GENETIC ANALYSES OF CLINICAL PROSTATE CANCER

P.C.M.S. Verhagen

Cover: X Chromosome (courtesy Indigo Instruments)

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GENETIC ANALYSES OF CLINICAL PROSTATE CANCER

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Wie met beide benen op de grond staat, komt niet vooruit.
(C. Weijnen)

Voor Marjo
Voor mijn ouders

CONTENTS

ABBREVIATIONS

BrdU	bromodeoxyuridine
BAC	bacterial artificial chromosome
CAP	college of American pathologists
cDNA	copy DNA
CGH	comparative genomic hybridization
CT	computerized tomography
DAPI	4′6′-diamino-2-phenylindole
DOP-PCR	degenerate oligonucleotide primer PCR
DRE	digital rectal examination
EGFP	enhanced green fluorescent protein
EST	expressed sequence tag
FISH	fluorescence in situ hybridization
FITC	fluorescein isothiocyanate
GSS	Gleason sum score
HD	homozygous deletion
HPC	hereditary prostate cancer
LCM	laser capture microdissection
LOD	logarithm of odds
LOH	loss of heterozygosity
MRI	magnetic resonance imaging
mRNA	messenger RNA
MSI	microsatellite instability
NCI	national cancer institute
NE	neuro endocrine
NIH	national institute of health
PAC	phage artificial chromosome
PC	prostate cancer
PCR	polymerase chain reaction
PIA	proliferative inflammatory atrophy
PSA	prostate specific antigen
PSAD	PSA density
PSAV	PSA velocity
SCPC	small cell prostate cancer
SKY	spectral karyotyping
SNP	single nucleotide polymorphism
SSCP	single strand conformation polymorphism
STS	sequence tagged site

TNM	tumour, nodes, metastasis
TRUS	transrectal ultrasound of prostate
TURP	transurethral resection of prostate
UCSC	University of California, Santa Cruz
WHO	world health organization
WPR	wild-type to pseudogene ratio
YAC	yeast artificial chromosome

GENES

Akt	serine/threonine protein kinase
AMACR	alpha-methylacyl-CoA racemase
BCL-2	B-cell CLL/lymphoma 2
BRCA1	breast cancer 1
BRCA2	breast cancer 2
DPC4	deletion target in pancreatic carcinoma 4; SMAD4
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
ELAC2	elaC homolog 2
EZH2	enhancer of zeste 2
FHIT	fragile histidine triad gene
HPC-1	hereditary prostate cancer 1
HPC-X	hereditary prostate cancer X
KAI1	kangai 1
MIB-1	mind bomb homolog 1
MMAC1	mutated in multiple advanced cancers 1 (PTEN)
MTS1	cyclin-dependent kinase inhibitor 2a; INK4
MSR1	macrophage scavenger 1
MXI1	MAX interacting protein 1
INK4	cyclin-dependent kinase inhibitor 2a, MTS1
P27(kip1)	cyclin-dependent kinase inhibitor 1b
P53	tumour protein p53
Phospho-Akt	phosphorylated Akt
PCNA	proliferating cell nuclear antigen
PSA	prostate specific antigen
PSMA	prostate specific membrane antigen
PTEN	phosphatase and tensin homologue (MMAC1, TEP1)
RB (1)	retinoblastoma 1
RNASEL	ribonucease L
SMAD4	mothers against DPP homolog 4; DPC4
TEP1	PTEN
TGF alfa	transforming growth factor alfa
TGF beta	transforming growth factor beta
WAF1/CIP1	cyclin-dependent kinase inhibitor 1A (p21, Cip1)
WT1	Wilms tumour 1

Chapter 1

GENERAL INTRODUCTION AND SCOPE OF THE THESIS

INTRODUCTION

The general scope of this thesis is to assess molecular changes in prostate cancer cells. This is important because of two reasons: 1) knowledge of molecular changes may contribute to the understanding of prostate cancer development and lead to new therapies and 2) molecular changes may have prognostic value.

Despite large changes in incidence and management of prostate cancer in the past decades, prostate cancer mortality has shown only minor changes. A modest drop in mortality is noted since 1992 in the USA [1]. For metastatic disease the corner stone of treatment remains hormonal therapy, originally described by Huggins and Hodges in 1941 [2]. This gives a response in most cases but after an average of 18 months hormone refractory disease develops. A better understanding of the molecular changes underlying prostate cancer is important to develop new, targeted therapies.

The incidence of prostate cancer has markedly increased since the introduction of the PSA serum test in 1986. Many early cancers develop very slowly, and are clinically not relevant. To date, it is not possible to accurately indicate for which cancers immediate therapy is required. It is estimated that 17 patients have to undergo curative treatment in order to prevent 1 patient from dying of prostate cancer [3]. In a screening population this number is even higher. Accurate prognostic factors are urgently needed.

GENETIC TECHNIQUES

The development of molecular techniques in the past decades have lead to an enormous increase in scale. A single experiment can provide information on thousands of genes. Changes in cancer cells can concern DNA (genomics), RNA or proteins (proteomics). Interpretation of large scale experiments can be difficult and often a focussed conventional analysis is necessary to confirm results from large scale experiments. Especially in clinical samples (tumours directly derived from patients) interpretation of results may be hampered by tumour heterogeneity and interfering normal (stromal) cells. It is important to study clinical samples because cell lines and other tumour models may have acquired genetic changes during their development.

In this thesis the focus is on genetic changes in clinical prostate cancer. DNA is a very stable molecule and it can be studied in tissues that have been paraffin embedded and stored for years. An advantage is that follow-up is available from these tumours. Furthermore, DNA changes are the hallmark of cancer cells. While epigenetic changes may be reversible in certain conditions, DNA abnormalities will be passed on to daughter cells. There are many indications that DNA changes are a crucial step towards the development of cancer. The following techniques can be used to document whole genome DNA changes in cancer cells.

CLASSICAL CYTOGENETICS

In classical cytogenetics (karyotyping) tumour cells are cultured and by adding an agent blocking cell division metaphase cells with condensed chromosomes accumulate. The banding pattern of DNA can be visualized by staining. Large deletions, amplifications and translocations can be made visible (Figure 1). The application of this technique to prostate cancer is hampered by the fact that these cancer cells grow very slowly and are usually outgrown by fibroblasts.

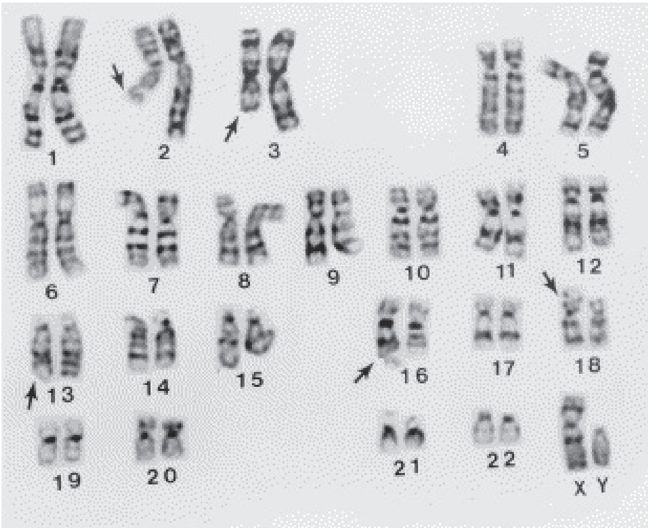

Figure 1. Leukocyte karyogram of a prostate cancer patient. Arrows indicate deletions and translocations [4] (for details see chapter 8)

SPECTRAL KARYOTYPING (SKY)

SKY was developed to unequivocally characterize chromosomes by colour. It is based on labelling of painting probes for each chromosome with different combinations of fluorochromes. These probes are hybridized to a metaphase. Computer analysis combines the data and displays each chromosome in a pseudo-colour (Figure 2). This method is especially suitable for the detection of translocations and for the identification of marker chromosomes. As in karyotyping, tumour cells have to be cultured, which limits the use for prostate cancer.

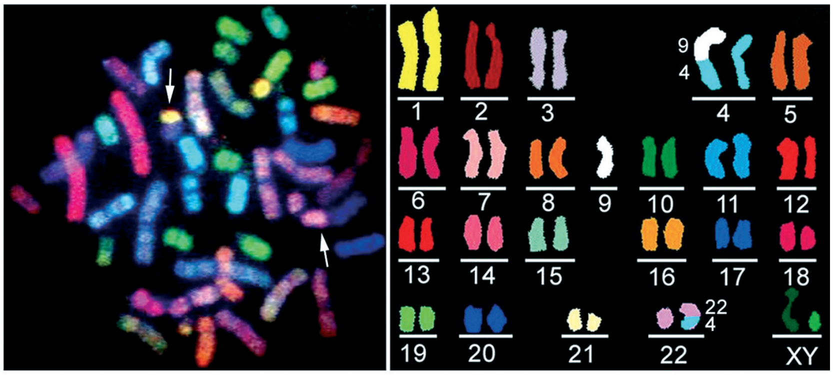

Figure 2. SKY of cultured tumour cells (variant of chronic myeloid leukaemia). Left: metaphase in true colours, arrows show translocations; right: karyogram in pseudo colours. (Courtesy of Jeremy Squire, Toronto, Canada)

ALLELOTYPE ANALYSIS

Allelotype analysis is based on PCR amplification of polymorphic microsatellite repeats. These mono-, di- and tri-nucleotide repeats are present throughout the genome and have an unknown function. The length of the repeat is highly variable. Amplification of DNA with primers spanning a repeat will result in accumulation of molecules derived from both DNA copies in the reaction mix. This product can be separated by electrophoresis and made visible by autoradiography or fluorescence. Normal DNA will give two signals of approximately the same intensity. Copy number changes, e.g. loss of one chromosome fragment, will lead to a loss of one signal (LOH), (Figure 3). In prostate cancer losses are more common than gains, but gains also occur. In clinical samples allelotype analysis shows a disbalance between alleles but it cannot always discriminate between a true loss and a gain. Tumour heterogeneity and interfering normal cells (stroma, vessels, leucocytes) will often cause a weak signal in stead of complete absence of the allele.

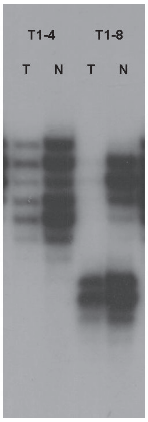

Figure 3. Autoradiography image of amplified microsatellite marker D10S1765 in prostate tumours T1-4 and T1-8. Retention of both alleles in T1-4, loss of the upper allele in the tumour lane of T1-8 (LOH). T: tumour; N: normal (blood) (see for details chapter 5).

Besides application in analysis of allelic imbalance, microsatellite analysis can also detect genetic instability, which can be visualized by variable microsatellite lengths.

