis.

Although most human cancers result from somatic genetic alterations in tumour cells, rare cancers can run in families with a hereditary pattern. In these families individuals are prone to develop tumours because of an inherited genetic alteration.

1.2.2 A genetic model of tumour development

Tumourigenesis is a multi-step process, as first proposed by Armitage and Doll (1954). The very first molecular events that underlie the initiation and progressive growth of human tumours were identified in the late eighties of the last century (reviewed by Bishop 1987; Weinberg 1989; Hanahan and Weinberg 2000). These observations constituted an essential contribution to a multi-step model for tumourigenesis. The first model describing a cascade of genetic changes was proposed for colorectal carcinogenesis (Fearon et al. 1990). This multi-step model described the sequential molecular and morphological transition of normal colon epithelium via hyperproliferating epithelium, benign adenoma, and malignant carcinoma to metastatic colon cancer. Furthermore, Fearon and Vogelstein (1990) assumed that (1) cancer involves the mutational activation of oncogenes and the inactivation of tumour suppressor genes, (2) the mutational activation or inactivation of four to five genes is required, and (3) although the genetic alterations may occur in a preferred sequence, the accumulation of changes rather than their order determines the biological properties of the tumour. These stepwise genetic alterations are currently well accepted as a general model for tumourigenesis.

The colorectal tumour progression model has been modified by Kinzler and Vogelstein (1997 and 1998), and Lengauer et al. (1998). They proposed that tumour suppressor genes could be sub-divided into three functionally distinct groups. Genes in these 3 groups are called: gatekeepers, caretakers, and landscapers. Gatekeepers are genes directly involved in neoplastic growth and prevent cancer by inhibiting cell growth or promoting apoptosis. Examples of this group of tumour suppressor genes are *RB1*, and *APC*. Restoration of a missing gatekeeper gene can result in suppression of neoplastic growth. Caretakers are indirectly involved in neoplastic growth. The normal function of these genes is to prevent DNA damage, chromosomal instability, and abnormal genetic recombination. Many genes with such a function are known nowadays

(Wood et al. 2001). Representatives of this group are the mismatch repair genes. Inactivation of certain caretaker genes results in a continuously increasing mutation rate in individual genes (domino effect) and thus genetic instability. Restoration of a defective caretaker function to cancer cells has no direct effect on neoplastic growth, because its loss has resulted in secondary mutations. Thus, inactivation of a caretakergene is not an immediate trigger to neoplastic growth. It is the increased mutation rate that results in a higher probability of mutational inactivation of gatekeeper genes.

Cancer research has predominantly focussed on cancer cells and their genomic properties. However, the importance of intercellular signalling between diverse cell types in cancer is also being recognized. Tumours are complex tissues in which mutant cells may have recruited normal non-neoplastic cells to serve as active collaborators in their neoplastic growth (Skobe and Fusenig 1998). Genes with a role in this partnership are designated "landscapers". An example of this group of tumour suppressor genes is *MADH4* (*SMAD4* / *DPC4*), which showed germline mutations in some JPS patients. Surprisingly, bi-allelic *MADH4* inactivation was only found in the stromal cells of the hamartomatous polyps of these patients (Koyama et al. 1999). Landscaper genes are thought to promote the gatekeeper- or caretaker-pathways in at least some tumour types (Jacoby et al. 1997).

1.3   GENETIC ALTERATIONS IN PROSTATE CANCER

The colorectal cancer models described above might be exemplary to tumour development in other human epithelial tumours, including PrCa. In PrCa, however, many of the molecular mechanisms underlying tumour development remain to be discovered. The focus of this thesis was to identify tumour suppressor genes that are involved in PrCa.

1.3.1 Methods to detect genes involved in cancer

In general, the genetic strategy to identify genes involved in cancer is (1) genome-wide screening, (2) chromosome mapping, (3) gene mapping, (4) mutation analysis of candidate genes, and (5) functional screening of candidate genes. Genes involved in hereditary and sporadic cancers are identified by different approaches. Hereditary cancer genes are genome-wide and fine-mapped by genetic linkage

analysis. This positional cloning technique is a powerful tool for the identification of inherited disease genes based solely on their location within the genome. Many currently known cancer susceptibility genes have originally been identified by linkage analysis of cancer-prone families. In a linkage analysis, a genotype of such families is generated using polymorphic markers scattered throughout the genome. Subsequent correlation of the genotype with the segregation pattern of the disease within the family may reveal linkage of the phenotype with a particular chromosomal locus.

Genes involved in sporadic cancers are genome-wide mapped by characteristic large chromosomal alterations (losses, gains, and translocations) in the tumour genomes. Chromosomal losses are an indication for the localization of a tumour suppressor gene, whereas gains and translocations are an indication for the location of an oncogene. These larger chromosomal alterations may be identified by karyotyping (Brothman et al. 1994), multicolour spectral karyotyping (SKY; Schrock et al. 1996; Speicher et al. 1996), comparative genomic hybridisation (CGH; Kallioniemi et al. 1992), and array CGH (Pinkel et al. 1998). Karyotyping provides a complete scan of banded mitotic chromosomes. It is suitable for the identification of all kinds of chromosomal alterations, including aneuploidy and iso-chromosome formation. However, the technique requires metaphase cells, and the identification of chromosomal aberrations may thus necessitate culturing of the tumour cells. Apart from difficulties like slowly growing cultures and cell selection, the induction of genetic alterations during culturing may lead to misinterpretation of the results. SKY is a modernized karyotyping showing each chromosome in a specific colour, which is particularly helpful for the identification of chromosomal translocations. For the identification of both losses and gains preference is given to CGH, in which normal and tumour DNA are each labelled with a different fluorescent dye and then hybridised to a normal metaphase chromosome spread. For CGH, DNA from frozen tumour samples as well as formalin-fixed, paraffin-embedded tumours can be utilized. Larger chromosomal aberrations can easily be characterized, but alterations like translocations, aneuploidy, and iso-chromosome formation can not be identified because CGH is based on the utilization of total tumour DNA. Nowadays, array CGH becomes a robust alternative for CGH analysis. Although allelotypical differences still can not be distinguished by this new technology it maps losses and gains more precisely than CGH.

Chromosomal regions of loss and gain can be mapped by chromosome transfer and genetically mapped in detail by analysis of allelic imbalances. Chromosome transfer provides a host cell with a complete set of genes from an added chromosome or chromosomal region, including the putative tumour suppressor gene or oncogene that induces the host-cell to lose or gain tumourigenic characteristics (Ichikawa et al. 1991). Chromosome transfer is a functional approach for the mapping of cancer genes to larger chromosomal regions. A potential problem of the technique is that transferred chromosomes tend to loose genomic fragments. Furthermore, results obtained by chromosome transfer can be misinterpreted due to the properties of the *in vitro* system used or the occurrence of mutations or deletions of multiple genes in the host tumour cell line.

Analysis of allelic imbalances does not easily discriminate between losses and gains of genomic DNA in the tumour cell. It can be carried out by analysis of restriction fragment length polymorphisms (RFLP; Vogelstein et al. 1989), analysis of polymorphic microsatellites (Weber and May 1989), or single nucleotide polymorphisms (SNP). Initially, analysis of allelic imbalance was done by RFLP analysis, which is a Southern blotting detection method for specific restricted genomic fragments. RFLP analysis is laborious and requires large amounts of tumour DNA sample. Much faster alternatives that require smaller amounts of sample DNA are microsatellite and SNP analysis. These two techniques are PCR-based detection methods of polymorphic mono-, di- and tri-nucleotide repeats (so-called microsatellites), and of single nucleotide polymorphisms (SNPs), respectively. SNPs can also be identified by oligo-hybridisation, allowing a high-throughput approach using microarrays. Allelic imbalance of informative markers specify the boundaries of a region of importance. Besides application in analysis of allelic imbalance, microsatellite analysis can also be informative in detecting genetic instability, which can be visualized by unstable microsatellite lengths (MSI, microsatellite instability).

A next step in fine-mapping regions of genetic alterations can be (fluorescent) *in situ* hybridisation ((F)ISH; Pinkel et al. 1986; Brothman et al. 1999) or screening for homozygous deletions. ISH is based on hybridisation of labelled DNA probes to chromosome spreads, cells in metaphase or interphase nuclei in histological sections. FISH is very powerful in detection of high-level amplifications.

Homozygous deletions are rare, but if found, they can map a tumour suppressor gene to a region of less than 1 Mbp. Homozygous deletions can be

identified in a defined chromosomal area by the absence of a PCR product, utilizing a large set of markers (Kamb et al. 1994; Hahn et al. 1996). A genome-wide search for homozygous deletions can be done by representational difference analysis (RDA). RDA identifies a small genomic fragment derived from normal DNA, which is not subtracted after reiterated hybridisation steps against tumour DNA (Lisitsyn and Wigler 1993). A search for homozygous deletions in DNA from primary tumour specimens can be severely complicated by the presence of contaminating DNA from normal cells. Nevertheless, a homozygous deletion narrows down a tumour suppressor gene region enormously.

Conclusive evidence for the identification of a tumour suppressor gene or oncogene can be given by mutational analyses and functional studies. In particular, for tumour suppressor genes and mismatch repair (MMR) genes, strongest evidence is provided by sequence analysis, showing a deletion, frame-shift or nonsense mutation. Functional evidence can be obtained by gene transfer or anti-sense RNA (RNAi) approaches. However, the efficacy of the introduced or inactivated gene will depend on the properties of the host cell line studied.

Alteration of gene expression may be a direct result from a mutation in a cancer gene. However, it also can be the result of an epigenetic event such as promoter hypermethylation. Therefore, down- or up-regulation of a candidate tumour gene provides indirect evidence for a role in cancer as it might be the result and not the cause of tumourigenesis.

1.3.2 Hereditary prostate cancer

1.3.2.1 Definition

Characteristic patterns for inherited cancer syndromes are familial aggregation of tumours and early disease onset (Bishop and Kiemeney 1997). Such patterns are found in nearly one quarter of all PrCa (reviewed by Carter et al. 1993; Narod 1999; Bratt 2000a, b; Karayi et al. 2000). By definition PrCa is familial if two first-degree relatives, father and son or brothers, develop the disease. Hereditary prostate carcinoma (HPC) refers to a subtype of familial PrCa in which there are either (1) at least three first degree cases of PrCa, (2) at least three successive generations of either maternal or paternal lineages with PrCa, or (3) a cluster of two relatives diagnosed with PrCa before the age of 55 years (Carter et al. 1993). Ninety percent of

PrCa cases are believed to be sporadic, the remaining ten percent displays a hereditary component (Carter et al. 1993; Keetch et al. 1996; Bratt et al. 1999).

1.3.2.2 Hereditary prostate cancer loci and genes

Various genome-wide scans of DNA samples from HPC families demonstrated linkage to loci on chromosomes 1p36, 1q42.2-q43, 1q24-q25, 8p22-p23, 11, 16q23, 17p11, 20q13, and Xq27-q28 (see Appendix A1 - Table I for references and locus names). Most linkages were confirmed in other studies. Some groups however, were unable to demonstrate linkage to a specific locus in their set of high-risk families. It is difficult to determine to which extent confounding factors, like late age of onset, lack of distinguishing features between the hereditary and sporadic forms of the disease, and the difficulty in identifying HPC families, might have affected these investigations (Smith et al. 1996).

Besides the age of onset, a few clinical features appear characteristic for HPC families. In families with linkage to 1p36, PrCa was found to aggregate with brain cancer (Gibbs et al. 1999; Xu et al. 2001b). In a subset of Swedish families, Gronberg et al. (2000) observed significant aggregation of PrCa with breast carcinoma and/or gastric carcinoma (Gronberg et al. 2000). In families with linkage to 1q24-q25, significantly more aggressive PrCa were diagnosed (Goode et al. 2001).

Although many HPC loci have been described, only a few high-penetrant susceptible genes were proposed (see Appendix A1 - Table I). In two HPC families that showed linkage to the HPC1 locus at chromosome 1q24-q25, a nonsense mutation and a mutation in an initiation codon of the *RNASEL* gene were found to segregate (Carpten et al. 2002). In three HPC families that showed linkage to the HPC2 locus at chromosome 17p11, a frame-shift and two missense mutations of the *ELAC2* gene were found to segregate (Rokman et al. 2001; Tavtigian et al. 2001; Wang et al. 2001). Besides these families, no additional *RNASEL* and *ELAC2* mutations or polymorphisms were found in HPC1 or HPC2 families, respectively. Subsequent studies of *ELAC2* failed to demonstrate an increased risk of a polymorphism described before (Rokman et al. 2001; Suarez et al. 2001; Vesprini et al. 2001; Wang et al. 2001; Xu et al. 2001a). Therefore, the relative importance of the *ELAC2* polymorphisms remains under debate. Other proposed high-penetrant susceptible genes are *HSD3B1* and *B2*, *TP73*, *PG1*, *LZTS1*, and *MSR1*, but the roles

of these genes are also disputable because the functional significance of the identified polymorphisms is not known.

HPC has also been associated with polymorphisms in genes involved in steroid hormone metabolism and steroid hormone signal transduction, including AR, SRD5A2, and cytochrome P450 isoforms (CYP17A1 and CYP3A4), vitamin D metabolism including VDR, and carcinogen metabolism including GSTM1 (Reviewed by Coughlin and Hall 2002). These polymorphisms, however, are not highly penetrant alleles in families at high risk for PrCa.

In summary, prostate cancer etiology involves several genetic loci, but so far no gene has been identified that accounts for a large proportion of susceptibility to the disease (reviewed by Nwosu et al. 2001).

1.3.3 Sporadic prostate cancer

1.3.3.1   Genome-wide search for sporadic prostate cancer loci

In the genome-wide search for chromosomal alterations in PrCa an important contribution was made by CGH analysis (Visakorpi et al. 1995; Cher et al. 1996; Nupponen et al. 1998b; Alers et al. 2000). Differences were identified between various clinical stages and histological grades of PrCa, implicating that certain chromosomal regions have their specific role in prostate tumourigenesis. Although CGH is not suitable to specify in detail a region of loss or gain, it provides a strong indication of the chromosomal alterations in the various stages of PrCa. Nevertheless, several discrepancies were found between the CGH-studies described above (see Appendix A2 and A3 - Tables II and III for more details). These differences may be due to the contamination of tumour DNA with DNA from normal cells, a small number of samples analysed, and by differences in interpretation of the data. For example, the chromosomal regions 1p36, 9q34, 11q13, 19, and 22q, are known to be problematic in CGH studies (Kirchhoff et al. 1998), therefore, the data of Nupponen et al. (1998a) describing loss of 1p36, 19 and 22q are of some concern.

In primary PrCa, chromosomal losses were found to predominate over gains in a 5 to 1 ratio (Visakorpi et al. 1995). Most frequently, deletions were found for chromosome arms 6q, 8p and 13q (Visakorpi et al. 1995; Alers et al. 2000). In metastatic PrCa a higher frequency of these three losses, and losses of 2q, 5q, 10q, and 16q were found. Furthermore, gains of 1q, 3q, 4q, 7q, 8q, 11p and 17q were

described (Cher et al. 1996; Alers et al. 2000). In local recurrences, that typically develop during endocrine therapy, a higher frequency of 8p and 16q losses, additional losses of 1p, 15q, 17p, 19, and 22q, and a higher frequency of 8q gain and gains of 7p, Xp, and Xq were found (Visakorpi et al. 1995; Nupponen et al. 1998b). The results of the studies described above are summarized in Figure 1.4A and 1.4B (more details are listed in Appendix A2 and A3 - Tables II and III). As shown in Figure 1.4 and Appendix A2 and A3 – Tables II and III, loss of 2q, 5q, and 6q, and gain of 1q, 3q, 4q, 9q, 11p, and 17q are more frequently found in metastases than in local recurrences.

So far, little attention has been given to chromosomal alterations in PIN lesions (Qian et al. 1998; Zitzelsberger et al. 2001). Although losses of 8p and 13q, and gains of 7 and 8q were found in PIN, these studies are not included in Figure 1.4 and Appendix A2 and A3 – Table II and III, because a limited number of samples was studied and because inconsistencies were described that were not seen in any other stage of PrCa. These discrepancies are likely due to the small number of patients in each study, and by small tissue samples that necessitated PCR amplification.

Genome-wide searches in PrCa have also been performed by allelic imbalance analysis (Cunningham et al. 1996). Cunningham et al. (1996) analysed DNAs from primary PrCa with a set of polymorphic markers scattered along all chromosomal arms. They reported an increased allelic imbalance for the same regions as described above in primary PrCa. Except for 8p, the frequencies found were less than those in the CGH studies. In addition, Cunningham et al. (1996) reported frequent allelic imbalance on 18q. Observed differences in frequency may be due to polymorphic markers with low heterozygosity, or to markers mapping outside the region of loss identified by CGH. If a marker maps in a region that is too small to be detected by CGH, allelotyping detects an additional genomic alteration. Furthermore, it is impossible to monitor by CGH loss of one allele combined with gain of the second copy.

% CGH losses

□ Primary PrCa   ■ Metastasis   ■ Recurrence

% CGH gains
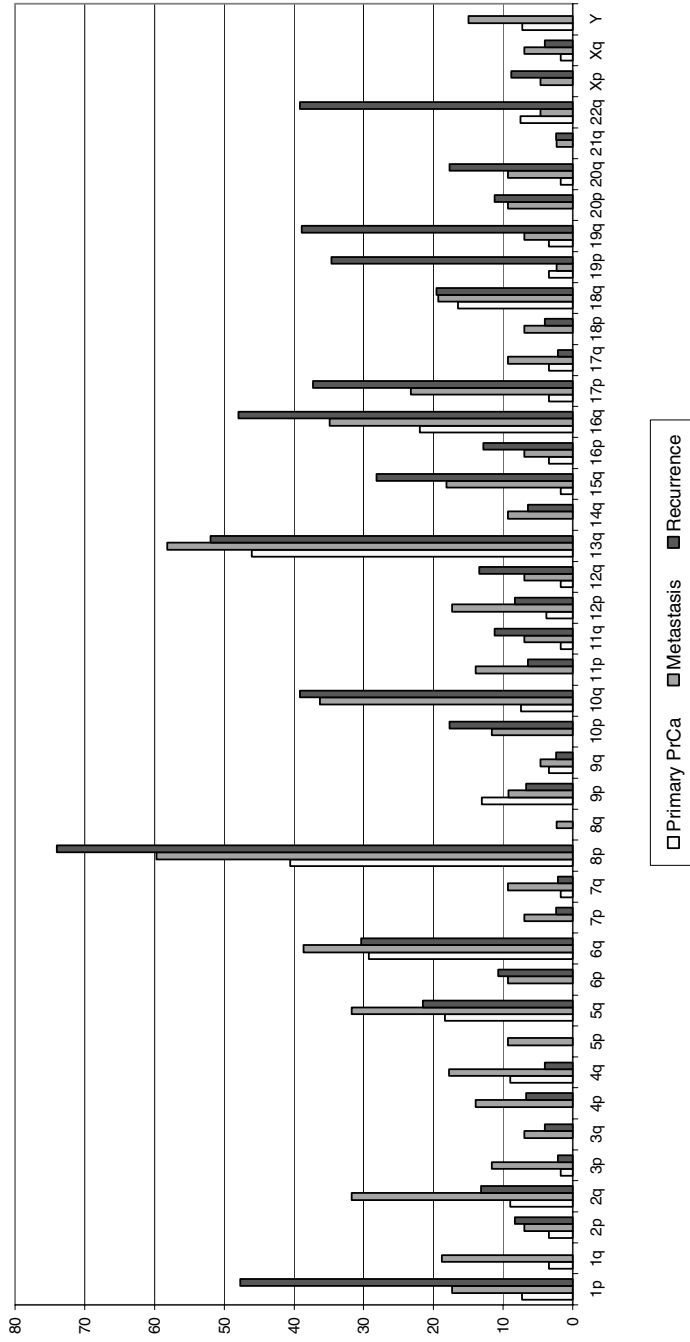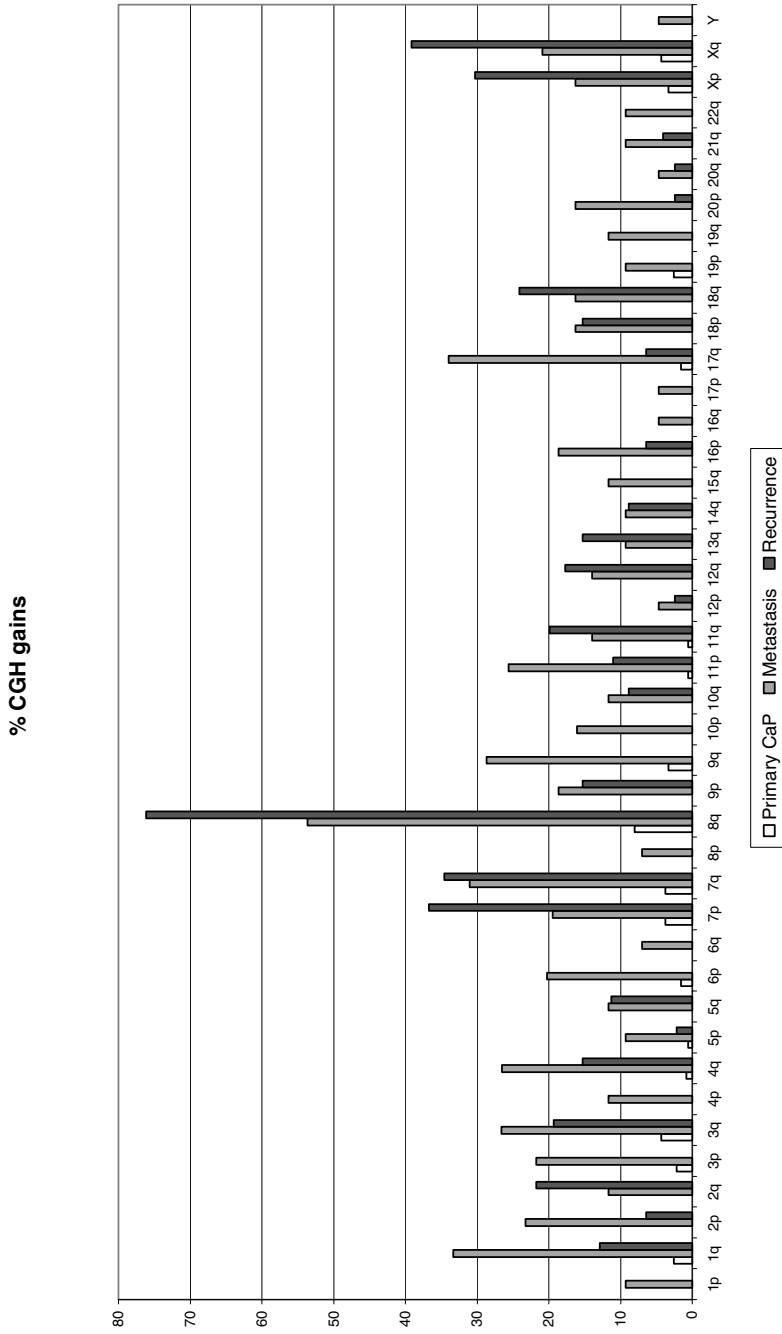
Figure 1.4. **Summary of chromosomal losses (A) and gains (B) in sporadic PrCa identified by CGH.** The percentages as shown are the mean of the studies of Visakorpi et al. (1995), Cher et al. (1996), Nupponen et al. (1998), and Alers et al. (2000) per tumour stage (see Appendix A2 and A3 - Table II and III for more details). High frequency of loss or gain was set at 25%.

1.3.3.2   Analysis of individual chromosomes

Deletion of 8p is one of the most common chromosomal alterations in PrCa. Loss of this chromosomal arm occurs already in early stage PrCa and increases during tumour progression to approximately 60-75% in advanced cancers. Deletion of 10q is observed in metastatic PrCa in particular, where it can be as high as 35-40%. Because this thesis focuses on chromosomes 8p and 10q, the remaining part of this chapter is limited to these two chromosomes.

1.3.3.2.1   Chromosome 8

The picture of 8p loss in PrCa that emerged from genome-wide CGH studies was supported by many independent studies using FISH analysis (Macoska et al. 1994; Oba et al. 2001), and analysis of allelic imbalance (Kunimi et al. 1991; Bova et al. 1993; Chang et al. 1994; MacGrogan et al. 1994; Trapman et al. 1994; Macoska et al. 1995; Suzuki et al. 1995; Vocke et al. 1996; Prasad et al. 1998, and reviewed in Isaacs 1995; Kallioniemi and Visakorpi 1996; Roylance et al. 1997; Abate-Shen and Shen 2000). Specifically, allelic imbalance studies demonstrated the presence of several separate regions of loss on this chromosomal arm. Comparison of these data indicated the presence of two, possibly three, regions of allelic loss at 8p, suggesting that this chromosomal arm contains several tumour suppressor genes that are of importance for PrCa tumourigenesis. The identified regions include 8p22-p23, 8p12-p21, and 8p11-p12 (Figure 1.5).

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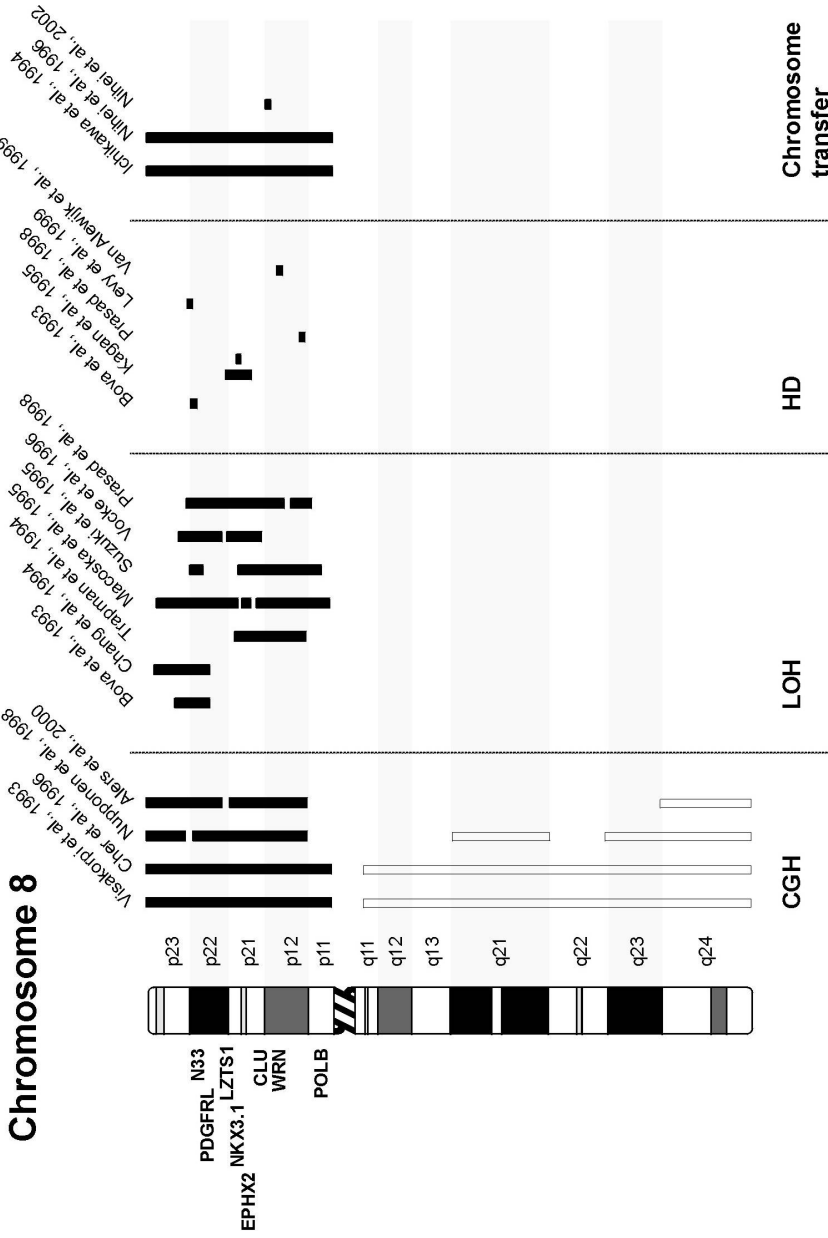
Figure 1.5. **Summary of smallest common lost or gained regions on chromosome 8 in PrCa identified by CGH, allelic imbalance analysis (LOH), HD screening, and chromosome transfer.** Losses are depicted by black bars, gains by open bars. Candidate tumour suppressor genes analysed in PrCa are listed to the left of the chromosome 8 pictogram, and arranged by their chromosomal localisation (April 2003 freeze UCSC database).

Gain of 8q is the most common chromosomal amplification in PrCa (Visakorpi et al. 1995; Cher et al. 1996; Nupponen et al. 1998b). Gain of this chromosomal arm is frequently seen in combination with loss of 8p. Gain of 8q occurs occasionally in early stage PrCa and increases during tumour progression to 75%. Nupponen et al. (1998b) identified two independently amplified regions on the q-arm, 8q21 and 8q23-qter, suggesting the presence of at least two PrCa-related oncogenes on 8q. The latter region was narrowed to 8q24 by Alers et al. (2000). Gain of either region on 8q was shown to correlate with tumour progression and poor prognosis (Van Den Berg et al. 1995; Sato et al. 1999; Alers et al. 2000), but the precise contribution of each region awaits the identification of the target genes. One of the strongest candidates is *MYC*, which maps at 8q24.2 (Reiter et al. 2000). A second candidate is *EIF3S3*, which maps at 8q24.1 (Saramaki et al. 2001). Detailed description of these target genes is beyond the scope of this thesis.

Part of the 8p losses and 8q gains in PrCa were found to occur through isochromosome 8q formation (Webb et al. 1996; Virgin et al. 1999; Macoska et al. 2000). However, there is also evidence for other mechanisms. In primary PrCa, loss of 8p was found more frequently than gain of 8q (Visakorpi et al. 1995; Alers et al. 2000), implicating that loss of 8p occurs earlier in prostate tumourigenesis than gain of 8q. Additional evidence was provided by Sato et al. (1999), who found frequent loss of 8p (24%) without gain of 8q in a large cohort of high-grade, advanced, non-metastatic PrCa (Sato et al. 1999).

Loss of 8p is not only common in PrCa, but also in many other tumour types, including carcinomas of the bladder (Kallioniemi et al. 1995; Takle and Knowles 1996; Wagner et al. 1997), breast (Kerangueven et al. 1997; Nishizaki et al. 1997; Anbazhagan et al. 1998), colon (Cunningham et al. 1993; Fujiwara et al. 1993; Chang et al. 1994), head and neck (Wu et al. 1997; El-Naggar et al. 1998), kidney (Schoenberg et al. 1995; Schullerus et al. 1999), lung (Ohata et al. 1993; Fujiwara et al. 1994; Lerebours et al. 1999), liver (Fujiwara et al. 1994; Pineau et al. 1999; Wang et al. 1999), ovary (Wright et al. 1998), stomach (Yustein et al. 1999; Baffa et al. 2000), and uterus (Fujino et al. 1994; Ahmed et al. 2000). Not unexpected, most of these regions of loss partially or completely overlap with the regions depicted in Figure 1.5. However, comparison of all data results in a complicated and rather confusing pattern. Besides experimental failures, these differences may reflect tissue specific

tumour suppressor genes. In this overview, only the smallest common deleted regions in PrCa will therefore be taken into account for further analysis.

The various regions of 8p loss were also found to correlate with tumour grade and progression (Matsuyama et al. 1994; Macoska et al. 1995; Suzuki et al. 1995; Jenkins et al. 1998; Sato et al. 1999; Matsuyama et al. 2001; Oba et al. 2001). Specifically, losses of 8p22 and 8p21.3 occur in early stage PrCa and correlate with tumour grade, indicating that these regions may harbour a tumour suppressor gene. On the other hand, deletion of 8p21.1-p21.2 occurs mainly in advanced prostate cancer, showing capsular penetration or positive nodal metastases, and was found to correlate significantly with tumour progression. In particular, deletion of 8p21.1-p21.2 was more frequently observed in PrCa cases with lymph node metastases than those without. These results implicate that this specific region may harbour a "metastatic" suppressor gene. No clinical pathological parameters were found to associate with deletions on 8p11-p12.

Functional evidence for a tumour suppressor gene at 8p involved in PrCa was initially obtained by chromosome transfer studies. Transfer of human chromosome 8p into a rat prostate cancer cell line, resulted in suppression of its metastatic capacity (Ichikawa et al. 1994; Nihei et al. 1996). Recently, Nihei et al. (2002) limited a tumour suppressor gene region to a surprisingly small region of 60 Kbp at 8p12-p21 in a chromosome transfer study. Although the human genome sequence of this region is known, the authors did not mention any candidate gene within this region. Genes located in and near this region are *RBPMS*, *1D12A*, *AK057533*, and *GTF2E2* (April 2003 freeze UCSC database). So far, none of these genes has been analysed in PrCa. No other small regions were identified in PrCa by chromosome transfer.

In a human colorectal cancer cell line, transfer of chromosome 8p22-p23 resulted in a less tumourigenic phenotype (Gustafson et al. 1996). Furthermore, transfer of 8p11.1-q11.1 restored the DNA-repair defect of murine SCID fibroblast cell lines SCVA2 and SCVA4 in response to ionising radiation (Kurimasa et al. 1994). Although both studies demonstrated reduction of tumourigenicity in a non-PrCa derived model system, the transferred tumour suppressor genes may also be involved in PrCa.

8p22-p23   The best effort to date to localize a tumour suppressor gene at 8p22-p23 was the identification of a 730-970 Kb homozygous deletion in a PrCa lymph node

metastasis (Bova et al. 1993; Bova et al. 1996). A candidate tumour suppressor gene located within this homozygously deleted region is the *N33* gene (Macgrogan et al. 1996). Although no mutations were found, *N33* transcripts were undetectable in cell lines derived from lung, liver and colon cancers (see Appendix A1 - Table I). Absence of expression was strongly correlated to hypermethylation of a CpG island in the promoter region of the *N33* gene, suggesting epigenetic inactivation of the gene. However, reintroduction of *N33* cDNA in these colorectal cancer cell lines did not change their growth or tumourigenic properties (MacGrogan and Bookstein 1997). In the PrCa derived cell lines DU-145, TSU-PR1, LNCaP, and PPC-1, *N33* inactivation was not detected. A second homozygous deletion (440-860 Kb) that partly overlapped with the deletion described by Bova et al. (1993) was found in a pancreatic tumour cell line (Levy et al. 1999). This overlap narrowed down the tumour suppressor gene region to less than 600 Kb and was found to contain, apart from *N33*, at least four ESTs that might represent a tumour suppressor gene (Bova et al. 1996; Macgrogan et al. 1996; Levy et al. 1999; Arbieva et al. 2000). To date however, no novel candidate gene has been identified in this region. Levy et al. (1999) speculated that this region of 8p22 simply is a fragile site without any biological significance for cancer cells, implicating a tumour suppressor gene elsewhere on 8p22-p23 in PrCa.

PDGFRL (platelet-derived growth factor receptor-like) is another candidate tumour suppressor gene at 8p22 (Fujiwara et al. 1995; Bova et al. 1996). However, only one missense mutation was found in a large series of prostate tumours (see Appendix A4 - Table IV), implicating a minor role for PDGFRL in PrCa. Consistent with this low mutation frequency, mutations in *PDGFRL* were not detected in the cohort of 43 prostate tumour DNA samples studied by us (Chapter 6; Van Alewijk et al., unpublished results).

A more recently identified candidate tumour suppressor gene at 8p22 is *LZTS1* (Ishii et al. 1999), which showed mutations in primary oesophageal cancers and in the prostate cancer cell line PC-3 (see Appendix A4 - Table IV). Intriguingly, *LZTS1* mRNA expression was undetectable in over 60% of epithelial tumours from various anatomical sites. Ishii et al. (1999) therefore suggested a role for LZTS1 in multiple human tumour types, including PrCa. We found down regulation of *LZTS1* expression in one xenograft, but no mutations in our cohort of 43 prostate tumour DNA samples and 15 PrCa derived xenografts and cell lines, including PC-3 (see Appendix A4 - Table IV). Although our results implicate a minor role for LZTS1 in PrCa, some support

for LZTS1 was provided by Cabeza-Arvelaiz et al. (2001). They demonstrated that transfer of YAC and BAC clones containing the *LZTS1* gene into rat prostate AT6.2 cells reduced their colony-forming efficiency. Subsequent experiments showed that over-expression of *LZTS1* cDNA inhibited colony-formation in soft agar of AT6.2, HEK-293, and LNCaP cells (Cabeza-Arvelaiz et al. 2001).

8p12-p21   Allelic imbalance studies strongly suggested that 8p12-p21 harbours a tumour suppressor gene involved in PrCa. The overall judgement of this region, however, is complicated by the heterogeneity of allelic losses (Figure 1.4). This may indicate that 8p12-p21 contains more than one tumour suppressor gene. The best evidence to date for a tumour suppressor gene in this region again is the identification of homozygous deletions. Four homozygous deletions were identified in PrCa (Kagan et al. 1995; Prasad et al. 1998; Van Alewijk et al. 1999). Kagan et al. (1995) reported two huge overlapping homozygous deletions, of 5 and 21 Mb, around the *NEFL* locus. The significance of these two homozygous deletions is unclear because of their size, but also because they are roughly defined, and because none of the deletions was flanked by a region of allelic loss. A homozygous deletion of the D8S87 locus at 8p11-p12 was reported by Prasad et al. (1998). This deletion however, needs to be defined in further detail. The only nearby located genes are *FLJ30656* and *UNC5D* (April 2003 freeze UCSC database).