SINGLE NUCLEOTIDE POLYMORPHISM (SNP)

Single nucleotide polymorphisms (SNPs) are normal genetic variants. They can be identified by PCR or by oligo-hybridization, allowing a high-throughput approach using microarrays (Figure 4).

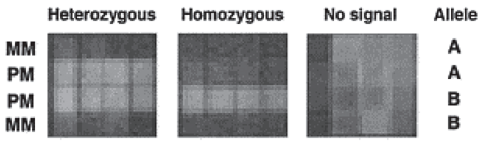

Figure 4. Example of SNP analysis. Experiments carried out in quadruplet (columns), for 2 alleles (A and B). Oligo's are designated mismatch (MM) and perfect match (PM). Example of heterozygous (left; AB) and homozygous (middle; BB) case. Tumour and normal DNA can be compared for heterozygous SNPs to show LOH [5].

FLUORESCENCE IN SITU HYBRIDIZATION (FISH)

FISH is based on hybridization of labelled DNA probes to chromosome spreads, cells in metaphase or interphase or nuclei in histological sections. FISH is especially powerful in the detection of high level amplifications. It can also be used to score loss or homozygous deletions. Tissue sections usually are 4 to 5 µm, which introduces a truncation effect of epithelial nuclei. About half of normal, diploid nuclei will show 2 signals. Approximately 15% will show no signal at all, the remainder will have 1 spot per nucleus. In order to score loss, a large number of cells have to be scored and the frequency distribution has be compared with that of normal tissue. Simultaneous hybridization of a second (centromere) probe with a different colour (dual colour FISH) allows an easy comparison of the number of centromere spots and the number of spots of the investigated sequence (Figure 5). Stromal cells within a tumour can serve as an internal control of the experiment.

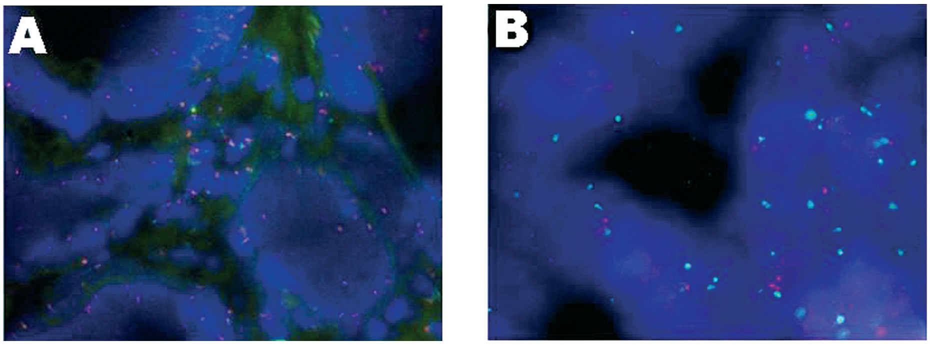

Figure 5: Dual colour FISH of prostatic cancer. Green: chromosome 10 centromere; red: *PTEN*. A: The central (stromal) region shows both green and reds signals. In the tubules only centromere signals are present indicating a homozygous deletion for *PTEN*. B: Both centromere and PTEN signals in another prostate tumour. (For details see chapter 5)

COMPARATIVE GENOMIC HYBRIDIZATION (CGH)

CGH can detect global gains and losses in the chromosomal content of solid tumours. In the CGH assay normal human metaphase chromosomes are competitively hybridized with two differentially labelled (red and green) DNAs, usually tumour and reference (normal) (Figure 6). Fluorescent ratios represent gains and losses of the tumour DNA relative to the reference DNA. DAPI counter stain is used to characterize the chromosomes in the metaphase. CGH can be performed with DNA derived from paraffin embedded tissues. A limitation is that CGH can only detect losses and gains, balanced translocations or inversions will not be detected. Another limitation of CGH is that it cannot detect intratumoural heterogeneity. The resolution of CGH is limited (~5-10 Mbp).

ARRAY-CGH

In array-CGH, the competitive hybridization (tumour versus normal) is performed on a microscopy slide containing many characterized DNA sequences (BACs), spotted in an array. Computerized analysis gives a tumour to normal ratio (expressed as log2 ratio) per BAC. This allows a detailed large scale experiment (high-throughput). In a normal, diploid case the green to red ratio is 1:1 = 1, with a log2 of 0. When one allele is lost, the expected log2 equals -1. In case of a homozygous deletion, the log2 ratio is theoretically -∞. In practice, low background staining and sequences from interspersed normal cells cause a higher value. For the interpretation of the results it is important to take the whole profile into account (Figure 7).

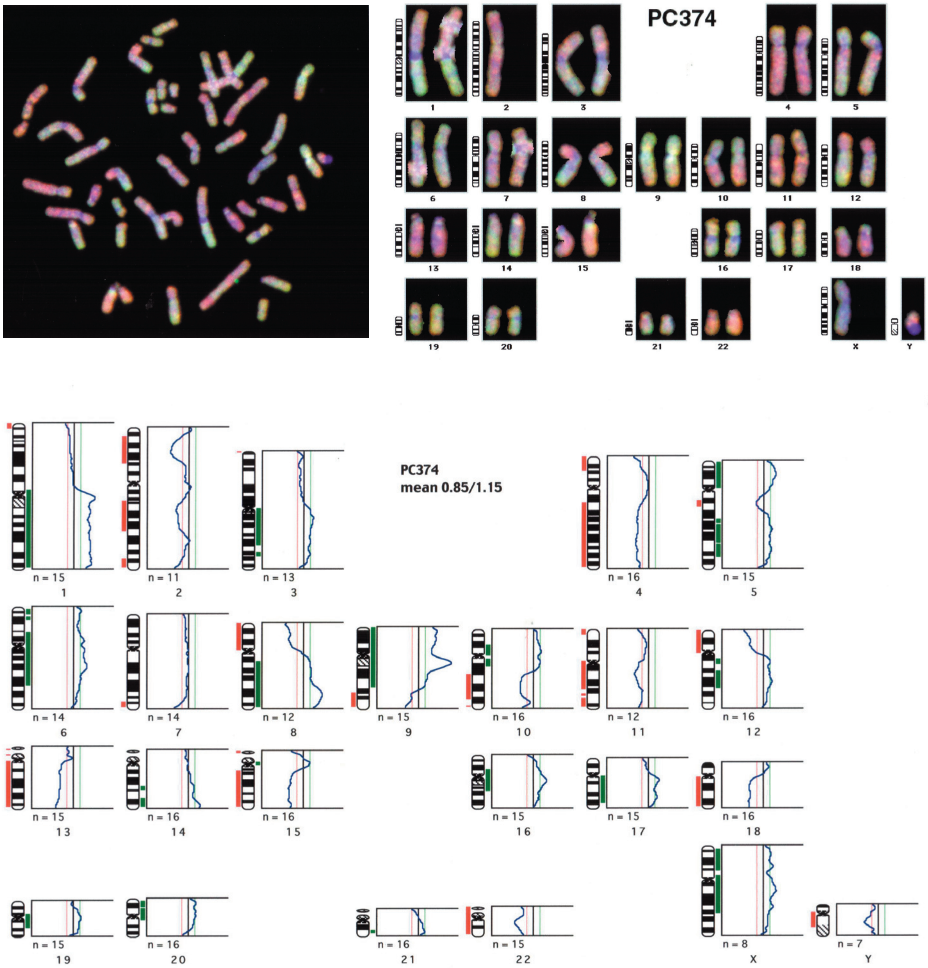

Figure 6. Upper left: normal leukocyte metaphase after hybridization with prostate cancer xenograft PC374 (green) and reference DNA (red). Upper right: karyogram. Bottom: average CGH profile derived from 8 metaphases. A loss is indicated as a red bar left to the chromosome, a gain as a green bar to the right of the chromosome. (For details see chapter 4)

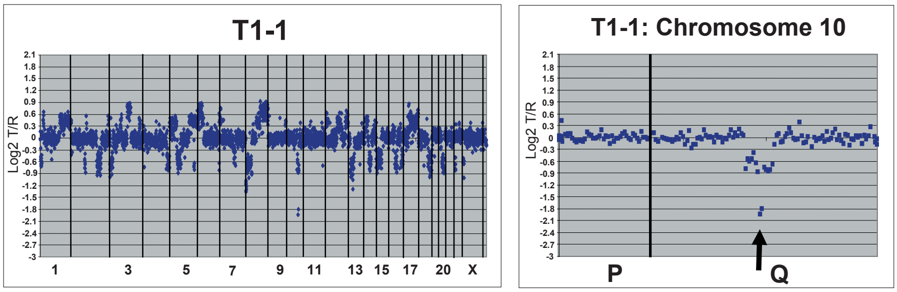

Figure 7: array CGH result of prostatic cancer T1-1. Left: log2 ratio for all BACs; chromosome numbers are represented on X axis. Right: detail for chromosome 10. Note low values indicating homozygous deletion in *PTEN* (arrow), positioned within a region of loss of one copy. For details see chapter 5.

Scope of the thesis

In this thesis molecular analyses are presented that focus on determination of chromosomal regions that appear critical in the development of prostate cancer. My position as clinical research fellow of the Dutch Cancer Society gave me the opportunity to work in Utah where I had access to prostate tumours that came from patients from high risk families (Chapter 3). In Rotterdam the focus was on chromosome 6 changes in prostate cancer (Chapter 4). The choice for chromosome 6 was because its involvement appeared to be frequent [6] and in contrast to chromosome 8 and 10, little attention was paid to this chromosome in the literature. Studies on the role and prognostic value of the tumour suppresser gene *PTEN* were also initiated in this period, and are presented in Chapters 5 and 6.

During the years I have worked on this thesis I became interested in the process of transdifferentiation: the capacity of pluripotent bone marrow-derived cells to change phenotype and become epithelial cells. If a blood cell has the ability to change into an epithelial cell, the process responsible for this can be important to the development of cancer, has been my hypothesis. The efforts to incorporate this topic in my thesis have not been very successful. However, the experimental evidence from Houghton et al. in 2004, who showed the direct link between transdifferentiation and the development of gastric cancer has lead to the contribution on transdifferentiation in Chapter 8 [7].

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Chapter 2

INTRODUCTION TO GENETIC ANALYSES IN CLINICAL PROSTATE CANCER

Based on:
Prognostic factors in
localized prostate cancer
With emphasis on the application of
molecular techniques

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Eur Urol, 2002. 41(4): p. 363-71.

ABSTRACT

Prostate cancer is the most prevalent malignancy in males in the Western world and the second leading cause of male cancer death. PSA based screening and case finding leads to identification of early stage prostate cancer. At present it is often difficult to discriminate between patients that need to be treated with curative intent and patients that can be managed conservatively. Genetic analyses increase our understanding of the molecular background of cancer, which may lead to a better prediction of outcome. Furthermore, knowledge of the molecular pathways involved in tumour initiation and progression may lead to the development of new, targeted therapies.