The fourth homozygous deletion on 8p12-p21 was reported by our group (Chapter 4 of this thesis; Van Alewijk et al. 1999). We identified a 890 Kb homozygous deletion in a PrCa derived xenograft (Chapter 5; Van Alewijk et al., submitted). This work became feasible after the construction of a continuous high-density physical and transcript map of 8p12-p21, encompassing the region between the markers D8S87 and D8S133 (Appendix A6 of this thesis). The interest for this specific region arose from previous results, showing frequent (69%) allelic loss in PrCa DNA samples (Trapman et al. 1994). Despite the availability of many markers, no second (overlapping) homozygous deletion was found in PrCa derived xenografts and cell lines. The Werner syndrome gene (*WRN*), which is disrupted by the homozygous deletion (Chapter 4; Van Alewijk et al. 1999), encodes a protein with DNA helicase and exonuclease activity involved in maintaining the integrity of the genome (Yu et al. 1996a; Gray et al. 1997; Suzuki et al. 1999; Hickson et al., 2003). Werner syndrome (WS) is an autosomal recessive disease characterised by accelerated aging and

predisposition to rare cancers, including soft tissue sarcoma, thyroid cancer, meningioma, and melanoma (Epstein et al. 1966; Goto et al. 1996; Yu et al. 1996a; Yu et al. 1997). However, no mutations or aberrant *WRN* gene expression patterns were identified in our set of PrCa derived xenografts and cell lines (Chapter 5; Van Alewijk et al., submitted). These results suggest that functional loss of two copies of *WRN* plays a minor role in PrCa, although it may be that the loss of the *WRN* gene contributed to the growth of xenograft PC133. Except for *WRN*, no other bona fide gene has been detected in the homozygous deletion region (see also Chapter 5 of this thesis).

The location and known function of clusterin (*CLU*; Fink et al. 1993), DNA polymerase ß (*POLB*; Cannizzaro et al. 1988; Dib et al. 1995), and epoxide hydrolase 2 (*EPHX2*; Larsson et al. 1995), made these genes good candidates for tumour suppressor genes at 8p12-p21 (see Appendix A4 - Table IV). To test this hypothesis, we analysed 15 PrCa derived xenografts and cell lines, and 43 PrCa tumour samples for mutations and altered expression (Van Alewijk et al., unpublished results). In *CLU*, apart from silent polymorphisms, no sequence variations were identified. Although 2 out of 12 PrCa samples were reported to have a mutation in *POLB* (Dobashi et al. 1994), no ORF mutations were found in our cohort of 43 prostate tumour DNA samples (Chapter 6; Van Alewijk et al., unpublished results). Thus, we found no clear evidence for a role of any of these two genes in PrCa. Expression of *EPHX2* was down-regulated in 2 samples. Furthermore, 6 other samples had retained an allelic variant with a unique amino acid substitution, which was also found in their normal tissue. Although 8p12-p21 has not been recognized as an HPC region, these results suggest a predisposition for PrCa due to *EPHX2* polymorphisms. Yet, there is no further evidence for a role of EPHX2 in PrCa.

To date, the most promising candidate tumour suppressor gene at 8p12-p21 is the homeobox gene *NKX3-1* (He et al. 1997). *NKX3-1* encodes a homeodomain-containing protein related to the Drosophila *NK-3* gene family. Northern blot analysis revealed that NKX3-1 had a unique tissue expression pattern. The 3.5 Kb *NKX3-1* transcript was abundantly expressed in the prostate, at lower levels in the testis, and absent from all other tissues tested (He et al. 1997). Furthermore, expression was seen in the hormone-responsive, AR-positive PrCa cell line LNCaP, and was markedly increased upon androgen stimulation. *NKX3-1* was not expressed in either of the two AR-negative cell lines PC-3 and DU-145 (He et al. 1997). Supported by frequent allelic

loss of 8p12-p21 in PrCa and the lack of expression in PC-3 and DU-145, He et al. (1997) suggested that *NKX3-1* was a candidate tumour suppressor gene. So far, no *NKX3-1* gene mutations or homozygous deletions have been identified in PrCa (Voeller et al. 1997; Ornstein et al. 2001; Chapter 6; Van Alewijk et al., unpublished results). However, we found low *NKX3-1* expression in 4 out of 15 PrCa derived xenografts and cell lines (PC133, PC135, PC324, and PC-3) (Chapter 6; Van Alewijk et al., unpublished results). All 4 xenografts and cell lines are hormone independent growing tumours.

The NKX-like transcription factors are implicated in many aspects of cell type specification and maintenance of the functions of differentiated tissues. Sciavolino et al. (1997) demonstrated that Nkx3-1 was involved in murine prostate organogenesis (Sciavolino et al. 1997). In line with expectations, homozygous *Nkx3-1* knockout mice showed defects in prostatic ductal morphogenesis and secretory protein production (Bhatia-Gaur et al. 1999). Notably, not only homozygous but also heterozygous *Nkx3-1* mutant mice displayed prostatic epithelial hyperplasia and dysplasia that increased in severity with aging (Bhatia-Gaur et al. 1999; Kim et al. 2002a), suggesting that loss of a single *Nkx3-1* allele may be sufficient to initiate PIN-like lesions. Due to the role of Nkx3-1 in prostate organogenesis, conventional knockouts do not represent lifelike tumourigenesis. Conditional knockouts simulate a more realistic situation inducing a tumourigenic effect in normal matured tissue. Conditional Nkx3-1 inactivation in adult mice induced hyperplasia and apparent PIN lesions in the prostate (Abdulkadir et al. 2002). Evidence for actual growth-suppressing activity of Nkx3-1 was recently provided by retroviral gene transfer of exogenous *Nkx3-1* into a human (PC-3) and rodent (AT6) PrCA derived cell line (Kim et al. 2002a).

1.3.3.2.2   Chromosome 10

The picture of 10q loss in PrCa that was obtained by genome-wide CGH studies was supported by many allelic imbalance analyses. Several of these studies found additional losses of 10p, which were not seen by CGH analysis (Gray et al. 1995; Ittmann 1996; Komiya et al. 1996; Trybus et al. 1996; Cairns et al. 1997; Feilotter et al. 1998; Chapter 3; Hermans et al., submitted). Comparison of the data revealed for both the 10p- and 10q-arm at least one, possibly two, regions of loss, suggesting that each chromosome 10 arm contains one or more PrCa tumour

suppressor genes. The identified regions are 10p12, 10p14-pter, 10q23, and 10q25-q26 (Figure 1.6).

Losses of 10p and 10q are not restricted to PrCa. High frequency loss of these chromosomal arms has also been described in bladder cancer (Cappellen et al. 1997), endometrial carcinoma (Peiffer et al. 1995; Nagase et al. 1997), hepatocellular carcinoma (Fujiwara et al. 2000), glioblastoma (Karlbom et al. 1993; Rasheed et al. 1995), malignant melanoma (Herbst et al. 1994; Reiffenberger 1999), meningioma (Rempel et al. 1993), non-Hodgkin's lymphoma (Speaks et al. 1992), renal cell carcinoma (RCC; Morita et al. 1991), and small lung cell cancer (Kim et al. 1998).

In many studies, losses of 10p and 10q were found to correlate with advanced clinical stages of PrCa, particularly metastases and local recurrences (Ittmann 1996; Komiya et al. 1996; Trybus et al. 1996; Dong et al. 1998; Ittmann 1998; Alers et al. 2000; Ozen et al. 2000; Srivastava et al. 2001; Leube et al. 2002).
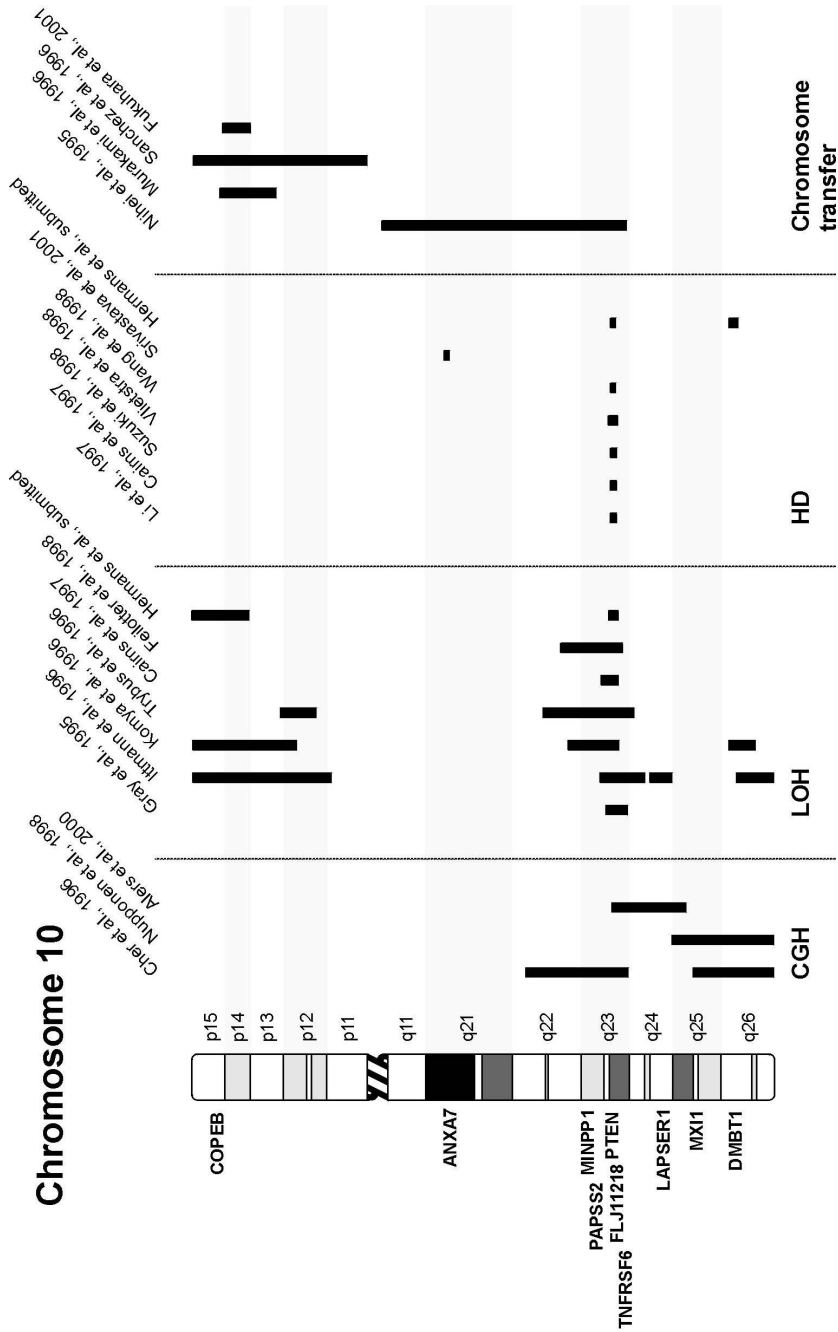
Figure 1.6. **Summary of smallest common lost regions on chromosome 10 in PrCa identified by CGH, allelic imbalance analysis (LOH), HD screening, and chromosome transfer.** Losses are depicted by black bars. Candidate tumour suppressor genes analysed in PrCa are listed at the left of chromosome 10 arranged by their chromosomal localisation (April 2003 freeze UCSC database).

10p12 and 10p14 Functional evidence for prostate tumour suppressor genes at 10p was initially obtained by chromosome transfer studies. Transfer of human chromosome 10p to human prostate cancer cell lines, resulted in suppression of their growth capacity (Murakami et al. 1996; Sanchez et al. 1996). Fukahara et al. (2001) narrowed down this tumour suppressor gene region to 1.2 Mb at 10p14 (Fukuhara et al. 2001). The authors did not refer to any candidate gene within this region. Genes located in or near this region are *GATA3* and *CUGBP2* (April 2003 freeze UCSC database). So far, none of these genes has been analysed in PrCa. Transfer of chromosome 10p14-p15 into a human glioblastoma cell line resulted in a markedly suppression of its colony forming ability in soft agar (Kon et al. 1998).

Within the overlapping region of loss at 10p12, no candidate tumour suppressor gene has been identified (Figure 1.6). The more telomeric loss at 10p14 encompasses the *COPEB* gene. This gene was found to be mutated in 55% of PrCa (Narla et al. 2001). The authors also showed that wild-type COPEB up-regulates CDKN1A in a p53-independent manner and significantly reduces cell proliferation, whereas tumour-derived *COPEB* mutants did not. Despite the high mutation frequency, the tumour suppressor gene functioning of *COPEB* has so far not been confirmed in any other study. Neither did we find any structural nor expression alterations in the panel PrCa derived xenografts and cell lines (Chapter 3; Hermans et al., submitted).

10q23 Transfer of 10q into a rat prostate cancer cell line resulted in suppression of its metastatic capacity (Nihei et al. 1995). No smaller regions of 10q were identified by chromosome transfer.

Shortly after the first allelic imbalance studies narrowed down the region of loss to 10q23 (see Fig. 1.8), the *PTEN* (phosphatase and tensin homologue deleted on chromosome 10) tumour suppressor gene was identified within this region (Li and Sun 1997; Li et al. 1997; Steck et al. 1997). Especially the finding of homozygous deletions has been pivotal in the identification of *PTEN* (Li et al. 1997; Steck et al. 1997). *PTEN* is also known as *MMAC1* (mutated in multiple advanced cancers (Steck et al. 1997) and *TEP1* (*TGF-β*-regulated and epithelial cell-enriched phosphatase (Li and Sun 1997).

The initial molecular cloning studies reported *PTEN* mutations in a large fraction of glioblastoma multiforme cell lines, xenografts, and primary tumours, as well as in smaller samples of breast and prostate cancers (Li et al. 1997; Steck et

al. 1997). Subsequent analyses confirmed that homozygotic inactivation of PTEN occurs in a large percentage of glioblastomas (at least 30% of primary tumours and 50-60% of cell lines) but not in lower-grade (i.e., less advanced) glial tumours. *PTEN* mutations also are very common in melanoma cell lines (>50%), advanced PrCa, and endometrial carcinomas (30-50%). Although *PTEN* mutations are predominantly found in advanced glial and prostate tumours, mutations occur with equal frequency at all stages of endometrial cancer, suggesting that *PTEN* activation is an early event in endometrial carcinogenesis. A significant percentage (~10%) of breast cancer cell lines has inactivated *PTEN. PTEN* mutations are rare in sporadic breast tumours, independent of severity. Although germ-line *PTEN* mutations lead to predisposition to breast cancer, *PTEN* mutations are not a frequent cause of familial breast cancer. Occasional *PTEN* mutations have been reported in head and neck cancers and in thyroid cancers (Cantley and Neel 1999).

Germ-line mutations in *PTEN* have been detected in Cowden disease, Lhermitte-Duclos disease, and Bannayan-Zonana syndrome, all of which are rare diseases with an autosomal dominant inheritance pattern. These syndromes are associated with hamartomas of the skin, thyroid, breast, oral mucosa, and intestine, and a predisposition to cancer (Cantley and Neel 1999).

Thus far, *PTEN* is the most frequently mutated tumour suppressor gene in PrCa. Li et al. (1997) reported homozygous deletions of *PTEN* in 2 out of 4 PrCa cell lines. We found a high frequency (60%) of *PTEN* deletions (5 out of 15) and mutations (4 out of 15) in PrCa derived xenografts and cell lines (Chapter 2; Vlietstra et al. 1998). *PTEN* mutations are most common in metastatic PrCa (30-60%; Cairns et al. 1997; Suzuki et al. 1998b), but less frequent in primary PrCa, (5-15%; Cairns et al. 1997; Teng et al. 1997; Feilotter et al. 1998; Whang et al. 1998). This implies that complete PTEN inactivation occurs mainly in advanced stage PrCa, concordant with 10q loss.

PTEN encodes a widely expressed 5.5-kb mRNA. The protein consists of 403 amino acids, it contains a catalytic domain of lipid and protein phosphatases and shows homology to the cytoskeletal proteins tensin and auxilin (Li and Sun 1997; Li et al. 1997; Steck et al. 1997). PTEN is involved in regulation of cell proliferation, cell survival, cell size, and chemotaxis (Figure 1.9; reviewed by Comer and Parent 2002).
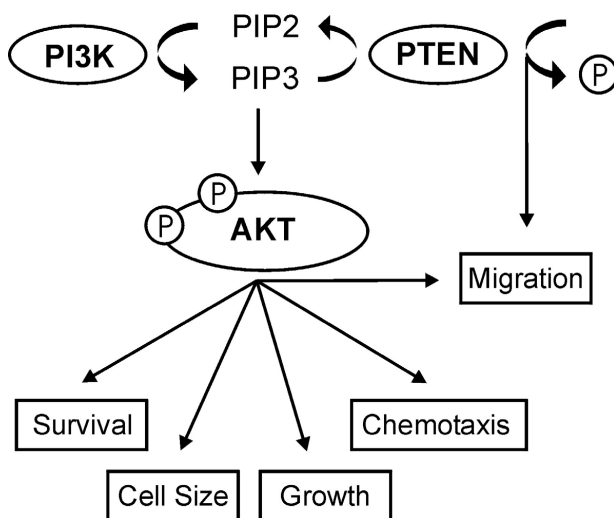
Figure 1.9. **Mechanisms of PTEN action.**

Although *PTK2* (protein tyrosine kinase 2) and the adaptor protein *Shc* have been postulated as targets of the weak *in vivo* protein phosphatase activity of *PTEN* (Gu et al. 1998; Tamura et al. 1998), the most prominent function of PTEN is the negative control of phosphoinositidyl 3-kinase (PI3K) signaling by dephosphorylation of the phospholipid PIP3 (phosphoinositol-3-phosphate; Maehama and Dixon 1998; Myers et al. 1998; see for recent reviews Cantley and Neel 1999; Dahia 2000). A prominent downstream target of PIP3 is AKT/PKB, which subsequently phosphorylates a large variety of target proteins, including BAD, CASP9, CREB, eNOS, FOXO3A, GSK3, IKK, MDM2, FRAP1, and CDKN1A. These targets have a characteristic R-x-R-xx-S/T-F/L – motif that is phosphorylated at the serine or threonine residues by activated AKT. Several of the AKT target proteins are involved in more than one of the biological functions of PTEN. The functioning of AKT is further complicated by cross-talk of some of its downstream targets with other tumour related pathways (e.g. TP53-, and WNT-pathway). In addition to the regulating mechanisms as mentioned above *PTEN* is thought to affect cell migration. Initially, FAK and Shc have been described as mediators for migration. However, evidence is growing that other downstream targets of Pi3k, like Rac / Cdc42, which might function upstream of Akt, have a more prominent role in cell migration (Higuchi et al. 2001). Little is known about the expression of *PTEN* in PrCa. Downregulation of *PTEN* expression was shown by McMenamin et al. (1999).

Recently, Yang et al. (2002) described an inverse correlation between PTEN (+) and CDKIN1B (+) expression, and the expression of the F-box protein Skp2 (-) (Yang et al. 2002).

Functional evidence for a role of PTEN in PrCa was primarily provided by Nihei et al. (1995), who showed that transfer of chromosome 10, particularily 10cen-q23 (including *PTEN*), suppressed the metastatic ability of a rat prostate cancer cell line (Nihei et al. 1995). Ever since, several studies provided direct and indirect evidence for a role of PTEN in PrCa. Transfer of *PTEN* cDNA into PC-3 cells induced G1 cell-cycle arrest, enhanced apoptosis, and reverted their invasive phenotype (Persad et al. 2000; Kotelevets et al. 2001). Accordingly, adenovirus-mediated expression of *PTEN* was found to inhibit the *in vitro* growth capability of PC-3 cells, primarily by blocking cell cycle progression. *In vivo* these transfected cells did not lose their tumourigenicity, but showed a significant reduction in tumour size and complete loss of their metastatic ability (Davies et al. 2002). In LNCaP cells, adenovirus-mediated expression of *PTEN* or cDNA transfection was found to negatively regulate the PI3K/AKT pathway and enhance apoptosis (Wu et al. 1998; Yuan and Whang 2002). Furthermore, *PTEN* was found to induce chemosensitivity in LNCaP cells by suppression of *BCL-2* expression. Specifically, the lipid-phosphatase activity of PTEN was found to be required for the inhibition of BCL-2. This inhibiting activity was blocked by overexpression of a constitutively active form of AKT (Huang et al. 2001).

Intriguingly, part of the losses at 10q23 included the *PTEN* gene, yet mutations were not identified in the remaining *PTEN* allele (Cairns et al. 1997; Teng et al. 1997; Feilotter et al. 1998; Whang et al. 1998). These observations suggested that a second tumour suppressor gene located nearby *PTEN* might be involved in PrCa. Alternatively, this could point to *PTEN* haplo-insufficiency in tumour growth. Nearby candidate tumour suppressor genes are *MINPP1, PAPSS2*, and *TNFRSF6* (see Appendix A5 - Table V). *MINPP1*, whose function overlaps with that of PTEN, had a missense mutation in a follicular thyroid tumour (Gimm et al. 2001). Gimm et al. (2001) therefore suggested a role for *MINPP1* in the pathogenesis of at least a subset of malignant follicular thyroid tumours. In a large inbred Pakistani family with severe chondrodysplasia, a nonsense mutation was found in *PAPSS2* (ul Haque et al. 1998). *TNFRSF6* was found to be inactivated by DNA methylation in a limited number of PrCa and advanced bladder carcinomas (Santourlidis et al. 2001). Additionally, specific inherited *TNFRSF6* mutations were correlated with a high risk factor for non-

Hodgkin and Hodgkin lymphomas (Straus et al. 2001). We screened 13 genes mapping in a 3 Mbp region around *PTEN* for expression alterations (Chapter 3; Hermans et al., submitted). Additionally, the genes *MINPP1, PAPSS2, FLJ11218* and *TNFRSF6*, were selected on the basis of altered expression, frequent deletion or by virtue of their function (see also Appendix A5 - Table V) for a mutation screen in our panel of xenografts and cell lines. Especially, *PAPSS2* and *FLJ11218* appeared interesting due to altered expressions, polymorphisms, a few missense mutations and a nonsense mutation. However, the functional relevance of these alterations in PrCa remains to be established.

Homozygous *Pten* knockout mice are embryonically lethal (Di Cristofano et al. 1998b; Suzuki et al. 1998a; Podsypanina et al. 1999). Heterozygous *Pten* knockout mice showed hyperplastic and dysplastic changes in the prostate, skin, and colon, which are characteristic for Cowden disease, Lhermitte-Duclos disease, and Bannayan-Zonana syndrome (Di Cristofano et al. 1998a). These mice also spontaneously developed germ cell, gonadostromal, thyroid, colon, T-cell and breast tumours. Analysis of PrCa progression in transgenic adenocarcinoma of mouse prostate (TRAMP) model mice crossed with *Pten* (+/-) heterozygous mice, revealed that haplo-insufficiency of the *Pten* gene promoted the progression of PrCa (Kwabi-Addo et al. 2001). Evidence is growing that not only in PrCa but also in other human malignancies loss of one *PTEN* allele can contribute to tumourigenesis (Velickovic et al. 2002; Byun et al. 2003).

10q25-q26 A fourth region of frequent loss of chromosome 10 includes 10q25-q26, which indicates a role for an additional 10q gene in PrCa (Cairns et al. 1997; Feilotter et al. 1998). This chromosomal arm harbours the candidate tumour suppressor genes *MXI1* (10q25.2), and *DMBT1* (10q26.1), which have been suggested to be involved in PrCa or other tumours (*MXI*: Eagle et al. 1995; Wechsler et al. 1996; Prochownik et al. 1998) (*DMBT1*: Mollenhauer et al. 1997; Somerville et al. 1998; Mori et al. 1999; Takeshita et al. 1999; Wu et al. 1999). In accordance with Gray et al. (1995), Kawamata et al. (1996), and Kuczyk et al. (1998), who doubted the role of *MXI1* in PrCa (Gray et al. 1995; Kawamata et al. 1996; Kuczyk et al. 1998), we did not find any altered expression or structure of *MXI1* (Chapter 3; Hermans et al., submitted). In one of our xenografts, analysis of *DMBT1* revealed an internal homozygous deletion, including a repetitive region from which several *DMBT1* variants are derived (Mollenhauer et al. 1999;

Chapter 3; Hermans et al., submitted). Although this deletion resulted in a shortened protein, its functional effect is as yet unknown (Mollenhauer et al. 1999). Down-regulation of *DMBT1* expression was found in lung, oesophageal, gastric and colon cancers, implicating a general role in cancer (Mori et al. 1999; Takeshita et al. 1999). In addition to these studies, we found expressional down-regulation in 2 samples of our panel of xenografts and cell lines (Chapter 3; Hermans et al., submitted).

1.4 SCOPE OF THIS THESIS

The aim of this thesis was the isolation and characterization of tumour suppressor genes located on chromosome 8p and 10q. Chapter 1 is a general introduction of PrCa, genetics of cancer, and genetic alterations in PrCa. Chapter 2 describes the expression and structural analysis of the *PTEN* gene in PrCa xenografts and cell lines. Chapter 3 describes the genetic analysis of chromosome 10. It also describes a refined characterization of the previously identified homozygous deletions disrupting the *PTEN* gene (Chapter 2), including the expression and structural analysis of genes flanking *PTEN*. Finally, chapter 3 describes the expression and structural analysis of the candidate tumour suppressor genes *MXI1* and *DMBT1* that are located on 10q. Chapter 4 describes the identification and characterization of a homozygous deletion at 8p12-p21 in xenograft PC133. Chapter 5 describes the genetic analysis of chromosome 8 in PrCa xenografts and cell lines using CGH and allelotyping. It further describes the screen for overlapping homozygous deletions and search for genes in the previously identified homozygous deletion. In addition, this chapter describes the expression and structural analysis of *WRN*. Chapter 6 describes the high-density screen for an homozygous deletion elsewhere on 8p12-p21 and the expression and structural analysis of the candidate tumour suppressor genes *LZTS1, NKX3-1,* and *EXPHX2* that are located on 8p. Chapter 7 discusses the results described in this thesis, its implications for PrCa and future directions of research.

CHAPTER 2

FREQUENT INACTIVATION OF *PTEN* IN PROSTATE CANCER CELL LINES AND XENOGRAFTS

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ABSTRACT

Loss of chromosome 10q is a frequently observed genetic defect in prostate cancer. Recently, the *PTEN/MMAC1* tumor suppressor gene was identified and mapped to chromosome 10q23.3. We studied *PTEN* structure and expression in four *in vitro* cell lines and eleven *in vivo* xenografts derived from six primary and nine metastatic human prostate cancers. DNA samples were allelotyped for eight polymorphic markers within and surrounding the *PTEN* gene. Additionally, the nine *PTEN* exons were tested for deletions. In five samples (PC3, PC133, PCEW, PC295, PC324) homozygous deletions of (parts of) the *PTEN* gene were detected. PC295 contained a small homozygous deletion, encompassing *PTEN* exon 5. In two DNAs (PC82 and PC346), nonsense mutations were found, and in two (LNCaP and PC374), frame-shift mutations were found. Missense mutations were not detected. *PTEN* mRNA expression was clearly observed in all cell lines and xenografts without large homozygous deletions, showing that *PTEN* down-regulation is not an important mechanism of *PTEN* inactivation. The high frequency (60%) of *PTEN* mutations and deletions indicates a significant role of this tumor suppressor gene in the pathogenesis of prostate cancer.

INTRODUCTION

Prostate cancer is the most frequently diagnosed tumor in men in the United States and in western and northern Europe, and the second leading cause of male cancer death (Parker et al. 1997). The molecular events leading to the development and the progressive growth of prostate cancer are poorly understood. The most frequent chromosomal aberrations are losses of chromosomes 8p, 10q, 13q and 16q (Bergerheim et al. 1991; Visakorpi et al. 1995; Cher et al. 1996), indicating the localization of tumor suppressor genes at these chromosomal sites. Detailed allelotyping implicated deletion of chromosome 10 region 10q23-25 in prostate cancer (Gray et al. 1995; Komiya et al. 1996; Li et al. 1997).

Recently, the *PTEN* gene, also known as *MMAC1* or *TEP1*, which is located at 10q23.3, has been found to be frequently mutated or deleted in glioblastomas (Li and Sun 1997; Li et al. 1997; Steck et al. 1997). *PTEN* encodes a dual-specific phosphatase and shows homology to the cytoskeletal proteins tensin and auxilin (Li and Sun 1997; Li et al. 1997; Myers et al. 1997; Steck et al. 1997). Structural analysis revealed mutations

of *PTEN* in many different tumor types. A high proportion of mutations was not only demonstrated in glioblastomas (Li et al. 1997; Rasheed et al. 1997; Steck et al. 1997; Wang et al. 1997) but also in endometrial carcinomas (Risinger et al. 1997; Tashiro et al. 1997). Germ-line mutations in *PTEN* have been detected in Cowden disease, an autosomal dominant cancer predisposition syndrome, associated with an increased risk of breast, skin, and thyroid cancer (Liaw et al. 1997; Nelen et al. 1997).

PTEN has also been implicated in prostate cancer. Li et al. (Li et al. 1997) described two homozygous deletions and one frame-shift mutation in prostate cancer cell lines. More recently, *PTEN* mutations and deletions were reported in metastatic prostate cancers (Cairns et al. 1997; Suzuki et al. 1998b). In primary tumors, *PTEN* mutations were less frequent (Cairns et al. 1997; Teng et al. 1997).

Detailed molecular genetic analysis of prostate cancer DNA and gene expression is complicated due to the contamination by normal cells. In this study, we demonstrate a high proportion of *PTEN* mutations and deletions in fifteen prostate cancer xenografts and cell lines. We characterized these mutations, and studied *PTEN* mRNA expression.

MATERIALS AND METHODS

Prostate Tumor Cell Lines and Xenografts.

The *in vitro* growing cell lines LNCaP, PC3, DU145 and TSU were cultured under standard conditions. *In vivo* xenografts PC82, PCEW, PC133, PC135, PC295, PC310, PC324, PC329, PC339, PC346 and PC374 were propagated in male nude mice (Noordzij et al. 1996; Van Weerden et al. 1996; and references therein).

DNA Preparation.

DNA from cell lines was isolated according to standard procedures (Sambrook et al. 1989). Genomic DNA from xenografts was isolated from 5 consecutive 5-µm cryostat tissue sections by overnight proteinase K incubation at 55°C, followed by phenol extraction and ethanol precipitation. DNA pellets were dissolved in TE [10 mM Tris.HCl (pH7.8)-1 mM EDTA].

<u>Allelotyping.</u>

DNAs from prostate cancer cell lines and xenografts were allelotyped by PCR amplification of eight polymorphic markers within or flanking the *PTEN* locus at chromosome 10q23.3: *D10S1687*, *D10S579*, *D10S215*, *PTENCA*, *AFMa086WG9*, *D10S541*, *D10S1753* and *D10S583*. *PTENCA* primers were from Ref. 12; other markers are described in Genome Data Base or in the Genethon database. PCR amplifications, using Taq polymerase (Promega, Madison, WI) were for 30 cycles of 1 min at 94°C, 1.5 min at 50°C or 55°C and 1.5 min at 72°C in a 15 µl reaction volume, containing 1.5 mM MgCl$_2$ and 1 µCi [alpha-^{32}P]dATP (Amersham, Buckinghamshire, UK). The radio-labelled PCR products were separated over a 6% polyacrylamide sequencing gel.

<u>Screening for Homozygous Deletions of *PTEN*.</u>

PTEN exons and flanking sequences were PCR amplified according to standard protocols: 30 cycles of 45 s at 94°C, 45 s at 50°C (exons 1 to 6) or 55°C (exons 7 to 9) and 45 s at 72°C in the presence of 1.5 mM (exons 1 to 6) or 2.0 mM MgCl$_2$ (exons 7 to 9). Exons 5 and 8 were amplified as two overlapping fragments. Primers were from (Steck et al. 1997) (1R, 5F1, 5R2, 7F and 7R, 8R2), and (Guldberg et al. 1997) (1F, 2F and 2R, 3F and 3R, 4F and 4R, 5R1 and F2, 6F and 6R, 9F and 9R). Primers 8R1 (5-CTTGTCATTATCTGCACGCT-3) and 8F2 (5-GAAAATGGAAGTCTATGTG-3) are novel. Control PCRs were with primers 791B9L-A (5-GAAGGTGGCAGTCTGATCTC-3) and 791B9L-B (5-GCAACTGGTTGAAACATACTC-3), which amplify a 410 bp fragment at chromosome 8p12-p21. Amplified products were separated over a 2% agarose gel.

<u>RT-PCR Analysis of *PTEN* Expression.</u>

RNA was isolated by standard guanidium isothiocyanate (cell lines) or LiCl (xenografts) protocols (Sambrook et al. 1989). cDNA synthesis, followed by PCR amplifications with *PTEN*-specific primers were performed on 500 ng RNA in the Access RT-PCR system (Promega) according to the instructions of the manufacturer. Primers used for cDNA synthesis were as follows: 9R-cDNA, 5-GGATGTGAACCAGTATATCACAA-3 (Fig. 3A); and 7R-cDNA, 5-CCGTCGTGTGGGTCCTGAATTAA-3 (Fig. 3B). cDNA synthesis was for 45 min at 48°C; amplification: 35 cycles of 30 sec at 94°C, 30 sec at 60°C, and 1 min at 68°C. Primer combinations for PCR amplification of cDNAs: 9R-cDNA and 8F-cDNA (5-AGCCAACCGATACTTTTCTCC-3), and 7R-cDNA and 1F-cDNA (5-

CCACCAGCAGCTTCTGCCATCTCT-3). Control RT-PCRs were carried out under the same conditions, using RNA polymerase II-specific primers: PolF-cDNA (5-GCTGAGAGAGCCAAGGATAT-3) and PolR-cDNA (5-CACCACCTCTTCCTCCTCTT-3). RT-PCR products were separated over a 2% agarose gel.

<u>PCR-SSCP Analysis.</u>

Fragments for PCR-SSCP analysis were obtained for all exons of the *PTEN* gene, utilizing primer sets and PCR conditions described above. PCR reactions were in a 15-µl volume in the presence of 1 µCi [alpha-^{32}P]dATP. Appropriate aliquots of the radiolabelled PCR products were separated over a 6% non-denaturing polyacrylamide gel containing 5 or 10% glycerol at 7 W overnight at room temperature.

<u>Structural Analysis.</u>

RT-PCR fragments and amplified exons were purified over QIAquick spin columns (Qiagen, Hilden, Germany), cloned into pGEM-T Easy (Promega) and sequenced according to the dideoxy chain termination method.

RESULTS

<u>Allelotyping of Prostate Cancer Cell Lines and Xenografts for Chromosome 10q23.</u>

Genomic DNAs from 11 prostate cancer xenografts and 4 *in vitro* propagated cell lines were allelotyped for eight highly polymorphic markers, spanning ~9 cM around the *PTEN* locus at chromosome 10q23 (*D10S1687, D10S579, D10S215, PTENCA, AFMa086wg9, D10S541, D10S1753,* and *D10S583*). The results are summarized in Fig. 1. For most markers, especially those mapping at 114 cM, only one amplified band was detected, suggesting hemizygosity. In DNAs from three xenografts (PC133, PC324 and PCEW) and one cell line (PC3), two or more polymorphic markers were completely deleted (see also (Li et al. 1997) for PC3 deletion). All four samples were negative for *AFMa086wg9*, which is situated at 114 cM, between exons 2 and 3 of the *PTEN* gene. Therefore, these tumors are expected to be completely or partially defective of *PTEN*. The homozygous deletion in PC3 is relatively large (>5 cM), other homozygous deletions seem to be much smaller.

Homozygous Deletion of the *PTEN* Gene.

To confirm *PTEN* losses and to determine more precisely the borders of the homozygous deletions, all nine exons of the *PTEN* gene, including flanking sequences, were individually amplified. Examples of exons 5 and 8 amplifications are shown in Fig. 2A; results are summarized in Fig. 2B. The four tumors that lacked polymorphic marker *AFMa086wg9* showed complete or partial deletion of *PTEN* (Fig. 2A, *Lanes 1,3,7* and *13*). In PC133 and PCEW, *PTEN* was completely deleted; in PC324, *PTEN* exons 2-9, and in PC3 exons 3-9 were absent (Fig. 2B). Importantly, PC295 contained a very small homozygous deletion, encompassing only *PTEN* exon 5, which could not be detected by allelotyping (Fig. 2A, *Lane 5*).

position →	D10S1687 (0.81)	D10S579 (0.59)	D10S215 (0.80)	PTEN CA	AFMa086WG9	D10S541 (0.78)	D10S1753 (0.74)	D10S583 (0.84)
	113cM	114cM	114cM	114cM	114cM	114cM	119cM	122cM
PC 3	1	1	1	1	0	0	0	1
PC 82	2	1	1	1	1	1	2	2
PC 133	1	1	1	0	0	0	1	1
PC 135	2	1	1	1	1	1	1	2
PC 295	1	1	1	1	1	1	1	1
PC 310	2	1	2	2	1	2	2	2
PC 324	2	1	1	1	0	0	2	1
PC 329	2	1	2	1	1	2	1	1
PC 339	2	2	2	1	1	1	1	1
PC 346	2	1	1	1	1	1	2	2
PC 374	1	1	1	1	1	1	1	1
DU 145	2	1	1	1	1	1	1	1
PCEW	2	0	0	0	0	1	1	1
LNCaP	1	1	1	1	1	1	1	1
TSU	1	1	1	1	1	1	1	1

Figure 1. **Allelotyping of prostate cancer cell lines and xenografts at chromosome 10q23.3.** Markers are described in more detail in Materials and Methods. Numbers between brackets indicate heterozygosity indices. Heterozygosity indices are not available for *PTEN CA* and *AFMa086WG9*. Genetic map positions of markers are from contig 10.7 of the Whitehead Institute map. The number of bands detected are indicated in each box (0, 1 or 2). Homozygous deletions are marked by shaded boxes.

A

B

	E1	E2	E3	E4	E5	E6	E7	E8	E9
PC 3	+	+	-	-	-	-	-	-	-
PC 82	+	+	+	+	+	+	+	+	+
PC 133	-	-	-	-	-	-	-	-	-
PC 135	+	+	+	+	+	+	+	+	+
PC 295	+	+	+	+	-	+	+	+	+
PC 310	+	+	+	+	+	+	+	+	+
PC 324	+	-	-	-	-	-	-	-	-
PC 329	+	+	+	+	+	+	+	+	+
PC 339	+	+	+	+	+	+	+	+	+
PC 346	+	+	+	+	+	+	+	+	+
PC 374	+	+	+	+	+	+	+	+	+
DU 145	+	+	+	+	+	+	+	+	+
PCEW	-	-	-	-	-	-	-	-	-
LNCaP	+	+	+	+	+	+	+	+	+
TSU	+	+	+	+	+	+	+	+	+
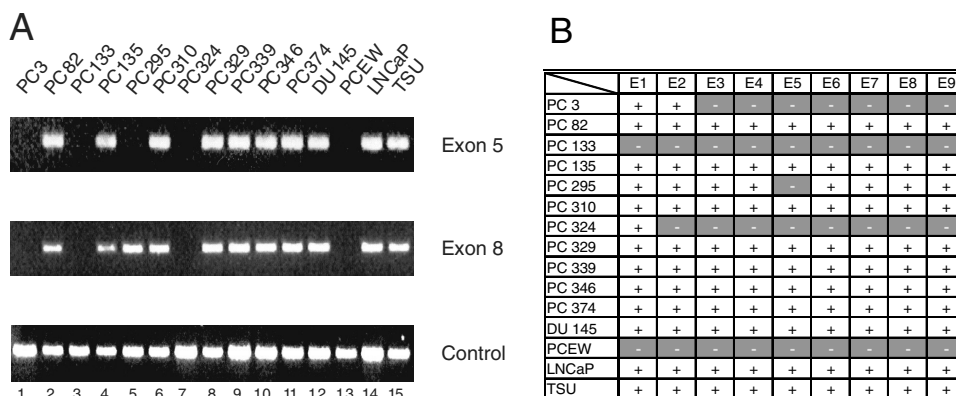
Figure 2. **Deletion analysis of *PTEN* in prostate cancer cell lines and xenografts.** (A) Agarose gel electrophoresis of PCR amplified exons 5 and 8. The control is YAC end fragment 791B9L, located at chromosome 8p12-p21. (B) Overview of the results obtained for all *PTEN* exons. Deleted exons are indicated by a minus in the shaded boxes.

Expression of *PTEN*.

Because of the absence of contaminating normal cells of human origin, xenografts and cell lines are preferable sources for the study of *PTEN* mRNA expression. In all RNA preparations from cell lines and xenografts without homozygous deletion, *PTEN* mRNA was easily detectable by RT-PCR, using human *PTEN* specific primers for amplification (Fig. 3A). In PC133 and PCEW, *PTEN* mRNA expression could not be visualized (Fig. 3A lanes 4, 15). However, PC3 and PC324, which also lack the *PTEN* gene showed a faint amplified band of the appropriate length (620 bp), which hybridized to a *PTEN* specific probe (data not shown). Sequencing of the RT-PCR product from PC324 and PC3 revealed that it was not identical to *PTEN,* but to a highly homologous processed *PTEN* pseudogene, located in a duplicated region of chromosome 7 to chromosome 9, near the T-cell receptor beta locus (GenBank, AF029308) (data not shown). This band was not detectable in controls, in which reverse transcriptase was omitted from the reaction mixture (data not shown). Therefore, we concluded that in PC324 and PC3 the pseudogene was expressed at a low level. Because *PTEN* pseudogene expression was low, or undetectable in the four tumors with chromosome 10q23 homozygous deletions, we presumed that the RT-PCR product from *PTEN* positive tumors was derived from the original *PTEN* gene. This was checked for PC295 (see below) and PC135. *PTEN* mRNA expression in PC135 is relatively low.

Digestions by slected restriction enzymes showed that most, if not all of the fragment amplified by RT-PCR was derived from wild type *PTEN* (data not shown).

Sequencing of a 530 bp exon 1 to exon 7 RT-PCR product of PC295 *PTEN* mRNA, confirmed the complete absence of exon 5 sequences in the transcript (Fig. 3B, and data not shown). The co-amplified 760 bp fragment was not derived from the human *PTEN* mRNA or the pseudogene, but from *PTEN* mRNA of mouse cells present in the transplanted tumor (data not shown).
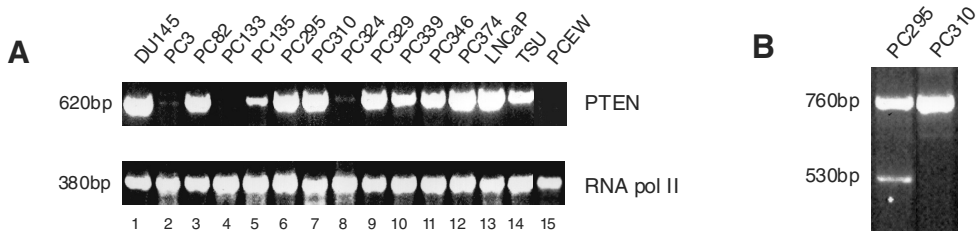
Figure 3. ***PTEN* mRNA expression in prostate cancer cell lines and xenografts. (A)** Agarose gel analysis of a 620 bp RT-PCR amplified fragment. Primers used are located in exons 8 and 9, and are specific for human *PTEN*. **(B)** *PTEN* RT-PCR analysis of PC295 RNA. Primers used are located in exons 1 and 7. PC310 RNA was used as a control. Primers detect both human and mouse *PTEN* mRNA.

Structural Analysis of the *PTEN* Gene.

From *PTEN* positive DNAs, all nine exons were analyzed for mutations by PCR-SSCP. Aberrant SSCP bands were sequenced. In four DNA samples, *PTEN* mutations were unambiguously established (PC82, PC346, PC374 and LNCaP). The exon 1, codon 6 AAA to A frame-shift mutation in LNCaP has been described previously (Li et al. 1997; Steck et al. 1997). The other three mutations are depicted in Fig. 4. PC82 *PTEN* contained a CAA to TAA nonsense mutation at codon 87; PC346 a CGA130TGA nonsense mutation, and PC374 a TAT76T frame-shift, directly resulting in a TGA stop codon at position 76. All mutations will lead to the synthesis of a truncated protein, lacking the phosphatase domain. In none of the sequenced samples the corresponding wild type sequence was detected, confirming that the second *PTEN* allele was deleted. Both LNCaP and TSU *PTEN* were found to contain GGT(Gly) instead of GGC(Gly) at codon 44. This presumed rare polymorphism could not be detected in 34 control DNAs from healthy individuals (data not shown). The previously described ATG134TTG missense mutation in DU145 *PTEN* could not be confirmed (Li et al. 1997).
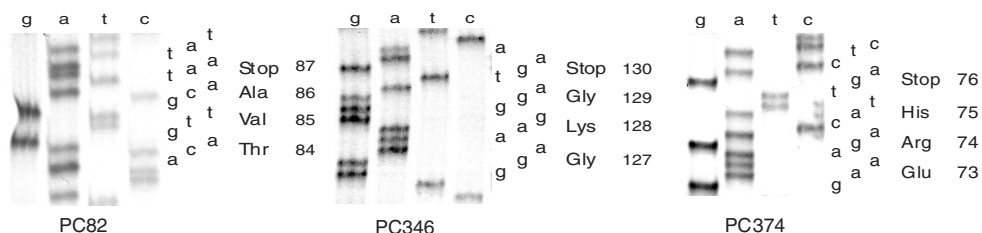
Figure 4. **Sequence analysis of *PTEN* mutations in prostate cancer xenografts.** In PC82 and PC346 nonsense mutations were detected; PC374 shows a TA deletion at codon 76, resulting in a TGA stop codon.

DISCUSSION

In this study we describe five homozygous deletions, two frame-shift mutations and two nonsense mutations in fifteen prostate cancer cell lines and xenografts. Homozygous deletions were established by the complete absence of polymorphic markers in the *PTEN* region and by failure of PTEN exon amplifications. RT-PCR showed *PTEN* expression in all tumors, in which the gene was not deleted.

The four tumors containing mutated *PTEN* (PC82, PC346, PC374 and LNCaP) showed one amplified band for all five polymorphic markers at 114 cM, suggesting deletion of one chromosome 10 copy at these sites. Absence of wild type *PTEN* at the mutated site confirmed this observation. Complete or almost complete deletion of both *PTEN* copies was detected in five tumor samples. The homozygous deletion in PC3 is large [>5cM, Fig. 1; see also (Li et al. 1997)], that in PC295 is probably less than 20 Kbp as judged from preliminary data of the lengths of introns 4 and 5.[5] In PC133, PC324 and PCEW, two or more markers at 114 cM are deleted. Minimal deleted regions in these tumors are estimated to be at least 100 Kbp.

Five out of six tumors, in which we could not find *PTEN* homozygous deletions or mutations (PC135, PC329, PC339, TSU and DU145), showed apparent allelic losses for the markers *PTENCA* and *AFMAa086WG9*, which are located within the *PTEN* gene. Three of these DNAs even showed one band for all five markers that map at 114 cM (Fig. 1). Although it cannot be completely ruled out, that a small proportion of point mutations in *PTEN* was missed, it is also possible that in these tumors a second *PTEN* deletion or mutation has not occurred, or that a gene different from *PTEN* is mutated. For

tumors with a large deletion, the *MXI1* gene at 10q24-25 might be a candidate, as suggested previously (Eagle et al. 1995). However, others exclude a major role of *MXI1* in prostate cancer (Gray et al. 1995).

Additionally to deletions and mutations, down-regulation of expression by promoter methylation, mutation or other processes can be a mechanism of gene inactivation. Xenografts and cell lines have the advantage that mRNA expression can easily be monitored. The RT-PCR experiments (Fig. 3A) clearly showed that down-regulation of *PTEN* expression is not a common mechanism of *PTEN* inactivation. An exception might be PC135, which shows a considerably lower *PTEN* mRNA level than the other samples. Additional experimental evidence must be collected to prove the physiological implication of this observation. The processed *PTEN* pseudogene, which most likely is expressed at a low level complicates expression studies. Previously, it has mistakenly been described as a mutated *PTEN* gene in breast cancer (Rhei et al. 1997).

Mutations or deletions were found in six out of nine xenografts/cell lines derived from metastatic sites and in three out of six, derived from primary tumors, obtained by prostatectomy or transurethral resection. Original tumor DNAs were available for PC346 (primary tumor) and PC374 (metastasis). In both samples the mutations as shown in Fig. 4 could be confirmed (data not shown), indicating that they were not introduced during propagation in nude mice.

During the course of our study, *PTEN* mutations were reported in primary and metastatic prostate cancer tissues (Cairns et al. 1997; Teng et al. 1997; Suzuki et al. 1998b). In the study of Cairns et al. (Cairns et al. 1997), in 3/60 primary cancers and in 7/20 lymph node metastases mutations or deletions were found. Teng et al. (Teng et al. 1997) could not detect *PTEN* mutations in six primary tumors; Suzuki et al. (Suzuki et al. 1998b) described *PTEN* abnormalities in twelve of nineteen tumors obtained from metastatic sites during autopsy. These numbers might be underestimations, because small deletions would have been missed. Like found in the present study, deletions, point mutations and frame-shifts were detected. In prostate cancer, *PTEN* mutations lead, almost without exception, to the synthesis of a truncated protein. The CGA130TGA nonsense mutation and the TAT76T frame-shift have now been reported in different studies in both glioblastomas and prostate cancer, indicating hot spots of mutation (Rasheed et al. 1997; Steck et al. 1997; Teng et al. 1997; Wang et al. 1997 and this study).

Most frequently, *PTEN* aberrations were found in DNAs from metastatic disease (Cairns et al. 1997; Suzuki et al. 1998b and this study). However, *PTEN* abnormalities do also occur at the primary tumor site (Cairns et al. 1997). Not unexpected, in the cell lines and xenografts derived from the primary tumor site, as studied here, the percentage of *PTEN* abnormalities is much higher than that found at the primary tumor site by Cairns et al. (Cairns et al. 1997) (3/6 and 3/60, respectively). The difference might be explained by a growth advantage of the original xenografted material in case of the absence of functional *PTEN*.

Because *PTEN* is frequently completely absent or severely truncated, immunohistochemical staining, utilizing specific antibodies could be applied to address in more detail the question whether *PTEN* is a tumor progression marker. Specific antibodies are also additional tools for the detection of the complete spectrum of *PTEN* mutations and deletions in patient samples, including small deletions such as found in PC295. Both complete absence and aberrant cellular distribution of the PTEN protein can be expected.

So far, *PTEN* is the most widely mutated tumor suppressor gene in prostate cancer. Therefore, further elucidation of its function in prostate cancer is of utmost importance.

ACKNOWLEDGMENTS

CHAPTER 3

CHROMOSOME 10 ALTERATIONS IN PROSTATE CANCER XENOGRAFTS AND CELL LINES: CANDIDATE TUMOUR SUPPRESSOR GENES AND THE *PTEN* FLANKING GENES

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ABSTRACT

Detailed genetic studies are a prerequisite in deciphering the molecular mechanisms of tumour cell growth. Xenografts and cell lines are excellent starting materials for such studies. We examined 11 xenografts and 4 cell lines derived from human prostate cancers for chromosome 10 alterations. Comparative Genomic Hybridisation (CGH) revealed a pattern of loss of distal 10p (3/15), gain of proximal 10p and proximal 10q (5/15), and loss of distal 10q (6/15). Allelotype analysis confirmed the CGH data in all samples, which contained 10p deletions (PC310, PC324 and PC3) and in 5 samples with large 10q deletions (PC133, PC295, PC339, PC374, PC3). Previously, we identified *PTEN* inactivation by homozygous deletion or mutation in 9 of the 15 xenografts and cell lines (Vlietstra et al. 1998). The present study excludes *MXI1* and *DMBT1* on distal 10q, and *KLF6* on distal 10p as important tumour suppressor genes in prostate cancer. Remarkably, study of the *PTEN* flanking region by allelotype analysis frequently predicted a very small region of allelic loss of 5.8 Mbp or less with or without *PTEN* inactivation. These regions were not detected by CGH. Sizes of homozygous deletions around *PTEN* ranged from approximately 1.2 Mbp (PC133) to less than 30 Kbp (*PTEN* exon 5 in PC295). In both *PTEN* positive and *PTEN* negative samples, 16 genes mapping in the *PTEN* region, were investigated for deletions and expression levels. Loss of 1 or 2 copies of *PTEN* was almost always accompanied by loss of the distal flanking gene *FLJ11218* and the proximal flanking genes *MINPP1, PAPSS2* and *FLJ14600.* Furthermore, differential expression was detected for *FLJ11218* and *PAPSS2*. Four genes were selected for mutation analysis, *MINPP1*, which like *PTEN* metabolises phospholipids, the sulfatase *PAPSS2*, the tumour necrosis factor receptor *TNFRSF6* and *FLJ11218*. Complete deletion or inactivating mutation of *PAPSS2* was found in at least 3 samples. *MINPP1* was deleted in PCEW, but not mutated in other samples. *TNFRFS6* structure was normal in all samples. Additional to 4 homozygous deletions, 1 missense mutation was detected in *FLJ11218*. In conclusion, our data provide evidence for inactivation of both *PTEN* alleles as the major genetic defect on chromosome 10 in prostate cancer cell lines and xenografts. *PTEN* haplo-insufficiency is observed in 1 or 2 samples. *PTEN* inactivation is in part of the samples accompanied by loss of one *MINPP1* allele, loss of one copy, mutation or low expression of *PAPSS2* and most frequently with mono- or bi-allelic loss, or low expression of *FLJ11218*.

INTRODUCTION

Prostate cancer is the most frequently diagnosed cancer and the second leading cause of male cancer death in Western and Northern Europe, North America and Australia (Greenlee et al. 2001). At present, an adequate therapy of metastatic prostate cancer is not available. In order to identify novel therapeutic targets, knowledge of the major molecular alterations is urgently needed.

In prostate cancer, most frequent deletions were found for 6q, 8p, 13q and 16q, indicating the localization of tumour suppressor genes on these chromosomal arms (Visakorpi et al. 1995; Cher et al. 1996; Nupponen et al. 1998b; Alers et al. 2000). Less frequent chromosomal losses were found for 5q, 10q and 17p. The most frequently gained chromosome arm was 8q, followed by 7p, 7q and 20q.

Loss of 10q is generally considered as a late step in prostate cancer progression. Allelic imbalance studies indicated separate regions at 10q22-q26 to be affected, suggesting the inactivation of more than one tumour suppressor gene (Gray et al. 1995; Ittmann 1996; Komiya et al. 1996; Trybus et al. 1996; Cairns et al. 1997; Feilotter et al. 1998; Ittmann 1998; Leube et al. 2002). Loss of 10q is not unique for prostate cancer. Frequent loss of distal 10q has also been described in renal cell carcinoma (Morita et al. 1991), non-Hodgkin's lymphoma (Speaks et al. 1992), glioblastoma (James et al. 1988; Fujimoto et al. 1989), meningioma (Rempel et al. 1993), malignant melanoma (Reifenberger et al. 2000), small lung cell cancer (Kim et al. 1998), bladder cancer (Cappellen et al. 1997), and endometrial carcinoma (Peiffer et al. 1995; Nagase et al. 1996).

The *PTEN* gene at 10q23.3, which encodes a lipid and protein phosphatase, is the most frequently altered tumour suppressor gene in prostate cancer (Cairns et al. 1997; Teng et al. 1997; Feilotter et al. 1998; Gray et al. 1998; Vlietstra et al. 1998; Wang et al. 1998; McMenamin et al. 1999). Complete *PTEN* inactivation was detected at varying frequency in primary tumours and in up to 60% of metastases, cell lines and xenografts. *PTEN* is even more frequently implicated in glioblastoma (Li et al. 1997; Rasheed et al. 1997; Steck et al. 1997; Teng et al. 1997; Wang et al. 1997) and endometrial carcinoma (Tashiro et al. 1997), and to a lesser extent in many other tumours. Less is known about 10p alterations in prostate cancer. Variable frequencies of loss of distal 10p in prostate cancer have been described (Ittmann 1996; Trybus et al. 1996; Fukuhara et al. 2001; Narla et al. 2001). Additional to *PTEN*, 10q harbors the

candidate tumour suppressor genes *MXI1* at 10q25.2 and *DMBT1* at 10q26.2 (Eagle et al. 1995; Mollenhauer et al. 1997). Recently, mutation of *KLF6* on 10p15 in prostate cancer has been described (Narla et al. 2001; Chen et al. 2003).

Xenografts and cell lines are powerful tools in the search for genetic alterations in human cancer. They are available in unlimited quantities and, importantly, they lack normal cells of human origin, which simplifies the analysis of chromosomal alterations, and structural alterations and expression levels of individual genes. Previously, we described frequent *PTEN* inactivation in prostate cancer xenografts and cell lines (Vlietstra et al. 1998). In the present study we analyse the role of chromosome 10 in prostate cancer by CGH and allelotype analyses. We present data on the expression and structure of the candidate tumour suppressor genes *MXI1*, *DMBT1* and *KLF6*. In addition, we studied the structure, deletion and expression of *PTEN* flanking genes. Furthermore, we address the issue of *PTEN* haplo-insufficiency in prostate cancer.

MATERIALS AND METHODS

Prostate Cancer derived Cell Lines and Xenografts

The *in vitro* growing cell lines LNCaP, PC-3, DU-145 and TSU were cultured under standard conditions. The *in vivo* xenografts PC82, PCEW, PC133, PC135, PC295, PC310, PC324, PC329, PC339, PC346 and PC374 were propagated on male nude mice (Hoehn et al. 1980; Hoehn et al. 1984; Van Weerden et al. 1996).

DNA and RNA preparation

Genomic DNA from cell lines and xenografts was isolated utilizing the Puregene system from Gentra Systems (Minneapolis, MN) according to the procedure described by the manufacturer.

Cell line RNA was isolated by the guanidium isothiocyanate procedure; xenograft RNA was isolated by the LiCl protocol (Sambrook and Russell, 2001). mRNAs from fetal brain and normal prostate tissue were purchased from Clontech Labs (Palo Alto, CA).

Comparative Genomic Hybridization

CGH was performed essentially as described (Kallioniemi et al. 1992). In brief, tumour DNA and normal male reference DNA samples were labeled by nick translation

(Nick translation system, Life Technologies, Rockville, MD) with bio-dUTP (Roche Diagnostics, Almere, The Netherlands) and digoxigenin (Roche Diagnostics), respectively. Labelled DNA samples (200 ng each) and 15μg COT-1 DNA was ethanol-precipitated and dissolved in 10 μl hybridisation mix (50% formamid, 0.1% Tween-20, and 10% dextran sulfate in 2xSSC at pH7.0). The probe mixture was denatured (10 min, $72^{o}C$), pre-hybridised (30 min, $37^{o}C$) and hybridized to normal male chromosome spreads (72 h, $37^{o}C$). Next, slides were washed, and fluorescent detection of the biotin- and dioxigenin-labelled DNA probes was by fluorescein isothiocyanate (FITC)-conjugated avidin (Vector Labs, Burlingame, CA) and anti-digoxigenin-rhodamine (Roche Diagnostics) staining, respectively. Chromosomes were DAPI counterstained (4'6'-diamidino-2-phenylindole) (Sigma, St. Louis, MO) in Vectashield anti-fade solution (Vector Labs).

Images were acquired with an epifluorescent microscope equipped with a cooled CCD camera (Photometrics Inc., Tuscon, AZ), a triple-band pass beam splitter emission filters (P-1 filter set, Chroma Technology, Brattleboro, VT), and a Quips XL image analysis system (version 3.1 Vysis Inc., Downers Grove, IL). Chromosomal regions were scored as lost if the mean green to red ratio was below 0.85 and gained if this ratio was above 1.15. Eight or more metaphases were analysed per sample.

PCR and PCR-SSCP

Standard PCR amplifications utilizing *Taq* polymerase (Promega, Madison, WI) included 35 cycles of 1 min at $95^{o}C$, 1 min at $50^{o}C$ or $55^{o}C$, and 1 min at $72^{o}C$. For allelotyping and PCR-SSCP, 1 μCi [alpha-^{32}P]dATP (Amersham, Buckinghamshire, UK) was added to a 15 μl reaction mix. Amplified, radio-labelled polymorphic microsatellite markers were separated on a sequence gel. SSCPs of radio-labeled gene specific PCR products were analysed on a 6% non-denaturing polyacrylamide gel containing 10% glycerol. Gels were run at 7W overnight at room temperature. For detection of homozygous deletions, PCR amplifications were performed in a 50 μl reaction volume. Amplified fragments were separated on a 2% agarose gel.

Allelotype analysis and screening for homozygous deletions

Polymorphic microsatellite markers applied for allelotype analysis and screening for homozygous deletions at 10p were: *D10S602, D10S1745,* CA237H5A, CA237H5B*, D10S591, D10S1729, D10S189, D10S547, D10S191, D10S595, D10S197, D10S193), and at 10q: D10S220, D10S581, D10S537, D10S1688, D10S1730, D10S1686, D10S1687,* CA163M19*, D10S579, D10S215, D10S1765, AFMa086WG9, D10S541,* CA13J3, CA80H5*, D10S1753, D10S583, D10S1680, D10S1726, D10S192, D10S187, D10S209, D10S217.* PCR primer sequences can be found in Genome Database (http://gdbwww.gdb.org/). Other primer sets are: CA237H5A: gcagagcagccttcagtaat and cacttggcaaactacagtgc; CA237H5B: caagagcatgagtcccattg and gaaccaatcagtcaccaagc; CA163M19: gttttgcccagttgaagtca and tccttccccaactattctatc; CA13J3: gattagcacaacactgggtag and accctctgggggaagtactat; CA80H5: accagattggatgtgcatgc and caaccagcagtatctgtcac. Positions of markers on chromosome 10 were derived from the April 2003 freeze of the UCSC human genome map (http://genome. ucsc.edu).

Primer sets utilized in screening for homozygous deletions of *KLF6, MINPP1, PAPSS2, FLJ14600, FLJ11218, LIPF, DKFZp761K1824, ACTA2, TNFRSF6, CH25H, LIPA, IFIT2, IFIT4, IFIT1, RI58, PANK, MPHOSPH1* and *MXI1* are available upon request.

The 74K, 36K, G14Ext, G14 and 60K primer sets for detection of homozygous deletions in *DMBT1* by PCR on genomic DNA are published previously (Mollenhauer et al. 1997). For more detailed analysis of the homozygous deletion in *DMBT1* by PCR-SSCP the primers DMBTME39-F (5'-ACTTCAGAGGTAGGAGGGT-3') and DMBTME39-R (AGGTAGAGAGTGAGCCCTAG-3') were utilized.

mRNA expression

Analysis of mRNA expression was performed by semi-quantitative RT-PCR. cDNA was synthesized on 1 μg RNA template utilizing 200 U M-MuLV-RT (Life Technologies) and a T_{12}-site primer (5-GCATGCGAATTCGGATCCT$_{12}$-3) in a buffer, containing 10 mM DTT, 1 mM dNTPs, and 40 U RNAsin (Promega) for 1 h at 37°C. RNA polymerase II was utilized as a control. Specific cDNA fragments were amplified by standard PCR. Gene specific RT-PCR primers are available upon request.

Structures of candidate tumour suppressor genes and *PTEN* flanking genes

For PCR-SSCP analysis, fragments of appropriate sizes were amplified of all exons of *MINPP1, PAPSS2, TNFRSF6, FLJ11218, MXI1* and *KLF6*. Primer sequences are available upon request. Selected amplified fragments were purified over QIAquick spin columns (Qiagen, Hilden, Germany), cloned into pGEM-T Easy (Promega), and sequenced according to the dideoxy chain termination method (Sambrook and Russell, 2001).

RESULTS

Alterations of chromosome 10 are among the most frequent chromosomal changes in prostate cancer as determined by allelotype analysis (Gray et al. 1995; Komiya et al. 1996; Trybus et al. 1996; Ittmann 1998; Leube et al. 2002). However, this is not the case for several CGH studies (Nupponen et al. 1998b; Alers et al. 2000). To increase our insight into the role of chromosome 10 in prostate cancer, we studied overall chromosome 10 alterations in prostate cancer xenografts and cell lines by both CGH and allelotype analysis.

Comparative Genomic Hybridisation

Eight out of 15 xenografts and cell lines showed gain or loss of specific parts of chromosome 10 by CGH (Figure 1). Deletion of the distal region of 10p was found in PC310 (p13-pter), PC324 (p14-pter) and PC3 (p13-pter). Small changes at the telomeres were not taken into account, because of limited reliability. The majority of amplifications were found around the centromere: PC135 (q11.2-q22), PC324 (p11.2-p12 and q21-q24), PC339 (p11.2-p14), PC374 (p12-q21) and PC3 (p11.2-q22). Losses of distal 10q were most frequent. They were present in PC133 (q22-qter), PC135 (q23-qter), PC295 (q21-q23), PC339 (q23-qter), PC374 (q22-q25) and PC3 (q23-qter). In PCEW, PC82, PC329, PC346, LNCaP, TSU and DU145 no clear chromosome 10 alterations were detected by CGH. Summarizing, chromosome 10 was found to be frequently altered in prostate cancer xenografts and cell lines with a characteristic pattern of loss of distal 10p, gain of proximal 10p and proximal 10q, and loss of distal 10q.

XENOGRAFTS
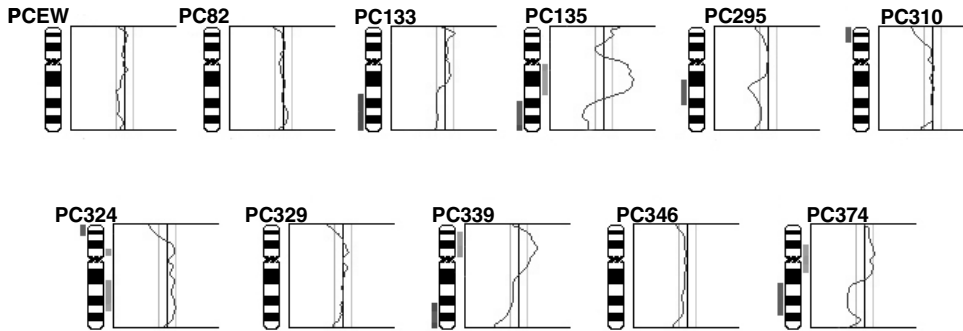
CELL LINES
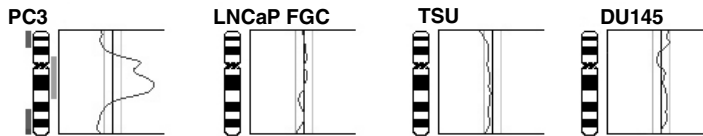
Figure 1.   Chromosome 10 alterations identified by comparative genomic hybridisation in prostate cancer xenografts and cell lines. A bar to the right of a chromosome ideogram indicates gain, a bar to the left indicates loss of the chromosomal region.

Allelotype analysis of chromosome 10

To obtain more detailed information on chromosome 10 alterations, the 15 genomic DNAs were screened for 35 polymorphic microsatellite markers along both chromosomal arms. A high marker density was chosen in a small region around the *PTEN* locus, which maps at 89.8 Mbp from the top of the p-arm, and around *KLF6* at 3.9 Mbp. The results are summarized in Figure 2. The previously published *PTEN* alterations are indicated at the bottom of Figure 2 (see also Vlietstra et al. 1998). Positions of markers were taken from the UCSC genome browser.

Because matching normal DNA samples were not available for comparison, 5 consecutive mono-allelic bands of highly polymorphic markers was taken as indicative for loss of one copy of the corresponding chromosomal region. According to this

definition, complete loss of one copy of 10p was found for TSU. Loss of distal 10p, including *KLF6*, was detected in PC310, PC324 and PC3. Large regions of apparent loss at 10q were present in PC133, PC295, PC339, PC374 and PC3. A remarkable large number of small regions of loss of 10q23.3 were detected in samples with or without complete *PTEN* inactivation (PCEW, PC82, PC135, PC324, PC346, LNCaP and DU145). Homozygous deletions and mutation of *PTEN* were found both in small and large regions of allelic loss (PCEW, PC82, PC324, PC346 and LNCaP, and PC133, PC295, PC374 and PC3, respectively; see Figure 2). In PC346, PC374, LNCaP and DU145, several polymorphic markers showed microsatellite instability (MSI), which limited the accuracy of allelotype analysis. Gains, which might be scored by allelic imbalance, were not taken into account.

Comparison of comparative genomic hybridisation and allelotype analysis

In general, large 10q deletions, as detected by CGH matched with allelotyping experiments. However, in contrast to allelotype analysis, CGH showed amplification of proximal 10q and deletion of distal 10q in PC135. CGH did not clearly detect loss of proximal 10q in PC339. The TSU cell line showed complete loss of one copy of chromosome 10 by allelotype studies and no loss by CGH. The large number of small mono-allelic regions or homozygous deletion of the *PTEN* locus explains the limited accuracy of CGH of 10q.

Marker	Heterozygosity	band	Mbp (Santa Cruz*)	PCEW	PC82	PC133	PC135	PC295	PC310	PC324	PC329	PC339	PC346	PC374	PC3	LNCaP	TSU	DU145
D10S602	73	10p15.3	2.2	2	2	1	2	1	1	1	1	2	2	2	1	1	1	MSI
D10S1745	85	10p15.3	2.5	2	2	1	2	2	1	1	2	1	2	MSI	1	MSI	1	MSI
CA237H5A	-	10p15.1	3.9	1	2	1	1	2	1	1	1	2	2	1	1	2	1	2
CA237H5B	-	10p15.1	4.0	2	2	1	2	2	1	1	2	2	1	MSI	1	MSI	1	MSI
D10S591	71	10p15.1	4.5	1	1	2	1	2	1	1	2	2	1	2	1	MSI	1	1
D10S1729	72	10p15.1	4.9	2	2	1	1	2	1	1	2	1	2	MSI	1	2	1	MSI
D10S189	73	10p14	6.9	2	1	1	2	1	1	1	2	1	2	2	1	2	1	1
D10S547	74	10p14	10.7	1	1	2	2	1	1	2	2	2	2	2	1	2	1	1
D10S191	81	10p13	14.7	1	1	1	2	2	1	2	2	2	2	MSI	1	MSI	1	MSI
D10S595	85	10p12.31	20.8	2	2	2	2	2	2	2	2	2	2	2	1	MSI	1	MSI
D10S197	75	10p12.1	26.7	1	2	2	2	2	2	2	1	2	MSI	MSI	1	2	1	MSI
D10S193	81	10p11.23	30.7	2	1	2	2	2	2	2	2	1	2	2	1	2	1	MSI
D10S220	84	10q11.23	52.2	1	2	1	1	2	2	1	2	1	2	2	2	1	2	MSI
D10S581	80	10q21.3	65.7	2	1	1	1	2	1	1	1	1	1	2	1	2	1	MSI
D10S537	83	10q22.1	72.3	2	2	1	1	1	2	2	2	1	MSI	1	1	MSI	1	MSI
D10S1688	86	10q22.1	72.5	1	1	2	1	1	1	2	2	1	2	1	1	MSI	1	1
D10S1730	83	10q22.3	78.8	1	2	2	1	1	2	1	1	1	MSI	MSI	1	2	1	MSI
D10S1686	86	10q23.1	85.7	2	2	1	2	1	2	2	2	1	2	MSI	1	2	1	MSI
D10S1687	81	10q23.2	88.8	2	2	1	2	1	2	2	2	2	2	1	1	1	1	2
CA163M19	-	10q23.2	89.1	1	2	1	2	1	2	2	2	2	1	1	1	1	1	MSI
D10S579	59	10q23.31	89.5	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1
D10S215	81	10q23.31	89.6	0	1	1	1	1	2	1	2	1	1	1	1	1	1	1
D10S1765	83	10q23.31	89.7	0	1	0	1	1	2	1	2	1	1	1	1	1	1	1
AFMa086WG9	-	10q23.31	89.8	0	1	0	1	1	1	0	1	1	1	1	0	1	1	1
D10S541	78	10q23.31	90.1	0	1	0	1	1	2	0	2	1	1	1	0	1	1	1
CA13J3	-	10q23.31	90.5	2	2	0	1	1	1	1	2	1	2	1	1	MSI	1	1
CA80H5	-	10q23.31	91.5	2	1	1	2	1	2	2	1	2	2	1	2	1	1	2
D10S1753	74	10q23.31	92.5	1	2	1	1	1	2	2	1	1	2	1	1	1	1	1
D10S583	84	10q23.33	94.5	1	2	1	2	1	2	1	1	1	2	1	1	1	1	1
D10S1680	82	10q23.33	95.7	2	2	1	1	2	2	1	2	1	MSI	1	1	1	1	MSI
D10S1726	76	10q24.2	100.8	2	1	1	2	2	2	1	1	1	MSI	MSI	1	MSI	1	MSI
D10S192	78	10q24.31	102.6	2	2	1	2	1	2	2	2	1	MSI	1	1	1	1	MSI
D10S187	84	10q25.3	118.8	2	2	1	1	2	2	2	2	1	2	MSI	1	MSI	1	MSI
D10S209	74	10q26.12	122.4	2	2	1	1	2	2	1	2	1	2	1	1	2	1	MSI
D10S217	81	10q26.2	129.5	1	2	1	1	2	2	2	2	1	MSI	MSI	1	2	1	MSI

* April 2003 freeze Genome Browser UCSC

PTEN

PTEN	10q23.31	89.8	-	-	-	+	-	+	-	+	+	-	-	-	-	+	+

Figure 2. Chromosome 10 alterations identified by allelotype analysis in prostate cancer xenografts and cell lines. If two bands of different length were detected, two allelic forms were retained in the DNA. One band indicates the presence of one allele or two alleles of identical length. One and two allelic forms are represented by "1" and "2", respectively. The homozygous deletions in PCEW, PC133, PC295, PC324, and PC-3 are represented by "0". The status of the *PTEN* gene is shown at the bottom of the figure. (+) indicates wild-type *PTEN*; (-) indicates inactivated *PTEN*.

Analysis of candidate tumour suppressor genes

Chromosomal studies (see Figures 1 and 2; Ittmann 1996; Komiya et al. 1996; Trybus et al. 1996; Leube et al. 2002) indicated that *PTEN* might not be the only tumour suppressor gene on chromosome 10. Distal to *PTEN* the candidate tumour suppressor genes *MXI1* (10q25.2) and *DMBT1* (10q26.2) have been mapped (Edelhoff et al. 1994; Shapiro et al. 1994; Mollenhauer et al. 1997). MXI1 antagonizes MYC in modulation of gene expression and tumorigenesis (Lahoz et al. 1994). *DMBT1* easily recombines in cancer cells and might play a role in immune defense and epithelial cell differentiation (Mollenhauer et al. 1997; Mollenhauer et al. 2000). We examined the expression and structure of both genes in the 15 prostate cancer cell lines and xenografts. No homozygous deletions of *MXI1* were detected, and *MXI1* mRNA was present in all RNA samples (data not shown). Structural analysis revealed polymorphisms, but no somatic mutations in the *MXI1* gene. One polymorphism was in the open reading frame (GGC-GGT, G23G), all others were in intron sequences. In the *DMBT1* gene, an intragenic homozygous deletion of the markers G14EXT and G14 was found in PC135 (Figure 3A). This homozygous deletion was further examined by PCR-SSCP, utilizing a primer set, which amplified the repeat units in *DMBT1* (Figure 3B; Mollenhauer et al. 1999). Sequencing of the amplified fragments combined with the presence of marker 36K (Figure 3A) indicated that the maximum size of the homozygous deletion in *DMBT1* is between exons 16 and 27 (Figure 3C). No deletions were detected in the other 14 DNA samples. Semi-quantitative RT-PCR indicated low expression of DMBT1 in PC82 and PCEW (data not shown).