Based on the classification proposed by the American College of Pathologists and the World Health Organization, we describe molecular factors in prostate cancer. Clinical applicable prognostic factors are stage, grade and serum PSA. Factors that have been shown to be predictive, but are at present not routinely used (for various reasons) are ploidy, histological type and cancer volume in needle biopsies. All other factors (including circulating tumour cells, angiogenesis, growth factors, proliferation rate, apoptosis, nuclear morphometry, neuroendocrine differentiation, loss of chromosomal regions, tumour suppressor genes (*p53, PTEN*) and adhesion molecules) are promising as prognostic factor although currently their use in clinical decisions is not recommended. The role of these factors in prostate cancer growth and their predictive value is discussed.

The rapid developments in molecular techniques allow assessment of structure or function of thousands of genes in a prostate biopsy sample. We expect that in the future molecular characterization of tumour material will become a clinically important tool to predict prognosis in patients with localized prostate cancer.

INTRODUCTION

Epidemiology

Prostate cancer is the most commonly diagnosed cancer in men in the Western world. It is the second leading cause of male cancer deaths [1] [2]. Below the age of 50, the incidence of prostate cancer is very low. Above the age of 55 there is a steep rise with age, to over 900 cases per 100.000 per year at the age of 80 [3, 4]. In the past decade the incidence of prostate cancer has shown marked changes. In the United States, the incidence rate peaked in 1992 at 240 cases per 100.000 per year, and has been declining since [1]. In the Netherlands, the incidence increased from 1989 to 1994 and has remained stable in 1995 and 1996 (at 87.7 European Standardized Rate (ESR) per 100.000 per year) [3]. This is mainly due to the introduction of Prostate Specific Antigen (PSA) serum tests and the increasing awareness of prostate cancer in the population.

The prostate cancer mortality rates have shown only small changes in the past decade. In the USA there was a modest increase until 1992, most likely attributable to the fact that men are living longer. From 1992 to 1995, the age adjusted mortality rate in the United States has shown a slight decline [2, 5]. The Netherlands Cancer Registry showed a constant age adjusted mortality rate at 34 (ESR per 100.000 per year) over the years 1993 to 1996 [3].

Need for new therapeutic targets

Several therapeutic options exist for localized prostate cancer. When regional or distant metastases have developed, no curative therapy is possible. In these patients hormonal therapy is initiated [6]. The goal of hormonal therapy is to withdraw androgens or to block their effect at the androgen receptor level. Hormonal therapy can be given in several ways, e.g. by surgical castration, or by administering LHRH analogs or anti androgens. The majority of patients responds well to this therapy, however the response is always temporarily. After an average period of 18 to 24 months progression occurs and only palliative procedures remain [7]. A better understanding of the molecular background of prostate cancer may lead to the development of new, targeted therapies.

Need for prognostic factors

The natural history of prostate cancer is not well known. Its course is variable and difficult to predict. In autopsy studies of asymptomatic men small foci of prostate cancer have been found in up to one third of men in their fifth decade [8, 9]. This figure increased up to 100% in men over 90 in one series [10]. These cases are clinically insignificant. In 1993 Coffey estimated that 1 out of 4 prostate cancers becomes clinically apparent and that 1 out of 3 of these apparent cancers will lead to death [11]. In Scandinavian countries, prostate cancer is predominantly managed conservatively. This means that in these patients no attempt is done to reach a cure. In case of tumour progression patients are treated by hormonal therapy, sometimes in combination with additional palliative procedures. Long-time survival has been reported in selected early stage prostate cancer patients after conservative management (disease specific survival over 80% after 10 to 15 years) [12-16]. Because of a selection-bias (patients with favourable characteristics are more likely to be managed conservatively), these series underestimate prostate cancer death risk in unselected early stage cases. A different approach was used by Aus et al. who traced all conservatively managed patients who died with prostate cancer in a certain period and concluded that death was attributable to prostate cancer in 50 % of originally M0 cases [17].

Two conclusions can be drawn from these studies: 1) a subset of patients will die from their cancer if managed conservatively and 2) it takes many years before a localized cancer leads to death. Many early cancers will never progress, which implies that at present there must be considerable overtreatment. All treatment options carry some complication risks, which will have to be endured for many years in these patients. Therefore it is important that prognostic factors are established that can identify the subset of patients with tumours that will cause clinical problems if managed conservatively.

Treatment options in localized disease

Established treatment options in localized disease consist of radical prostatectomy, radiotherapy (external beam or brachytherapy) or watchful waiting. Radical prostatectomy gives a good tumour control but there are no randomized studies to confirm that its outcome is superior to other treatment alternatives. Radical prostatectomy and radiotherapy carry the risk of erectile dysfunction and incontinence. In deciding on a treatment modality, three factors must be taken into account: 1) the expected biological behaviour of the tumour, 2) the life expectancy of the patient and 3) quantity versus quality of life trade-offs. All three items are difficult to assess. This review will focus on the difficulties in prediction of tumour behaviour. It will be restricted to clinically localized prostate cancer for the sake of comprehensibility, and because in these patients treatment decisions have to be made which may be based on prognostic factors.

Prognostic factors

A prognostic factor is 'a qualitative or quantitative alteration or deviation from normal of a molecule, substance or process that can be detected by some type of assay' [18]. Factors include genes, RNAs, proteins, carbohydrates, lipids or processes. They may be assessed in body fluids (blood, urine, semen), tumour samples (biopsy), by imaging (ultrasound, computerized tomography (CT), magnetic resonance imaging (MRI)) or by other procedures (e.g. magnetic resonance spectroscopy). To classify factors the College of American Pathologists (CAP) defined 3 categories, based on clinical applicability. Table 1 shows the classifications proposed by the 1999 CAP Conference on Solid Tumour Prognostic Factors and the 1999 World Health Organization (WHO) Second International Consultation on Prostate Cancer [19, 20].

Category I: **Factors that have been proven to be prognostic or predictive based on evidence from multiple published trials and are recommended for routine reporting**	
1999 CAP	*1999 WHO*
TNM stage	TNM stage
Histological grade (Gleason)	Histological grade (Gleason, WHO)
Surgical margin status	Surgical margin status
Perioperative PSA	Perioperative PSA
	Pathological effects of treatment
	Location of cancer within prostate
Category II: **Factors that show promise as predictive factors based on evidence from multiple published studies but that require further evaluation before recommendation or are recommended despite incomplete data as diagnostic or prognostic markers.**	
1999 CAP and 1999 WHO classification identical:	
DNA ploidy	
Histological type	
Cancer volume in biopsy	
Cancer volume in RP	
Category III: **Factors that have some scientific evidence to support their adoption as diagnostic or prognostic agents but are not currently recommended; also, factors of uncertain significance.**	
1999 CAP and 1999 WHO classification identical:	
Prostate-specific membrane antigen	
Other serum tests (RT-PCR, PSM, hK2, IGF)	
Perineural invasion	
Vascular/lymphatic invasion	
Microvessel density	
Stromal factors (TGF-β, integrins)	
Proliferation markers and apoptosis	
Nuclear morphometry and karyometric analysis	
Androgen receptor	
Neuroendocrine markers	
Genetic markers	
All other factors	

Table 1: Classification of prognostic factors by CAP and WHO

Many potential factors have been studied and have been reported to show a correlation with the conventional prognostic factors grade and stage. This does not imply that they have independent prognostic value. Tumours acquire more genetic and phenotypic abnormalities during their development. It is therefore not surprising to find more abnormalities in more progressed cases. A multivariate analysis can assess the independent prognostic value of a factor.

The value of factors assessed on tissue (needle biopsies) is hampered by the multifocal and heterogeneous nature of prostate cancer. Miller and Cygan found the number of tumour foci per radical prostatectomy specimen ranging from 1 (43.7%) to 6 (0.7%) [21]. The average number of separate tumour foci in an other series of 160 radical prostatectomy specimens was 2.3 [22]. These foci usually show variable growth patterns and heterogeneity is reported in many molecular studies [22-25]. Some factors may have prognostic value when assessed in radical prostatectomy specimens, but not when assessed in corresponding needle biopsies due to the sampling error [26]. Currently applicable factors and a selection of interesting additional factors will be discussed based on the classification given above.

I FACTORS THAT HAVE BEEN PROVEN TO BE PROGNOSTIC OR PREDICTIVE BASED ON EVIDENCE FROM MULTIPLE PUBLISHED TRIALS AND ARE RECOMMENDED FOR ROUTINE REPORTING

Stage

The clinical stage of prostate cancer is assessed by digital rectal examination, transrectal ultrasound (TRUS), CT, MRI and bone scintigraphy. The TNM system (tumour, nodes, metastasis) is the most widely used staging system [27].

Clinical TNM stage is inaccurate. 20-49% of patients with a clinical T1c cancer (not palpable and not visible by imaging) appear to have extracapsular disease (pT3) [28-30]. Clinical T stage is therefore of limited value as prognostic factor. Pathological T stage performs much better, but can only be assessed after radical prostatectomy. Absence of pathological staging information following radiotherapy hampers comparison of outcome of this treatment with surgical techniques.

Grade

The most widely used grading system is the Gleason sum score (GSS) [31]. This score is determined by assigning a number (1 to 5) to the histological patterns of the tumour. The numbers of the 2 most common patterns are added to form the GSS. Many reports have shown the strong and independent prognostic value of the GSS [32-35]. Several factors limit the use of this system in an individual patient:

1) It is subjective; there is intra- and inter-observer variability [36, 37].
2) The sampling error in biopsies can produce considerable differences in the GSS compared to the final score on the radical prostatectomy specimen [38, 39].
3) The prognostic value is more pronounced when the GSS is low (2-4) or high (8-10); the majority of patients have intermediate scores and within this large group the GSS does not differentiate very well.

In Europe also other grading system are used, (e.g. M.D. Anderson, WHO, Mostofi). These systems also correlate with outcome, but there application in an individual patient is limited by the problems listed above [36]. There is one exception: poorly differentiated cancer identified in needle biopsies almost invariably predicts a poor outcome and this information should play a role in the choice of treatment [19]. Additional histopathological factors are perineural invasion and vascular or lymphatic invasion. Some authors report these factors to have some predictive value [40, 41], others failed to show prognostic significance in multivariate analysis [42, 43].

Serum PSA

Pretreatment serum PSA correlates with pathological stage. Partin et al. studied 4133 men who underwent radical prostatectomy. Preoperative serum PSA, clinical stage and biopsy GSS were found to predict pathological stage. These 'Partin tables' are widely used to instruct patients on there chances of being cured by radical prostatectomy [32, 44]. High serum PSA can be used to identify a subset of patients that will probably not benefit from radical treatment. Elevated PSA levels can also be caused by several benign disorders, e.g. benign prostatic hyperplasia or prostatitis. Low values cannot be used to select patients that do not need curative treatment because of the decreasing production of PSA in higher grade lesions [45].