Recently, inactivating mutations in *KLF6* on 10p15 has been described in prostate cancer (Narla et al. 2001; Chen et al. 2003). However, examination of *KLF6* in the 15 xenografts and cell lines revealed clear expression and absence of homozygous deletions or inactivating mutations (data not shown).
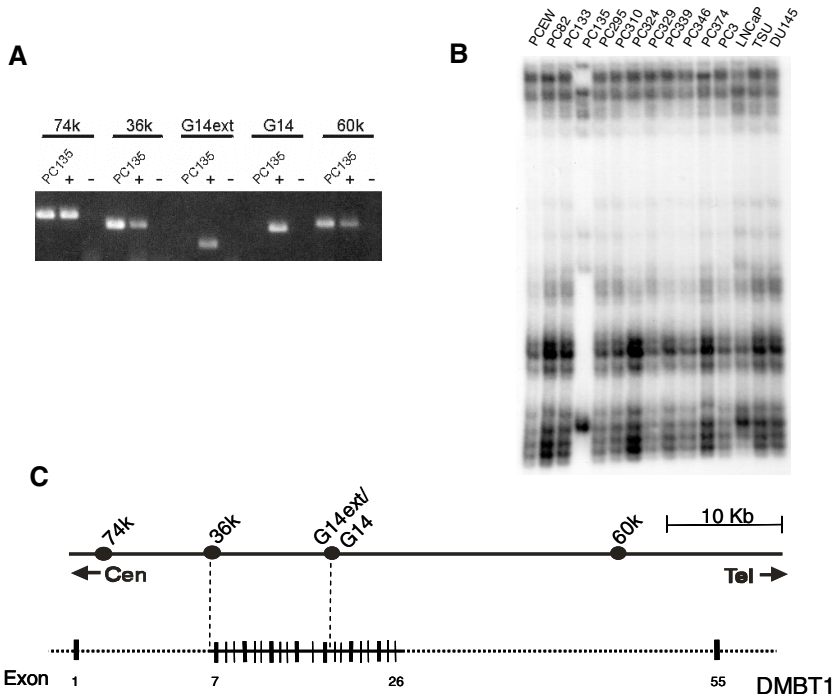
Figure 3.   Characterization of the homozygous deletion in *DMBT1* in PC135. (A) Agarose gel analysis of the indicated amplified *DMBT1* genomic fragments. (B) PCR-SSCP of the repeat units of *DMBT1* in prostate cancer xenografts and cell lines. (C) Schematic representation of the deleted segment in *DMBT1.*

Loss of the *PTEN* flanking region at 10q23.3

In mouse prostate cancer models not only complete *PTEN* inactivation, but also *PTEN* haplo-insufficiency has been implicated in tumorigenisis (Di Cristofano et al. 2001; Kwabi-Addo et al. 2001; Kim et al. 2002b; You et al. 2002). The role of *PTEN* haplo-insufficiency in human prostate cancer is not well defined. Investigation of the *PTEN* locus (Figure 2), compared with *PTEN* inactivation data (Figure 2, bottom; Vlietstra et al. 1998) learned that the xenografts PC310 and PC329 contain two wild-type *PTEN* alleles. In 9 DNA samples both *PTEN* alleles were inactivated by homozygous deletion (PCEW, PC133, PC295, PC324 and PC3) or deletion of one allele combined with a point mutation (PC82, PC346, PC374 and LNCaP). Loss of 1 copy of *PTEN* might have occurred in PC135, PC339 and DU145 (Figure 2). TSU was not taken into account,

because all chromosome 10 markers showed 1 allelic form. More detailed studies also excluded PC339 (see below). So, *PTEN* haplo-insufficiency might be present in 2 out of 15 xenograft and cell line DNAs.

Flanking genes might be complementary or independent candidate genes involved in prostate cancer. We searched 16 genes flanking *PTEN* for homozygous deletions, and the borders of small regions of allelic loss or homozygous deletion were accurately mapped. The order of genes and candidate genes was taken from the UCSC genome browser (see also Figure 5A). Figure 4 illustrates the genes at 10q23.3, which were completely deleted in the xenografts and cell lines, as determined by PCR. The first distal *PTEN* flanking gene, *FLJ11218*, was completely or partially deleted in all samples with *PTEN* deletion, except for PC295. *MINPP1, PAPSS2, FLJ14600* and *LIPF* were deleted in part of the samples. None of the DNAs without complete deletion of *PTEN* contained a homozygous deletion of one or more of 16 flanking genes (data not shown; see for genes investigated Materials and methods and Figure 5A).
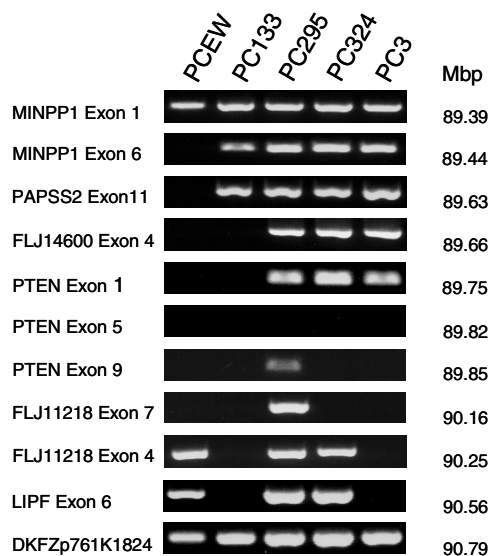
Figure 4. Genes inactivated by homozygous deletion in the prostate cancer xenografts PCEW, PC133, PC295 and PC324 and the cell line PC3. The figure indicates agarose gel electrophoresis of amplified exons of the indicated genes. The positions of the genes on chromosome 10 in Mbp from the top are indicated at the right.

Figure 5 summarizes the calculations of the lengths of homozygous deletions and mono-allelic regions in the *PTEN* region. These calculations not only took into account the outcome of microsatellite repeat analyses, but also single nucleotide polymorphisms in *MINPP1, PAPSS2, FLJ11218* and *TNFRSF6* genes (see Materials and Methods). The homozygous deletions in PCEW, PC133, PC295, PC324 and PC3 ranged in size from more than 1.2 Mbp (PC133) to less than 30 Kbp (PC295) (Figure 5B, see also Figures 2 and 4). In both PC324 and PCEW, the telomeric border of the deletion is in intron 5 of *FLJ11218*. In PCEW, the deletions in both copies of 10q are small and almost identical in lengths. In PC324, the mono-allelic region around *PTEN* is less than 2 Mbp. The mono-allelic regions in PC82 and PC346, which both contain a *PTEN* point mutation, and in DU145 and PC135, which do not show structural alterations of *PTEN* , are also small (Figures 2 and 5C,D). So, in many samples small mono-allelic regions around the *PTEN* locus could be precisely determined. All samples had in common that they lost one or two copies of *PTEN*. Most DNAs lost one or two copies of *FLJ11218* and one copy of *MINPP1, PAPSS2* and *FLJ14600*. Most likely, PC339 is an exception, because the mono-allelic region starts in *FLJ11218*, distal from *PTEN*.
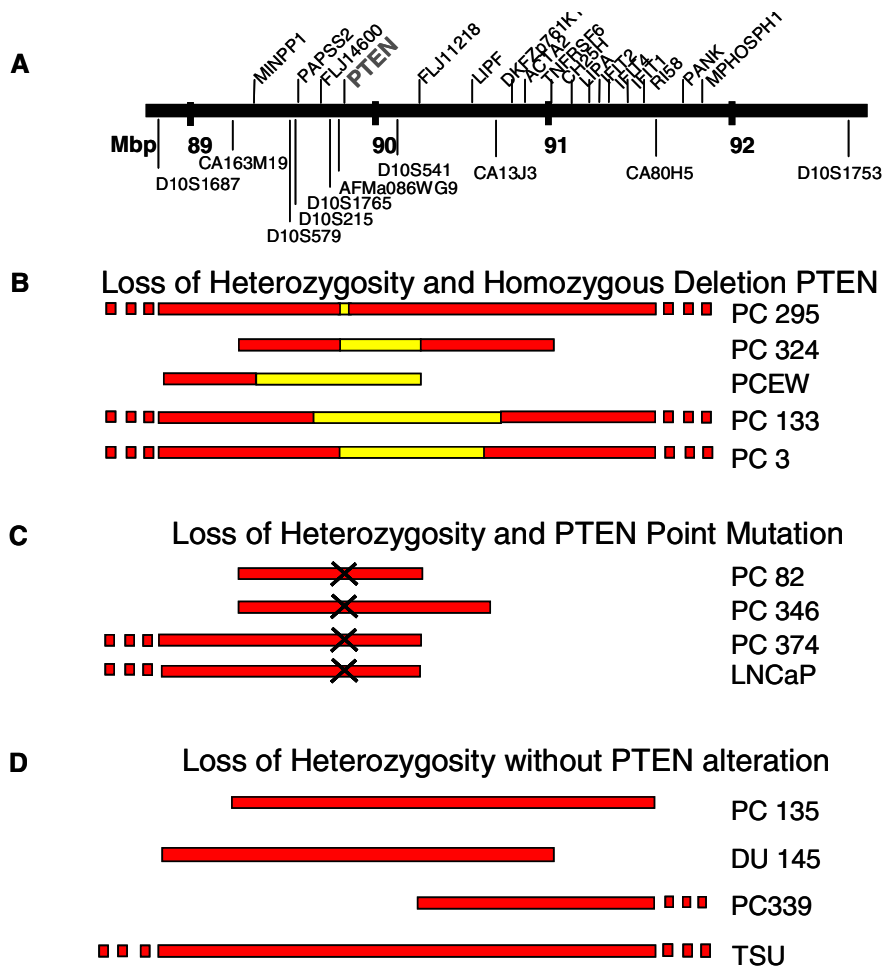
Figure 5. Schematic representation of the homozygous deletions and mono-allelic regions flanking the *PTEN* locus in prostate cancer xenografts and cell lines. (A) Genes flanking *PTEN*. Data are from the April 2003 freeze UCSC gene map. (B) Homozygous deletions (open bars) and mono-allelic regions (gray bar) in PC295, PC324, PCEW, PC133 and PC3. Grey blocks indicate unknown border (C) Mono-allelic regions (gray bars) in PC82, PC346, PC374 and LNCaP. A cross indicates *PTEN* inactivation by point mutation. (D) Mono-allelic regions in PC135, DU145, PC339 and TSU, which lack complete *PTEN* inactivation.

Expression and structure of genes flanking the *PTEN* locus

To investigate further a possible contribution of *PTEN* flanking genes in prostate cancer, the expression patterns of 14 genes in 3 Mbp flanking the *PTEN* locus, bordered by the polymorphic markers *D10S1687* and *D10S1753* (see Figure 5A) were analysed by semi quantitative RT-PCR The results are summarized in Table I. Two 1 exon genes, *DKFZp761K184* and *CH25H* were omitted. *MINPP1, PAPSS2* and *FLJ11218* expressions are shown as examples in Figure 6. A mixed pattern of expression profiles was found. Obviously, in all examples except for *PTEN* in PC295, homozygous deletion of a gene correlated with absence of expression. In the semi-quantitative RT-PCR no clear-cut correlation was found between the copy number and expression level of a gene, indicating gene-copy independent regulatory mechanisms to be more important. The stomach-specific gene *LIPF* was hardly expressed in the prostate tumour cells. Most other genes showed a rather stable expression level. Potentially interesting variable expression patterns were seen for *PAPSS2*, *FLJ11218*, and the interferon-regulated gene family *IFIT1, 2, 4, RI58*. High expression of the latter gene family almost perfectly correlated with androgen independence of a xenograft or cell line (PC82 is the exception; see Table I). *PAPSS2* not only showed absence of expression due to homozygous deletion of the gene (PCEW), but expression was also undetectable or very low in PC133, PC324, PC329 and LNCaP. *FLJ11218* expression was not only absent in the 4 samples, where the gene was completely deleted, but also absent or very low in PC135, PC346, LNCaP and TSU.

Figure 6. Expression of MINPP1, PAPSS2 and FLJ11218 mRNA in prostate cancer xenografts and cell lines. Expression was monitored by agarose gel electrophoresis of semi-quantitative RT-PCR products, indicated by "+" or "-" reverse transcriptase. RNA pol II was used as a control.

Table I

Expression of the PTEN flanking genes in prostate cancer xenografts and cell lines

	Xenografts											Cell lines			
	PCEW	PC82	PC133	PC135	PC295	PC310	PC324	PC329	PC339	PC346	PC374	PC3	LNCaP	TSU	DU145
Androgen dependent	+	+	-	-	+	+	-	+	-	+	+/-	-	+	-	-
mRNA															
MINPP1	-	+++	+++	+++	++	+++	+++	+++	+++	+++	+++	+++	++	+++	+++
PAPSS2	-	+++	+/--	+++	+++	+++	-	+/-	+++	+	++	++	+/--	+++	+++
FLJ14600	-	+++	+	+++	+++	+++	+++	+++	++	+++	+++	+++	+++	+++	+++
PTEN	-	+++	-	+	+++	+++	-	+++	++	+++	+++	-	+++	++	+++
FLJ11218	-	+++	-	-	+++	++	-	+	++	-	+	-	+/--	-	++
LIPF	+/-	+	-	-	+	-	-	-	-	-	+/-	-	-	-	-
ACTA2	++	++	-	++	++	++	++	++	++	-	++	++	++	++	++
TNFRSF6	+++	+++	+++	+++	+++	+++	+++	+++	+++	+	+++	+++	+++	+++	+++
LIPA	+++	+++	+++	+++	+++	+++	+++	+++	+++	+/-	+++	+++	+++	+++	+++
IFIT2	+/-	+	+	++	+/-	+	+++	+/-	+	+/-	+++	+++	+/-	+	++
IFIT4	-	++	++	++	-	+/-	+++	+/-	++	-	++	+++	-	+	+++
IFIT1	-	++	++	+++	-	+	+++	-	+++	+/-	+++	+++	+/-	+++	+++
RI58	-	++	++	++	-	+	+++	-	++	-	++	+++	+	++	++
PANK	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
MPHOSPH1	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++

Four flanking genes, which were selected on the basis of frequent deletion and low expression (*FLJ11218*) or function, the phospholipid phosphatase *MINPP,* the sulfatase *PAPSS2* and the tumour necrosis factor receptor *TNFRSF6*, were searched for point mutations. In *MINPP1* and *TNFRSF6*, polymorphisms, but no somatic mutations were detected. In *PAPSS2* a polymorphism, which is presumed to decrease its function (Met295 in PC133; see Xu et al. 2002b), one frame-shift (deletion C in codon 355 in LNCaP) and a missense mutation [GTG to ATG (V45M) in PC346] were found. In *FLJ11218* we frequently observed the polymorphism GAG/GAC (E37D) in exon 1, and polymorphisms in introns 4 and 6. In addition, we detected in PC374 the missense mutation ATT to AGT (N232S) in exon 5.

Summarizing, inactivation of *PTEN* always paralleled low expression or inactivation of 1 or 2 copies of the telomeric flanking gene *FLJ11218*. Less frequent alterations in *PAPSS2* copy number, structure or expression were detected. In approximately half of the DNAs one copy of *MINPP1* was lost.

DISCUSSION

In this study, we describe the genetic characterization of chromosome 10 in xenografts and cell lines derived from human prostate cancer. Although it can be argued that xenografts and cell lines acquire extra genetic alterations during *in vivo* and *in vitro* culturing, it is our experience (Vlietstra et al. 1998; Trapman, unpublished data), that as far as we were able to check for xenografts, most genetic alterations are already present in the tumour tissue from which they are derived. We choose for xenogafts and cell lines because they lack normal human cells, which enabled accurate study of homozygous deletions, regions of allelic loss and gene expression.

First, overall chromosome 10 losses and gains were investigated by CGH. Our findings indicated a pattern of loss of distal 10p, gain of proximal 10p and 10q and loss of distal 10q. Previous CGH studies of DNA from prostate cancer patients showed a similar pattern, although at a lower frequency and less clear (Cher et al. 1996; Nupponen et al. 1998b; Alers et al. 2000). Like in this study, in previous studies loss of distal 10q was the most frequent chromosome 10 alteration. Absence of chromosome 10 alterations in LNCaP and TSU, and the loss-gain-loss pattern in PC3 were in accordance with previous CGHs (Nupponen et al. 1998a). In PC3, chromosome 10

seems to be involved in several translocations (Pan et al. 1999). A frequently detected breakpoint in PC3 and other prostate cancer cell lines maps at band 10q22 (Pan et al. 2001). A similar breakpoint at 10q22, combined with amplification of centomeric 10q and loss of telomeric 10q might be present in xenograft PC135. A recent CGH study on a different panel of xenografts provided an overall picture as shown here, loss of distal 10p and distal 10q and gain of the middle part of chromosome 10 (Laitinen et al. 2002).

Allelotype analysis confirmed loss of distal 10p in PC310, PC324 and PC3. We did not find alterations in *KLF6* at 10p15, as previously reported (Narla et al. 2001; Chen et al. 2003). Although it can be argued that the number of samples studied is small, we favour the hypothesis that the most frequently affected tumour suppressor gene on 10p remains to be detected.

CGH data did not match allelotype data for 10p and 10q in TSU. No chromosome copy change was seen in CGH, but allelotype analysis showed one allelic form for all markers. This is strong evidence for chromosome 10 isodisomy in this cell line. A similar observation has been made for chromosomes 6 and 8 (Verhagen et al. 2002; Van Alewijk, unpublished), suggesting defective chromosome segregation in TSU.

CGH data for 10q are less informative than allelotype studies. Although in some DNAs hampered by microsatellite instability, allelotype analysis was more informative, due to the occurrence of many small regions of chromosomal loss at the *PTEN* locus. Six small deletions were not detected by CGH. It can be assumed that CGH of prostate cancer tissues also frequently misses loss of *PTEN* alleles, underestimating complete inactivation or haplo-insufficiency of *PTEN* in patients.

Allelotype analysis of 10q23.3 in tumours from patients frequently showed a higher percentage of allelic loss at the *PTEN* locus than *PTEN* alterations (Feilotter et al. 1998; Pesche et al. 1998). There might be several explanations for this discrepancy. First of all, complete inactivation of *PTEN* by point mutation or homozygous deletion might have been missed, because many studies were incomplete. Secondly, it has long been thought that in these samples not *PTEN*, but a more distal gene was inactivated. Although this cannot be excluded completely, our present study supports the assumption that this is not the case. We could not detect alterations in *MXI1,* a gene at 10q25.2, previously implicated at low frequency in prostate cancer (Eagle et al. 1995; Prochownik et al. 1998). Neither did we detect at

high frequency deletions in the unstable *DMBT1* gene, which has been described as a candidate tumour suppressor gene in several other tumour types (Mollenhauer et al. 1999). We also could not find complete inactivation of one of the *PTEN* flanking genes in the absence of *PTEN* alteration. Therefore, an attractive alternative is *PTEN* haplo-insufficiency as the underlying genetic defect on 10q in early stage prostate cancer. *PTEN* haplo-insufficiency seems to be present in only 1 xenograft (PC135) and 1 cell line (DU145), which represent late stage prostate cancer. Careful examination of DNA from micro-dissected prostate cancer samples with a variety of experimental approaches should be carried out to address this important question. Complementary to mutation analysis, array CGH on a distal 10q tilted BAC contig should provide this important information. In favour of an important role of *PTEN* haplo-insufficiency in human prostate cancer are mouse model studies. In 4 prostate cancer models based on prostate specific SV40-Tag expression (TRAMP model), inactivation of *Ink4a/Arf*, *Cdkn1b* or *Nkx3.1 Pten* haplo-insufficiency contributed to tumorigenesis (Di Cristofano et al. 2001; Kwabi-Addo et al. 2001; Kim et al. 2002b; You et al. 2002).

Although we could not find evidence for a *PTEN* independent role of flanking genes in tumourigenesis, a complementary role cannot be excluded. Two observations are important in this regard: the high frequency of *PTEN* inactivation by homozygous deletion, and the remarkable large number of small mono-allelic regions that accompany *PTEN* inactivation. The latter observation limits the candidate genes to those directly flanking *PTEN*. We did not study in detail *FLJ14600*, because it is clearly expressed in all xenografts and cell lines except PCEW and PC133. *MINPP1* would have been an interesting candidate, because like *PTEN* it is able to affect phospholipid metabolism. However, the specificity of MINPP1 seems different from PTEN, and *MINPP1* is, with the exception of PCEW only affected by loss of one gene copy in part of the samples. The sulfatase gene *PAPSS2* is more frequently affected by loss of one copy of the gene. In addition, PAPPS2 is not expressed or expressed at a low level in several xenografts and cell lines. Interestingly, also 2 presumed inactivating mutations were detected, although both are in samples (PC133 and LNCaP), which already show a low level of PAPSS2 expression. Arguing against a role of PAPSS2 deficiency in prostate cancer is the expression of the related gene *PAPSS1* in all cell lines and xenografts (data not shown). The strongest candidate to complement *PTEN* in tumourigenesis is the candidate gene *FLJ11218*. It is inactivated

by homozygous deletion in 4 DNAs, and loss of 1 copy or low expression is found in many other samples (Figures 5 and 6). Unfortunately, so far the function of FLJ11218 is unknown. Elucidation of this function, combined with study of deletion and expression of the gene in prostate cancer tissues should be carried out to substantiate the hypothesis.

ACKNOWLEDGEMENTS

CHAPTER 4

IDENTIFICATION OF A HOMOZYGOUS DELETION AT 8P12-21 IN A HUMAN PROSTATE CANCER XENOGRAFT

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ABSTRACT

One of the most frequent genetic abnormalities in prostate cancer is loss of the complete, or part of the short arm of chromosome 8, indicating the localization of one or more tumor suppressor genes on this chromosomal arm. Using allelotyping, a frequently deleted region in prostate cancer in a genetic interval of approximately 17cM between sequence tagged sites *D8S87* and *D8S133* at chromosome 8p12-21 was previously detected. A detailed physical map of this region is now available. Using known and novel polymorphic and nonpolymorphic sequence tagged sites in this interval, a search for homozygous deletions in DNAs from fourteen prostate cancer derived cell lines and xenografts was carried out. In DNA from xenograft PC133, the presence of a small homozygously deleted region of 730-1320 Kb was unambiguously established. At one site, the deletion disrupts the Werner syndrome gene. Data from allelotyping were confirmed and extended by fluorescence *in situ* hybridization analysis of PC133 chromosome spreads using centromere, YAC, and PAC chromosome 8 probes.

INTRODUCTION

Prostate cancer is the most frequently diagnosed cancer in men in Western countries, and the second leading cause of male cancer death (Parker et al. 1997). Frequent chromosomal abnormalities in sporadic prostate cancers are deletions of chromosome arms 8p, 10q, and 16q (Carter et al. 1990; Bergerheim et al. 1991; Kunimi et al. 1991), which indicate the localization of tumor suppressor genes (TSGs) at these regions. So far, only the TSG at 10q (*PTEN / MMAC1*) has been identified (Cairns et al. 1997; Vlietstra et al. 1998).

Fluorescence in situ hybridization (FISH) analysis with chromosome specific probes and comparative genomic hybridization (CGH) studies have confirmed and extended the previous allelotyping data showing frequent deletion of 8p (Isaacs 1995; Cher et al. 1996; Kallioniemi and Visakorpi 1996). Several groups have focused on more precise mapping of a deleted region at 8p, as a first step to identify candidate TSGs (Isaacs 1995; Kallioniemi and Visakorpi 1996). In many tumor DNAs, the complete or almost complete short arm of one chromosome 8 was deleted. Furthermore, comparison of the different data showed a complex pattern of overlapping and non-overlapping regions of allelic imbalance, suggesting the presence of at least two separate regions involved in prostate cancer, 8p22 and 8p12-21, respectively. Chromosome arm 8p deletions have also been found in cancers derived from colon, lung, liver, and breast. Functional evidence for a TSG on 8p has been provided by transfer of the complete or part of chromosome arm 8p to colorectal or prostate cancer cell lines, resulting in a less tumorigenic phenotype (Ichikawa et al. 1994; Gustafson et al. 1996; Nihei et al. 1996; Tanaka et al. 1996).

To fine-map the position of a TSG, the search for a homozygous deletion (HD) is an important first step. This procedure was pivotal in the identification of the *BRCA2* gene in hereditary breast cancer (Wooster et al. 1995), the *DPC4* gene in pancreatic cancer (Hahn et al. 1996), and the *PTEN/MMAC1* gene in neuroblastoma (Li et al. 1997; Steck et al. 1997). In prostate cancer, an HD of approximately 1 Mb at 8p22 has been described by Bova et al. (1993, 1996) (Bova et al. 1993; Bova et al. 1996). Additionally, Kagan et al. (1995) reported less defined large HDs in three different prostate tumors (Kagan et al. 1995). In the present study, the identification of a small HD at 8p12-21 in a human prostate cancer xenograft is presented.

MATERIALS AND METHODS

Prostate Cancer Cell Lines and Xenografts

The in vitro growing cell lines DU-145, PC-3, and LNCaP were cultured under standard conditions. PC82, PC-EW, PC133, PC135, PC295, PC310, PC324, PC329, PC339, PC346, and PC374 were grown by serial transplantation as xenografts on male nude mice (van Weerden et al. 1996).

Genomic DNA

Standard protocols were used to isolate genomic DNA from DU-145, PC-3, and LNCaP cell pellets (Sambrook et al. 1989). Genomic DNA from xenografts was isolated from five consecutive 5 μm cryostat tissue sections according to standard procedures (overnight proteinase K incubation at 55°C, phenol extraction, and ethanol precipitation). DNA was dissolved in TE (10 mM Tris.HCl, pH 7.8; 1 mM EDTA).

Chromosome Markers

D8S278, *D8S283*, *D8S540*, *D8S1733*, *D8S1769*, AFMa224wh5 (Dib et al., 1996), *D21S275* (Chumakov et al. 1992), *D8S87* (Weber et al. 1991), *D8S133* (Wood and Schertzer 1992), *D8S136* (NIH/CEPH Collaborative Mapping Group 1992), and *D8S2162* (Yu et al. 1996b) are polymorphic STSs. Other STSs are from YAC end fragments: 721D7-R (forward: aggtttgtatcccagcttttccag; reverse: caggctctatactaagctcttctc), 896F4-L (forward: tccatcaccctatttggcca; reverse: ttctacaggcaagaagcagg), 721D7-L (forward: ctaactcaaggcacaggcggca; reverse: gtgttggcatctgtggaaaggag), and 761A2-L (forward: gcatcgatttgtaagtcaacatgac; reverse: tgagtttcaagtcctgggtatca). *MSR* exon 4 (forward: gatgtgacagtggaagctatgg; reverse: ggaaaaatgtggtatatctgaagctc), *POLB* exon 12 (forward: ttaagccttaagtttagaacatc; reverse: gagggagaaaacgagacaag), *WRN* exon 1 (forward: tgctgatttggtgtctagcct; reverse: cgagaagatccagtccaacag), exon 26 (forward: cttgtgagaggcctataaactgg; reverse: ggtaaacagtgtaggagtctgc) (Yu et al. 1996a), and exon 35 (forward: tcttctgggagcctacgtgag; reverse: tgcggtttcattttcactgccctg) are gene specific markers. Detailed mapping of novel chromosome 8 STSs will be published elsewhere.

PCR Analyses

PCR amplifications, using *Taq* polymerase (Promega, Madison, WI), included 30 cycles of 1 min at 95°C, 2 min at 55°C, and 2 min at 72°C in a 50 μl reaction volume. In radioactive multiplex PCRs, 1 μCi [α^{32}P]dATP (Amersham, Buckinghamshire, UK) was added to the 15 μl reaction mix. Radio-labeled PCR products were separated on a sequence gel. Gels were dried and overnight exposed. Unlabeled PCR fragments were separated on a 2% agarose gel.

YACs and PACs

YACs y721D7, y953H12, and y888D12 were identified and selected by PCR screening and Southern blotting of the CEPH-YAC libraries (Albertsen et al. 1990; Chumakov et al. 1992). YAC DNA was isolated as described by Green and Olson (1990) (Green and Olson 1990). PACs were obtained from Genome Systems (St. Louis, MO) after screening gridded filters with specific hybridization probes derived from the markers *MSR* (p23G19), AFMa224wh5 (p243O21), *D8S1769* (p81H5), 761A2-L (p252H7), and *POLB* (p65P22), respectively. PAC DNA was isolated as described by the protocol of the manufacturers.

Fluorescence In Situ Hybridization

Chromosome preparations were made from phytohemagglutinin stimulated lymphocytes and from xenograft PC133 according to standard protocols. Slides containing chromosome spreads were washed in PBS for 5 min at room temperature. Next, they were incubated in 0.01 N HCl, containing 5 mg pepsin (Serva, Heidelberg, Germany) per 100 ml, for 10 min at 37°C, subsequently rinsed in 2xSSC, directly denatured in 70% formamide (Merck, Darmstadt, Germany)/2xSSC for 5 min at 75°C, and serially dehydrated. YAC and PAC DNAs were either biotin-14-dATP or digoxigenin-11-dUTP labeled by nick translation (nick translation kit, Gibco-BRL, Gaithersburg, MD). The biotin labeled chromosome 8 specific probe was obtained from Cambio LTD (Cambridge, UK). The chromosome-specific α-satellite probe D8Z2 (Donlon et al. 1986) was directly labeled with tetra methyl rhodamine-6-dUTP by nick translation (nick translation kit, Boehringer Mannheim, Germany). Hybridizations were done after preannealing with 1-3 μg human Cot-1 human DNA (Boehringer Mannheim, Germany) for 1 h at 37°C. All hybridizations were performed in 50%

formamide, 2xSSC, and 10% dextran sulfate in a moist chamber for 16 h at 37°C. Slides were washed twice in 50% formamide / 2xSSC for 5 min at 45°C, twice for 5 min in 2xSSC at 45°C, and once in 0.1xSSC for 5 min at 45°C. The hybridization signal was visualized by incubation with FITC conjugated avidin (Vector, Burlingame, CA), or anti-dig-rhodamine (Boehringer Mannheim, Germany). The FITC signal was amplified with two additional layers of biotinylated goat anti-avidin and FITC conjugated avidin. The slides were mounted in Vectra Shield (Vector, Burlingame, CA) containing 0.1 ng/µl DAPI (Sigma, St. Louis, MO). Analyses were performed with a Leica DM-RXA microscope equipped with a PowerGene image analyses system (PSI, Chester, UK).

RESULTS

Screening of Human Prostate Cancer Cell Line and Xenograft DNAs for Homozygous Deletions

Previously, the 8p12-21 region between markers *D8S87* and *D8S133* was found to be frequently deleted in prostate cancer (Trapman et al. 1994). Ten sequence tagged sites (STSs) that mapped between *D8S87* and *D8S133* were used to screen for HDs in DNAs from fourteen prostate cancer cell lines and xenografts (Fig. 1A, and J. Trapman, unpublished observations). For nine STSs, an amplified fragment could be detected in all DNA samples in multiplex PCRs, using chromosome 21 STS *D21S275* as an internal control (see as an example the polymorphic marker *D8S1733*; Fig. 1B). In contrast, in PC133 DNA a specific fragment was absent in the *D8S1769* amplification reaction, suggesting that part of 8p12-21, encompassing the *D8S1769* fragment, was lacking in this DNA. In some DNAs, two bands of different length were detected, showing that two allelic forms are retained in the tumor DNA. One band indicates the presence of one allele or two alleles of identical lengths. In PC133 DNA, each polymorphic marker gave rise to one fragment, with the exception of the negative *D8S1768* (Fig. 1B), and *D8S87*, which showed two bands (data not shown).

A

B
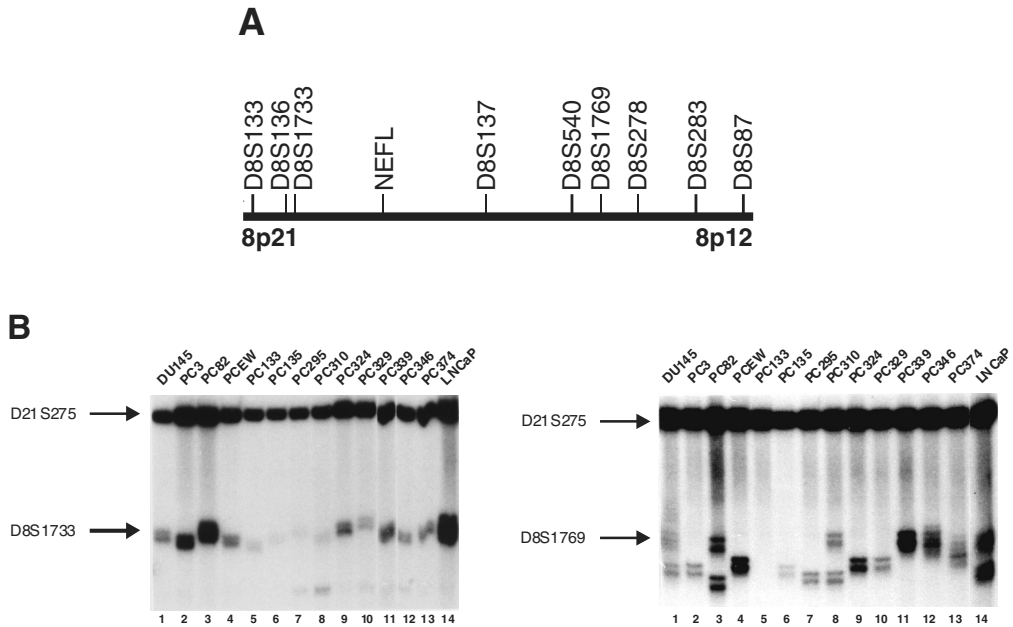
Figure 1.(A) Physical map of the region between markers *D8S133* and *D8S87* at 8p12-21, which is frequently deleted in prostate cancer. **(B)** Radioactive multiplex PCR analysis of *D8S1733* (telomeric of HD region) and *D8S1769* (in HD region) in fourteen prostate cancer cell lines. *D21S275* is used as the internal control.

Fine-mapping of the HD Region in PC133

To obtain more detailed information about the HD in PC133 DNA, additional amplification reactions were carried out with STSs flanking *D8S1769*, and between *D8S540* and *D8S278*, the two markers which were found to be present in PC133 DNA (Fig. 2). AFMa224wh5 and 761A2-L, located telomeric and centromeric to *D8S1769*, respectively (Fig. 2A), gave a PCR product in PC133. In contrast, PC133 genomic DNA was negative for STSs 721D7-R, *D8S2162*, 896F4-L, and 721D7-L, which are more closely linked to *D8S1769* (Fig. 2B). From these data it was concluded that the boundaries of the deleted region in PC133 DNA were given by AFMa224wh5 and 761A2-L. YAC 721D7 has an estimated length of 730 Kb (CEPH-Genethon database). Both end-fragments of this YAC could be mapped at 8p, showing that it was not chimeric. Both end-fragments were absent in PC133 DNA, which indicates that the HD has a length of at least 730 Kb (Fig. 2A).
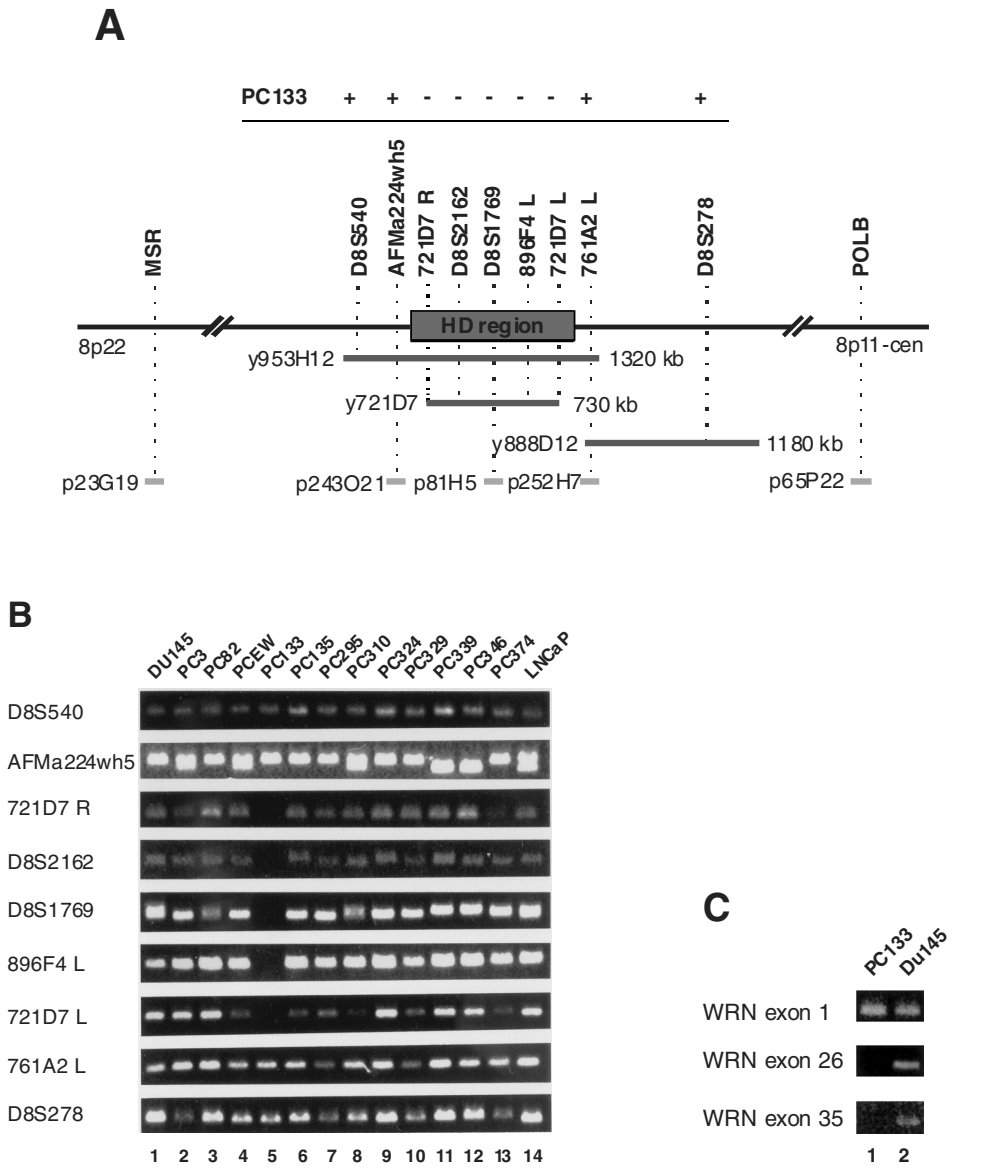
Figure 2. (A) Schematic representation of the markers used in the fine mapping of the HD region in PC133. **(B)** Fine-mapping of the HD region in PC133. **(C)** Mapping of *WRN* in the HD region by amplification of specific exons.

Genes in the HD Region

The marker *D8S2162* has been mapped close to the Werner syndrome gene (*WRN*) (Yu et al. 1996a; Yu et al. 1996b). Using *WRN* exon 1, 26, and 35 specific primers, the position of *WRN* to the HD region was determined. No PCR products could be detected for *WRN* exon 26 or 35, whereas *WRN* exon 1 was found to be present (Fig. 2C). This finding shows that the HD in PC133 interrupts the *WRN* gene.