PSA density (PSA level divided by prostatic volume), PSA velocity (rate of PSA change) and PSA free to total ratio (free PSA divided by free PSA + α1-chymotrypsin bound PSA) are methods applied to enhance the use of PSA for tumour detection. These methods are also investigated for prognostic value [28, 46-48]. A rise in serum PSA level of more than 2.0 ng/ml in the year preceding a radical prostatectomy appeared to be associated with an increased risk of prostate cancer death [49].

II FACTORS THAT SHOW PROMISE AS PREDICTIVE FACTORS BASED ON EVIDENCE FROM MULTIPLE PUBLISHED STUDIES BUT THAT REQUIRE FURTHER EVALUATION BEFORE RECOMMENDATION OR ARE RECOMMENDED DESPITE INCOMPLETE DATA AS DIAGNOSTIC OR PROGNOSTIC MARKERS.

Ploidy

Normal cells have a diploid DNA content, which can be determined by static or flow cytometry. Prostate tumours of low grade and stage are usually also diploid, whereas tumours of higher grade and stage are more often aneuploid. Since 1966 this topic has received much attention [50-52]. The majority of papers reported a decrease in survival in patients with aneuploid tumours. In several reports ploidy was found to be an independent prognostic factor in multivariate analysis. The issue of sampling error in biopsies has been addressed in studies that reported a high concordance between ploidy of biopsies and surgical specimens [53]. In the individual patient, the application of this information in deciding on a treatment modality is still limited [19, 54, 55].

Histological type

Over 95% of prostatic tumours are adenocarcinomas and studies on prognostic factors in general apply to this category. Other histological types include transitional cell carcinoma, squamous cell carcinoma, undifferentiated carcinoma, rhabdomyosarcoma and leiomyosarcoma. Neuroendocrine cells can be identified in benign and malignant prostatic tissues. They are usually recognized by their immunoreactivity for chromogranin A and neuron specific enolase. Neuroendocrine differentiation in prostate tumours refers to 3 different entities: 1) small cell prostate cancer (SCPC), 2) prostatic carcinoid and 3) adenocarcinoma with scattered neuroendocrine cells [56]. SCPC accounts for <5% of prostate tumours and is reported to follow an aggressive course [57]. Prostatic carcinoid is very rare. Adenocarcinomas are reported to contain scattered NE cells in 10-100%. Also lymph node and bone metastases are reported to contain these cells [58]. Neuroendocrine cells differentiate from the epithelial tumour cells. Reports on possible prognostic significance of these cells are not conclusive [59-61].

Cancer volume in needle biopsies

Prostate cancer volume is difficult to assess because macroscopically the tumour is hard to recognize and often multifocal. In radical prostatectomy specimens volume can be measured and is reported to correlate with grade, stage and outcome [62, 63]. Peller e.a. counted the number of positive sextant biopsies and found a correlation with volume, grade and stage [64]. Irwin and Trapasso tried to identify insignificant cancers by evaluation of preoperative parameters. The definition of insignificant cancer is controversial. These authors considered a cancer insignificant when the total volume was < 0.5 ml with well differentiated histology (GSS 1+1; 1+2; 2+1; 2+2). They concluded that the combination of a cancer of <= 3 mm in sextant biopsies and a Gleason grade (not GSS) <= 2 and a PSA density (PSAD) of <= 0.1 pointed to 'a substantial risk (82%) of having a probably insignificant cancer' [65]. The difficulty to apply information from prognostic factors in individual patients is well illustrated by this description. Also others tried to predict tumour volume by investigating biopsies [66-71]. The number of positive biopsies and the percent of biopsies positive for cancer is reported to correlate with tumour volume and other pathological variables [66, 72, 73]. While one small focus of tumour in sextant biopsies does not imply the presence of a limited cancer [67, 70], extensive involvement of multiple biopsies points towards extensive disease and this can contribute to treatment decisions[72, 73].

III FACTORS THAT HAVE SOME SCIENTIFIC EVIDENCE TO SUPPORT THEIR ADOPTION AS DIAGNOSTIC OR PROGNOSTIC AGENTS BUT ARE NOT CURRENTLY RECOMMENDED; ALSO, FACTORS OF UNCERTAIN SIGNIFICANCE.

Chromosomal regions involved in prostate cancer

● *Classical cytogenetics*

Chromosome aberrations found by classical cytogenetics include structural changes in chromosome 1, 2, 7, 3p, 6p, 8p, 10q, 13q, 15q and 16q. Whole chromosome gain of 7, 14, 20 and 22 and loss of 1, 2, 4, 5 and Y are reported. These changes seem independent of stage and grade [74-78].

● *CGH*

CGH has the advantage that it can be performed with tumour DNA, it is not necessary to culture cells, as is the case for karyotyping. Culturing may lead to selection of certain abnormalities and therefore, CGH results may differ from cytogenetic analysis [79]. Comparative genomic hybridization (CGH) of primary prostate cancers revealed frequent loss of the chromosomal regions 6q, 8p, 13q, and 16q and gain of 7 and 8q [80, 81]. Prostatic intraepithelial neoplasia (PIN) is considered a prostate cancer precursor lesion. PIN lesions also contain some of these chromosomal changes: loss of 4q, 5q, 8p, 13q and 18q and gain of 7, 8q and Xq [82]. CGH applied on biopsy material revealed loss at 8p,13q, and 16q and gain at 8q. Loss at 16q appeared to be correlated to grade and stage [83]. Gain at 8q was associated with stage and progression [84]. Progressors after radical prostatectomy appeared to have more genetic abnormalities compared to non-progressors, especially gain of 8q24-qter [85].

● *Array-CGH*

In array-CGH detailed genomic information can be obtained in a single hybridization experiment. In radical prostatectomy specimens with Gleason 3 and 4 pattern, frequent deletions were seen on 8p (60%), 6q (30%), 1p (20%), 2q (20%), proximal 8q (20%), 10q (20%), 13q (20%), 16q (20%), and 18q (20%). Gain was frequently detected on distal 13q (20%) [86]. In a cohort of 64 radical prostatectomy patients, half of whom recurred postoperatively, loss at 8p23.2 was associated with advanced stage disease, and gain at 11q13.1 was found to be predictive of postoperative recurrence, independent of stage and grade [87].

● *Allelotype analysis*

Microsatellite studies can demonstrate loss of heterozygosity (LOH) in a precise chromosomal location. LOH is reported in 6q13-21, 7q31.1, 8p12-p22, 10p, 10q23-q25, 13q14, 16q, 17q21 and 18q [88-94]. Loss at 7q31.1 was present in 30% of primary prostate cancer which correlated with tumour grade and lymph node metastasis [95]. Loss at proximal 6q appeared not to be related to established prognostic factors [90, 96]. In 48 radical prostatectomy specimens, the rate of LOH was between 40% and 47% for chromosomes 7, 8, 12 and 16. Loss at 12p13 and 16q23.2 were correlated to PSA and Gleason score. Perineural invasion was significantly associated with LOH on 8p22 [97]. Von Knobloch et al., also investigating radical prostatectomy specimens, found LOH at 8p, 9p, 13q, and 17p to be associated with advanced tumour stage, while the combination of LOH at 8p and 13q was strongly associated with advanced tumour stage [98].

● *SNP analysis*

Oligonucleotide arrays can detect single nucleotide polymorphisms and can be used to generate genome-wide LOH maps. Application to 50 radical prostatectomy samples revealed distinct patterns of loss, suggesting distinct genetic subsets. The most frequently involved regions were 1p33, 3q27, 8p21, 10q23, 14q12, 16q23 and 17p13 [99]. Another study in 11 prostate cancer cases pointed to loss at 3p12, 5q23, 8p21, 10q22, 13q14 and 16q23 [100].

● *Hereditary prostate cancer*

A positive family history is among the strongest epidemiological risk factors for prostate cancer. Carter et al. estimated that approximately 10% of all prostate cancers are hereditary with an autosomal dominant pattern of inheritance [101]. Others found a pattern consistent with X-linked segregation [102]. Current experimental evidence better supports the hypothesis that familial risk may be due to inheritance of multiple moderate-risk genetic variants. Linkage analysis suggests several regions to be linked to hereditary prostate cancer, e.g. 1q24 [103], Xq27-28 [104] and 1q42-43 [105]. In a combined analysis of 426 families from 4 study populations, additional regions with evidence for linkage were identified, namely: 17q22, 2q32, 15q11, Xq25 and 6q22 [106].

Three candidate susceptibility genes have been described, namely ELAC2 (chromosome 17p11) [107], 2′-5′-oligoadenylate-dependent ribonuclease L (RNASEL) (chromosome 1q24-25) [108], and Macrophage Scavenger Receptor 1 (MSR1) (chromosome 8p22-23) [109]. ELAC2 was mutated in 2 families with high risk prostate cancer [107]. All three susceptibility genes appear to play a minor role in sporadic prostate cancer [110].

BRCA2, one of the breast cancer susceptibility genes is also associated with increased prostate cancer risk (odds ratio, 4.78) whereas the risk in BRCA1 mutation carriers is not significantly increased [111].

Gene expression studies

cDNA microarray expression studies have shown two genes that appear to be consistently increased in prostate cancer; hepsin, located at 19q11-13.2 encoding a transmembrane serine protease, normally expressed at high levels in liver and other tissues and AMACR (α-Methylacyl-CoA racemace), a mitochondrial and peroxisomal enzyme [112-116]. A third molecule with over expression in prostate cancer is the polycomb group protein enhancer of zeste homolog 2 (EZH2) [117]. The exact role of these molecules in prostate cancer is not clear.

Classical tumour suppressor genes

• *p53 and WAF1/CIP1*

The *p53* tumour suppressor gene has a function in cell cycle control and regulates the WAF1/CIP1 gene. The proteins of these genes are referred to as Tp53 and p21 respectively. The *p53* gene is mapped on chromosome 17p. A *p53* germ-line mutation causes the Li-Fraumeni syndrome, characterized by the development of several malignancies during infancy [118].

Abnormalities in the *p53* gene can be demonstrated by single strand conformational polymorphism (SSCP) and sequencing and/or immunohistochemistry. The truncated Tp53 protein has a longer half life than the wild-type and accumulates in the nucleus. Therefore, mutations in the gene lead to an increased nuclear staining of the protein. Some reports mention an excellent correlation between immunohistochemical results and DNA analysis [119-121]. Others warn for false positive results in immunohistochemical staining [122].