Characterization of Chromosome 8 in PC133

To extend our knowledge of the chromosomal composition of PC133, especially focussing on chromosome 8, FISH analyses of chromosome spreads with a variety of hybridization probes was done. First, the number of chromosome 8 copies in PC133 was determined by chromosome 8 painting and hybridization with the centromere probe D8Z2. This resulted in the detection of two apparently intact chromosomes 8, one 8p-, one copy of a dicentric chromosome 8, one solitary chromosome 8 centromere fragment, and a chromosome 8 fragment in a marker chromosome (Fig. 3A).

In a second experiment, YAC 721D7, which is in the HD region, and YAC 888D12, which is centromeric to the HD (J. Trapman, unpublished observations) were used as hybridization probes (Fig. 2A). YACs 721D7 and 888D12 are linked by YAC 953H12, which has a length of 1320 Kb (Fig. 2A and CEPH-Genethon database). Although 721D7 provided a clear signal at normal control lymphocyte chromosomes (Fig. 3B), as expected, no signal could be detected in PC133 chromosome spreads (Fig. 3C). Hybridization with the control YAC 888D12 gave rise to a signal at the two apparently intact chromosomes 8 and two signals at the dicentric chromosome 8. Other chromosome 8 fragments were negative for 888D12 (Fig. 3C).

Subsequently, PACs 243O21 and 252H7, which are located directly telomeric and centromeric to the HD region, respectively (Fig. 2A), were used as hybridization probes. In a double FISH experiment on PC133 chromosome spreads, probes derived from both PACs gave signals on the two apparently intact chromosome 8 copies (Fig. 3D). However, other chromosome 8 fragments, including the dicentric chromosome 8, which was positive for YAC 888D12, were negative. PAC 81H5, which is completely located within the HD region (Fig. 2A) did not hybridize with any of the chromosome 8 copies (data not shown). A double FISH experiment with PAC 23G19, which contains the more telomeric *MSR* gene, and with the more centromeric *POLB* positive PAC

65P22 (Fig. 2A) provided additional information about the different chromosome 8 fragments, including the dicentric chromosome (Fig. 3E). PAC 23G19 (*MSR*; green) gave only a signal on the two apparently intact chromosomes 8, whereas 65P22 (*POLB*; red) hybridized to all chromosomes 8, including the small centromeric fragment, but excluding the chromosome 8 fragment in the marker chromosome.
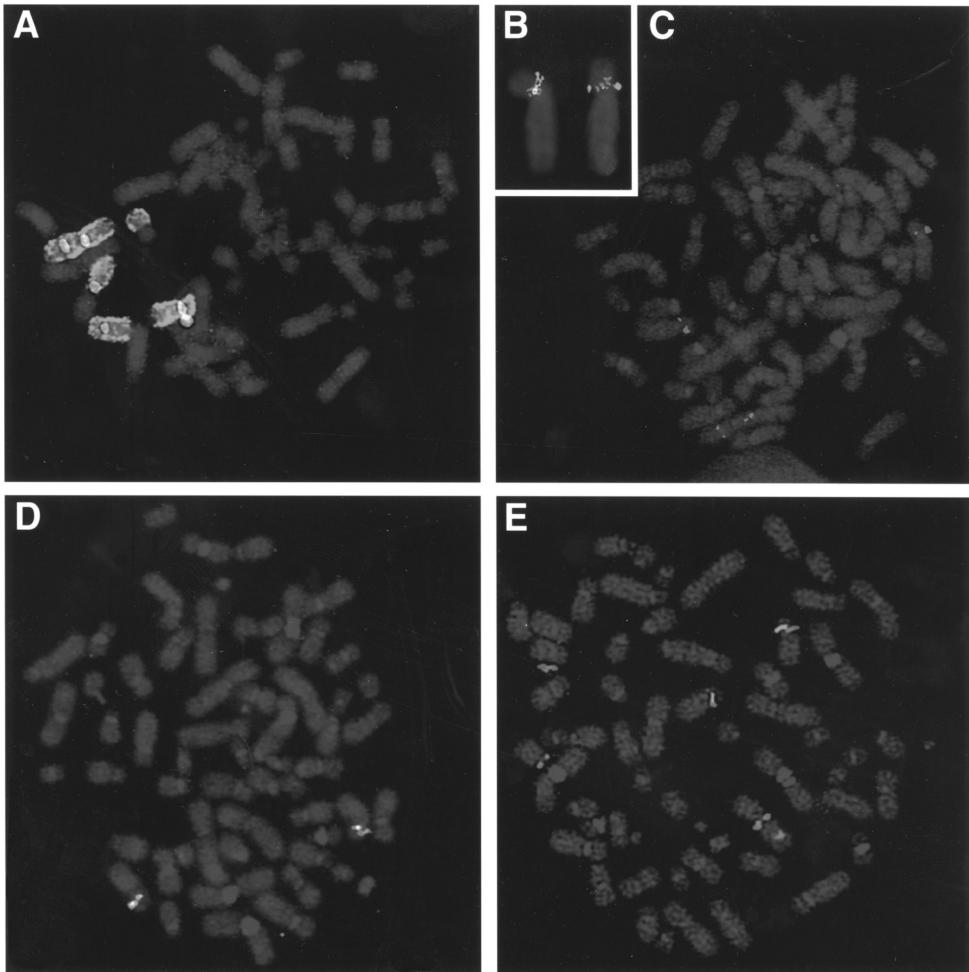
Figure 3.Characterization of chromosome 8 fragments in PC133 by FISH analysis. (A) A paint of chromosome 8 (green) in combination with the D8Z2 (red) chromosome 8 centromere probe. **(B)** Double FISH of YAC 721D7 (green) and YAC 888D12 (red) on lymphocyte and **(C)** on PC133 chromosome spreads. **(D)** Double FISH of PAC 243O21 (green) and PAC 252H7 (red) on a PC133 metaphase. **(E)** Double FISH of PAC 23G19 (green) and PAC 65P22 (red) on PC133 chromosomes.

A summary of the FISH data is schematically depicted in Figure 4. Six chromosome 8 fragments were detected (see also Fig. 3A). Two copies seemed completely identical, and contained the small 8p12-21 deletion (A and B in Fig. 4). One chromosome 8 had a deletion of the major part of 8p (C in Fig. 4). The dicentric chromosome contained a duplicated 8p fragment, and most likely an intact q arm (E in Fig. 4). The 8p deletion in E (Fig. 4) was much smaller than in C (Fig. 4), but larger than in A and B (Fig. 4). Furthermore, two less well defined small fragments (D and F in Fig. 4) were found to be present.
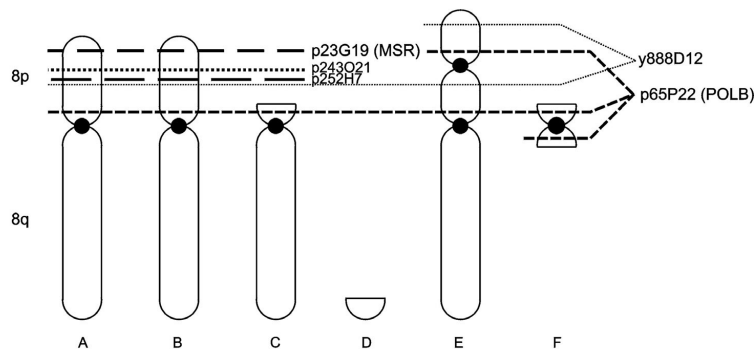
Figure 4. **Schematic representation of the chromosome 8 composition of xenograft PC133.**

DISCUSSION

In this study, we describe a homozygous deleted region at chromosome arm 8p in the prostate cancer xenograft PC133, derived from a skeletal metastasis. Original patient material was not available for further study. However, the HD was already found in the first passage of PC133 on nude mice, showing that it was not the result of selection during subsequent transplantation steps. The HD was detected by PCR on genomic DNA, using five different markers, and confirmed by FISH analyses. The HD is located at 8p12-21 between *D8S133* and *D8S87*, a region frequently showing allelic

loss in prostate cancer (Trapman et al. 1994). The length of the HD is less than 1320 Kb and more than 730 Kb, as judged from the lengths of YACs 953H12 and 721D7.

FISH analyses showed that the HD is present in a duplicated, but otherwise intact chromosome 8. Further, PC133 shows a complex chromosome 8 composition: 8p-, a dicentric chromosome 8, one solitary chromosome 8 centromere, and a small fragment in a marker chromosome. All these chromosome 8 copies lack parts of the p arm, which are larger than the minimal HD, as judged from the absence of p23G19 (*MSR*) and p252H7 (Fig. 4). The centromeric breakpoints in the dicentric chromosome must be close to the centromeric side of the HD, because FISH results with YAC 888D12 are positive. It is not possible to deduce with certainty from the complex chromosome 8 profile the order of genetic changes. Obviously, the small HD has arisen prior to duplication of this particular chromosome 8 copy, suggesting that the deletion is a relatively early event (Fig. 4 A and B). Furthermore, because two allelic forms of the polymorphic marker *D8S87* could be detected, some of, or all chromosomes C-F in Figure 4 must be derived from the other chromosome 8 homologe.

So far, we have not been able to identify an HD, overlapping the PC133 HD, in a different xenograft or cell line. This suggests that HDs at 8p12-21 are relatively rare events in prostate cancer and that inactivation of the putative TSG located in this region by complete or partial deletion is infrequent. Small or overlapping larger HDs have been pivotal in the identification of TSGs like *BRCA2* (Schutte et al. 1995; Wooster et al. 1995), *DPC4* (Hahn et al. 1996), and, more recently, *PTEN/MMAC1* (Li et al. 1997; Steck et al. 1997). Four other HD regions at 8p have been described in prostate cancer. A small (0.7 to 1 Mb) HD region has been identified at the *MSR* locus (8p22). Several genes have been mapped within this HD region (Macgrogan et al. 1996). However, there is, as yet, no evidence that one of these genes is a TSG. Additional HDs have been described at 8p21 and 8p22 by Kagan et al. (1995) (Kagan et al. 1995). However, these HDs are very large, and the experiments lack proper controls.

The HD region in PC133 is expected to contain approximately 30 genes. One of the 8p prostate cancer TSGs is expected to be one of these genes. So far, the only gene found to be located within the HD is the *WRN* gene. One copy is completely

absent, whereas the second copy misses at least the last 10 exons. Werner syndrome is an autosomal recessive disease characterized by accelerated aging and by predisposition to certain cancers. WRN shows significant similarity to DNA helicases (Yu et al. 1996a; Yu et al. 1997). Because of its function and the localization in the HD region, *WRN* might be a candidate TSG involved in prostate cancer. The structure of *WRN* in other xenografts and tumor DNA samples is under investigation.

ACKNOWLEDGMENTS

CHAPTER 5

GENETIC ANALYSIS OF CHROMOSOME INTERVAL 8P12-P21 IN PROSTATE CANCER XENOGRAFTS AND CELL LINES: CHARACTERIZATION OF A HOMOZYGOUS DELETION IN XENOGRAFT PC133 DISRUPTING THE WERNER SYNDROME GENE

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ABSTRACT

Chromosome 8p deletions have frequently been described in prostate cancer. Previously, we identified a homozygous deletion at 8p12 in prostate cancer xenograft PC133, which disrupts the Werner Syndrome gene (*WRN*). In the present study we extend our search for the role of chromosome 8 alterations in prostate cancer, mainly focused on 8p12-p21. Eleven xenografts and four cell lines derived from human prostate cancers were searched for chromosome 8 alterations by Comparative Genomic Hybridisation (CGH) and allelotyping. CGH showed 8p loss in ten DNAs. In eight of the samples loss of 8p included the 8p12-p21 interval. In most DNAs allelotyping matched CGH data. The homozygous deletion in PC133 was found to be 890 Kbp. Thirty-seven STSs mapping in the homozygous deletion were selected for screening of the xenografts and cell lines for overlapping homozygous deletions. However, further homozygous deletions were not detected. To identify candidate tumour suppressor genes in the homozygous deletion, two approaches were used. Search for genomic sequences encoding novel genes identified processed pseudogenes of IDH3A, SMT3H2 and FLJ20038, but no functional genes. RT-PCR showed that EST sts-N22494 was part of the 3'-UTR of a long *WRN* transcript. Also, exon trapping with appropriate restriction fragments from six PACs overlapping the homozygous deletion failed to detect novel genes. Finally, to test *WRN* as a candidate tumour suppressor gene in prostate cancer, mutation analysis of this gene in the xenografts and cell lines was performed. Except for PC133 no alteration in *WRN* were found. So, although complete absence of functional *WRN* might contribute to PC133 tumour growth, in none of the other xenografts and cell lines evidence was found for a role of complete inactivation of *WRN* in tumour development.

INTRODUCTION

Prostate cancer is the most frequently diagnosed cancer and the second leading cause of male cancer death in countries with a Western lifestyle (Greenlee et al. 2001). At present, an adequate therapy of metastatic prostate cancer is not available. In order

to develop novel, targeted therapeutic regimens, knowledge of the major molecular defects in prostate cancer is urgently needed.

Tumour cells are characterized by specific chromosomal alterations, mutations in tumour suppressor genes and oncogenes, and modulated gene expression patterns by chromatin modification, promoter hypermethylation and epigenetic events. Comparative Genomic Hybridization (CGH) has been utilized for genome-wide screening of chromosomal amplifications and deletions in sporadic prostate tumours (Visakorpi et al. 1995; Cher et al. 1996; Nupponen et al. 1998b; Alers et al. 2000; El Gedaily et al. 2001). Most frequent deletions have been found for chromosome arms 6q, 8p, 13q and 16q, indicating the localization of important tumour-associated genes on these chromosome arms. Other common chromosomal deletions in prostate cancer were losses of 5q, 10q, 17p and 18q. The most frequently amplified chromosome arm was 8q, followed by 1q, 3q, 7p, 7q, 17q and 20q. Frequent deletion of 8p is not only found in prostate cancer, but also in many other tumours, including colon, lung, liver and breast cancer (Emi et al. 1992; Emi et al. 1993; Fujiwara et al. 1993; Becker et al. 1996; Yaremko et al. 1996; Chughtai et al. 1999; Pineau et al. 1999; Wistuba et al. 1999).

Although allelotyping has been used in genome-wide screening studies of prostate cancer (Kunimi et al. 1991; Cunningham et al. 1996; Saric et al. 1999), it is generally applied in the accurate mapping of a deleted or amplified chromosomal region. At least two separate regions of allelic loss on chromosome 8p have been found, which is indicative for the localization of two or more distinct tumour suppressor genes on this chromosome arm (Bova et al. 1993; MacGrogan et al. 1994; Trapman et al. 1994; Macoska et al. 1995; Suzuki et al. 1995; Vocke et al. 1996; Prasad et al. 1998). Deletion of 8p22-p23 has been implicated in tumour initiation, whereas 8p12-p21 deletion may play a more significant role in tumour progression (Oba et al. 2001).

An important first step in the identification of tumour suppressor genes can be the detection of a homozygous deletion. Well known examples of tumour suppressor genes in sporadic tumours isolated by this approach are *DPC4* at 18q and *PTEN* at 10q (Hahn et al. 1996; Li et al. 1997; Steck et al. 1997). In prostate cancer tissues, homozygous deletions of chromosome arm 8p have been identified at 8p22 (Bova et al. 1993; Levy et al. 1999), 8p21 (Kagan et al. 1995), and at 8p12 (Prasad et al. 1998). Although the gene *N33* mapping in the homozygous deletion at 8p22 has been suggested as a candidate tumour suppressor gene, conclusive evidence for such a role is lacking (Macgrogan et al. 1996; Arbieva et al. 2000).

Xenografts and cell lines are powerful tools in the analysis of human cancer, because they are available in unlimited quantities, and they can be manipulated by modifications of growth conditions. Additionally, xenografts and cell lines facilitate reliable mRNA and protein expression profiling. Importantly, they lack contaminating normal cells of human origin, which simplifies the detection of homozygous deletions and small mutations in their genomic DNAs. Previously, we identified a small homozygous deletion at 8p12-p21 in the prostate cancer xenograft PC133 (Van Alewijk et al. 1999). This deletion disrupted the Werner syndrome gene (*WRN*).

In this study we extend our search for the role of chromosome region 8p12-p21 in prostate cancer, by detailed comparison of CGH and allelotyping in human prostate cancer derived xenografts, which were generated in our laboratory (Hoehn et al. 1980; Hoehn et al. 1984; Noordzij et al. 1996; Van Weerden et al. 1996), and in prostate cancer cell lines. In addition, we present the characterization of the homozygous deletion in PC133, including the gene content search, and the expression and structural analysis of *WRN* in prostate cancer xenografts and cell lines.

MATERIALS AND METHODS

Prostate Cancer derived Xenografts and Cell lines

The *in vitro* growing cell lines LNCaP, PC-3, DU-145 and TSU were grown under standard cell culture conditions. The *in vivo* xenografts PC82, PCEW, PC133, PC135, PC295, PC310, PC324, PC329, PC339, PC346 and PC374 were propagated on male nude mice as described (see Van Weerden et al. 1996).

DNA and RNA preparation

Genomic DNA from xenografts and cell lines utilized in PCR was isolated according to standard procedures, including overnight proteinase K incubation at 55°C, phenol extraction and ethanol precipitation. DNA pellets were dissolved in TE buffer (10 mM Tris.HCl, pH7.8; 1 mM EDTA). Genomic DNA for CGH was further purified by RNAse treatment. Normal mouse DNA was used as a control in allelotyping studies.

RNA was isolated by standard guanidinium isothiocyanate (cell lines) or LiCl (xenografts) protocols (Sambrook et al. 2001). Total RNAs from fetal brain and normal prostate were purchased from Clontech (Palo Alto, CA).

Comparative Genomic Hybridisation

CGH was performed essentially as described (Kallioniemi et al. 1992). In brief, tumour DNA and normal male reference DNA samples were labeled by nick translation (nick translation system, Life Technologies, Rockville, MD) with biotinylated dUTP (Roche Diagnostics, Almere, The Netherlands) and digoxigenin (Roche Diagnostics), respectively. Labeled DNA samples (200 ng each) and 15 mg Cot-1 DNA (Roche Diagnostics) were ethanol-precipitated and dissolved in 10 ml hybridization mix (50% formamide, 0.1% Tween-20, and 10% dextran sulphate in 2x SSC, pH7.0). The probe mixture was denatured and hybridized to normal male chromosome spreads (Vysis Inc., Downers Grove, IL) for 72 h at 37°C. After the slides were washed, fluorescent detection of the biotin- and digoxigenin-labelled DNA probes was accomplished by fluorescein isothiocyanate (FITC)-conjugated avidin (Vector Labs, Burlingame, CA) and anti-digoxigenin-rhodamine (Roche Diagnostics), respectively. Samples were counterstained with DAPI (4'6'-diamidino-2-phenylindole) (Sigma, St. Louis, MO) in Vectashield anti-fade solution (Vector Labs).

Analyses were performed with a Leica epifluorescent microscope, equipped with a cooled CCD camera (Photometrics Inc., Tuscon, AZ), triple-band pass beam splitter emission filters (P-1 filter set, Chroma Technology, Brattleboro, VT), and a Quips XL image analysis system (version 3.0.1; Vysis Inc.). Chromosomal regions were scored as deleted if the mean green to red ratio was below 0.85, and gained if the ratio was above 1.15.

PCR amplification, allelotyping and SSCP

PCR amplifications, using *Taq* polymerase (Promega, Madison, WI) included 30 cycles of denaturation for 1 min at 95°C, 1 min annealing at 50°C or 55°C, and extension for 1 min at 72°C. For allelotyping and PCR-SSCP, 1 µCi [alpha-^{32}P]dATP (Amersham, Buckinghamshire, UK) was added to the 15 µl reaction mixture. For allelotyping with polymorphic microsatellite markers, radiolabeled PCR products were separated on a polyacrylamide sequence gel. For allelotyping with single nucleotide polymorphisms (SNPs), and for SSCP, radiolabeled PCR products were separated on a 6% non-

denaturing polyacrylamide gel containing 10% glycerol at low power, overnight at room temperature. Gels were dried and exposed to X-ray film for an appropriate period. Non-radioactive PCR amplifications were performed in a 50 µl reaction volume. Amplified fragments were separated on a 2% agarose gel.

Markers for allelotyping of chromosome 8

Genomic DNA was allelotyped by twenty-seven polymorphic microsatellite markers at 8p: *D8S264*, *D8S1742*, *D8S1819*, *D8S1706*, *D8S503*, *D8S549*, *D8S1731*, *D8S261*, *D8S258*, *D8S282*, *D8S133*, *D8S1733*, *D8S1752*, *D8S1739*, *D8S1771*, *D8S131*, *D8S1820*, *D8S1809*, *D8S339*, *AFMa224wh5*, *D8S1770*, *D8S1769*, *D8S1711*, *D8S1810*, *D8S283*, *D8S1722* and *D8S532*, and seven markers at 8q: *D8S260*, *D8S1797*, *D8S1705*, *D8S273*, *D8S267*, *D8S1774* and *D8S272*. Primer sequences and fragment lengths are available in the Genome Database (http://www.gdb.org). SNP markers utilized were Wiaf-3724, Wiaf-1897, Wiaf-3834, Wiaf-3345, Wiaf-283, Wiaf-72, Wiaf-3420, Wiaf-4228, and Wiaf-3809. For details see http://www.ncbi.nlm.nih.gov/SNP.

Screening for homozygous deletions

Screening for homozygous deletions directly downstream of *WRN* in genomic DNAs were done by standard PCR with thirty-seven STSs. Amplified fragments were separated on a 2% agarose gel. Details of the primer sets are available upon request.

Gene prediction of genomic DNA sequences

Human genome sequences in GenBank were searched for candidate genes by the on-line gene prediction programs GRAIL, Pipeline Analysis (Oak Ridge National Laboratory; http://compbio.ornl.gov), and Genefinder (Sanger Center; http://genomic.sanger.ac.uk/gf/gf.html). In addition, the UCSC database (http://www.cse.ucsc.edu) was searched for annotated candidate genes.

Generation of a PAC/cosmid contig of the region deleted in PC133

Gridded PAC filters (Genome Systems, St. Louis, MO), were hybridized with ^{32}P-labeled probes derived from the markers *AFMa224wh5*, 721D7R, WRN exon 26, 721C, WRN exon 35, 107C2-T7, 721A, *D8S1770, D8S1769, D8S124, D8S1711*, 978B9R,

872E4R, 896F4L, 192D20-T7, 13P8-Sp6, 721D7L, 693D8L, *D8S1810* and 761A2L. Sequences of primers utilized to generate the fragments are available upon request. DNA of individual PACs was isolated according to the manufacturers' protocol.

<u>Exon trapping and structural analysis of novel fragments</u>

Candidate exons were isolated from genomic DNA fragments, essentially according to the protocol of the manufacturer of the utilized exon trap kit (Life Technologies, Rockville, MD). In short, PACs 281D2, 107C2, 33H11, 22O23, 57E9 and 13P8 were digested with BamHI/BglII and PstI. Fragments were ligated into the exon trapping vector pSPL3. The libraries were propagated in JM109. COS cells were transfected with pSPL3 libraries by electroporation. After short-time culturing, total RNA was isolated from transfected cells and trapped exons were amplified by RT-PCR. PCR fragments of appropriate lengths were cloned in pGEM-T Easy (Promega, Madison, WI) and sequenced by the dideoxychain termination method. GenBank was searched for homologous sequences by BLAST programs (http://www.ncbi.nlm.nih.gov/blast).

For candidate exons, which contained an open reading frame, and which lacked repetitive sequences, a forward primer was developed and used in combination with the site-primer (described below) in a semi-nested PCR on cDNA from fetal brain and normal prostate RNA to try to generate a downstream flanking fragment.

<u>mRNA expression</u>

mRNA expression was assayed by RT-PCR. First-strand cDNA synthesis was on 1 µg RNA for 1 h at 37°C using 200 U M-MLV-RT (Life Technologies) and a T_{12}-site primer (5-GCATGCGAATTCGGATCCT$_{12}$-3) in first-strand buffer (Life Technologies), containing 10 mM DTT, 1 mM dNTPs, and 40 U RNAsin (Promega). The first PCR reaction was performed on 1/20th of the cDNA reaction mixture, utilizing a specific forward (F) primer in combination with site-primer 5-GCATGCGAATTCGGATCC-3. Next, semi-nested PCR fragments were obtained with gene specific forward and reverse primers (F/R) on 1/500th of the first PCR product. Gene specific primer combinations for RT-PCR of *WRN* were: WRN-F 5-TCTTCTGGGAGCCTACGTGAG-3 and WRN-R 5-TGCGGTTTCATTTTCACTGCCCTG-3, respectively. Primer combinations for RT-PCR of EST sts-N22494 was; sts-N22494-F 5-ACTGAGAACAGCAGCATTGT-3 and sts-N22494-R 5-GGAAACTATTACTGGTCCAC-3. Control RT-PCRs were carried out under

the same conditions, using RNA polymerase II specific primers: Pol-F (5-GCTGAGAGAGCCAAGGATAT-3) and Pol-R (5-CACCACCTCTTCCTCCTCTT-3).

Structural analysis of ESTs

Sequences overlapping with sts-N22494 were found in three other cDNA clones derived from different libraries (AI248120, fetal liver spleen; BG029683 and BI850480, mammary carcinoma; AW779829, kidney).

EST sts-N22494 RT-PCR fragments were obtained according to the RT-PCR protocol as described above. Specific primer combinations for the first PCR amplification were: 34F 5-GGTTCTGAAGAGATCTGTTC-3 and 35R 5-CCCTGGTCAACTAATACCA-3 or WRN/N22494 5-CCAACTCTGTGTCACAGTATAG-3. The primer set for the nested amplification was: 34F2 5-GCAAGGAAGAAGTAGGCATC-3 and 35R2 5-CCAGAAGACCCAGAAACTAC-3.

Structural analysis of *WRN*

For PCR-SSCP analysis of *WRN*, overlapping 700-800 bp cDNA fragments, encompassing the complete open reading frame, were amplified. Next, smaller overlapping fragments were obtained by (semi)nested PCR and analyzed for mutations and polymorphisms by SSCP and sequencing. Primer sequences are available upon request. For sequencing, amplified fragments were purified over QIAquick spin columns (Qiagen, Hilden, Germany) and ligated into pGEM-T Easy.

RESULTS

Identification of chromosome 8 alterations in prostate cancer derived xenografts and cell lines

Chromosome 8 alterations are the most frequent genetic defects in prostate cancer. Because xenografts and cell lines are important starting materials for detailed molecular genetic studies, chromosome 8 was investigated by CGH and allelotyping in these specimens. We utilized genomic DNA from the xenografts PCEW, PC82, PC133, PC135, PC295, PC324, PC329, PC310, PC329, PC339, and PC374, and the cell lines LNCaP, PC3, DU145 and TSU.

Comparative Genomic Hybridisation

In all samples, except for xenografts PCEW and PC82, and the LNCaP cell line, chromosome 8 alterations were detected by CGH (Fig. 1A). In PC295, PC324, PC329, and PC374 loss of 8p paralleled amplification of 8q. In PC133 and PC3 apparent chromosome break points could be found in the short arm or in the long arm, respectively (Fig. 1B; see also Van Alewijk et al. 1999). In PC310, only the distal parts of 8p and 8q were deleted and amplified, respectively (Fig. 1B). Loss of 8p without 8q amplification was detected in TSU; 8q amplification without 8p deletion was present in PC346. The largest deviations from the general pattern of 8p loss and 8q gain were found for PC135 and PC339. CGH indicated in both xenografts additionally to gain of 8q, amplification of parts of 8p (Fig. 1C).
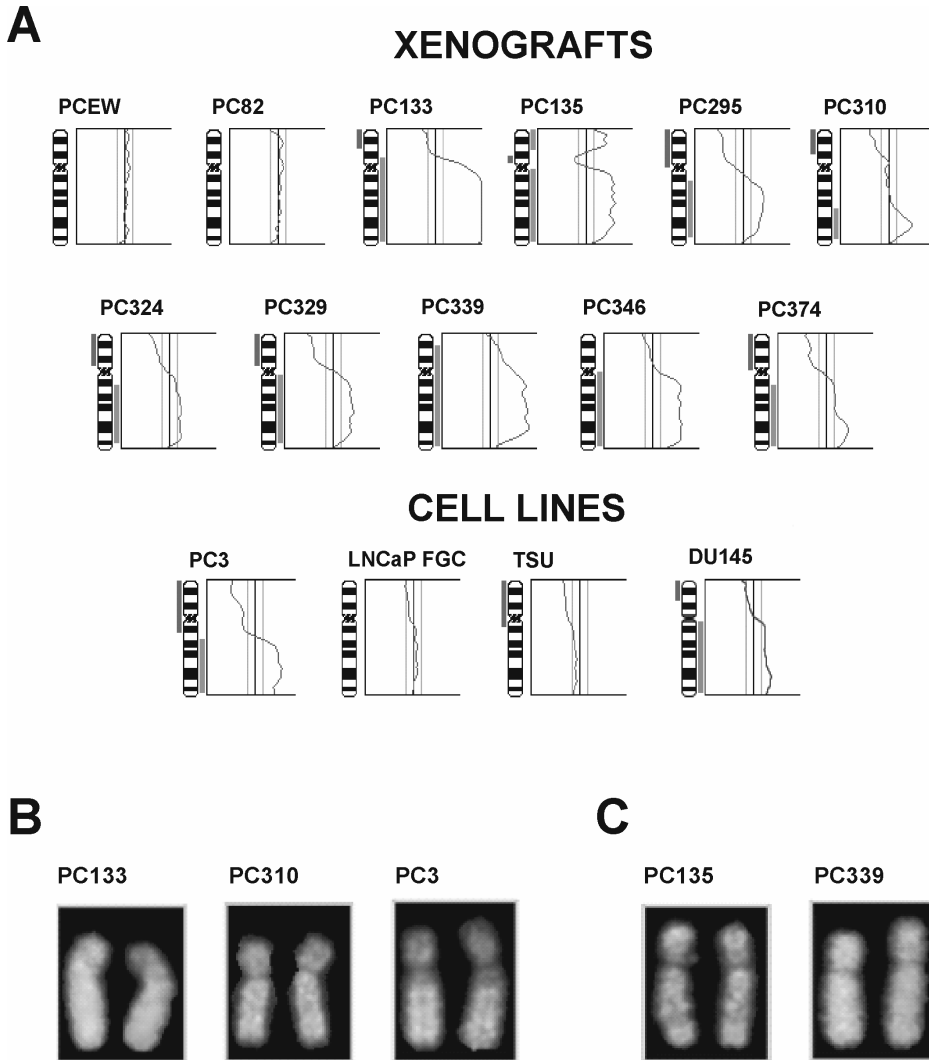
Figure 1. **(A)** Chromosome 8 alterations identified by comparative genomic hybridisation in fifteen prostate cancer xenografts and cell lines (CGH: mean 0.85-1.15; n=12-17). A bar to the right of an ideogram indicates gain; a bar to the left indicates loss. **(B)** Samples showing gain of 8q and proximal 8p, and loss of distal 8p (PC133), gain of 8q and loss of 8p (PC310), and gain of distal 8q and loss of proximal 8q and 8p (PC3). **(C)** Samples showing gain of whole chromosome 8 (PC339), and gain of distal 8p and distal 8q (PC135).

Allelotyping

To obtain independent complementary, more detailed information on chromosome 8 alterations, the same genomic DNAs were allelotyped by thirty-five highly polymorphic microsatellite markers. The highest marker density was chosen for 8p12-p21, where in previous studies loss of heterozygosity in DNA from prostate tumour samples, and a homozygous deletion in xenograft PC133 have been described by us (Trapman et al. 1994; Van Alewijk et al. 1999). Allelotyping results are summarized in Fig. 2A; some informative examples are depicted in Fig. 2B. In PC346, PC374, LNCaP and DU145 30-65% instability of the microsatellite markers was detected, which limited the accuracy of the analyses in these samples [see *D8S1731* in PC346 and LNCaP as an example (Fig. 2B)].

Almost all xenografts and cell lines showed two allelic forms of at least one polymorphic marker on 8q. For many 8q and some 8p markers a clear imbalance of the two alleles was found, which is indicative of gain [see *D8S1731* (PC135), *D8S1722* (PC133), *D8S273* (PC135) and *D8S272* (PC339) as examples in Fig. 2B]. In PC3 and TSU all seven 8q and twenty-eight 8p markers demonstrated one allelic form, indicating the loss of one copy of the whole chromosome. In PC133, PC135, PC295, PC310, PC324, PC329, PC339, PC346 and P374 large series of consecutive markers on 8p showing apparent homozygosity were detected, strongly suggesting loss of one copy of the corresponding chromosomal regions. The maximum number of consecutive markers showing one band in PCEW was five: from *D8S258* till *D8S1752*, in a 5 Mbp interval. This might represent a small region of chromosomal loss. In the region between *AFMa224wh5* and *D8S283*, which is completely deleted in PC133, no clear evidence for allelic loss was found in PCEW, PC82, PC310, PC346, LNCaP and DU145.

A

Chromosome 8

Marker	Mbp (UCSC Apr 2003 freeze)	PC EW	PC 82	PC 133	PC 135	PC 295	PC 310	PC 324	PC 329	PC 339	PC 346	PC 374	PC-3	LNCaP	TSU	DU-145
		Xenografts											Cell lines			
D8S264	2,3	1	2	1	2	1	1	1	1	1	1	MSI	1	MSI	1	2
D8S1742	6,4	1	2	1	1	1	1	1	1	1	MSI	MSI	1	MSI	1	MSI
D8S1819	6,9	2	1	1	1	1	1	1	1	1	MSI	MSI	1	MSI	1	MSI
D8S1706	7,1	1	2	1	2	1	1	1	1	1	MSI	MSI	1	2	1	MSI
D8S503	9,1	1	1	1	1	1	1	1	1	1	1	1	1	MSI	1	MSI
D8S1731	15,0	1	1	1	2	1	1	1	1	1	MSI	1	1	MSI	1	MSI
D8S549	15,4	1	1	1	1	1	1	1	1	1	1	1	1	2	1	1
D8S261	17,6	2	2	1	1	1	1	1	1	1	MSI	MSI	1	MSI	1	MSI
D8S258	20,2	1	2	1	1	1	1	1	1	1	1	1	1	MSI	1	MSI
D8S282	21,2	1	2	1	1	1	1	1	1	1	MSI		1	2	1	1
D8S133	21,8	1	2	1	1	1	1	1	1	1	1		1	MSI	1	1
D8S1733	22,3	1	1	1	1	1	1	1	1	1	1		1	1	1	1
D8S1752	22,5	1	2	1	1	1	1	1	1	1	1		1	MSI	1	MSI
D8S1739	24,8	2	2	1	1	1	1	1	1	1	1		1	MSI	1	2
D8S1771	25,3	2	1	1	1	1	1	1	1	1	1		1	MSI	1	MSI
D8S131	27,2	2	2	1	1	2	1	1	1	1	MSI	MSI	1	MSI	1	MSI
D8S1820	27,8	2	1	1	1	2	1	1	1	1	MSI		1	MSI	1	MSI
D8S1809	28,0	2	2	1	1	2	1	1	1	1	MSI	1	1	1	1	MSI
D8S339	30,2	2	2	1	1	2	1	1	1	1	1		1	1	1	2
AFMa224wh5	30,6	2	1	1	1	2	1	1	1	1	1		1	1	1	2
D8S1770	31,0	1	2	0	1	1	2	1	1	1	1	1	1	2	1	1
D8S1769	31,0	1	2	0	1	1	2	1	1	1	MSI	MSI	1	2	1	MSI
D8S1711	31,2	2	1	0	1	1	1	1	1	1	1	1	1	MSI	1	2
D8S1810	31,5	2	1	0	1	1	2	1	1	1	MSI	1	1	MSI	1	MSI
D8S283	33,5	2	2	1	1	1	2	1	1	1	1	1	1	MSI	1	MSI
D8S87	35,2	1	1	2	1	1	2	1	1	1	1	1	1	1	1	MSI
D8S1722	37,5	2	2	2	1	1	2	1	1	1	MSI	1	1	2	1	MSI
D8S532	40,5	2	2	1	2	1	2	1	1	1	MSI	1	1	MSI	1	MSI
D8S260	61,5	2	1	2	2	2	2	2	2	1	MSI	1	1	MSI	1	MSI
D8S1797	67,0	1	2	2	2	1	2	2	2	2	MSI	2	1	2	1	MSI
D8S1705	80,0	2	1	2	2	1	2	2	2	2	MSI	MSI	1	MSI	1	MSI
D8S273	88,6	2	2	2	2	1	2	1	2	1	2	2	1	MSI	1	2
D8S267	104,5	2	2	2	1	2	2	1	1	1	2	2	1	MSI	1	MSI
D8S1774	127,1	2	2	2	1	2	1	2	2	2	MSI	MSI	1	MSI	1	MSI
D8S272	137,4	2	1	2	2	1	1	1	1	2	2	MSI	1	MSI	1	2

p

q

B

D8S1731	D8S1722	D8S273	D8S272
PC133 PC135 PC339 PC346 LNCaP	PC82 PC133 PC310 PC324	PC135 PC310 PC329	PC324 PC329 PC339
1 2+ 1 MSI MSI	2 2+ 1 1	2+ 1 1	1 1 2+

Figure 2. **(A)** Chromosome 8 alterations as identified by allelotyping of fifteen prostate cancer xenografts and cell lines. Two allelic bands are given as 2; 1 indicates one band, which might represent one allele or two alleles of identical length. Occasionally, one of two allelic bands was stronger, indicative of gain (data not shown). The homozygous deletion in PC133 (REF) is represented by "0". MSI: microsatellite instability. The previous described LOH region (Trapman et al. 1994) between *D8S133* and *D8S87* at 8p12-p21 is represented by a vertical bar. **(B)** Selection of samples demonstrating one and two allelic forms, amplification (2+), and MSI.

To obtain more information on loss of chromosome 8 in the four samples with frequent MSI (PC346, PC374, LNCaP and DU145), they were typed by nine single

nucleotide polymorphisms (SNPs) scattered along 8p22-pter (see Materials and Methods). In both LNCaP and DU145, several SNP markers revealed two alleles across 8p22-pter, which is indicative of absence of 8p loss in these cell lines. PC346 showed two allelic forms of a clusterin (*CLU*) SNP, which mapped between *D8S1771* and *D8S131*, but one allelic form for all more distal SNPs, indicating loss of the distal part of 8p (data not shown). In PC374 none of the 8p SNPs showed two alleles.