In prostate cancer varying frequencies of *p53* mutations have been reported. In general, the frequency of *p53* mutations in primary prostate cancers is reported to be low (0-14%) [23, 119-121, 123-125]. Mutations in *p53* were shown to vary within one tumour [23]. Foster et al reported a considerable higher proportion of immunohistochemical aberrant Tp53 expression in primary cancers, but also noted positive staining in benign prostatic tissues [126]. Also others found 65 to 80% of clinically localized cancers to be immunohistochemically positive for Tp53 [127, 128].

Studies on the prognostic significance of *p53* mutations give conflicting results. Several studies found prognostic value of abnormal Tp53 expression [123, 127-129]. The data in the study by Bauer et al. were obtained from radical prostatectomy specimens [127]. Corresponding needle biopsies analyzed for Tp53 expression did not show prognostic significance [130]. Others found no prognostic significance in localized cancer [131]. Taken together, assessment of prognostic value of Tp53 expression is hampered by variability in techniques and criteria for positivity, heterogeneous expression within a tumour and limited number of prospective studies performed.

WAF1/CIP1 is activated by p53. The gene product, p21, inhibits proliferation and directs the cell into apoptosis. Mutations have been reported in 17% of primary prostate cancers [132]. p21 expression is increased in cancers compared to benign prostatic tissue [133, 134]. Several studies found increased p21 expression to be associated with other prognostic factors (stage, grade) [135, 136] or survival [137-139]. In one study immunohistochemical p21 over-expression was an independent predictor of PSA failure after radical prostatectomy [140].

● *PTEN and Akt*

PTEN/MMAC1 (PTEN; phosphatase and tensin homologue deleted from chromosome 10) was identified in 1997 and mapped to 10q23.3 [141]. Germ line mutations in the PTEN gene cause Cowden disease, characterized by hamartomas and a predisposition to various tumours. The biological target of PTEN appeared to be inositol phospholipids. PTEN dephosphorylates these lipids. Absence of PTEN causes an accumulation of phosphorylated lipids (PIP3) which in turn cause increased levels of phosphorylated Akt. Phosphorylated Akt plays a central role in various cellular processes including cell cycle regulation and cell survival [142]. PTEN is frequently inactivated in prostate cancer [143-146]. Fenic et al. studied PTEN mRNA and protein expression in PIN, prostate tumours and metastasis [147]. In this study, total or partial loss of PTEN protein occurred with tumour progression but this association was not statistically significant.

Loss of PTEN expression (determined by polyclonal antiserum) was assessed in 109 paraffin embedded primary prostate cancers. Loss of PTEN was shown to correlate with Gleason score and advanced stage [146]. In another series of 104 radical prostatectomies PTEN expression was also assessed by immunohistochemistry. Combined loss of PTEN and p27 was an independent predictor of biochemical progression [145].

Increased levels of phospho-Akt appeared to be associated with high grade prostate cancer [148]. In 53 radical prostatectomy cases phospho-Akt was an excellent marker of biochemical progression [149].

The downstream target of phospho-Akt, mTOR (mammalian target of rapamycin) can be inhibited by rapamycin (sirolimus). This drug and some derivatives are being tested for activity in prostate cancer.

● *p27(Kip1)*

The p27(Kip1) (p27) encoding gene is located at 12p13.2. p27(kip1) is a cyclin-dependent kinase inhibitor with a role in cell cycle arrest and apoptosis [150]. p27(Kip1) is thought to have a downstream connection to the PTEN phospho-Akt pathway. However, this relationship is not clear [147]. Decreased expression in prostate cancer is reported to correlate with grade, stage and prognosis [151-156]. Expression of p27 in needle biopsies is reported to correlate with the expression level in radical prostatectomy specimens [157]. Several studies found low p27 expression to be an independent predictor of biochemical recurrence after radical prostatectomy [154, 156, 158-160].

● *Rb*

The *Rb* gene (responsible for hereditary retino blastoma) has drawn attention because it is located on 13q14.1 which is frequently deleted in prostate cancer. In a mouse model a conditional somatic deletion of a single *Rb* allele in the epithelial cells of the prostate causes focal hyperplasia, resembling early stage prostate cancer [161]. Bookstein found abnormal Rb expression in 2 out of 7 advanced cancers [162]. In a series of 26 prostate cancers Ittmann et al. found LOH in 35% and decreased or absent Rb protein was only present in cases with loss [163]. Vesalainen et al., however, in a larger study (118 cases), found no correlation between LOH in the Rb region and absence of the Rb protein suggesting the presence of another tumour suppressor gene in this region [164].

● *INK4/MTS1*

The *INK4/MTS1* gene (p16) is a regulator of *Rb* and maps to 9p21. In prostate cancer mutations are rare. Tamimi et al. found mutations in 2 out of 4 prostate cancer cell lines and 20 primary tumours [165]. In 104 radical prostatectomy cases Halvorsen et al. quantitated the levels of the p16 protein by immunofluorescence flow cytometry. Surprisingly, they report an elevated p16 to be an independent predictor of biochemical failure [166]. Also Jarrard et al. report paradoxically over-expressed p16 to correlate with a high grade and treatment failure [167]. In contrast, Chakravarti et al. reported that in 67 cases of locally advanced prostate cancer treated by radiotherapy loss of immunohistochemical p16 expression was significantly associated with adverse clinical outcome [168].

● *MXI1*

MXI1 is located on 10q24-25. The protein product negatively regulates the oncogene myc. Mutations were shown in primary tumours with concomitant 10q24-25 deletions [169, 170]. Hermans et al. failed to find genetic inactivation of the MXI1 gene in a panel of 4 cell lines and 11 xenografts [171].

Oncogenes

● *Apoptosis; bcl-2*

Programmed cell death (apoptosis) is essential in normal development and can occur in prostate cancer cells following hormonal treatment. In prostate cancer oncogenes appear to play a minor role, although . The apoptosis inhibitor bcl-2 and the EGFR related oncoprotein C-erbB-2 (HER-2/neu) are two examples. The bcl-2 oncoprotein inhibits apoptosis [172, 173]. In primary prostate cancer, overexpression has been reported in 32-41% [174, 175]. Elevated Bcl-2 expression is reported to correlate with tumour progression [176, 177].

In radical prostatectomy series bcl-2 expression was an independent predictor of PSA recurrence [127, 178]. 47/175 patients had bcl-2 over-expression. Of these men, 67% recurred at 5 years, compared to 30,5% in bcl-2 negative patients. Bcl-2 expression in needle biopsies from this same cohort of patients was not predictive of recurrence [130]. Also Oxley et al. reported bcl-2 in needle biopsies to have no independent prognostic value in predicting disease recurrence after radical prostatectomy [179]. Huang et al. found pre-treatment bcl-2 expression in biopsies to be predictive of recurrence in patients treated by radiotherapy [180].

● *C-erbB-2 (HER-2/neu) and other growth factors*

Normal prostate epithelial cells exhibit dependence on epithelial and stromal cell-derived growth factors. Epidermal growth factor (EGF) and transforming growth factor-α (TGF-α) both activate the same receptor (EGFR) which leads to proliferation of normal epithelial cells. Transforming growth factor-β (TGF-β) has an inhibitory effect on normal epithelial cells in vitro. In contrast, in vivo TGF-β enhances cancer growth and metastasis [181].

C-erbB-2 (HER-2/neu) is an oncoprotein related to EGFR, with prognostic value in breast and ovarian cancer. Sadasivan et al. detected this protein in prostate cancer but not in benign prostatic tissues [182]. Ross et al. found its presence to be associated with high tumour grade and stage [183]. In 70 patients with metastatic disease survival was 33 months in patients with C-erbB-2 over-expression in the primary tumours, compared to 54 months in C-erbB-2 negative tumours [184]. However, C-erbB-2 appeared not to be an independent prognostic factor for the prediction of metastatic disease [183] or biochemical failure after radical prostatectomy [185].

EGFR over-expression was reported to correlate with progression free, cancer specific and overall survival in 147 prostatic cancers, but had no independent prognostic value in multivariate analysis [186]. In another study, EGFR expression was an independent predictor of disease recurrence in radical prostatectomy patients [185].

Loss of expression of TGF-β receptor R1 correlated with tumour stage and survival [187]. TGF-β R1 was a independent predictor of biochemical progression after radical prostatectomy [188]. TGF-β1 levels in blood were associated with advanced disease and metastasis [188, 189].

Therapeutic strategies based on blocking the EGF pathway are being developed. The EGFR tyrosine kinase inhibitor gefitinib (Iressa ®) is one example. This oral, well tolerated medicine appeared effective in non-small cell lung cancer [190] but results for prostate cancer have been disappointing [191]. Also recombinant antibodies targeting ErbB-1/HER-1/EGFR and ErbB-2/HER-2/neu are being tested for anti prostate cancer activity.

● *RAS and c-Myc*

There are 3 *RAS* oncogenes (*HRAS, KRAS* and *NRAS*), human DNA sequences homologous to cloned DNA fragments containing the oncogenic nucleic acid sequences of a type C mammalian retrovirus. The viral transforming genes are collectively called v-onc and their normal cellular counterparts are collectively called c-onc. Studies on the *RAS* oncogenes report variable frequency of mutations in primary prostate cancers: 2 out of 29 (HRAS) [192], 1 out of 22 (*HRAS*) [193]), 3 out of 9 (*HRAS and KRAS*) [24].

The *c-Myc* oncogene is located on chromosome 8q24, a region that shows frequent gain in prostate cancer. Sato et al. reported amplification of *c-myc* in high grade advanced cancers to be predictive of outcome [194].

Factors associated with proliferation

Tumour size is determined by cell proliferation and cell death. Several markers are applied to measure proliferation and apoptosis (programmed cell death). Methods to measure proliferative activity include: counting of mitotic figures, determination of the S-phase fraction by flowcytometry and labelling of DNA by incorporation of tritiated thymidine or bromodeoxyuridine (BrdU). These methods have now largely been replaced by applying antibodies to proliferation related antigens: Ki-67, MIB-1 (both directed to the same antigen) and proliferating cell nuclear antigen (PCNA) [195]. Ki-67 is a nuclear antigen present throughout the cell cycle, but not present at rest (G0 or early G1 phase [196].

The proliferation rate of prostate cancer in general is low. Several studies found an increased proliferation rate (assessed by Ki67 immunoreactivity) in cancer compared to benign prostatic hyperplasia [197-199]. A correlation of proliferative activity with grade and stage was reported for PCNA [131, 200-202] and Ki-67/MIB-1 [203, 204]. Others did not find this correlation for PCNA [200] and Ki67/MIB-1 [198, 199, 205, 206]. Several studies report the Ki-67/MIB-1 index to be an independent predictor of progression after radical prostatectomy [203, 206, 207]. Coetzee et al. assessed proliferative index by Ki67 (on frozen tissue) and MIB1 (on paraffin embedded tissue) in 244 radical prostatectomy specimens. A high proliferative index added little above GSS, pathological stage and ploidy [208]. Moul et al. reported Ki-67 to have no independent prognostic value in 162 radical prostatectomy cases [209].

Angiogenesis

Microvessel density can be studied by applying antibodies to factor VIII, CD31 or CD34 to prostatic tissues. Microvessel density was higher in tumours compared to adjacent normal tissue [210, 211]. Prognostic value has been reported after radiotherapy [212] and radical prostatectomy [213-215]. Silberman et al. found it to be an independent predictor of progression after radical prostatectomy in intermediate grade cancers [214]. Bostwick et al. studied needle biopsies from 186 patients who subsequently underwent radical prostatectomy. Microvessel density improved prediction of extraprostatic disease when combined with Gleason score and serum PSA [215]. Rubin et al. concluded that microvessel density (assessed by CD31 antibodies) did not predict biochemical failure after radical prostatectomy [216].

Other molecular markers in the tumour

- *E-cadherine/α-catenin*

E-cadherin is a cell to cell adhesion molecule, mapped to chromosome 16q22.1. The protein is located at the cell membrane and forms a complex with the cytoplasmatic catenins. An E-cadherin germ line mutation is the cause of hereditary gastric cancer [217]. In normal prostate, membranous staining of E-cadherin in the epithelial cells can be demonstrated. In tumours this staining pattern is often aberrant or absent [89, 218-220]. There is frequent loss of 16q22 in prostate cancer, but concomitant mutations of E-cadherin are not often found. Decreased expression appears to be due to hypermethylation of CpG islands in the E-cadherin promotor region [221]. All studies describe an inverse correlation between E-cadherin expression and grade and stage. After radical prostatectomy, a significantly higher biochemical failure rate was reported for patients with abnormal E-cadherin expression [178]. Because E-cadherin depends on the presence of α-catenin to form a functional complex, some studies analyzed these molecules together and report improved prognostic value if they are both taken into account [222, 223].

● *CD44*

CD44, located on 11p13 encodes a transmembrane glycoprotein with a function in intercellular interactions. The gene has 19 exons. Standard CD44 (CD44s) is composed of exons 1-5 and 15-19; several splice variants contain one or more of the exons 6-14. In gastrointestinal epithelia, CD44 is not expressed. In tumours of the gastrointestinal tract, CD44 expression is correlated with adverse outcome [224]. In contrast, Noordzij et al. found expression of CD44s in normal prostatic epithelium [225]. In radical prostatectomy specimens, loss of CD44s expression was an independent predictor of poor prognosis [156, 225]. The concordance in CD44s assessment between the biopsies and matched radical prostatectomy specimens appeared low, implying limited prognostic value of CD44s in needle biopsies [160]. Aaltomaa et al. also assessed CD44s on archival needle biopsies of 209 prostate cancer patients of various stages and concluded that low expression was related to high T classification, metastasis, high Gleason score, DNA aneuploidy, high S-phase fraction, high mitotic index, perineural growth, dense amount of tumour infiltrating lymphocytes and poor outcome [226].

● *PSMA*

Prostate-specific membrane antigen (*PSMA*), mapped to 11p11-p12, is a surface glycoprotein with expression mainly restricted to normal prostatic tissue and prostate cancer. Anti-PSMA antibodies are used for diagnostic and therapeutic targeting strategies. Immunohistochemical staining for PSMA on formalin-fixed paraffin-embedded radical prostatectomy sections revealed that high PSMA expression correlated with tumour grade, pathological stage, aneuploidy, and biochemical recurrence [227].

● *KAI1/CD82*

The *KAI1* gene or CD82, located on 11p11.2 is a transmembrane protein with a putative role in regulation of cell development, activation, growth and motility. Dong et al. reported that this gene suppressed metastasis when introduced into rat AT6.1 prostate cancer cells [228]. Several studies showed an inverse correlation between KAI1 expression and tumour grade and/or stage [229, 230].

● *GSTP1 hypermethylation*

Somatic hypermethylation of CpG island sequences at *GSTP1*, the gene encoding the pi-class glutathione S-transferase, appears to be characteristic of human prostatic carcinogenesis. *GSTP1* CpG island hypermethylation was present in DNA from approximately 90% of prostate cancer cases [231]. *GSTP1* CpG island hypermethylation changes have been detected in DNA from candidate prostate cancer precursor lesions proliferative inflammatory atrophy (PIA) and prostatic intraepithelial neoplasia (PIN). but not in DNA from normal prostate tissues, or from benign prostatic hyperplasia tissues [231].

● *Androgen receptor*

Amplification of the *androgen receptor* (mapped to Xq11-q12) is a frequent aberration (28%) present in hormone independent prostate cancer, and appears to be associated with a better survival [232]. A high androgen receptor content (assessed by immunohistochemistry) was also found to predict a better outcome in patients with advanced cancers [233].

● *Telomerase*

During normal replication chromosomes shorten and this limits the life span of the cell. Telomerase is an enzyme that reverses this chromosome shortening. Telomerase activity was found to be absent in benign prostatic tissues and present in prostate cancer [234, 235]. Athanassiadou et al, studied telomerase expression in cancerous prostate smears, using an in situ hybridization procedure. Telomerase expression was significantly correlated with the Gleason score and PSA serum levels, but not to histopathological staging [236].

● *PSA in tissue*

PSA is expressed by normal prostatic epithelial cells and also by the majority of the malignant prostatic cells. There is a strong inverse correlation between Gleason grade and the PSA content of prostate cancer [237]. The prognostic value of PSA expression in biopsies or TURP specimens was reported to be limited [238, 239] or absent [240].

Factors in blood and urine

● *Circulating prostate cells*

Reverse transcriptase-polymerase chain reaction (RT-PCR) can be used to detect a small number of cells with a specific messenger RNA (mRNA) in a tissue that does not express this particular mRNA. In prostate cancer patients this technique has been applied to detect prostate cells in peripheral blood, bone marrow and lymph nodes. PSA-mRNA, prostate specific membrane antigen (PSMA)-mRNA and human kallikrein 2 mRNA have been used for this purpose, assuming that these are entirely prostate specific. Other tissues are reported to express small quantities of these mRNAs as well, which may lead to false positive results [241, 242].

The first studies published on this issue reported a correlation between a positive RT-PCR result of blood and grade and stage [243, 244]. Manipulation of the prostate (biopsy or radical prostatectomy) was reported to change a negative result into positive [245, 246]. Several studies tried to correlate pre-operative RT-PCR results with biochemical recurrence after radical prostatectomy. Some found such a correlation [247-249] and others did not [250]. Some found superior results using PSA mRNA [251], others claimed optimal results with PSMA mRNA [252, 253]. Shariat et al. found that the pre-operative use of PSA RT-PCR had no value as prognostic factor, however, the test predicted biochemical failure following radical prostatectomy if performed on blood taken after surgery [254]. The conflicting results may in part be explained by the variety of procedures used. Standardization of the molecular test is an important issue. A quantitative RT-PCR procedure appeared to have no additional value compared to previously used method [255].

- *DD3(PCA3)*

DD3(PCA3) is an RNA specific to prostate cancer, that can be detected in urine after digital rectal examination. This RNA does not encode for a protein. Its biological function is unknown. DD3(PCA3) in urine can be used to detect prostate cancer and is reported to be superior to total PSA [256, 257].

CONCLUSION

The factors grade, stage, serum PSA, ploidy and cancer volume in needle biopsies have been studied extensively in clinical settings and can to some extent predict the course of localized prostate cancer in an individual patient. For various reasons these prognostic factors have limited power in discriminating patients with early prostate cancer in those who need a cure and those who do not. The majority of patients in a screening population have a cancer that will not change their life expectancy, which underscores the great need for more precise prognostic factors.

The multifocal and heterogeneous nature of prostate cancer makes it difficult to obtain a representative biopsy sample. Improvements in biopsy procedures will be mandatory in order to make progress on this issue.

Molecular techniques have made it possible to study genetic abnormalities in detail. This contributes to the understanding of the multiple steps of prostate cancer initiation and progression, which may ultimately lead to new therapies for metastatic disease, for which at present no cure is possible. In addition these steps can serve as prognostic factors that provide us with information on the nature of the cancer. More advanced tumours have more genetic abnormalities. This is reflected in numerous reports that show progressive abnormalities of a marker with advancing tumour grade and stage. This does not imply that these markers are prognostically important. Prognostic significance can only be shown in multivariate analysis of prospective trials, were new markers are compared with established prognosticators and outcome. Most trials currently available used radical prostatectomy specimens to investigate a marker. We need markers that can be assessed before the treatment decision is made. For tissue factors this implies that they have to be tested on biopsy materials.

Standardization of techniques is an important issue before promising markers can be used in a clinical setting. For example the wide variability of reported p53 staining in primary prostate cancer (0-80%, see above) must be attributed to differences in technique and scoring. We need markers in patients with localized cancers which means that a very long follow-up is necessary before a correlation with outcome can be established. These aspects make this kind of research difficult. It is not surprising therefore, that molecular factors at present cannot be advocated to contribute in clinical decisions.

It is our conviction that this will change. It is unlikely that a single marker will emerge, which can provide all the information necessary. cDNA microarrays make it possible to obtain information on expression of thousands of genes from one biopsy sample in a single experiment [112, 258]. It is likely that the extensive molecular characterization of tumours will lead to a better understanding of prostate cancer development and the prediction of its biological behaviour. A better understanding of the cellular pathways involved in the initiation and progression of prostate cancer is expected to lead to the development of new, targeted therapies. Finally, a major improvement in prostate cancer therapy will be possible if patients with localized disease can be adequately selected for expectant or less invasive management. The life expectancy of the patient and quality versus quantity of life trade-offs are the complementary issues that still may form a difficult subject for debate.

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Chapter 3

MICRODISSECTION, DOP-PCR AND COMPARATIVE GENOMIC HYBRIDIZATION OF PARAFFIN EMBEDDED FAMILIAL PROSTATE CANCERS

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ABSTRACT

There is a clear genetic component to prostate cancer susceptibility. Regions reported to be linked to prostate cancer include 1q24-25 (HPC-1), 1q42.2-43 and Xq27-28. There is limited genetic information on familial prostate tumours. We used the Utah Population Database to identify familial prostate cancer cases and selected 35 cases from high risk families. Tissue blocks containing discernable tumour were available from 19 cases and 13 of these yielded adequate specimen for analysis. Six cases came from families with linkage to HPC-1, three were known to have linkage to Xq27-28 and four had no linkage to a known locus; seven cases were analyzed from patients who showed no known linkage (sporadic tumours) as controls. These paraffin embedded tumours were laser microdissected, DOP amplified and labelled for fluorescence detection by comparative genomic hybridization (CGH). Loss of 7q, 10q and 16q and gain of 8q were common abnormalities present in both familial and sporadic tumours. Distinctive abnormalities included loss of 3p12-3p22 in three of six HPC-1 linked cases and in two of three X-linked cases and gain of 6q11-6q21 in two each of HPC-1 and X-linked tumours. In conclusion, laser microdissection, DOP-PCR and CGH is a feasible method for analysis of paraffin embedded prostate tumours. This study provides preliminary data suggesting that familial prostate cancer harbours some unique genetic changes when compared with sporadic prostate tumours.