Comparison of Comparative Genomic Hybridization and allelotyping of prostate cancer xenografts

In general, the CGH and allelotyping data matched well, although there were some discrepancies. Most remarkable are TSU, for which allelotyping of the whole chromosome showed the presence of single alleles, without indication of loss of 8q, PC3 with 8q amplification by CGH analysis and loss by allelotyping, and PC339 with 8p amplification by CGH and 8p deletion by allelotyping.

The homozygous-deleted region in xenograft PC133

Length of the homozygous-deleted region in PC133, and search for novel, overlapping homozygous deletions

As shown previously (Van Alewijk et al. 1999) and in this study (Fig. 2A and 3), xenograft PC133 contained a homozygous deletion at 8p12. The deletion disrupted at its telomeric border *WRN*. As deduced from YAC lengths, the length of the homozygous-deleted region was previously estimated to be between 0.7 and 1.3 Mbp (Fig. 3C; Van Alewijk et al. 1999).

The borders of the deleted region were more accurately determined with STS markers. The telomeric border mapped in *WRN* intron 8 (Fig. 3B). Centromeric, *D8S1810* was found to be located in the deleted region, whereas 13P8-T7 was just proximal to the deletion (Fig. 3B). Comparison with the human genome sequence showed that the homozygous deletion spanned approximately 890 Kbp (Fig. 3E). Within this region, thirty-seven STS markers, together with markers that mapped at the borders of the homozygous-deleted region, were applied in screening for overlapping homozygous deletions in all available prostate cancer xenografts and cell lines. However, in none of the DNAs a novel homozygous deletion could be detected.

Figure 3. (A) Physical map of chromosome 8p12-p21, which is frequently deleted in prostate cancer. HD: homozygous deletion. (B) Physical map of the homozygous deletion identified in PC133. (C) YACs spanning the homozygous deletion in PC133. (D) PACs utilized for exon trapping. (E) Sequenced BACs from UCSC genome browser. (F) Genes and pseudogenes mapping in the investigated region.

Gene search in the homozygous deletion by sequence analysis

Annotation of the human genome sequence by different gene prediction programs (UCSC, Ensembl, NCBI) showed *WRN* as the only known gene in the homozygous deletion. Gene finding programs like GRAIL, Genefinder, and Pipeline Analysis also failed to detect *bona vide* genes, except *WRN*. Sequence homology searches identified processed pseudogenes of *IDH3A, SMT3H2* and *FLJ20038* in the homozygous deletion (Fig. 3F).

Many ESTs mapped in the homozygous deletion. However, critical study of EST sequences and their flanking genomic sequences, absence of a polyadenylation signal, presence of a flanking genomic A-track, presence of a repeat sequence, absence of a long open reading frame, combined with low expression made it very unlikely that they were parts of genes. The exception was sts-N22494, which is discussed below.

Gene search in the homozygous-deleted region by exon trapping

We isolated the 890 Kbp genomic segment in a PAC/Cosmid contig. Appropriate genomic DNA fragments from six overlapping PACs (see Fig. 3D) that covered the homozygous deletion were screened for novel genes by exon trapping. Approximately one hundred different genomic fragments were identified. From PAC P281D2 *WRN* exons 14 to 34 were isolated. None of the other trapped fragments was identical to a known gene or EST sequence. Twenty fragments had an open reading frame and did not contain repetitive sequences. To study whether these fragments were present in mRNA, semi-nested RT-PCRs were done on RNA from normal prostate and from fetal brain. However, none of the PCRs was positive, accordingly none of the fragments were part of a *bona fide* mRNA.

Characterization of sts-N22494

Sts-N22494 mapped just downstream of the last *WRN* exon (Fig. 3F). Overlapping sequences were found in 4 other ESTs (see Materials and Methods). All contained a polyadenylation signal, but lacked a long open reading frame (data not shown). The genomic sequence was identical to the cDNA sequence, but lacked a polyA-stretch downstream of the polyadenylation signal. Sts-N22494 expression was analyzed by RT-PCR (Fig. 4A). Sts-N22494 was easily detected in all RNA samples from prostate cancer xenografts and cell lines, with the exception of PC133. The genomic sequence revealed that sts-N22494 was located approximately 2.2 Kbp downstream of the *WRN* gene, in the same orientation. Because no convincing open reading frame could be found in the genomic sequence between *WRN* and sts-N22494,

the possibility that sts-N22494 was part of a longer *WRN* transcript was considered (Fig. 4B and 4C). First, a RT-PCR reaction from *WRN* exon 34 across the initially described *WRN* exon 35 was done (34F-WRN/N22494R, Fig. 4B). Subsequently, the product was visualized by a nested PCR from *WRN* exon 34 to exon 35 (34F2-35R2 in Fig. 4B; 335 bp fragment in 4C); the 34F-35R RT-PCR followed by 34F2-35R2 nested PCR was carried out as a control (*WRN* in Fig. 4C). The positive result provided strong evidence that indeed sts-N22494 was derived from a longer *WRN* transcript.

Expression and structural analysis of *WRN*

Because *WRN* turned out to be the only gene present in the homozygous deletion in PC133, *WRN* expression and structure were investigated in all other prostate cancer xenografts and cell lines. In all samples, except PC133, *WRN* mRNA was detectable by RT-PCR (Fig. 4). The entire open reading frame of *WRN* mRNA was analyzed for mutations by RT-PCR-SSCP. All fragments showing an altered PCR-SSCP pattern were sequenced. The altered sequences turned out to be previously described polymorphisms: TGT(Cys)171TGC(Cys); ATG(Met)387ATA(Ile); CTT (Leu)787CT(Leu); TTT(Phe)1074TTG(Leu); AGC(Ser)1361AGT(Ser) and TGT(Cys)1367CGT(Arg) (Bernardino et al. 1997; Meisslitzer et al. 1997; Ye et al. 1997). So, no evidence could be found for substantial downregulation of *WRN* expression or inactivating mutations in the open reading frame.
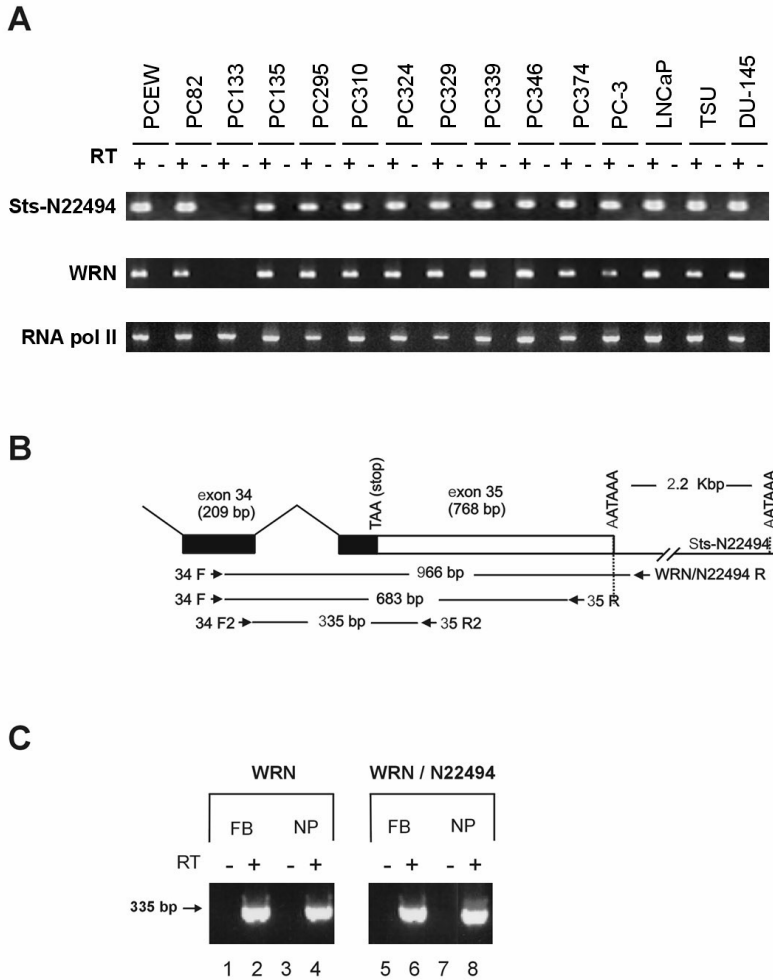
Figure 4. **(A)** RT-PCR analysis of STS-N229494 and *WRN* mRNA expression in prostate cancer cell lines and xenografts and cell lines. RNA pol II was utilized as a control. RT: reverse transcriptase. **(B)** Schematic representation of RT-PCR and nested PCR to detect *WRN* and STS-N22494 transcripts. **(C)** Amplified fragments obtained by nested PCR (34F2/35R2) of *WRN* and STS-N22494 transcripts, showing that sts-N22494 represents a longer *WRN* transcript. FB: fetal brain; NP: normal prostate; RT: reverse transcriptase.

DISCUSSION

In this study, we describe the genetic characterization of chromosome 8 in fifteen human prostate cancer derived xenografts and cell lines, mainly focused on the homozygous deletion at 8p12 in xenograft PC133 (Van Alewijk et al. 1999). Xenografts and cell lines are unique starting materials for the identification of genes associated with prostate cancer and the functional characterization of such genes.

Loss of 8p and gain of 8q are among the most frequently observed chromosomal alterations in clinical prostate cancer samples (Bova et al. 1993; Trapman et al. 1994; Visakorpi et al. 1995; Cher et al. 1996; Vocke et al. 1996; Nupponen et al. 1998b; Prasad et al. 1998; Alers et al. 2000). Loss of 8p indicates the localization of one or more tumour suppressor genes and amplification of 8q suggests the presence of oncogenes. By CGH, ten out of fifteen xenografts and cell lines showed loss of 8p, encompassing the region 8p12-pter in eight samples. Furthermore, in eleven samples gain of 8q was found. So, the xenografts and cell lines reflect the chromosome 8 alterations found in patient materials.

Concomitant 8p loss and 8q gain has been attributed to the presence of isochromosome 8q (Macoska et al. 2000). In at least four xenografts this mechanism might have occurred (PC295, PC324, PC329, and PC374). However, in four xenografts and cell lines, different chromosome alterations must have taken place. CGH data indicate loss of 8p without gain of 8q in TSU, amplification of 8q13-qter in PC3, loss of distal 8p and gain of distal 8q in PC310, and a dicentric chromosome 8 in PC133 (Van Alewijk et al. 1999).

In general, CGH data matched those obtained by allelotyping. Major discrepancies were found for PC339, PC3, TSU and DU145. In PC339, CGH indicated gain of the entire chromosome 8. However, all polymorphic markers on 8p showed one allelic form, which is strong evidence for 8p loss. Probably, one copy of chromosome 8 lost its p-arm, followed by amplification of the second, intact, copy. Similarly, CGH indicated gain of 8q in PC3, but all seven 8q markers showed one allelic band. Possibly, one copy of chromosome 8 was lost and the q-arm of the second copy was amplified. In DU145, CGH showed 8p loss and by allelotyping two alleles for the most distal marker *D8S264* and four 8p SNPs were found. The most likely explanation for the discrepancy

is loss of 8p in the partially triploid cell line DU145. In TSU, CGH indicated loss of 8p, but allelotyping suggested loss of one entire copy of chromosome 8. Interestingly, a similar observation was made by allelotyping of chromosomes 6 and 10 (Verhagen et al. 2002; Hermans, unpublished), suggesting a defect in chromosome segregation in this cell line. Our CGH data of the cell lines PC3, LNCaP, TSU and DU-145 were in accordance with previous CGH and SKY studies (Bernardino et al. 1997; Nupponen et al. 1998a; Pan et al. 1999). The complementary allelotyping showed that genomic alterations in these cell lines might be more complex than indicated by CGH. Small regions of amplification or loss might be most accurately identified by array CGH (Pinkel et al. 1998). This might lead to detection of high-level amplifications on 8q and small regions of loss in those xenografts and cell lines that seem to contain a normal 8p content. Array CGH might also be applied to confirm the proposed small region of loss in PCEW (Fig. 2) and to identify novel small homozygous deletions on 8p.

In PC346, PC374, LNCaP and DU145 we observed a high frequency of MSI, implicating defects of the mismatch repair machinery. In DU145 a splice acceptor site mutation has been found in *MLH1* (Boyer et al. 1995; Chen et al. 2001). LNCaP contained a homozygous deletion of *MSH2* (Leach et al. 2000; Chen et al. 2001); Trapman, unpublished). The defects in mismatch repair genes in PC346 and PC374 remain to be established. MSI was also detected in the clinical specimen from which xenograft PC346 was derived (data not shown). The frequency of MSI or aberrant expression of mismatch repair genes in clinical prostate cancer specimens, as reported so far, is very variable, ranging from 0% to 65% (Egawa et al. 1995 ; Uchida et al. 1995; Cunningham et al. 1996; Rohrbach et al. 1999). Our observations warrant renewed search for the role of mismatch repair genes in prostate cancer.

Although we carried out an extensive search, we were unable to find in any of the xenografts and cell lines a homozygous deletion overlapping the one previously detected in PC133 (Van Alewijk et al. 1999). The deletion in PC133 has a length of approximately 890 Kbp and disrupts *WRN*. The complete sequence of this genomic region is known. Except for *WRN*, no genes mapped in this region, utilizing a variety of experimental and gene prediction approaches. Because in PC133 the homozygous deletion was selected during tumour growth, it is very likely that *WRN* plays a role in the development of this tumour. *WRN* encodes a RecQ helicase that also contains DNA exonuclease activity. It is believed to function in maintaining the integrity of the genome (Moser et al. 2000a) reviewed by (Hickson 2003). Werner Syndrome patients are characterized by premature

aging and by predisposition for tumour development. In mouse models, *Wrn* deficiency accelerates tumour growth and broadens the tumour profile of *p53* null mice (Lombard et al. 2000; Lebel et al. 2001). Although prostate cancer has never been associated with Werner Syndrome these properties make it a candidate tumour suppressor gene in PC133. *WRN* seems not to be involved in the other xenografts and cell lines, because no deletions or point mutations have been found. However, it cannot be excluded that diminished *WRN* expression, due to haplo-insufficiency contributes to tumour formation. Targeted inactivation of *Wrn* in appropriate mouse cancer models will be instrumental to investigate this hypothesis.

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CHAPTER 6

PRELIMINARY DATA ON SCREENING FOR HOMOZYGOUS DELETIONS, AND EXPRESSION AND MUTATION ANALYSIS OF CANDIDATE TUMOUR SUPPRESSOR GENES ON CHROMOSOME 8P12-P21 IN PROSTATE CANCER

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INTRODUCTION

One of the most frequent genomic alterations in prostate cancer is partial or complete loss of chromosome arm 8p, which indicates the localization of one or more tumour suppressor genes on this chromosomal arm (Kunimi et al. 1991; Visakorpi et al. 1995; Cher et al. 1996; Cunningham et al. 1996; Nupponen et al. 1998b; Saric et al. 1999; Alers et al. 2000). An important first step in the identification of tumour suppressor genes is the identification of a homozygous deletion. In prostate cancer, several homozygous deletions have been identified at chromosome 8p (Bova et al. 1993; Kagan et al. 1995; Prasad et al. 1998; Levy et al. 1999; Van Alewijk et al. 1999). However, this has so far not resulted in the isolation of a *bona fide* tumour suppressor gene. The homozygous deletion that we identified disrupted the Werner syndrome gene (*WRN*) in xenograft PC133 (Van Alewijk et al. 1999). However, structural alterations or absence of WRN expression could not be found in any of 11 prostate cancer xenografts, generated in Rotterdam (Noordzij et al. 1996; Van Weerden et al. 1996; and references therein), or the cell lines PC-3, LNCaP, TSU and DU-145. It was concluded that complete inactivation of *WRN* had no significant role in prostate cancer (Chapter 5; Van Alewijk et al. submitted).

We continued to search for a tumour suppressor gene on 8p12-p21 by two parallel experimental approaches. First, screening for novel homozygous deletions, and secondly, structural and expression analysis of candidate genes. As a start we investigated *LZTS1/FEZ*, *NKX3-1*, *EPHX2* and *CLU* on 8p12-p21, *POLB* on 8p11 and *PRLTS* on 8p22. Except for *EPHX2* and *CLU*, all genes were previously implicated in prostate cancer (Dobashi et al. 1994; Komiya et al. 1997; Ishii et al. 1999; Kim et al. 2002a). *EPHX2* and *CLU* were selected because of their physiological function.

In both the screening for homozygous deletions, and the expression and mutation analysis we used the xenografts and cell lines as described above. Xenografts and cell lines are powerful tools for the genetic analysis of cancer because they lack contaminating normal cells of human origin. Like in prostate cancer tissues, deletion of 8p in xenografts and cell lines is a frequent chromosomal defect (Chapter 5; Van Alewijk et al., submitted). If the expression and mutation analysis of the xenografts and cell lines showed a possible tumour related alteration, we continued by screening for mutations in DNAs from 43 locally progressive prostate tumour samples.

MATERIALS AND METHODS

Prostate Cancer derived Xenografts and Cell lines

The *in vitro* growing cell lines LNCaP, PC-3, DU-145 and TSU were grown under standard cell culture conditions. The *in vivo* xenografts PC82, PCEW, PC133, PC135, PC295, PC310, PC324, PC329, PC339, PC346 and PC374 were propagated on male nude mice as described (Van Weerden et al. 1996). Prostate tumour tissues were from patients with a confirmed clinical history of locally progressive prostate cancer. Tissue samples were obtained by transurethral resection (TUR).

DNA and RNA preparation

Genomic DNA from xenografts and cell lines was isolated according to standard procedures, including overnight proteinase K incubation at 55°C, phenol extraction and ethanol precipitation. DNA pellets were dissolved in TE buffer (10 mM Tris.HCl, pH7.8; 1 mM EDTA). Tumour DNA was isolated from five consecutive 5 µm cryostate tissue sections according to the standard procedure. In the vast majority of cases, control DNA was from white blood cells. For a minority of tumours, control DNA was from non tumour regions of prostate tissue.

RNA was isolated by standard guanidinium isothiocyanate (cell lines) or LiCl (xenografts) protocols (Sambrook et al. 2001). Total RNA from foetal brain and normal prostate were purchased from Clontech (Palo Alto, CA).

PCR amplification and SSCP

PCR amplifications, using *Taq* polymerase (Promega, Madison, WI) included 30 cycles of denaturation for 1 min at 95°C, 1 min annealing at 50°C or 55°C, and extension for 1 min at 72°C. For PCR-SSCP, 1 µCi [alpha-^{32}P]dATP (Amersham, Buckinghamshire, UK) was added to the 15 µl reaction mixture. For SSCP, radio-labelled PCR products were separated on a 6% non-denaturing polyacrylamide gel containing 10% glycerol at low power, overnight at room temperature. Gels were dried and exposed to X-ray film for an appropriate period. Standard PCR amplifications were performed in a 50 µl reaction volume. Amplified fragments were separated on a 2% agarose gel.

Screening for homozygous deletions

The 15 xenograft and cell line DNAs were analysed by PCR for homozygous deletions in a 17 Mbp region on 8p12-p21, utilising approximately 300 specific markers (see Appendix A6 - supplementary Table I). Amplified fragments were separated on a 2% agarose gel. Primer sets are available upon request.

<u>mRNA expression</u>

mRNA expression was assayed by RT-PCR. First-strand cDNA synthesis was on 1 µg RNA for 1 hr at 37°C using 200 U M-MLV-RT (Life Technologies, Rockville, MD) and a T_{12}-site primer (5-GCATGCGAATTCGGATCCT$_{12}$-3) in first-strand buffer (Life Technologies), containing 10 mM DTT, 1 mM dNTPs, and 40 U RNAsin (Promega). The first PCR reaction was performed on 5% of the cDNA reaction mixture, utilizing a specific forward (F) primer in combination with site-primer 5-GCATGCGAATTCGGATCC-3. Next, semi-nested PCR fragments were obtained with gene specific forward and reverse primers (F/R) on 1/500 of the first PCR product. Gene specific primer combinations for RT-PCR of *PDGFRL*, *LZTS1*, *NKX3-1*, *EPHX2*, *CLU*, *POLB*, and *RNA pol-II* are available upon request.

<u>Structural analysis</u>

Amplified fragments were purified over QIAquick spin columns (Qiagen, Hilden, Germany), ligated into pGEM-T Easy, and sequenced by the dideoxy chain termination method.

RESULTS AND DISCUSSION

Here, the preliminary results of the search for homozygous deletions at chromosome 8p12-p21, and the expression and mutation analysis of the genes *PDGFRL*, *LZTS1*, *NKX3-1*, *EPHX2*, *CLU*, and *POLB* in prostate cancer are described.

<u>High-density screening for homozygous deletions at 8p12-p21</u>

To screen the xenografts and cell lines for a homozygous deletion in a 17 Mbp region on 8p12-p21, approximately 300 markers mapping between LPL and BC028701 were selected (see Appendix A6 - supplementary Table I). Except for the previously described homozygous deletion in PC133 (Van Alewijk et al. 1999), no homozygous deletion could be identified, despite the high marker density.

Expression and mutation analysis of candidate tumour suppressor genes

In previous studies, 4 genes have been claimed to be involved in prostate cancer, *PDGFRL* (8p22), *LZTS1/FEZ* (8p21), *NKX3-1* (8p21) and *POLB* (8p11). *PDGFRL* was suggested as a candidate tumour suppressor gene because of 1 missense mutation in a series of 69 prostate tumour samples (Fujiwara et al. 1995). In addition, missense and frameshift mutations were found in 2 out of 48 hepatocellular cancer and 1 out of 28 colorectal cancers (Fujiwara et al. 1995). *LZTS1* was suggested to be a tumour suppressor gene, because expression was undetectable in more than 60% of epithelial tumours, whereas it is ubiquitously expressed in normal tissues (Ishii et al. 1999). In addition, in a series of 194 epithelial tumours, Ishii et al (1999) found 2 missense mutations in primary oesophageal cancers and 1 nonsense mutation in the prostate cancer cell line PC-3. Further, LZTS1 expression levels were significantly reduced in gastric carcinomas (Vecchione et al. 2001). Germ line sequence variants of the *LZTS1* gene were associated with an increased prostate cancer risk (Hawkins et al. 2002). Because of its function and prostate specific expression the homeobox gene *NKX3-1* was associated with prostate cancer (He et al. 1997; Sciavolino et al. 1997; Bhatia-Gaur et al. 1999). *POLB* showed 1 frameshift and 1 missense mutation in a series of 12 prostate cancer samples (Dobashi et al. 1994). In addition, 1 missense and 1 frameshift mutation were found in 2 out of 6 colorectal cancers (Wang et al. 1992).

In addition to candidate tumour suppressor genes from literature searches, *EPHX2* (8p21) was studied because of its function as a cytosolic member of the family of the epoxide hydrolases (Larsson et al. 1995; Sandberg and Meijer 1996). In various organisms epoxide hydrolases have an important protective function because they are able to convert potentially harmful epoxide-containing compounds into diols, which are less reactive and easier to secrete (Meijer and DePierre 1988). Interestingly, a significant association was found between polymorphisms and activity of microsomal epoxide hydrolase 1 (*EPHX1*), and cancers of the respiratory tract (Benhamou et al. 1998; Jourenkova-Mironova et al. 2000). The authors postulated that *EPHX1* was an important genetic determinant for smoking-induced lung cancer.

CLU (8p21) has also been investigated as a candidate tumour suppressor gene because of its function. Clusterin has been implicated in several diverse physiological

processes, one of these is apoptosis of normal and prostate cancer cells (Trougakos and Gonos 2002; Miyake et al. 2003).

For neither *PDGFRL*, nor *CLU* or *POLB* any alteration in expression, as analysed by RT-PCR, or structure, as determined by PCR-SSCP and sequencing, was detected in the 15 xenografts and cell lines, indicating an insignificant role in prostate cancer (data not shown).

As shown in Table I, expression of *LZTS1* mRNA was detectable in all RNA samples, except for PC133. *NKX3-1* mRNA was detectable in all RNA samples from prostate. As expected *NKX3-1* mRNA expression was undetectable in foetal brain (He et al. 1997). Except for TSU, the expression of *EPHX2* mRNA was detectable in all RNA samples. In summary, no significantly altered expression pattern in prostate cancer was found for these 3 genes.

Table I

Expression of candidate tumour suppressor genes on chromosome 8p in prostate cancer

	PC82	PCEW	PC133	PC135	PC295	PC310	PC324	PC329	PC339	PC346	PC374	PC3	LNCaP	TSU	Du145	Normal prostate	Fetal Brain
LZTS1	++	++	-	++	++	++	++	++	++	++	++	++	++	++	++	++	++
NKX 3.1	++	++	+	+	++	++	+	++	++	++	++	+	++	++	++	++	-
EPHX2	+++	+++	+	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	-	+++	+++	+++
RNA pol II	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++

LZTS1 and *EPHX2* were initially analysed for mutations by PCR-SSCP on genomic DNA of the xenografts and cell lines. Subsequently, samples showing aberrant SSCP bands were sequenced. Because paired control DNA was not available, 50 independent normal DNAs were used as controls. *NKX3-1* sequence was analysed by PCR-SSCP of overlapping amplified cDNA fragments, encompassing the complete open reading frame. *NKX3.1* single nucleotide polymorphisms present in databases were used as controls. The experimental results are listed in Table II. Frequently found polymorphisms are not included.

LZTS1

As shown in Table II, we identified in *LZTS1* 1 silent polymorphism and mutations or rare polymorphisms in DU-145 (AAC>AGC, codon 223) and in LNCaP (CAG>CGG, codon 556), respectively. In both DU-145 and LNCaP the wild type allele was retained. We could not find the previously by Ishii et al. (1999) reported nonsense mutation in *LZTS1* at codon 501 in PC3. Possibly, the PC3 cell line analysed by Ishii and co-workers has gained the mutation during culturing. Although our results implicated a minor role for *LZTS1* in PrCa, support for *LZTS1* as a tumour suppressor gene was published by Cabeza-Arvelaiz et al. (2001). These authors demonstrated that transfer of YAC and BAC clones containing the *LZTS1* gene into rat prostate AT6.2 cells reduced their colony-forming efficiency. Subsequent experiments showed that over-expression of *LZTS1* cDNA inhibited colony-formation in soft agar of AT6.2, HEK-293 and LNCaP cells (Cabeza-Arvelaiz et al. 2001).

NKX3-1

Two different *NKX3-1* sequences were detected. Previously, these were identified as single nucleotide polymorphisms (Voeller et al. 1997). Although no structural indications were found, evidence is growing for a role of *NKX3-1* in prostate cancer. The mechanism through which NKX3-1 functions in prostate cancer appears not to be by bi-allelic inactivation but by haplo-insufficiency (Bhatia-Gaur et al. 1999; Abdulkadir et al. 2002; Kim et al. 2002a).

EPHX2

Although minor differences were found in *EPHX2* mRNA expression, structural analysis revealed interesting data. As shown in Table II, we detected in the xenografts PC133 and PC324 and in the cell line PC3 unique sequences, which were not present in the 50 control DNAs. The wild type allele was lost in all 3 DNAs. For PC324, the corresponding tumour tissue (T1.7) and paired control DNA were available. These DNAs also contained the unique TCT codon 344.

Subsequent study of *EPHX2* in DNAs from 43 prostate tumour tissues showed 4 rare polymorphisms, leading to amino acid substitutions. Remarkably, in all cases the wild type allele was lost (Table II). Some of these amino acid residues were in conserved regions, suggesting that they could affect EPHX2 function. In a Swedish (n=25) and a Japanese (n=48) study (Sandberg et al. 2000; Saito et al. 2001) the

polymorphisms that we described were not found in normal control DNAs. Although absence of these rare polymorphisms might be explained by a difference in ethnic background and by the low number of samples, they support a possible role of *EPHX2* as a susceptibility gene in prostate cancer. This hypothesis however needs to be substantiated by screening of a significant larger cohort of preferably Dutch individuals, and by functional studies of mutated EPHX2.

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Table II

Mutation analysis of candidate tumour suppressor genes on chromosome 8p in prostate cancer

LZTS1	Sample	Rare polymorphisms / somatic mutations	Frequency of allele in control samples	Allelotyping	Remarks
Xenografts and cell lines	DU-145	AAC > AGC: Asn 223 Ser	0 / 50	MSI	both alleles identified
	PC374	GAT > GAC: Asp 296 Asp	3 / 50	MSI	silent
	LNCaP	CAG > CGG: Gln 556 Arg	0 / 50	MSI	both alleles identified

NKX3.1	Sample	Rare polymorphisms / somatic mutations	Frequency of allele in control samples	Allelotyping	Remarks
Xenografts and cell lines	PC-3	CGC > TGC: Arg 52 Cys	nt	loss	
	PCEW	ACC > ACT: Thr 78 Thr	nt	no loss	silent / both alleles identified

EPHX2	Sample	Rare polymorphisms / somatic mutations	Frequency of allele in control samples	Allelotyping	Remarks
Xenografts and cell lines	PC133	TGT > CGT: Cys 141 Arg	0 / 50	loss	rather conserved region
	PC324	GCT > TCT: Ala 344 Ser	0 / 50	loss	close to catalytic domain / PC324 = T1.7 (PC324 lacks wt)
	PC-3	AAA > AGA: Lys 55 Arg	unknown	loss	difficult to amplify by PCR
Tumour samples	T 1.8	CGG > TGG: Arg 52 Trp	1 / 50	loss	tumour lacks wild type allele (normal is heterozygous)
	T 10.4	CGG > TGG: Arg 52 Trp	0 / 50	loss	tumour shows same pattern as normal / contaminated (normal is heterozygous)
	T 9.4	CGG > CAG: Arg 52 Gln	0 / 50	loss	tumour lacks wild type allele (normal is heterozygous)
	T 8.13	CGC > TGC: Arg 103 Cys	0 / 50	loss	within conserved region / tumour lacks wild type allele (normal is heterozygous)
	T 1.2	GCC > ACC: Ala 376 Thr	0 / 50	loss	tumour shows same pattern as normal, contaminated (normal is heterozygous)

nt = not tested
MSI = Micro Satellite Instability

CHAPTER 7

GENERAL DISCUSSION

The work described in this thesis covers the search for tumour suppressor genes located in chromosomal regions at 8p and 10q that are frequently deleted in prostate cancer (PrCa). The study was originally guided by the paradigm that cancer is a genetic disease involving dominant mono-allelic activation of proto-oncogenes and recessive bi-allelic inactivation of tumour suppressor genes. Many tumour suppressor genes and oncogenes have indeed been identified that follow this classical principle. The number of tumour suppressor genes as well as the number of tumour types in which they are shown to be involved is however still relatively limited. Evidence is growing that the general genetic and molecular mechanisms underlying tumourigenesis is more complex and that the classical principles require adjustment. This seems also true for PrCa.

PTEN: a classical PrCa tumour suppressor gene at chromosome 10q

Deletions of chromosome 10q are among the most frequent chromosomal alterations observed in advanced PrCa (50-70%). In 1997, Li and co-authors identified bi-allelic inactivating mutations in the *PTEN* gene in multiple tumour types, including PrCa. *PTEN* is located at chromosome 10q and is now known as the classical PrCa tumour suppressor gene. We and others found frequent bi-allelic *PTEN* inactivation in PrCa. We identified *PTEN* deletions or mutations in 7 out of 11 human PrCa xenografts (Vlietstra et al. 1998). Overall, *PTEN* mutations are observed in a small proportion of primary PrCa (5-15%) and in a majority of metastatic PrCa (30-60%). The frequencies of bi-allelic inactivations of the *PTEN* gene in PrCa are however consistently lower than the rates of 10q loss of heterozygosity in these tumours.

Is there a classical PrCa tumour suppressor gene at chromosome 8p?

Loss of chromosome 8p is the most common chromosomal alteration in PrCa, occurring already in PIN (20-60%) and increasing during PrCa tumour progression (45-85%), indicative for the presence of more than one tumour suppressor gene on this chromosomal arm. The location of a tumour suppressor gene involved in PrCa at 8p was substantiated by chromosomal transfer experiments. Many independent studies, including several from our laboratory, have revealed a complicated picture of the allelic losses at 8p. There are multiple regions of frequent loss of heterozygosity at this chromosomal arm, including 8p22-p23, 8p12-p21, and 8p11-p12. The losses of 8p22-

p23 and 8p21.3 correlate with tumour grade and the losses of 8p21.1-p21.2 correlate with tumour progression, suggesting that chromosome 8p may contain multiple tumour suppressor genes involved in PrCa. Obviously, the presence of multiple tumour suppressor genes at 8p will complicate the identification of any of these genes. Although we and others have taken this possibility into account and have adjusted our gene searches accordingly, not a single classical tumour suppressor gene at 8p has been found as yet. This raises the question if there exists such genes at 8p. If not, why then would 8p losses correlate with tumourigenesis?

Haplo-insufficiency as an additional mechanism in tumourigenesis

Like for chromosome 8p, searches for PrCa tumour suppressor genes were also unsuccessful for other frequenty deleted chromosome arms such as 5q, 13q and 16q. Similar difficulties were encountered for the characteristic losses in other tumour types. In 2000, Cook and McCaw proposed an important extension of the paradigm in cancer genetics by suggesting that mono-allelic inactivation of a tumour suppressor gene may eventuate in a selective growth advantage of the mutant cell. Such haplo-insufficiency would result in a lower dosage of the encoded protein, presumably enough to effectuate clonal outgrowth. Ever since, a growing number of reports has provided evidence for a role of haplo-insufficiency in tumourigenesis (reviewed by Quon and Berns 2001; Fodde and Smits 2002). Importantly, haplo-insufficiency would very well explain the inability to find a classical PrCa tumour suppressor gene at 8p, as mono-allelic inactivation through loss of heterozygosity would suffice (Kim et al. 2002a).

Is haplo-insufficiency a common mechanism in PrCa?

Although it has been shown that *PTEN* is a classical tumour suppressor gene in PrCa and in many other human malignancies, the rate of bi-allelic inactivation seems significantly lower than the rate of loss of heterozygosity at the *PTEN* locus (Cairns et al. 1997; Teng et al. 1997; Feilotter et al. 1998; Whang et al. 1998). Apart from a second tumour suppressor gene located nearby *PTEN*, this could point to a role of *PTEN* haplo-insufficiency in tumour growth. In mice, concomitant inactivation of a single *Pten* allele with one or both of Cdkn1b, Cdkn2a or Nkx3-1 alleles was found to accelerate spontaneous neoplastic transformation (Di Cristofano et al. 2001; Kim et al. 2002b; You et al. 2002). Crosses of TRAMP mice, having SV40 early genes driven by

the prostate-specific probasin promoter, with heterozygous *Pten* (+/-) mice also revealed that mono-allelic inactivation of the *Pten* gene promoted the progression of PrCa (Kwabi-Addo et al. 2001). Together these findings provide evidence that *Pten* haplo-insufficiency indeed is involved in PrCa in mice. Similarly, loss of heterozygosity at the *PTEN* locus in human PrCa may promote tumourigenesis by haplo-insufficiency of the gene, thus explaining the discordance between the *PTEN* mutation rate and the rate of loss of heterozygosity at 10q.

Whereas haplo-insufficiency may perhaps be of minor importance for the *PTEN* gene, it could be a particularly important mechanism for the multiple PrCa tumour suppressor genes thought to be located at 8p. It may be that the additive effect of mono-allelic inactivation of several tumour suppressor genes at the same time by loss of a large part of the chromosomal arm has a stronger effect on tumour growth than the bi-allelic inactivation of just a single tumour suppressor gene at this arm. In my opinion, at least four candidate tumour suppressor genes at 8p deserve attention, in particular because of their potential to promote PrCa by haplo-insufficiency. In mouse models, unambiguous evidence has been provided that the *NKX3-1* gene at 8p21.2 promotes PrCa tumour growth by haplo-insufficincy. Mono-allelic inactivation of *Nkx3-1* in adult mice results in the development of hyperplasia and apparent PIN lesions in the prostate (Abdulkadir et al. 2002; Bhatia-Gaur et al. 1999; Kim et al. 2002a). Several alterations have been observed for the *LZTS1* gene at 8p21.3, including significantly reduced mRNA expression levels, sparce missense mutations and germline sequence variants. In addition, restoration of LZTS1 function by gene transfer experiments implicated *LZTS1* as a tumour suppressor gene (Hawkins et al. 2002; Ishii et al. 1999; Vecchione et al. 2001). We found the *WRN* gene at 8p12 to be disrupted by a homozygous deletion in one of our PrCa xenografts. The WRN protein belongs to the highly conserved family of RecQ helicases. WRN and other family members like BLM and RECQ4 are associated with rare chromosomal instability disorders that also include a predisposition to develop cancer (Hickson 2003). Heterozygous human *WRN* (+/-) cells expressed reduced levels of WRN protein and had reduced helicase activity, which was associated with an increased sensitivity to DNA damaging agents and genetic instability (Moser et al. 2000b). Haplo-insufficiency may also be of importance for other RecQ helicases, because *Blm* (+/-) heterozygous mice showed an enhanced tumour formation (Goss et al. 2002). Lastly, I believe the *EPHX2* gene at 8p21.1 to be a promising candidate tumour suppressor gene in PrCa. We identified germline sequence

variants of the *EPHX2* gene in 4 out of 43 PrCa samples, whereas no variations were identified in 50 control samples. Importantly, all of 4 PrCa samples with a variant *EPHX2* allele had lost the wild-type *EPHX2* allele, suggesting bi-allelic inactivation of the gene. Although the numbers are small, it is tempting to speculate that *EPHX2* is a low-penetrant susceptible gene in PrCa.

How to hunt for genes inactivated by haplo-insufficiency across the genome?

The majority of tumour suppressor genes identified to date follow the classical paradigm of bi-allelic inactivation. Bi-allelic inactivation of a tumour suppressor gene is however presumed to have a stronger effect on clonal outgrowth than mono-allelic inactivation of the gene (Fero et al. 1998, Smits et al. 2000), which inevitably will have biased the identification of the classical tumour suppressor genes. Indeed, the tumour types in which they have been identified generally are rather aggressive in their growth pattern. In this respect, clinical diagnosed PrCa generally has a late age of onset and is a slow growing tumour type. It may be that the tumour suppressor genes involved in PrCa commonly exert their tumour promoting effect by haplo-insufficiency. If so, the identification of these genes may require a different approach. The more subtle the tumour promoting effect of a mutation in a tumour suppressor gene, the larger the amount of tumour samples necessary to prove such an effect. In other words, searches for PrCa genes should exploit high-throughput screening procedures.