INTRODUCTION

Prostate cancer (PC) is the most commonly diagnosed cancer in men in the Western world and the second leading cause of male cancer death [1]. Risk factors for PC include age, race, country of origin and familial history. Familial clustering of PC can be caused by genetic and/or environmental factors. Carter et al. reported that there is an autosomal dominant inheritance of a rare high risk allele which accounts for 9% of all prostate cancers [2]. Monroe et al. reported a higher risk for brothers of PC cases but not for fathers which suggests a recessive or X-linked inheritance [3]. Although the model of inheritance is not defined, there appears to be a clear genetic component for PC susceptibility.

The first genetic region reported to be linked to PC was designated HPC-1 on chromosome 1, at band q24-25 [4, 5]. Other regions are Xq27-28, 1q42.2-43 and 1p36 [6-8]. No genes involved in hereditary PC have yet been identified. Dunsmuir and co-workers reported allelic imbalance at HPC1 in approximately 7.5% of 32 familial tumours. This percentage was equal in 40 sporadic controls suggesting that cellular changes in tumours at this locus are not different regardless of genetic predisposition [9].

In breast cancer it was demonstrated that tumours from patients with BRCA1 or BRCA2 germline mutations showed an almost 2 times higher number of genetic changes compared to unselected controls. Loss of 13cen-q21, containing the BRCA2 locus, was the most frequently involved abnormality in BRCA2 cases [10]. Thus, by characterizing familial prostate cancer cases, a clue may be found to chromosomal regions involved in these cancers.

Chromosomal abnormalities reported in sporadic primary prostate cancer consist predominantly of losses. More specifically, these changes include loss of portions of 6q, 8p, 10q, 13q, 16q, 17q, 18q, and gain of portions of 8q, 7q and X [11-14]. In contrast to the previous reports, Sattler found predominantly gains in primary prostate tumours [15].

Comparative genomic hybridization (CGH) allows screening of the entire genome for chromosomal aberrations [16]. Degenerate oligonucleotide PCR (DOP-PCR) is a procedure for whole genome amplification [17]. Several authors reported the feasibility of combining laser microdissection with DOP-PCR and CGH [18-23]. We applied these techniques on paraffin embedded familial prostate cancer tissues. The Utah Population Database was used to identify familial PC cases [24]. Ten cases showing no linkage (sporadic cancers) were obtained as controls.

PATIENTS AND METHODS

Patient selection and specimen preparation

35 patients from high risk PC families were selected from the Utah Population Database. Selection criteria included: 1) 2 first- or 4 second-degree relatives with PC; 2) in the absence of first

or second-degree male relatives, family clustering (affected sons or siblings of female second degree relatives). In 16 patients no tumour focus could be identified in the available blocks. Thirteen of the remaining 19 were used for these studies (6 were lost to technical limitations in specimen preparation). It is impossible to determine the involvement of any particular predisposition gene (HPC1 or HPCX) in a single case without knowledge of the genes and specific mutations observed. For this study, to assign a single case to HPC1 or HPCX or consider it unlinked, we observed the segregation of haplotypes consisting of multiple markers for each region. Cases were categorized as linked to HPC1, Xq27-28, non-linked, or non-familial (sporadic) based on observed haplotype sharing. There may be some inaccuracy in this method, since we observed that some cases showed sharing in more than one region. The assignment was then made based on the shared haplotype which best explained all cases in the pedigree. Six cases came from families with linkage to the HPC-1 locus on 1q24-25. Three cases were selected from families with linkage to Xq27-28. Four cases came from families that showed no linkage to these regions. Seven of 10 unselected sporadic controls (with no known family history) could be analyzed. Sequential 5 μm sections were cut from paraffin blocks. One section was routinely processed and stained with hematoxylin and eosin (H&E). All specimens were reviewed by one pathologist (R.R.) and marked to discern tumour foci. Two sections were left uncovered and also stained with H&E for laser capture microdissection (LCM) [25].

Table 1 gives an overview of the tumours in which the analysis could be completed. The majority of tumours were obtained by transurethral resection. Because microdissection was performed, the single Gleason pattern score is given in Table 1.

Microdissection, DNA extraction and purification

A laser microdissection (LCM) system (Arcturus Pix Cell, Arcturus Engineering, Mountain View CA) was used to isolate approximately 100 tumour cells from each specimen according to manufacturers instructions. Both tumour and normal cells were taken from the same slide. First the laser was used to remove the surrounding cells from an area of interest. Then, the target cells were attached to the cap by firing the laser. The cap was transferred to a sterile microcentrifuge tube, containing 40 μl proteinase K extraction buffer (10mM TRIS HCl, pH 8.0; 1mM EDTA; proteinase K 40 μg/ml; 1% Tween 20, pH 8.0). The tubes were stored inverted at 55 °C overnight. Proteinase K was inactivated by heating samples to 95 °C for 8 minutes. DNA was purified by the phenol:chloroform:isoamyl alcohol (25:24:1) method. For precipitation 1/10 volume 3M sodium acetate, pH 5.5, 1 μl glycogen, 1/100 volume 1M $MgCl_2$ and 2.5 volume 100% ethanol was added. DNA pellets were washed in 70% ethanol, dried and dissolved in 6 μl water.

nr	tissue	Gleason	age	affected relatives
HPC-1 linked cases				
1	TURP	3	73	3 sons, 3 brothers, 2 nephews
2	RP	2	71	father, 2 brothers
3	RP	4	74	2 brothers
4	TURP	3	62	brother
5	RP	3	55	father
6	RP	3	73	2 brothers, 2 sons, 2 nephews
X-Linked cases				
1	Biopsy	5	74	brother, nephew
2	RP	3	43	father, 2 brothers, 3 uncles
3	RP	3	69	5 brothers, 1 nephew
Non-linked cases				
1	RP	2	64	2 uncles
2	TURP	3	77	father, 1 nephew
3	RP	3	70	father, 2 brothers
4	RP	3	74	father, 2 brothers
Sporadic cases (unknown history)				
1	TURP	3	82	
2	TURP	4	85	
3	TURP	3	73	
4	TURP	2	79	
5	TURP	4	56	
6	TURP	4	82	
7	RP	3	56	

Table 1: Patient information. TURP = transurethral resection of prostate, RP = radical prostatectomy, Gleason = single growth pattern score.

DOP-PCR and DNA labelling

DOP-PCR was performed using the DOP-PCR master kit (Boehringer Mannheim, Mannheim, Germany) according to manufacturers instructions with minor modifications. Briefly, in a total volume of 50 μl, the sample was heated to 95 °C for 5 minutes followed by 5 cycles of 94 °C 1 minute; 35 °C 1.5 minutes; ramp 35 °C – 72 °C at 14 °C/min; 72 °C 3minutes. Then 35 cycles followed at 94 °C 1 minute; 60 °C 1 minute and 72 °C 2 minutes +1 sec/cycle. After a final extension period of 7 minutes the sample was cooled to 4 °C. 10 μl of the product was used to check the fragment size on a 2% agarose ethidium bromide gel. Samples were taken into analysis if a smear could be detected after the first round of DOP-PCR.

One μl of the DOP-PCR product was used as template in the labelling PCR. The reaction was performed in a total volume of 20 μl with a final concentration of the following reagents: 1x

PCR buffer (Perkin Elmer), 2.5 mM $MgCl_2$, 0.2 mM of dATP, dCTP, dGTP, 0.13 mM dTTP, 1.5 μM DOP primer, 1U Taq polymerase and 0.07 mM direct labelled dUTP (tumour: SpectrumGreen (Vysis inc., Downers Grove, Il.); normal: Cy5, (Amersham, Arlington Heights, IL). A total of 25 cycles was completed at temperature and time settings identical to the second part of the DOP-PCR. An aliquot of 2 μl of this product was run on a 2% agarose gel to determine fragment size and quantity of product. 1 ng DNA from a healthy male, with a known normal karyotype, was amplified and labelled to serve as reference DNA in all CGH experiments. In control experiments unamplified DNA was used, labelled by nick translation (Nick Translation System, Gibco BRL, Rockville MD) according to manufacturers instructions.

CGH

Metaphase slides were prepared from phytohemagglutinin-stimulated peripheral blood lymphocytes from a healthy male according to standard procedures [26]. CGH experiments were performed using software and protocols supplied by the manufacturer (Vysis, Downer's Grove, Il). Briefly, 18 μl of SpectrumGreen labelled tumour DNA and 4 μl of Cy5 labelled normal DNA were hybridized at 37 °C with 15 μg Cot-1 DNA (Gibco BRL) on normal metaphase spreads during 48 hours. Slides were counterstained with 4',6'-diamidino-2-phenylindole (DAPI) in antifade.

Image analysis

Images were captured on an Olympus epifluorescence microscope equipped with Cy-5, SpectrumGreen and DAPI filters (Chroma Technology, Brattleboro VT). For each experiment a minimum of 10 chromosomes were combined for analysis. DNA extracted from histologically normal regions in the tumour slides were used as negative controls. Based on these control experiments a ratio between 0.85 to 1.15 was excepted as normal. Telomeric regions, heterochromatic regions and the chromosome Y were all excluded from analyses, due to 'background' amplitude signal changes in control studies. Samples were not taken into analysis if a faint hybridization image was obtained requiring an automated exposure time exceeding 20 seconds.

RESULTS

Validation of the procedure

The procedure was tested by taking 1 ng of DNA from blood of a male patient with trisomy 18 (47,XY,+18). This was DOP amplified, labelled (SpectrumGreen, Vysis) and cohybridyzed with 0.4 μg of nick translated normal male DNA (Cy5, Amersham, Arlington Heights, IL) and 10 μg Cot-1 DNA. The resulting profile showed additional signal on chromosome 18, consistent with trisomy, with some minor other abnormalities (primarily at centromeric or telomeric sites), likely due to unequal amplification by DOP-PCR. Similarly, DOP amplification of 1 ng of the reference male DNA resulted in a balanced profile with an excess of chromosome 18 when co-hybridized with the specimen from the trisomy 18 patient in the test sample (Figure 1).