Recent technological developments, in particular microarray analysis, now allow the performance of many tests in parallel. On a microarray, DNA or protein fragments are organized in a gridded way at a flat surface, such as a glass slide or a porous substrate. Microarray analysis can be applied to identify both larger chromosomal alterations and smaller genomic changes at the nucleotide level (e.g. CGH and SNP microarrays, respectively). Large-scale expression analysis can be performed on RNA and protein level using cDNA and oligo microarrays and protein microarrays, respectively. The integration of such high-throughput genomics and proteomics analysis with functional studies should eventually result in the identification of the PrCa tumour suppressor genes on chromosomes 5q, 8p, 13q and 16q.

APPENDIX

Table I.
HPC loci and candidate genes

Symbol	Susceptible locus	Family origin (age of onset)	References	Gene analyzed in HPC	Gene alterations	References
	1p13	North Amerika (±61)	(Chang et al. 2002)	HSD3B1 HSD3B2	Combination of polymorphisms	(Chang et al. 2002)
CAPB (BC-PC)	1p36	North America and Canada (<66)	(Gibbs et al. 1999 ; Xu et al. 2001b)	P73	Silent Intron (>= 16bp from splice sites)	(Peters et al. 2001a)
PCAP	1q42.2-q43	South and West Europe (<60-66)	(Berthon et al. 1998; Berry et al. 2000a; Cancel-Tassin et al. 2001)	-	-	-
HPC1	1q24-q25	North America, Sweden, Finland (<55-69)	(Smith et al. 1996; Cooney et al. 1997; Hsieh et al. 1997; Gronberg et al. 1999; Neuhausen et al. 1999; Berry et al. 2000a; Goode et al. 2000; Xu 2000)	RNASEL	Nonsense	(Carpten et al. 2002)
-	8p22-p23	Ashkenazi Jews (>65)	(Xu et al. 2001c)	PG1	Homozygous polymorphisms	(Xu et al. 2001c)
		Sweden (<65)	(Wiklund et al. 2003)	LZTS1	Missense	(Hawkins et al. 2002)
				MSR1	Missense	(Xu et al. 2002a ; Xu et al. 2003)
-	11	North America (>65)	(Gibbs et al. 2000)	-	-	-
-	16q23	North America	(Paris et al. 2000; Suarez et al. 2000)	-	-	-
HPC2	17p11	North America (<>)	(Tavtigian et al. 2001)	ELAC2	Frame-shift Missense Combination of Ser217Leu and Ala541Thr polymorphisms	(Rebbeck et al. 2000: Rokman et al. 2001; Tavtigian et al. 2001; Vesprini et al. 2001; Xu et al. 2001a)
HPC20	20q13	North America (>65)	(Berry et al. 2000b; Zheng et al. 2001)	-	-	-
HPCX	Xq27-q28	North America, Sweden, Finland (>60)	(Xu et al. 1998; Schleutker et al. 2000; Peters et al. 2001b)	-	-	-

Table II
Details on chromosomal losses observed by CGH analysis in PrCa

Loss	PIN Zitz'01 (n=12)	Primary PrCa Vis'95 (n=31)	Primary PrCa Alers'00 (n=23)	Metastasis Cher'96 (n=20)	LN-metastasis Alers'00 (n=18)	Distant metastases Alers'00 (n=5)	Recurrence Vis'95 (n=9)	Recurrence Nupp'98 (n=37)
1p	nd	6% (1p32-pter)	9%	25%	8%	20%	22% (1p21-p31)	54% (1p31 + 1p36-pter) *
1q	nd	3% (1q25-qter)	4%	30%	6%	20%	nd	nd
2p	8%	6% (2p16)	13%	15%	nd	nd	22% (2p15-p21)	5% (2p24-pter)
2q	8%	6% (2q23-q33)	13%	42% * (50%)	11%	33%	22% (2q11-q31)	11% (2cen-q22)
3p	8%	3% (3cen-p21)	nd	25%	nd	nd	11% (3p22-pter)	nd
3q	nd	nd	nd	15%	nd	nd	nd	5% (3qcen-q23)
4p	nd	nd	nd	30%	nd	nd	22% (4p15-pter)	3% (4cen-pter)
4q	17%	6% (4q28-q34)	13%	20%	11%	33%	nd	5% (4q34-qter)
5p	nd	nd	nd	20%	nd	nd	nd	nd
5q	8%	6% (5q14)	35%	39% * (45%)	11%	53%	44% (5q14-q23)*	16% (5q15-q23)*
6p	nd	nd	nd	20%	nd	nd	22% (6p23-pter)	8% (6pter)
6q	8%	22% (6cen-q21)*	39%	39% * (45%)	22%	73%	44% (6q13-q21)*	27% (6q16 + q24-qter)*
7p	nd	nd	nd	15%	nd	nd	nd	3% (7p14-pter)
7q	nd	3% (7q21-q35)	nd	20%	nd	nd	11% (7q22-qter)	nd
8p	8%	32% (8p12-qter)*	52%	80% * (80%)	39%	53%	78% (8p21-pter)*	73% (8p12-p22 + p23)*
8q	nd	nd	nd	5%	nd	nd	nd	nd
9p	nd	16% (9p23-pter)*	9%	5%	11%	20%	22% (9p21-pter)	3% (9pter)
9q	nd	6% (9q34)	nd	10%	nd	nd	nd	3% (9cen-qter)
10p	nd	nd	nd	25%	nd	nd	nd	22% (10p11)*
10q	8%	10% (10q22-q23)	4%	50% * (50%)	22%	33%	11% (10cen-q23)	46% (10cen-q21 + q26)*
11p	nd	nd	nd	30%	nd	nd	nd	8% (11p15-pter)
11q	nd	3% (11q14-q24)	nd	15%	nd	nd	nd	14% (11q24-qter)
12p	nd	nd	nd	25%	nd	nd	22% (12cen-pter)	5% (12p12-pter)
12q	8%	3% (12q24-qter)	nd	15%	6%	27%	11% (12q24)	14% (12q24-qter)
13q	25%	32% (13q21-q31)*	65%	75% * (75%)	39%	60%	56% (13cen-q21)*	51% (13q12 + q21)*
14q	nd	nd	nd	20%	nd	nd	nd	8% (14q31)
15q	nd	3% (15q22-qter)	nd	39% * (45%)	nd	nd	nd	35% (15cen-q21 + q25-qter)*
16p	nd	6% (16cen-pter)	nd	15%	nd	nd	nd	16% (16p12-pter)
16q	nd	19% (16cen-q23)*	26%	55% * (55%)	11%	40%	56% (16q22-qter)*	46% (16q24)*
17p	nd	6% (17cen-pter)	nd	50% * (50%)	nd	nd	22% (17p12-p13)	41% (17p) *
17q	nd	6% (17cen-pter)	nd	20%	nd	nd	11% (17q21-q24)	nd
18p	nd	nd	nd	15%	nd	nd	nd	5% (18cen-pter)
18q	8%	19% (18q22-qter)*	13%	15%	22%	27%	22% (18q12-qter)	19% (18q22-qter)*
19p	nd	6% (19cen-pter)	nd	5%	nd	nd	nd	nd
19q	nd	6% (19cen-qter)	nd	15%	nd	nd	22% (19cen-qter)	43% (pter-q13)*
20p	nd	nd	nd	20%	nd	nd	nd	14% (20cen-pter)
20q	nd	3% (20cen-qter)	nd	20%	nd	nd	nd	22% (20cen-q22)*
21q	nd	nd	nd	5%	nd	nd	nd	3% (21cen-pter)
22q	nd	13% (22qcen-qter)	nd	10%	nd	nd	11% (22cen-qter)	46% (22q13)*
Xp	nd	nd	nd	10%	nd	nd	nd	11% (Xpter)
Xq	nd	3% (Xq21-qter)	nd	15%	nd	nd	nd	5% (Xq27-qter)
Y	8%	nd	17%	20%	6%	27%	nd	nd

* percentages and regions determined by Visakorpi, Cher, and Nupponen
nd = not detected

Table III
Details on chromosomal gains observed by CGH analysis in PrCa

Gain	PIN	Primary CaP	Primary Cap	Metastasis	LN-metastasis	Distant metastases	Recurrence	Recurrence
	Zitz'01 (n=12)	Vis'95 (n=31)	Alers'00 (n=23)	Cher'96 (n=20)	Alers'00 (n=18)	Alers'00 (n=5)	Vis'95 (n=9)	Nupp'98 (n=37)
1p	8%	nd	nd	20%	nd	nd	nd	nd
1q	nd	nd	13%	52% * (55%)	11%	27%	nd	16% (1q25-q32)
2p	nd	nd	nd	45% * (50%)	nd	nd	nd	8% (2p23-pter)
2q	nd	nd	nd	25%	nd	27%	nd	27% (2q33)*
3p	nd	nd	11%	40%	nd	27%	nd	nd
3q	nd	nd	22%	52% * (45%)	6%	27%	nd	24% (3q25-q26)*
4p	8%	nd	nd	25%	nd	nd	nd	nd
4q	nd	nd	4%	40%	17%	7%	nd	19% (4q13-q23)*
5p	nd	3% (5cen-pter)	nd	20%	nd	nd	11% (5cen-pter)	nd
5q	nd	nd	nd	25%	17%	13%	nd	14% (5q14-q31)*
6p	nd	nd	8%	25%	17%	nd	nd	nd
6q	nd	nd	nd	15%	nd	nd	nd	nd
7p	25%	6% (7cen-pter)	13%	35%	nd	27%	56% (7p13)*	32% (7p15-p21)*
7q	25%	6% (7q32-qter)	13%	45%	11%	47%	nd	43% (7q21 + q31)*
8p	nd	nd	nd	15%	nd	nd	nd	nd
8q	42%	6% (8q)*	35%	85% * (85%)	17%	60%	89% (8q)*	73% (8q21 + q23-qter)*
9p	nd	nd	nd	40%	nd	nd	nd	19% (9p21-pter)
9q	nd	nd	17%	45%	11%	27%	nd	nd
10p	nd	nd	nd	25%	25%	20%	nd	nd
10q	nd	nd	nd	20%	5%	20%	nd	11% (10q21)
11p	nd	3% (11cen-p14)	nd	52% * (55%)	nd	nd	11% (11cen-pter)	11% (11p14)
11q	8%	3% (11q14-q22)	nd	30%	nd	nd	11% (11cen-q22)	22% (11q22)*
12p	nd	nd	nd	10%	nd	nd	nd	3% (12p12-pter)
12q	17%	nd	nd	30%	nd	nd	nd	22% (12q21)*
13q	nd	nd	nd	20%	nd	nd	nd	19% (13q31)*
14q	17%	nd	nd	20%	nd	nd	nd	11% (14q21)
15q	17%	nd	nd	25%	nd	nd	nd	nd
16p	25%	nd	nd	40%	nd	nd	nd	8% (16cen-pter)
16q	nd	nd	nd	10%	nd	20%	nd	nd
17p	25%	nd	nd	5%	nd	nd	nd	nd
17q	25%	nd	8%	35%	33%	33%	nd	8% (17q22-qter)
18p	nd	nd	nd	35%	nd	nd	nd	19% (18pter)
18q	nd	nd	nd	35%	nd	nd	nd	30% (18q12)*
19p	33%	13% (19cen-pter)	17%	20%	nd	nd	nd	nd
19q	33%	nd	nd	25%	nd	nd	nd	nd
20p	25%	nd	nd	35%	nd	nd	nd	3% (20cen-pter)
20q	25%	nd	nd	10%	nd	nd	nd	3% (20q12-qter)
21q	nd	nd	nd	20%	nd	nd	nd	5% (21q22-qter)
22q	17%	nd	nd	20%	nd	nd	nd	nd
Xp	nd	nd	17%	20%	11%	20%	56% (Xp11-q13)*	24% (Xp21-pter)*
Xq	17%	nd	22%	25%	11%	40%	nd	35% (Xcen-q13)*
Y	nd	nd	nd	10%	nd	nd	nd	nd

* percentages and regions determined by Visakorpi, Cher, and Nupponen
nd = not detected

Table IV

Candidate tumour suppressor genes on chromosome 8 analysed in PrCa

Gene	Summary	Tumour related alterations in PrCa		References	Tumour related alterations in other tumours		References
N33	**Locus:** 8p22 (chr8:15.2-15.4) **Function:** unknown **Similarity:** belongs to the ost3 family.	HD Nonsense Frameshift Missense Low expr.	1 / 52 metastases - - - -	(Bova et al. 1993)	HD Mutation Low expr.	1 / 3 pancreatic tumor cell line - 14 / 15 colorectal cancer cell lines 1 / 8 lung cancer cell lines 1 / 4 liver cancer cell lines	(Levy et al. 1999) (Macgrogan et al. 1996)
PDGFRL	**Locus:** 8p22 (chr8:17.5-17.6) **Function:** unknown **Similarity:** 27% and 25% identity with the extracellular domain of platelet-derived growth factor receptor-beta (PDGF-β) and FMS-like tyrosine kinase.	HD Nonsense Frameshift Missense Low expr.	- - - 1 / 68 primary PrCa 0 / 15 xenografts and cell lines 0 / 15 xenografts and cell lines	(Komiya et al. 1997) (van Alewijk et al., unpublished results)	HD Mutation Low expr.	- 2 / 48 hepatocellular carcinomas 1 / 28 colorectal carcinomas -	(Fujiwara et al. 1995)
LZTS1	**Locus:** 8p21.3 (chr8:20.1) **Function:** unknown **Similarity:** DNA-binding domain of the cAMP-responsive activating transcription factor-5	HD Nonsense Frameshift Missense Low expr	nt 1 / 3 cell lines - 0 / 15 xenografts and cell lines 1 / 15 xenografts and cell lines	(Ishii et al. 1999) (van Alewijk et al., unpublished results)	HD Mutation Low expr.	- 2 / 53 esophageal squamous cell carcinomas 1 / 39 gastric carcinomas 60 % epithelial tumors 8 / 8 gastric cancer cell lines 39 / 88 gastric carcinomas breast cancer cell line MCF7	(Ishii et al. 1999) (Vecchione et al. 2001) (Ishii et al. 1999) (Ishii et al. 1999)
NKX3.1	**Locus:** 8p21.2 (chr8:23.9) **Function:** transcription factor, which binds preferentially the consensus sequence 5'-taagt[ag]-3' and can behave as a transcriptional repressor. Could play an important role in regulating proliferation of glandular epithelium and in the formation of ducts in prostate. **Similarity:** NK-3 family of homeobox proteins.	Mutation Low expr	0 / 50 prim PrCa and metastases 0 / 13 prim PrCa 0 / 15 xenografts and cell lines 11 / 52 prim PrCa 78 % metastases 4 / 15 xenografts and cell lines	(Voeller et al. 1997) (Ornstein et al. 2001) (Van Alewijk et al., submitted) (Xu et al. 2000) (Bowen et al. 2000) (van Alewijk et al., unpublished results)	nt	-	-

Gene	Locus / Function / Similarity	Type	Result	Reference	Type	Result	Reference
EPHX2	**Locus:** 8p21.1 (chr8:27.9) **Function:** converts potentially harmful epoxide-containing compounds into diols, which are less reactive and easier to excrete (Meijer and DePierre 1988). **Similarity:** belongs to the epoxide hydrolase family.	Mutation Low expr.	0 / 15 xenografts and cell lines 2 / 15 xenografts and cell lines	(van Alewijk et al., unpublished results)	nt	-	
CLU	**Locus:** 8p21.1 (chr8:28.0) **Function:** not yet clear, it is known to be expressed in a variety of tissues and it seems to be able to bind to cells, membranes, and hydrophobic proteins. It has been associated with programmed cell death (apoptosis). **Similarity:** belongs to the clusterin family.	Mutation Low expr.	0 / 15 xenografts and cell lines 0 / 15 xenografts and cell lines	(van Alewijk et al., unpublished results)	nt	-	
WRN	**Locus:** 8p12 (chr8:31.1-31.5) **Function:** essential for the formation of DNA replication focal centers; stably associates with foci elements generating binding sites for rp-a. Exhibits a magnesium-dependent ATP-dependent DNA- helicase activity. May be involved in the control of genomic stability. **Similarity:** belongs to the helicase family (RECQ subfamily).	HD Mutation Low expr.	1 / 15 xenografts and cell lines 0 / 15 xenografts and cell lines 0 / 15 xenografts and cell lines	(Van Alewijk et al. 1999) (Van Alewijk et al., submitted)	nt	-	
POLB	**Locus:** 8p11.21 (chr8:42.2-42.3) **Function:** repair polymerase, which conducts "gap-filling" DNA synthesis in a stepwise distributive fashion rather than in a processive fashion as for other DNA polymerases. **Similarity:** belongs to DNA polymerase type-x family.	Mutation Frameshift Missens Low expr.	0 / 15 xenografts and cell lines 1 / 12 prim PrCa 1 / 12 prim PrCa 0 / 15 xenografts and cell lines	(van Alewijk et al., unpublished results) (Dobashi et al. 1994) (van Alewijk et al., unpublished results)	HD Mutation Low expr.	- 0 / 14 bladder cancer cell lines 0 / 90 bladder carcinomas 2 / 6 colorectal carcinomas -	- (Eydmann and Knowles 1997) (Wang et al. 1992)

Locus is obtained from UCSC April 2003 freeze database
Function and similarity are obtained from Weizman GeneCards (red NCBI LocusLink)

Table V

Candidate tumour suppressor genes on chromosome 10 involved in PrCa

Gene	Summary	Tumour related alterations in PrCa	References	Tumour related alterations in other tumours	References
COPEB	**Locus:** 10p15.2 (chr10: 3.9) **Function:** transcriptional activator (by similarity), which binds a GC box motif. Could play a role in b-cell growth and development. **Similarity:** belongs to the krueppel family of C2H2-type zinc- finger proteins	HD - Nonsense 1 / 33 primary PrCa Frameshift 17 / 33 primary PrCa Missense - Low expr. -	(Narla et al. 2001)	HD - Mutation - Low expr. -	
ANXA7	**Locus:** 10q22.2 (chr10: 75.0-75.1) **Function:** calcium-dependent membrane-binding protein, fuses membranes and acts as a voltage-dependent calcium channel **Similarity:** belongs to the annexin familiy of calcium-dependent phospholipid binding proteins	HD 0 / 15 xeno. and cell lines Nonsense 1 / 7 (20) prim. PrCa Frameshift - Missense - Low expr. -	(Trapman et al., unpublished results) (Srivastava et al. 2001)	HD - Mutation - Low expr. -	
MINPP1	**Locus:** 10q23.31 (chr10 : 89.4) **Function:** dephosphorylates Ins(1,3,4,5)P4. hydrolyzes inositol pentakisphosphate and inositol hexakisphosphate **Similarity:** is a distinct group within the histidine phosphatase family.	HD 1 / 15 xeno. and cell lines Nonsense - Frameshift - Missense - Low expr. -	(Hermans et al., submitted)	HD - Mutation 1 / 4 (15) follicular thyroid carc. Low expr. -	(Gimm et al. 2001)
PAPSS2	**Locus:** 10q23.31(chr10: 89.6) **Function:** Bifunctional polypeptide; has ATP sulfurylase and adenosine 5'-phosphosulfate kinase activites, required for the synthesis of sulfonate donor 3'-phosphoadenosine 5'-phosphosulfate **Similarity:** the N-terminal section belongs to the APS kinase family. The C-terminal section belongs to the sulfate adenylyltrasferase family.	HD 1 / 15 xeno. and cell lines Nonsense - Frameshift - Missense - Low expr. 5 / 15 xeno. and cell lines	(Hermans et al., submitted)	HD - Mutation - Low expr. -	

Gene	Description	Type	Finding	Reference	Type	Finding	Reference
PTEN	**Locus:** 10q23.31 (chr10: 89.8-89.9) **Function:** dual specific phosphatase: antagonizes signal transduction of PI-3 kinase by dephosphorylating Ins(3,4,5)P3; also active as a phosphatase on tyrosine, serine and threonine residues. **Similarity:** belongs to the non-receptor class of the protein-tyrosine phosphatase family.	HD	2 / 4 cell lines	(Li et al. 1997)	HD	Glioma, prostate, kidney, endometrium and breast carcinoma cell lines or tumour specimens	(Cantley and Neel 1999)
		Nonsense	6 / 23 (80) prim. PrCa & LNM; 1 / 10 cell lines	(Cairns et al. 1997) (Whang et al. 1998)	Mutation	-	
		Frameshift	1 / 23 (80) prim. PrCa & LNM; 1 / 3 cell lines	(Cairns et al. 1997) (Steck et al. 1997)			
		Missense	3 / 23 (80) prim. PrCa & LNM	(Cairns et al. 1997)	Low expr.	-	
		Low expr.	1 / 25 (51) prim. PrCa	(Feilotter et al. 1998)			
FLJ11218	**Locus:** 10q23.31 (chr10: 90.2-90.5) **Function:** unknown **Similarity:** none, hypothetical protein	HD	4 / 15 xeno. and cell lines	(Hermans et al., submitted)	HD	-	
		Nonsense	-		Mutation	-	
		Frameshift	-		Low expr.	-	
		Missense	1 / 15 xeno. and cell lines				
		Low expr.	4 / 15 xeno. and cell lines				
TNFRSF6	**Locus:** 10q23.31 (chr10: 90.9) **Function:** signals apoptotic cell death in sensitive cells **Similarity:** member of the tumour necrosis/nerve growth factor receptor family	HD	-		HD	-	-
		Nonsense	1 / 111 high grade PIN	(Takayama et al. 2001)	Mutation	3 / 21 burn scar-related squamous cell carcinomas	(Lee et al. 2000)
		Frameshift	-		Low expr.	3 / 15 bladder carcinomas	(Santourlidis et al. 2001)
		Missense	3 / 111 high grade PIN; 1 / 15 xeno. and cell lines	(Takayama et al. 2001) (Hermans et al., submitted)			
		Low expr.	4 / 15 prim. PrCa	(Santourlidis et al. 2001)			
MXI1	**Locus:** 10q25.2 (112.1-112.2) **Function:** transcriptional repressor, which heterodimerizes with MAX to antagonize MYC activity **Similarity:** belongs to the basic helix-loop-helix (bHLH) family of transcription factors.	HD	-		HD	-	-
		Nonsense	1 / 21 (40) prim. PrCa; 2 / 10 (10) prim. PrCa	(Prochownik et al. 1998) (Eagle et al. 1995)	Mutation	2 / 16 bladder carc. cell lines	(Wang et al. 2000)
		Frameshift	1 / 21 (40) prim. PrCa	(Prochownik et al. 1998)	Low expr.	-	
		Missense	2 / 10 (10) prim. PrCa; 6 / 21 (40) Prim. PrCa	(Eagle et al. 1995) (Prochownik et al. 1998)			
		Low expr.	-				
DMBT1	**Locus:** 10q26.13 (gap) **Function:** Little is known about its function but a transcript variant has been shown to bind surfactant protein D independent of carbohydrate recognition. This indicates that DMBT1 may not be a classical tumour suppressor gene, but rather play a role in the interaction of tumour cells and the immune system. **Similarity:** Member of the scavenger receptor cysteine-rich (SRCR) superfamily	HD	1 / 15 xeno. and cell lines	(Hermans et al., submitted)	HD	2 / 20 medulloblastomas; 9 / 39 glioblastomas; 8 / 21 glioblastomas; 2 / 23 lung carc. cell lines; 4 / 40 SCLC cell lines; 1 / 8 NSCLC cell lines; 5 / 79 gliomas	(Mollenhauer et al. 1997) (Somerville et al. 1998) (Takeshita et al. 1999) (Wu et al. 1999) (Takeshita et al. 1999) (Wu et al. 1999) (Mueller et al. 2002)
		Nonsense	-		Mutation	4 / 5 brain tumour cell lines; 23 / 43 oesophageal carc.; 12 / 15 oesopha. carc. cell lines; 5 / 40 gastric carc.; 4 / 24 colorectal carc.	(Mollenhauer et al. 1997) (Mori et al. 1999)
		Frameshift	-		Low expr.	20 / 23 lung carc. cell lines; 20 / 20 SCLC cell lines; 6 / 14 NSCLC cell lines	(Takeshita et al. 1999) (Wu et al. 1999) (Wu et al. 1999)
		Missense	2 / 15 xeno. and cell lines				
		Low expr.	-				

A HIGH-RESOLUTION PHYSICAL AND TRANSCRIPT MAP OF CHROMOSOME 8P12-P21

To facilitate search for homozygous deletions and mapping of candidate tumour suppressor genes we isolated the 8p12-p21 region in a large YAC contig. The contig was finished in 2000. YACs were identified and selected by standard PCR screening and Southern blotting of the CEPH YAC library (Albertsen et al. 1990) and the CEPH mega YAC library (Chumakov et al. 1992). Single YACs were tested for the presence of specific markers by PCR. The contig is bordered by the telomeric marker LPL at 8p21.3 and the centromeric marker BC028701 at 8p12. It is composed of 172 overlapping YACs and 303 markers, including polymorphic microsatellite markers, sequence-tagged sites (STSs), ESTs, genes and pseudogenes, were mapped in the contig (Table I). From selected YACs the end fragments were isolated and mapped. Chimeric YACs were included in the contig, because the 8p part of these YACs turned out to be of value for the construction of the contig and for accurate marker mapping. The final map was constructed such that a minimal number of YACs contained an internal deletion.

In 2001, the first draft sequence of the human genome was published (Lander et al. 2001; Venter et al. 2001). Recently, the completion of the human genome was announced by The International Human Genome Sequencing Consortium (Nature April 27, 2003). We compared the several consecutive releases of the gene maps of the University of California Santa Cruz (UCSC) database (http://genome.ucsc.edu) with the map deduced from the YAC contig. A minority of the genes and markers from the YAC contig were present in the 2001 UCSC map. Although the UCSC November 2002 map was detailed, some regions were differently ordered as compared to the YAC map. The UCSC April 2003 release and the YAC map show a good fit (Table I). As calculated from the human genome sequence, the YAC contig spans approximately 17 Mbp. However, as shown in Table I by grey-boxed areas, there are some small discrepancies between both maps. The most obvious discrepancy is the region around the *NEF3* and *NEFL* genes. Some of differences might be due to previously unrecognized internal deletions in the YACs. However, although the human genome sequence from which the UCSC map is derived, is considered as over 99.9% accurate, it might still contain some alignment errors. The UCSC genome sequence contains one small gap, between Mbp 29.28 and 29.78.

Table II summarizes the ordered genes in the UCSC map and the YAC map. The UCSC map contains 95 genes, 72 of these were previously mapped in the YAC contig. For 9 genes the positions in the YAC map and UCSC map slightly differ. MGC29816 could not be located on the UCSC map. Most likely, MAPKK maps in the gap present in the UCSC map. 8p12-p21.3 contains 3 gene poor regions, between Mbp 19.9 –21.3, 30.7-32.4 and 33.3-35.5, respectively. The homozygous deletion in PC133 maps in the Mbp 30.7-32.4 segment.

LEGENDS TO THE TABLES

Supplementary Table I. YAC-contig of chromosome 8p12-p21 spanning the region between the markers LPL and BC028701. Gene names are listed in the first column. Alternative names, ESTs, ESTs by which genes were mapped, and STSs without a locus number are listed in the second column. In case a definite order could not be established, markers are boxed by a dark line. In the third column, the marker position in the UCSC April 2003 database are listed (Mbp from the top of 8p). In the fourth column, marker types are listed: (p) polymorphic marker; (s) STS; (e) EST; (g) gene; (pg) pseudogene.

Supplementary Table II. Alignment of known genes from the YAC-contig and the UCSC April 2003 database. Known genes that were mapped in the YAC contig are listed in the first column, the UCSC genes are listed in the second column. In the third column, the marker positions in the UCSC April 2003 database are listed. In the fourth column the chromosomal band is indicated.

Supplementary Table I

YAC contig of chromosome 8p12-p21 spanning the region between the markers LPL and BC028701

Locus	Alias	UCSC Apr 03	Type	904D7	913F10	713G1	220G1	690F5	746H6	66C7	923G7	900G12	675A7	57D1	467E2	238C6	777G4	730H9	672H10	670G5	287C5	810A1
LPL	WI-21956	19.61	g	904D7																		
	stSG30884	19.14	e	904D7																		
	WI-19010	19.76	e	904D7																		
ATP6V1B2		19.89	g	904D7																		
LZTS1	stSG10118	19.91	g	904D7	913F10																	
D8S258	AFM107xb6	20.18	p	904D7	913F10																	
D8S280	AFM205wc5	20.24	p	904D7	913F10																	
D8S1983	WI-10327	21.04	s	904D7	913F10	713G1	220G1	690F5	746H6		923G7											
D8S1509	WI-6088	21.16	s			713G1	220G1	690F5	746H6		923G7											
D8S282	AFM234if4	21.22	p			713G1	220G1	690F5	746H6	66C7	923G7	900G12										
D8S1116	CHLC.ATA18B	21.24	p			713G1	220G1	690F5	746H6	66C7	923G7	900G12										
D8S196	cCI8-281		p			713G1	220G1	690F5	746H6	66C7	923G7											
GFRA2	SHGC-31819	21.36	g			713G1	220G1	690F5	746H6	66C7	923G7											
D8S560	AFMa127ye5	21.41	p			713G1	220G1	690F5	746H6	66C7	923G7											
D8S232	cCI8-448		s			713G1	220G1	690F5	746H6	66C7	923G7											
	57D1R						220G1	690F5	746H6	66C7	923G7			57D1								
	E-1077-2		s							66C7	923G7			57D1								
D8S298	AFM234yh10	21.58	p								923G7			57D1								
DOK2	stSG36099	21.59	g								923G7			57D1								
D8S133		21.79	p								923G7		675A7	57D1								
D8S1937	WI-8527	21.62	e										675A7	57D1								
	stSG16422	21.77	g										675A7	57D1								
RAI16	R30665	21.78	g										675A7	57D1								
RAI16	A005V15	21.78	g										675A7	57D1								
HR	WI-6304	21.79	g										675A7	57D1								
HR		21.79	s										675A7	57D1								
	467E2 R												-	57D1	467E2							
EPB49	WI-20118	21.76	s										675A7	57D1	467E2							
HR	stSG44685	21.79	g										675A7	57D1	467E2							
XPO7	SGC33989	21.68	g											57D1	467E2							
XPO7	R20754	21.68	g											57D1	467E2							
LGI3	WI-15206	21.82	g											57D1	467E2							
SFTPC	E-1025-5	21.84	s											57D1	467E2	238C6						
BMP1	stSG9073	21.84	g											57D1	467E2	238C6						
FLJ22246	stSG47781	21.82	g											57D1	467E2	238C6						
PHYHIP	WI-6273	21.90	g											57D1		238C6		730H9				
	57D1L		s											57D1		238C6		730H9				
	WI-7676	21.93	g													238C6	777G4	730H9	672H10	670G5		
	stSG52127	21.93	e													238C6	777G4	730H9	672H10	670G5		
	stSG53946	22.03	g													238C6	777G4	730H9	672H10	670G5		
PIWIL2	A009B38	22.10	e													238C6	777G4	730H9	672H10	670G5		810A1
D31887	WI-6536	22.10	e													238C6	777G4	730H9	672H10	670G5		810A1
PPP3CC	stSG3913	22.22	g													238C6	777G4		672H10	670G5	287C5	810A1
SCAM-1	SHGC-30970	22.22	g													238C6			672H10	670G5	287C5	810A1
PDLIM2	stSG8968	22.27	g													-			672H10	670G5	287C5	810A1
FLJ34715	stSG60062	22.28	g													238C6			672H10	670G5	287C5	810A1

Gene	Marker	Position	Type
	sts-AA031259	22.28	e
	sts-H96791	22.29	e
DBC-1	stSG63380	22.29	g
DBC-1	stSG62344	22.29	g
DBC-1	WI-15087	22.30	g
D8S136	stSG28683	22.25	s
BIN3	238C6R	22.30	g
D8S1733	AFM197yc1	22.34	p
D8S220	cCI8-319		p
	stSG26923	22.36	e
EGR3	WI-15547	22.37	g
	SHGC-36225	22.44	e
	stSG31244	22.43	e
D8S1752	AFMb055za9	22.49	p
RHOBTB2	WI-16696	22.69	g
RHOBTB2	WI-14836	22.70	g
TNFRSF10B	WI-15306	22.70	g
MGC29816	sts-W49732	22.94	g
TNFRSF10C	WI-11701	22.80	g
	stSG34952	22.80	e
TNFRSF10D	SHGC-15994	22.81	g
	H93300	22.87	e
	AA102383	22.87	e
LYSAL1	SGC32556	23.11	g
	sts-H92890	23.13	e
	WI-16880	23.16	e
LOXL2	sts-U89942	22.98	g
	SHGC-52491	23.17	e
	WI-18184	23.17	e
	stSG68140	23.22	e
	stSG63330	23.23	e
	stSG8498	23.23	e
MSCP	stSG8797	23.24	g
MSCP	WI-14743	23.25	g
MSCP	sts-H40450	23.25	g
D8S1077	WI-961	23.29	s
	sts-R98014	23.25	e
NKX3-1	stSG52445	23.26	e
NKX3-1	SGC35548	23.26	g
STC1	T97343	23.52	g
		?	? e
ADAM28	stSG21122	23.80	e
D8S2210	stSG42867	24.03	g
	767G4L		s
	981G5L		s
NEF3	WI-7612	24.60	g
D8S1670	WI-5833	24.58	s
	stSG47039	24.56	e
	stSG28797	24.54	e
NEFL		24.63	g / p
D8S1739	AFMa346ze5	24.75	p

The following table is a dense STS / BAC‑contig map (rotated 90° in the original). It has been transcribed into horizontal reading order. Each marker (STS) row lists the cM position, a flag, and the BAC clone addresses (grouped by contig band) that contain the marker.

Gene	STS	cM	f	791B9	774C1	943C4	803C1	240G10	758H6	733C10	257D9	93A5	65D3	810D6	100B4	308A5	486F12	799G9	617F5	620A7	757B1	965E1	949B8	926H8	984A3	767G4	808F9	981G5	848A7
D8S1725	848A7L	24.92	p		774C1	943C4	803C1	240G10	758H6	733C10											757B1	965E1	949B8	926H8	984A3	767G4	808F9	981G5	848A7
D8S1489	AFMa226zh5	24.98	s		774C1	943C4	803C1	240G10	758H6												757B1	965E1	949B8	926H8	984A3	767G4	808F9	981G5	848A7
	WI-4475	24.98	s		774C1	943C4	803C1	240G10													757B1	965E1	949B8	926H8	984A3	767G4	808F9	981G5	848A7
NEFL	WI-16152	24.63	g		774C1	943C4	803C1															965E1					808F9	981G5	848A7
GNRH1	SGC34359	25.09	g		774C1	943C4	803C1				257D9		65D3			308A5	486F12					965E1			984A3	767G4	808F9	981G5	848A7
	WI-15182	25.10	g		774C1	943C4	803C1	90F11		209G10	257D9		65D3			308A5	486F12	799G9		620A7		965E1			984A3	767G4	808F9	981G5	848A7
D8S1771	AFMb320va5	25.25	e		774C1	943C4	803C1	90F11	394D12	209G10	257D9		65D3			308A5	486F12	799G9	617F5	620A7	757B1							981G5	848A7
	SHGC-36250	25.26	p		774C1	943C4	803C1			209G10	257D9		65D3			308A5	486F12	799G9	617F5	620A7	757B1							981G5	848A7
FLJ25804	WI-11501	25.18	e		774C1	943C4	803C1		394D12	209G10	257D9		65D3			308A5	486F12	799G9	617F5	620A7	757B1							981G5	848A7
	stSG46607	25.19	g		774C1				394D12	209G10	257D9		65D3			308A5	486F12	799G9	617F5	620A7	757B1					421C4		981G5	848A7
D8S5	TL11	25.37	p		774C1																					421C4		981G5	848A7
	848A7R		s		774C1																								
	sts-T69732	25.55	e																										
	A009ZO1	25.94	e																										
	SHGC-36329	26.02	e																										
PPP2R2A	WI-18898	26.05	g						854B8	935G11																	808F9	981G5	
PPP2R2A	stSG63671	26.05	g					819B4	854B8	935G11																		981G5	
	stSG15253	26.08	e					819B4	854B8	935G11																		981G5	
BNIP3L	WI-8264	26.09	g					819B4	854B8	935G11																		981G5	
BNIP3L	sts-Z40756	26.09	g					819B4	854B8	935G11																		981G5	
	sts-W93086	26.14	e					819B4	854B8	935G11																			
PNMA2	SHGC-36722	26.18	g			741B6	741B7	819B4	854B8	935G11																			
DPYSL2	WI-6663	26.34	g			741B6	741B7	819B4	854B8	935G11				810D6															
ADRA1A	WI-17142	26.43	g			741B6				935G11				810D6															
ADRA1A	SGC35558	26.43	g							935G11																			
	stSG41355	26.76	g							935G11																			
	93A5R		s							935G11		93A5																	
STMN4	TIGR-A002J23	26.91	g	791B9								93A5		810D6															
D8S2299	791B9L		s	791B9								93A5		810D6					771G9										
TRIM35	A009Z35	26.96	g	791B9								93A5	65D3	810D6		308A5	486F12	799G9	771G9										
TRIM35	stSG26015	26.96	g	791B9								93A5	65D3	810D6		308A5	486F12	799G9	771G9										
TRIM35	SGC33557	26.97	g	791B9								93A5	65D3	810D6		308A5	486F12	799G9	771G9										
	stSG62910	26.97	e	791B9								93A5	65D3	810D6		308A5	486F12	799G9	771G9										
PTK2	cCI8-535	27.14	g	791B9								93A5	65D3	810D6	100B4	308A5	326D7		771G9	444E7									
D8S239A	AFMa083xd5		s	791B9								93A5	65D3	810D6	100B4	308A5	326D7		771G9	444E7									
D8S1839	sts-AA011480	27.20	p	791B9								93A5	65D3	810D6	100B4	308A5	326D7		771G9	444E7									
D8S242A	cCI8-554	27.21		791B9								93A5	65D3	810D6	100B4	308A5	326D7	189C9	771G9	444E7									
EPHX2 / GULOP	SGC35790	27.22	p	791B9								93A5	65D3	810D6	100B4	308A5	326D7	189C9	771G9	444E7									
CLU / ADRA1A / D8S131	stSG21579	27.28	g	791B9											100B4		326D7	189C9	771G9	444E7		755B3							
FLJ10853	WI-12131	27.41	g	406H12													326D7	189C9	771G9	444E7									
	stSG320266	27.44	e																771G9	444E7									
	stSG447247	27.46	e																771G9	444E7									
D8S137	sts-H67282	27.48	p																771G9	444E7		755B3							
	stSG54076	27.48	e																771G9	444E7									
TOPK	WI-1172	27.49	g																771G9	444E7		755B3							
D8S1161		27.53	s																										

Locus	Marker	cM	Type	Clones (tiling path, left → right)
	E-705-1	27,64	s	791B9
D8S1820	stSG47267	27,82	p	791B9, 406H12, 195D7, 305C12, 897C5, 572A3, 755B3, 771G9
ELP3	AFMa051zb1	27,84	p	791B9, 406H12, 195D7, 305C12, 514E7, 897C5, 572A3, –, 771G9
ELP3	stSG9277	27,87	g	791B9, 406H12, 195D7, 305C12, 514E7, 897C5, 572A3, 755B3, 771G9
ELP3	A002C05	27,87	g	791B9, 406H12, 195D7, 305C12, 514E7, 897C5, 798E4, 572A3, 771G10
D8S2211	WI-19683	27,87	g	791B9, 406H12, 195D7, 305C12, 514E7, 897C5, 798E4, 805B8, 771G9
	791B9R	27,87	s	791B9, 406H12, 195D7, 305C12, 514E7, 897C5, 798E4, 805B8
	A002O09	28,02	g	406H12, 195D7, 305C12, 514E7, 897C5, 798E4, 805B8
	Cda1ca05	28,03	g	406H12, 195D7, 305C12, 514E7, 897C5, 798E4, 805B8
LOC55893	stSG34833	28,03	g	406H12, 195D7, 305C12, 514E7, 897C5, 798E4, 805B8
D8S1809	AFM333yg9	28,01	p	406H12, 195D7, 305C12, 897C5, 798E4, 805B8
PNOC	WI-15701	28,02	g	406H12, 195D7, 305C12, 897C5, 798E4, 805B8
D8S2029	SHGC-10443	27,98	s	406H12, 195D7, 897C5, 798E4, 805B8
FZD3	N33122	28,13	e	897C5, 798E4, 805B8
	sts-AA024552	28,18	g	897C5, 798E4, 805B8
FZD3	SGC31127	28,24	g	897C5, 798E4, 805B8
	stSG2251	28,25	g	281F12, 897C5, 798E4, 805B8
	A007B14	28,25	e	281F12, 897C5, 798E4, 805B8
EXTL3	Cda13d01	28,43	e	281F12, 798E4, 805B8
FLJ10871	WI-6274	28,45	g	281F12, 645B9, 699D4, 798E4, 805B8
FLJ10871	SHGC-31253	28,53	g	281F12, 645B9, 699D4, 778B12, 798E4, 805B8, 887C7
	stSG22385	28,70	e	281F12, 645B9, 699D4, 778B12, 798E4, 805B8, 887C7
	A006K04	28,74	e	778B12, 798E4, 805B8, 887C7, 750E9
FLJ21616	SHGC-15326	28,69	g	750E9, 887C7, 768D6
FLJ21616	WI-20805	28,73	g	750E9, 887C7, 768D6
FLJ21616	stSG62535	28,73	g	750E9, 887C7, 768D6
KIF13B	WI-14186	28,75	g	750E9, 887C7, 768D6
	stSG39502	28,76	e	750E9, 887C7, 768D6
KIF13B	SHGC-52529	28,87	g	750E9, 887C7, 768D6, 936F7
KIF13B	WI-16549	28,87	e	750E9, 887C7, 768D6, 936F7
	stSG15815	28,89	e	750E9, 887C7, 768D6, 936F7
	SHGC-36290	28,91	e	814E11, 750E9, 887C7, 768D6, 936F7
	A009U09	28,91	s	814E11, 750E9, 887C7, 768D6, 936F7
DUSP4	750A	29,01	g	750E9, 887C7, 936F7, 768D6, 844E2
	WI-21790	28,94	e	750E9, 887C7, 936F7, 768D6, 816C10, 844E2
	A009Q36	28,94	e	750E9, 887C7, 936F7, 768D6, 816C10, 844E2
DUSP4	WI-22140	29,02	g	750E9, 887C7, 936F7, 768D6, 816C10, 844E2
DUSP4	A004W39	29,01	g	750E9, 887C7, 936F7, 768D6, 816C10, 844E2
	750C	29,24	s	750E9, 936F7, 816C10, 844E2
	SGC33982	29,24	e	936G4, 750E9, 936F7, 816C10, 844E2
	stSG30527	29,24	e	936G4, 750E9, 936F7, 816C10, 844E2
	A004P36	29,28	e	936G4, 750E9, 936F7, 816C10, 844E2
MAPKK	E-1051-1		gap	936G4, 814E11, 750E9, 936F7, 816C10, 824C8, 914B12, 844E2
MGC8721	WI-20115	29,78	g	936G4, 814E11, 807F11, 750E9, 936F7, 816C10, 824C8, 914B12, 844E2
MGC8721	GAAT-P33725	29,80	g	936G4, 814E11, 807F11, 750E9, 936F7, 816C10, 824C8, 914B12, 654A12, 844E2
LEPROTL1	W11-6E	29,82	g	936G4, 814E11, 807F11, 750E9, 936F7, 816C10, 824C8, 914B12, 710C3, 654A12, 844E2
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	750B		s	936G4	814E11	807F11						750E9		936F7		816C10	-		914B12	654A12	844E2			
	stSG31257	29,34	e	936G4	814E11	807F11						750E9		936F7		816C10	-				844E2	729A6		
1D12A	SHGC-11047	30,13	g	-	814E11	-						750E9		936F7			-				844E2	729A6		
RBPMS		30,10	g	-	814E11	-					762F7	750E9		936F7			-				844E2	729A6		
	E-1046-2		s	-	814E11	-					762F7	750E9		936F7			-				844E2	-		
TUBB	SHGC-10364	30,07	pg	936G4	814E11	807F11					762F7	750E9		936F7			-				844E2	-		
GSR	W3-5	30,43	g	936G4	814E11	807F11					762F7	750E9					-				844E2	729A6		
	WP2252B42	30,16	e	-	814E11	807F11	896F4				762F7	750E9					-				844E2	729A6		
	stSG16429	30,19	e	-	814E11	807F11	896F4				762F7	750E9					-				844E2	729A6		
D8S339	WT251	30,21	p	-	814E11	807F11	896F4				762F7	750E9					-				844E2	729A6		
RBPMS	WT10905	30,22	g	-	814E11	807F11	896F4	693D8			762F7						-				844E2	729A6		
	SGC34085	30,13	e	-	814E11	807F11	896F4	693D8									-				844E2	729A6		
	SGC30104	30,23	e	-	814E11	807F11	896F4	693D8									-				844E2	729A6		
	E-1055-1		s	-	814E11	807F11	896F4	693D8									-					729A6		
	W1-6	30,27	e	-	814E11	807F11	896F4	693D8									824C8					729A6		
RBPMS	W1-25	30,28	g	-	814E11	807F11	896F4	693D8														729A6		
RBPMS	W11-4D	30,28	g	-	814E11	807F11	896F4	693D8														729A6		
	stSG60069	30,28	e	-	814E11	807F11	896F4	693D8														-		
	stSG48118	30,30	e	-	814E11	807F11	896F4	693D8														-		
GTF2E2		30,29	g	-	814E11	807F11	896F4	693D8										953H12				729A6		
GTF2E2	W13-5A	30,31	g	936G4	814E11	807F11	896F4	693D8										953H12						
	stSG62551	30,36	e	936G4	814E11	807F11	896F4	693D8			780E6	672C9						953H12						
	763A7L		s	936G4	814E11	807F11	896F4	693D8	786D10	763A7	780E6	672C9						953H12						
	stSG28623	30,36	e	936G4	814E11	807F11	896F4	693D8	-	763A7	780E6	672C9		804A1				953H12						
D8S540	AFM281yb9	30,37	p	936G4	814E11	807F11	896F4	693D8	-	763A7	780E6	672C9	686G9	-				953H12						
GSR		30,39	g	936G4	814E11	807F11	896F4	693D8	-	763A7	780E6	672C9	686G9	-				953H12						
GSR	WI-13416	30,39	g	936G4	814E11	807F11	896F4	693D8	786D10	763A7	780E6			-				953H12						
GSR	WI-21746	30,39	g	936G4	814E11	807F11	896F4	693D8	786D10	763A7	780E6			-				953H12						
	A006C09	30,41	e	936G4	814E11	807F11	896F4	693D8	786D10	763A7	780E6			804A1				953H12						
	765D9L		s	936G4	814E11	807F11	896F4	693D8	786D10	763A7	780E6			804A1				953H12		765D9				
D8S1630	WI-3666	30,46	s	936G4	814E11	807F11	896F4	693D8	786D10	763A7	780E6			804A1				953H12		765D9				
D8S2298E	D8S2298E	30,48	g	936G4	814E11	807F11	896F4	693D8	786D10	763A7	780E6			804A1				953H12		765D9				
PPP2CB		30,50	g	936G4	814E11	807F11	896F4	693D8	786D10	763A7	780E6			804A1				953H12		765D9				
	W13-3D-II		e	936G4	814E11	807F11	896F4	693D8	786D10	763A7	780E6			804A1				953H12		765D9				
	stSG22284	30,51	e	936G4	814E11	807F11	896F4	693D8	786D10	763A7	780E6			804A1				953H12		765D9				
	AFMa224wh5	30,64	p		814E11	807F11	896F4	693D8	786D10	763A7		791A1	234H4	804A1	901E10			953H12		765D9				
	SHGC-30538	30,72	e			807F11	896F4	693D8	786D10	763A7		791A1	234H4	804A1	901E10			953H12		765D9				
	815E8L		s				896F4	693D8	786D10	763A7		791A1	234H4	804A1	901E10			953H12		765D9		815E8		
WRN	WI-17945	30,78	g				896F4	693D8	786D10	763A7		791A1	234H4	804A1	901E10			953H12		765D9		815E8	798B11	
	721D7R		s				896F4	693D8	786D10	763A7		791A1	234H4	804A1	901E10			953H12		765D9	721D7	815E8	798B11	
WRN	WRN exon 35		g				896F4	693D8	786D10	763A7	613E1	791A1	234H4	804A1	901E10		336D12	953H12		765D9	721D7	815E8	798B11	
	sts-N22494	30,89	e				896F4	693D8	786D10	763A7	613E1	791A1	234H4	804A1	901E10		336D12	953H12		765D9	721D7	815E8	798B11	
	721C		s				896F4	693D8	786D10	763A7	613E1	791A1	234H4	804A1	901E10		336D12	953H12		765D9	721D7	815E8	798B11	
	721A		s			82D5	896F4	693D8	786D10	763A7	613E1	791A1	234H4	804A1	901E10	554G3	336D12	953H12	147C10	765D9	721D7	815E8	798B11	
	721B		s		848D12	82D5	896F4	693D8		763A7	613E1	791A1	234H4	804A1	901E10	554G3	336D12	953H12	147C10	765D9	721D7	815E8	798B11	
D8S1770	AFM238vd10	31,00	p	600D8	848D12	82D5	896F4	693D8		763A7	613E1	791A1	234H4	804A1	901E10	554G3	336D12	953H12	147C10	765D9	721D7	815E8	798B11	
D8S1769	AFM220yf8	31,00	p	600D8	848D12	82D5	896F4	693D8		763A7	613E1	791A1	234H4	804A1	901E10	554G3	336D12	953H12	147C10	765D9	721D7	815E8	798B11	
D8S124	pBS8	31,06	s	600D8	848D12	82D5	896F4	693D8		763A7	613E1	-	234H4	804A1	901E10	554G3	336D12	953H12	147C10	765D9	721D7	815E8	798B11	788H6
D8S1711	AFM158xg7	31,18	p				896F4	693D8		763A7		-			901E10	554G3	336D12	953H12	147C10	765D9	721D7	815E8	798B11	788H6
	978B9R		s		978B9		896F4	693D8		763A7		-				554G3	336D12	953H12	147C10	765D9	721D7	815E8	798B11	788H6
	872E4R		s	872E4	978B9		896F4	693D8				-						953H12	147C10	765D9	721D7	815E8	798B11	788H6
D8S2217	896F4L		s	872E4	978B9		896F4	693D8				-						953H12	147C10	765D9	721D7	815E8	798B11	788H6

	Name	Pos.	783C11	847B12	872E4	978B9	960B4	761A2	888D12	742F2	693D8	952D5	905E5	956H3	726G12	627D5	711E1	716G9	49A2	907D5	939G12	909A11	898G11	951G11	79F6	791A1	751E7	420C10	306H11	955C4	847F9	756E1	908C1	798G8	798E11	347B2	215D1	953H12	849C2	808C7	802F7	765D9	721D7	268E1	710C12	815E8	798B11	845G8	788H6	815B2	750H12
	stSG2166	31,42 e			872E4	978B9																																	953H12				765D9	721D7			815E8	798B11		788H6		
D8S1810	stSG30639	31,42 e			872E4	978B9																																	953H12				765D9	721D7			815E8	798B11		788H6		
D8S2226	721D7L	s			872E4	978B9																																	953H12				765D9	721D7			815E8	798B11		788H6		
	693D8L	s			872E4	978B9					693D8																												953H12				765D9				815E8	798B11		788H6		
	AFM345td5	31,55 p			872E4	978B9					693D8															791A1													953H12				765D9				815E8	798B11		788H6		
	761A2L	s			872E4	978B9	761A2			693D8																791A1													953H12				765D9				815E8	798B11		788H6		
	742F2R	s			872E4	978B9	761A2	888D12	742F2																	791A1													953H12				765D9				815E8	798B11		788H6		
	WI-12979	31,76 s			872E4	978B9	761A2	888D12	742F2																	791A1													953H12								815E8	798B11		788H6		
AFMa143yg9		31,84 p			872E4	978B9	761A2	888D12	742F2																																		765D9				815E8	798B11		788H6		
stSG46856		31,85 e			872E4	978B9	761A2	888D12	742F2																																						815E8			788H6		
815E8R		s			872E4	978B9	761A2	-	742F2																																									788H6		
872E4L		e			872E4	978B9	761A2	888D12	742F2																																									788H6		
sts-Z40930		32,06 e				978B9	761A2	888D12	742F2																		751E7																									
D8S1125	CHLC.ATA9G02	32,22 p				978B9	761A2	888D12	742F2	443B9																	751E7																									
D8S278	AFM200ye1	32,40 p				978B9	761A2	888D12	742F2	443B9	754D11														79F6		751E7																		710C12	268E1					
NRG1		32,48 g				978B9	761A2	888D12	742F2		754D11														79F6		751E7	420C10																	710C12	268E1					
	888D12L	s				978B9	761A2	888D12	742F2																79F6		751E7	420C10																	710C12	268E1					
D8S2221	742F2L	s				978B9	761A2	888D12	742F2																79F6		751E7	420C10	306H11											849C2	808C7				268E1	710C12					
	SGC33877	32,76 e				978B9	761A2																				751E7	420C10	306H11											849C2	808C7										
	sts-N33323	32,76 e				978B9	761A2																				751E7	420C10	306H11											849C2	808C7										
D8S71	pL8	32,81 s				978B9	761A2																				751E7	420C10	306H11							347B2	215D1		849C2												
D8S1758	AFMb292zf1	32,91 p				978B9	761A2																				751E7	420C10	306H11	955C4						347B2	215D1		849C2												
D8S259	AFM107yb2	32,92 p				978B9	761A2																				751E7	420C10	306H11	955C4						347B2	215D1		849C2												
sts-A028099		33,07 e																									751E7										215D1		-												
MGC1136	stSG44713	33,31 g																																			215D1		849C2	808C7	802F7									
MGC1136	stSG10073	33,31 s																																			215D1		849C2	808C7	802F7									
D8S1845	E-1030-2	33,33 p	783C11																																		215D1		849C2	808C7	802F7									
D8S35	AFM127xe5	33,55 p	783C11												726G12	627D5																					215D1		849C2	808C7	802F7							845G8			
LOC84549	AFM266zc9	33,21 g	783C11												726G12	627D5		716G9																			215D1		849C2	808C7	802F7							845G8			
D8S1709	SHGC-32777	33,48 p	783C11												726G12	-	711E1	716G9																					849C2	808C7	802F7							845G8			
D8S283	AFMa163xg5	33,48 p	783C11												726G12	-	711E1	-																					849C2	808C7	802F7							845G8			
D8S513	AFM238yh12	33,53 p	783C11												726G12	627D5	711E1	-																					849C2	808C7	802F7							845G8			
PIP5K2B	AFM211wa9	chr17 g																716G9																					849C2	808C7	802F7							845G8			
D8S2220	WI-6601	34,31 p																716G9	49A2			909A11																	849C2	808C7	802F7							845G8			
D8S505	849C2L	e																	49A2			909A11	898G11	951G11																808C7	802F7							845G8			
	AFM198tb2	34,74 e																		939G12			898G11	951G11																											
	stSG47682	34,93 s																		939G12			898G11	951G11																											
D8S1501	WI-5543	35,00 s		847B12																		909A11	898G11	951G11							847F9	756E1	908C1																		
D8S1486	SHGC-32224	35,14 s		847B12																											847F9	756E1	908C1																		
D8S7	WI-4330	35,16 p		847B12					956H3																						847F9	756E1	908C1	798G8																		
D8S1719	AFMa192xd5	35,25 p		847B12		960B4		905E5	956H3		952D5								907D5	939G12		898G11	951G11							847F9	756E1	908C1	798G8	798E11																	
D8S1766	AFMb311yb9	35,30 p		847B12		960B4		905E5	956H3		952D5								907D5	939G12		898G11	-							847F9	756E1	908C1	798G8	798E11															815B2	
D8S1121	CHLC.ATA22A0	35,66 p		847B12		960B4		905E5	956H3		952D5								907D5		930F3	898G11							847F9	756E1	908C1	798G8	798E11															815B2	
UNC5D	AFM283xf1	35,50 p		847B12		960B4		905E5	956H3		952D5								907D5	939G12	930F3								847F9	756E1		798G8	798E11															815B2	
D8S1803	AFM312zf1	35,51 g		847B12		960B4		905E5	956H3		952D5								907D5		930F3			951G11					847F9	756E1		798G8	798E11															815B2	750H12
BC028701	WI-11522	36,65 g		847B12		960B4		905E5	956H3		952D5								907D5		930F3								847F9	756E1		798G8	798E11															815B2	750H12

Supplementary Table II
Alignment of known genes from the YAC-contig and the UCSC April 2003 database

JNI - 2000	Mapping UCSC Apr 03	Mbp	Band
LPL	LPL	19.60	8p21.3
	SLC18A1	19.81	
ATP6V1B2	ATP6V1B2	19.88	
LZTS1	LZTS1	19.91	
GFRA2	GFRA2	21.35	
DOK2	DOK2	21.58	
	XPO7	21.68	
	FGF17	21.72	
	EPB49	21.76	
RAI16	RAI16	21.78	
	FLJ22494	21.78	
HR	HR	21.79	
	FLJ22246	21.81	
EPB49			
XP07			
LGI3	LGI3	21.82	
SFTPC	SFTPC	21.84	
BMP1	BMP1	21.84	
FLJ22246			
PHYHIP	PHYHIP	21.89	
	POLR3D	21.92	
PIWIL2	PIWIL2	22.03	
PPP3CC	PPP3CC	22.21	
SCAM-1	SCAM-1	22.25	
PDLIM2	PDLIM2	22.27	
FLJ34715	FLJ34715	22.28	
DBC-1	DBC-1	22.29	
BIN3	BIN3	22.29	
	FLJ14107	22.32	
EGR3	EGR3	22.36	
	MGC22776	22.39	8p21.2
RHOBTB2	RHOBTB2	22.68	
TNFRSF10B	TNFRSF10B	22.69	
MGC29816			
TNFRSF10C	TNFRSF10C	22.79	
TNFRSF10D	TNFRSF10D	22.81	
	TNFRSF10A	22.87	
	MGC29816	22.93	
LYSAL1			
LOXL2	LOXL2	22.97	
	LYSAL1	23.10	
	MSCP	23.24	
	PRO1496	23.21	
MSCP			
NKX3-1	NKX3-1	23.35	
STC1	STC1	23.52	
ADAM28	ADAM28	24.03	
	ADAMDEC1	24.06	
NEF3	NEF3	24.59	
NEFL	NEFL	24.63	
	FLJ21034	25.07	
GNRH1	GNRH1	25.10	
	FLJ20038	25.11	

JNI - 2000	Mapping UCSC Apr 03	Mbp	Band
FLJ25804	FLJ25804	25.18	
	EBF2	25.52	
PPP2R2A	PPP2R2A	26.04	
BNIP3L	BNIP3L	26.08	
PNMA2	PNMA2	26.18	
DPYSL2	DPYSL2	26.33	
ADRA1A	ADRA1A	26.43	
STMN4	STMN4	26.91	
TRIM35	TRIM35	26.96	
PTK2B	PTK2B	27.13	
	CHRNA2	27.14	8p21.1
EPHX2	EPHX2	27.22	
CLU	CLU	27.27	
ADRA1A		26.43	
	SCARA3	27.31	
FLJ10853	FLJ10853	27.41	
TOPK	TOPK	27.48	
	MGC45780	27.58	
ELP3	ELP3	27.83	
	PNOC	28.02	
	DKFZp434K1210	28.02	
LOC55893	LOC55893	28.02	
PNOC			
	FBX16	28.11	
FZD3	FZD3	28.18	
EXTL3	EXTL3	28.43	
FLJ10871	FLJ10871	28.44	
FLJ21616	FLJ21616	28.68	
KIF13B	KIF13B	28.74	8p12
DUSP4	DUSP4	29.01	
MAPKK		gap	
MGC8721	MGC8721	29.77	
LEPROTL1	LEPROTL1	29.81	
DCTN6	DCTN6	29.89	
	RBPMS	30.09	
1D12A	1D12A	30.13	
RBPMS			
GTF2E2	GTF2E2	30.31	
GSR	GSR	30.39	
D8S2298E	D8S2298E	30.48	
PPP2CB	PPP2CB	30.49	
	TEX15	30.55	
	PURG	30.74	
WRN	WRN	30.78	
NRG1	NRG1	32.47	
	FUT10	33.09	
	LOC84549	33.21	
	FLJ23263	33.21	
	FLJ12526	33.26	
MGC1136	MGC1136	33.30	
LOC84549			
UNC5D	UNC5D	35.50	
BC028701	BC028701	36.64	

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SUMMARY

Prostate cancer (PrCa) is the most frequently diagnosed cancer in men, and the second leading cause of male cancer death in Western countries (Chapter 1-1). In order to design novel targeted therapies, knowledge of the molecular mechanisms underlying PrCa growth are urgently needed. Like other cancers, PrCa is believed to result from genomic instability, leading to mutation, deletion, amplification and translocation of a large variety of genes that regulate cell growth and survival. Chromosomal loss is an indication for the localization of a tumour suppressor gene, whereas amplification and translocation are an indication for the localization of an oncogene (Chapter 1-2). One of the most frequent genetic abnormalities in PrCa is loss of the complete, or part of the short arm of chromosome 8. Loss of the long arm of chromosome 10 is another frequently observed genetic defect in PrCa (Chapter 1-3). The aim of this thesis is to further characterise the regions of frequent loss at chromosome 10q and 8p, and to identify the tumour suppressor genes located in these regions (Chapter 1-4).

This study mainly used a set of PrCa derived xenografts (11) and cell lines (4). Human tumours, propagated as xenografts in nude mice, or as *in vitro* growing cell lines are unique sources of tumour DNA that aid the identification of genetic defects in human cancer. They are available in unlimited quantities. Importantly, they lack normal cells of human origin, which increases the detection of chromosomal alterations, homozygous deletions and point mutations in individual genes and their expression level. An additional set of 43 prostate tumour samples was used for chromosome 8p gene mutation analyses.

The set of xenografts and cell lines was searched for chromosome 10 alterations by Comparative Genomic Hybridisation and allelotyping (Chapter 3). CGH revealed a pattern of loss of 10q in six out of fifteen samples, which were confirmed by allelotyping. These were all large deletions. In another seven samples, allelotyping revealed small regions of loss of 10q23.3, in particular. Recently, the *PTEN* tumour suppressor gene was identified and mapped to this chromosomal locus. A *PTEN* structure and expression analysis of the xenografts and cell lines demonstrated five homozygous deletions, two nonsense mutations, and two frame-shift mutations (Chapter 2). Except for the samples with a large homozygous deletion, no aberrant *PTEN* mRNA expression was found. This

indicates that expressional down-regulation is no important mechanism of PTEN inactivation. The high percentage (60%) of deletions and mutations implicates a significant role for PTEN in the pathogenesis of PrCa. Nevertheless, not all losses of 10q23.3 were found in combination with a PTEN inactivation. Four samples with loss of 10q showed an intact PTEN gene (Chapter 3). Apart from haplo-insufficiency, which has been assigned as a mechanism of PTEN inactivation, another 10q tumour suppressor gene may also be involved. We therefore analysed the deletion and down-regulation of expression of genes flanking the *PTEN* locus in both PTEN positive and negative samples (Chapter 3). Candidate gene FLJ11218 appeared particularly interesting because of its differential expression in several samples and a missense mutation in one sample, and because it accompanied all losses and homozygous deletions of PTEN. A search for tumour suppressor genes on distal 10p and 10q did not reveal alteration of expression or structure of the KLF6 and MXI genes. The DMBT1 gene at 10q was deleted in one xenograft and down-regulated in two.

The set of xenografts was also searched for chromosome 8 alterations by Comparative Genomic Hybridisation (CGH) and allelotyping (Chapter 5). CGH showed 8p loss in ten DNAs. In eight of the samples 8p loss included the 8p12-p21 interval. In most DNAs allelotyping matched with the CGH data. Lack of a previously identified tumour suppressor gene on 8p forced us to fine map the position of a tumour suppressor gene. In the past, the search for a homozygous deletion has been pivotal in the identification of a number of tumour suppressor genes, including *BRCA2*, *DPC4*, and *PTEN*. We searched for homozygous deletions in the 8p12-p21 interval by using known and novel polymorphic and nonpolymorphic sequence tagged sites (STSs) between the markers D8S87 and D8S133. The interest for this specific region arose from our previous results, showing allelic loss in 69% of the PrCa samples. In xenograft PC133, the presence of a small homozygous deleted region of 730-1320 Kb was unambiguously established (Chapter 4). At one site, the deletion was found to disrupt the major part of the Werner Syndrome gene (*WRN*). To facilitate the identification and isolation of candidate tumour suppressor genes in this area, we generated a contiguous PAC/cosmid contig (Chapter 5). Thirty-seven STSs were localized in this map that were subsequently used to screen the panel of xenografts and cell lines for an overlapping homozygous deletion. No additional homozygous deletion was found. Exon trapping, database analysis of sequenced genomic DNA, or expressed sequence tag (EST) characterization did not reveal a novel *bona fide* gene other than

the WRN gene. A single EST, sts-N22494, was identified as part of the 3'-UTR of a longer *WRN* transcript. To test *WRN* as a candidate tumour suppressor gene in PrCa, a mutation and expression analysis was performed. Except for PC133, no alteration in *WRN* was found. Although absence of *WRN* may have contributed to PC133 tumour growth, no further evidence was found for a role of *WRN* in PrCa tumour development. This suggests another tumour suppressor gene on 8p12-p21. To facilitate the identification and isolation of candidate tumour suppressor genes in this area, we isolated the BC028701 - LPL segment, which completely contains the D8S87-D8S133 region, in a single contig of approximately 17 Mbp, composed of 172 YACs (Appendix). We then ordered 303 markers within this contig, including polymorphic sequence tagged sites (STSs), nonpolymorphic STSs, expressed sequence tags (ESTs), genes, and pseudogenes. The availability of this robust physical and transcript map facilitated and justified an extensive search for a homozygous deletion on 8p12-p21 (Chapter 6). Again no homozygous deletion was found. We therefore continued our search for tumour suppressor genes by analysis of positional and functional candidate genes at 8p. An extensive mutation and expression analysis of the *LZTS1*, *PRLTS*, *NKX3-1*, *EPHX2*, *CLU* and *POLB* genes was performed. We did not find convincing evidence that any of these genes are frequently bi-allelic inactivated in human PrCa, although the *EPHX2* gene may require further analysis.

In conclusion, except for *PTEN* not a single gene on chromosome 8p or elsewhere on 10q was found to be frequently inactivated due to a double hit deletion and or mutation (Chapter 7). Haplo-insufficiency as an additional mechanism of gene inactivation and its possible implications for PrCa tumorigenesis are discussed in a larger context.

SAMENVATTING

Prostaatkanker is de meest voorkomende vorm van kanker bij mannen in Westerse landen, en de op een na meest frequente oorzaak van overlijden aan kanker bij mannen (Hoofdstuk 1.1). Om nieuwe therapieën te kunnen ontwikkelen is kennis van de moleculaire mechanismen die ten grondslag liggen aan het ontstaan en de groei van de tumor noodzakelijk. Net zoals bij andere tumoren, wordt gedacht dat prostaatkanker het gevolg is van instabiliteit van het genoom. Deze instabiliteit resulteert in mutatie, deletie, amplificatie en translocatie van een grote variëteit aan genen die betrokken zijn bij de regulering van celgroei en celdood. Verlies, amplificatie en translocatie van specifieke delen van chromosomen zijn karakteristieken van tumorcellen. Verlies is een aanwijzing voor de locatie van een gen dat de groei van een tumor remt, oftewel een tumorsuppressorgen. Een amplificatie of translocatie is daarentegen een aanwijzing voor de locatie van een gen dat de groei van een tumor stimuleert, oftewel een oncogen (Hoofdstuk 1.2). Een van de meest voorkomende afwijkingen van het genoom in prostaatkanker is geheel of gedeeltelijk verlies van de korte arm van chromosoom 8. Verlies van de lange arm van chromosoom 10 is eveneens een veel voorkomend defect van het genoom in prostaatkanker (Hoofdstuk 1.3). Doel van dit proefschrift is de karakterisering van de delen van chromosoom 8p en 10q, die het meest frequent verloren gaan, en de identificatie van de tumorsuppressorgenen in deze regio's (Hoofdstuk 1.4).

In deze studie is voornamelijk gebruik gemaakt van een set xenograften (11) en cellijnen (4) die zijn afgeleid van prostaattumoren van de mens. Xenograften zijn tumoren, die als een transplantaat groeien in muizen met een verzwakt immuunsysteem (naakte muizen). Xenograften en *in vitro* groeiende cellijnen zijn unieke bronnen van tumor-DNA, die een belangrijk hulpmiddel zijn bij de identificatie van veranderingen in het genoom van humane tumoren. Ze zijn in principe in onbeperkte hoeveelheden beschikbaar. Nog belangrijker is dat ze, in tegenstelling tot direct van de mens afkomstig tumorweefsel, geheel vrij zijn van normale cellen van humane oorsprong zoals de cellen van steun- en vaatweefsel. Dit vergroot de kans op het vinden van veranderingen in chromosomen en het detecteren van homozygote deleties en puntmutaties in individuele genen. Om dezelfde reden kan genexpressie beter bestudeerd worden. Voor de analyse van mutaties in genen op chromosoom 8p is tevens gebruik gemaakt van weefsel van 43 prostaattumoren.

De xenograften en cellijnen zijn onderzocht op afwijkingen van chromosoom 10 door middel van CGH (Comparative Genomic Hybridization) en allelotypering (Hoofdstuk 3). In zes van de vijftien monsters werd verlies van 10q aangetoond. Dit werd bevestigd met allelotypering. Daarnaast werden met allelotypering in zeven andere monsters verliezen van een klein deel van de chromosomale band 10q23.3 gevonden. Hierin is het *PTEN* tumorsuppressorgen gelegen. Analyse van de structuur en expressie van *PTEN* toonde vijf homozygote deleties aan, twee nonsense-mutaties en twee frame-shift-mutaties (Hoofdstuk 2). Behalve in monsters met een grote homozygote deletie werd geen afwijking in de expressie van *PTEN* gevonden. Dit is een aanwijzing dat vermindering van expressie geen belangrijk mechanisme van inactivering van *PTEN* is. Het hoge percentage (60%) deleties en mutaties suggereert nochtans een significante rol voor *PTEN* bij prostaatkanker. Vier DNA's met verlies van 10q bezaten een intact tweede kopie van het PTEN-gen (Hoofdstuk 3). Mogelijk kan verlies van één kopie van *PTEN* al een rol spelen bij de groei van de tumor. Dit staat bekend als haplo-insufficientie. Daarnaast is het mogelijk dat een ander tumorsuppressorgen op 10q een rol speelt in prostaatkanker. Genen die het *PTEN*-gen flankeren zijn daarom onderzocht op deleties en verlaagde expressie. Hiervoor zijn zowel *PTEN*-positieve als -negatieve samples gebruikt (Hoofdstuk 3). Met name kandidaatgen *FLJ11218* lijkt interessant omdat het betrokken is bij homozygote deleties en het differentieel tot expressie komt in normaal en tumorweefsel. Bovendien is een missense-mutatie aangetoond in het DNA van een prostaat carcinoom. Andere kandidaten zijn *MINPP1* en *PAPSS2*. Onderzoek naar de eerder beschreven kandidaat-tumorsuppressorgenen *KLF6* en *MXI1*, gelegen op respectievelijk het uiteinde van chromosoom 10p en 10q, heeft geen afwijkingen laten zien. Het *DMBT1*-gen op 10q bevat in een xenograft een intragenetische homozygote deletie. Daarnaast is de expressie van dit gen laag in twee xenograften.

De xenograften en cellijnen zijn eveneens onderzocht op afwijkingen van chromosoom 8 door middel van CGH en allelotypering (Hoofdstuk 5). In het DNA van tien verschillende tumoren/cellijnen werd verlies van 8p aangetoond met CGH, waarbij in acht gevallen het gebied 8p12-p21 verloren was gegaan. In de meeste gevallen kwamen de resultaten van allelotypering en CGH overeen. Het ontbreken van een eerder geïdentificeerd tumorsuppressorgen op 8p noodzaakte ons de positie van zo'n gen te bepalen. In het verleden zijn homozygote deleties cruciaal gebleken voor de

identificatie van tumorsuppressorgenen zoals *BRCA2*, *DPC4* en *PTEN*. Daarom werd in het gebied 8p12-p21, met behulp van polymorfe en niet polymorfe markers (STSs), gezocht naar homozygote deleties. Specifiek is gezocht tussen de markers *D8S87* en *D8S133*. De interesse voor dit deel van 8p kwam voort uit eerder onderzoek waarin frequent verlies (69%) van dit gebied werd aangetoond in prostaattumoren. In xenograft PC133 werd een kleine homozygote deletie (730 – 1320 Kb) vastgesteld (Hoofdstuk 4). Deze deletie omvatte een deel van het Werner Syndroom-gen (*WRN*). Om andere kandidaat-tumorsuppressorgenen in dit gebied te kunnen ontdekken, werd hiervan een PAC/cosmide contig gegenereerd (Hoofdstuk 5). In het gedeleteerde gebied werden 37 markers (STSs) gelokaliseerd. Deze werden vervolgens gebruikt bij het onderzoek op kleine homozygote deleties in de andere xenograften en cellijnen. Dit was zonder succes. Exon trapping en onderzoek van de sequentie van het genomische DNA en analyse van fragmenten van onbekende genen (ESTs), leverde alleen het WRN-gen op. Een EST, sts-N22494, bleek een deel van het 3'UTR van een langer WRN transcript te zijn. Om te testen of het *WRN*-gen een kandidaat tumorsuppressorgen is in prostaatkanker, werd een analyse van mutatie en expressie van dit gen uitgevoerd. Behalve bij PC133 werd er geen afwijking in *WRN* gevonden. Hoewel afwezigheid van WRN een bijdrage kan hebben geleverd aan de groei van PC133, zij geen nieuwe argumenten gevonden voor een rol van WRN in prostaatkanker. Dit suggereert dat één of meer andere genen op 8p12-p21 hierbij van belang zijn. Om de identificatie en isolatie van kandidaat-tumorsuppressorgenen te vergemakkelijken is het gebied tussen BC028701 en LPL geïsoleerd in een YAC contig (Appendix). Dit gebied bevat de gehele regio tussen D8S87 en D8S133, in een contig van 172 YACs met een omvang van 17 Mbp (Appendix A6). In dit contig konden 303 markers worden geordend, waaronder polymorfe markers, STSs, ESTs, genen en pseudogenen. Deze gedetailleerde fysische- en transcriptie-kaart maakte het vervolgens mogelijk en verantwoord chromosoom 8p12-p21 in detail te screenen op homozygote deleties (Hoofdstuk 6). Wederom werd geen homozygote deletie gevonden. Het zoeken naar tumorsuppressorgenen is daarom voortgezet middels mutatie- en expressieanalyse van de 8p kandidaatgenen *LZTS1*, *PRLTS*, *NKX3-1*, *EPHX2*, *CLU* en *POLB*. Behalve vanwege hun locatie werden deze genen geselecteerd omdat het biologisch plausibel is dat ze een rol spelen in prostaatkanker. We vonden voor geen van deze genen

overtuigend bewijs dat zij frequent bi-allelisch geinactiveerd zijn in prostaatkanker. Alleen aan *EPHX2* is verder onderzoek gerechtvaardigd.

In conclusie, behalve *PTEN*, is op 8p en 10q geen enkel gen gevonden dat frequent is gedeleteerd en / of gemuteerd in beide allelen (Hoofdstuk 7). Gezien deze negatieve bevindingen moet worden overwogen dat in prostaatkanker haplo-insufficiency vaker een rol speelt dan tot nu toe werd aangenomen.

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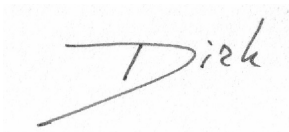
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