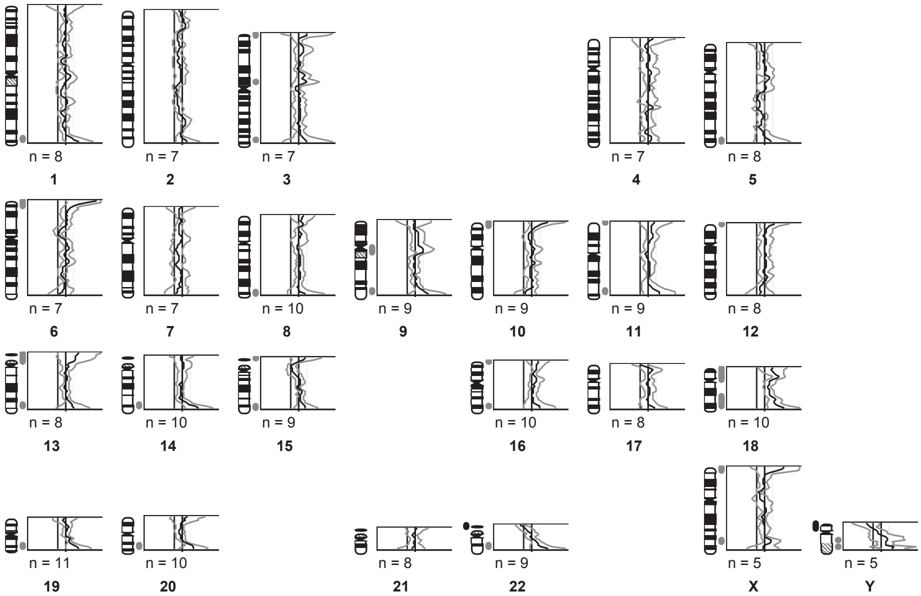

Figure 1: CGH profile obtained after DOP amplification of 1 nanogram 47, XY, +18 DNA (test) versus DOP amplified 46,XY DNA (reference). Excess of a chromosomal region is depicted as a bar to the right side of the chromosome ideogram.

Blank controls

Blanks were tested with all DOP-PCR and labelling reactions. Product in the blank lane wasn't found after the initial DOP-PCR. When 1 μl from the blank of the first DOP-PCR was added to the mix of the labelling PCR a smear was consistently observed. The intensity and size of this smear was approximately equal to the products in the positive lanes. This finding was consistent in different kits and batches but specific PCR primers failed to amplify a product and several hybridization experiments (both with SpectrumGreen and Cy5 labelled products) indicated that this product did not hybridize to human chromosomes.

Detected abnormalities

The most commonly observed abnormalities included loss of portions of 3p, 7q, 10q, 11q, 16q and gain of 4q, 8q, 21q and portions of the X chromosome. Figure 2 shows examples of some specific CGH profiles.

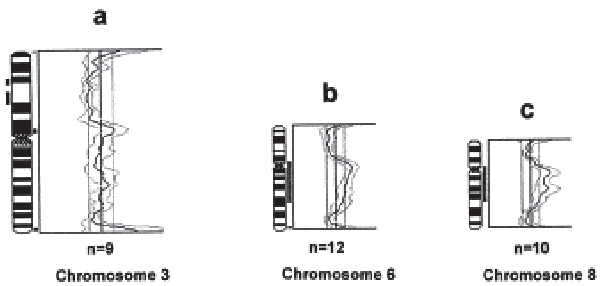

Figure 2: The CGH profiles showing specific abnormalities. Aberrations detected only in the *HPC-1-* and *X-linked* cases: (a) 3p12–3p22 loss; (b) 6q11–6q21 gain; and the typical profile seen in all cases showing 8q11.2–8q22 gain (c). Signal ratios outside the defined 0.85–1.15 range were considered significant and are depicted as a bar to the left of the chromosome ideogram for loss (a) or to the right of the chromosome ideogram for gain
(b and c).

Figure 3 shows overall gains and losses detected at selected loci. Distinctive abnormalities include loss of 3p12-22 in three of six HPC-1 linked cases and in two of three X-linked cases. The seven sporadic cases showed no loss at this region. Five of six HPC-1 linked cases showed loss at 6p21.1-6p22, which was not found in any of the other groups. Gain of 6q11-6q21 was seen in two each of HPC1-linked and X-linked cases but not in any sporadic cases. Xp21 showed gain in three of six HPC-1 linked cases, two of three X linked cases, one non-linked case and none of the sporadic cases.

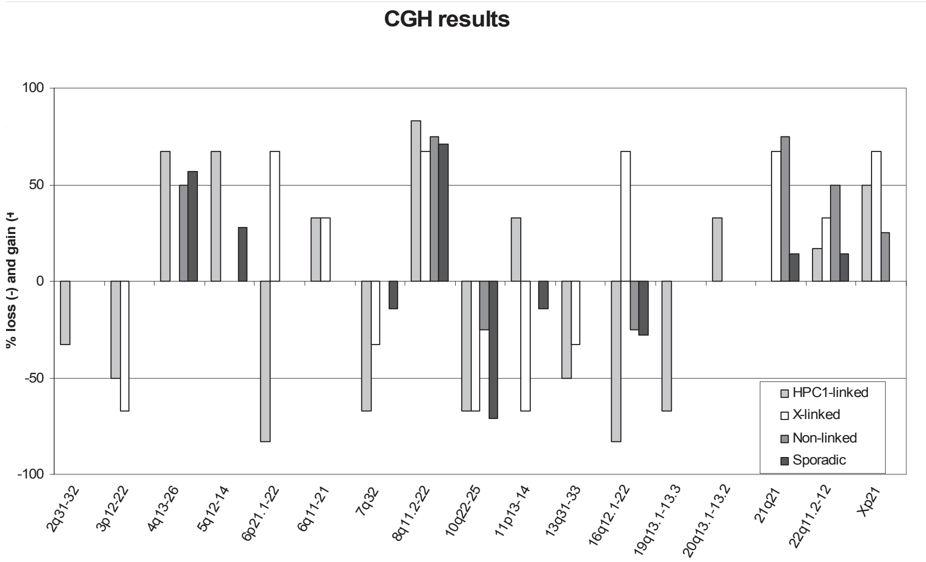

Figure 3: Documented abnormalities at selected chromosomal regions.

DISCUSSION

The application of LCM, DOP-PCR and CGH to paraffin embedded tissues opens the possibility to obtain information on DNA copy number changes in rare and valuable archival tissues. Several reports showed the feasibility of this procedure in paraffin embedded tissue [18-21, 23]. In these reports the DOP-PCR was shown to amplify DNA sequences equally throughout the genome. We consistently found unequal amplification of certain chromosomal regions in the DOP product when tested against nick translated DNA (+4q, +5q, -6p, +7q). A balanced profile was obtained when both test and reference DNA were DOP amplified.

The DOP-PCR procedure is very sensitive to contamination. This may be explained by the degenerate primers in the reaction, which will amplify DNA from any source present in the tube. In fact, Telenius et al. reported to consistently find a product in control lanes at certain conditions [27]. Another explanation for finding a product in control reactions is that at the low temperatures in the DOP-PCR, the DOP primers attach to each other and are fused to a double stranded product by the taq polymerase. Other reports mention the sensitivity of DOP-PCR to contamination but don't report to test blanks sequentially. We recommend to test sequential blanks if a second PCR is used for labelling and perform a control hybridization if a product is detected.

Familial clustering of PC occurs in about 10% of prostate cancer patients [2] and therefore some familial cancers may actually have been inadvertently included in previous studies of sporadic tumours. Due to the high prevalence of PC in the general population, an extended genealogy is necessary to identify high-risk prostate cancer families. We used the Utah Population Database to identify PC cases which belong to these high-risk families. The paraffin embedded tissues were collected from different hospitals. Accordingly, these tissues were fixed and embedded following routine procedures in the various hospitals and there was thus no standardization between facilities. This process can influence the quality and characteristics of the tissues. The failure to complete the analysis in 6 out of 19 available familial tumours may be attributed to these differences.

We show that LCM, DOP-PCR and CGH is feasible in a subset of routinely collected tissues. Our results suggest that loss of 3p12-3p22 and gain of 6q11-6q21 may be a novel change in HPC-1 and X linked cases but not in sporadic cases of prostate cancers, since these sites have not been previously reported. Loss of 6p21.1-6p22 and gain of chromosome region Xp21 were also novel changes observed only in HPC-1 linked cases. It is interesting to speculate whether novel tumour suppressor genes may reside at these loci. Further studies are currently underway to further characterize these observations.

In summary, we present results that show the feasibility of LCM, DOP-PCR and CGH in a subset of routinely fixed, paraffin embedded prostate tumours. Our observations, particularly involving chromosomes 3 and 6 in tumours from individuals showing genetic linkage suggest that familial PC may contain unique cellular changes when compared with sporadic tumours.

ACKNOWLEDGMENTS

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Chapter 4

DELETION OF CHROMOSOMAL REGION 6q14-16 IN PROSTATE CANCER

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ABSTRACT

A detailed analysis of chromosome 6 in DNAs from prostate cancers was performed, in order to define a region for subsequent search for cancer genes. DNA from four prostate cancer cell lines and eleven xenografts was used for comparative genomic hybridization (CGH) and whole chromosome Allelotyping with polymorphic microsatellite markers. Loss of proximal 6q was studied in more detail by high-density allelotyping of xenografts, cell lines and nineteen prostate tumour specimens from transurethral resections (TURP). Seven out of fifteen xenografts and cell lines showed deletion of proximal 6q by CGH. Gain of 6q was found in two samples. Six samples showed 6p gain, and one had 6p loss. Allelotyping results were consistent with CGH data in eleven of fifteen DNAs. In LNCaP and DU145 cells, CGH showed 6p loss and 6q loss, respectively, but two allelic bands were detected for many polymorphic markers on these chromosome arms. These apparent discrepancies might be explained by aneuploidy. In cell line TSU, allelotyping demonstrated chromosome 6 deletion, which was not clearly detected by CGH, indicating loss of one copy of chromosome 6, followed by gain of the retained copy during progressive tumour growth. Loss of heterozygosity was detected in nine out of nineteen TURP specimens. Combining all data, we found a common minimal region of loss at 6q14-16 with a length of 8.6 Mbp flanked by the markers D6S1609 and D6S417. One hundred and twenty-three Sequenced Tag Sites (STSs), genes and candidate genes mapping in this interval were used to screen the xenografts and cell lines for homozygous deletions, but none was detected. In summary, chromosome region 6q14-16 was deleted in approximately 50% of the prostate cancer specimens analysed. The high percentage of loss underscores the importance of genes within this region in prostate cancer growth.

INTRODUCTION

Prostate cancer is the most frequent malignancy in men in the Western world and the second leading cause of male cancer death [1]. Following the introduction of PSA serum tests and the increasing awareness of prostate cancer in the general population, the incidence of prostate cancer has shown a marked rise in the passed decade. The mortality rate has shown modest changes in this period, with a recent small decline in the United States [2]. A substantial portion of the PSA-detected cancers is biologically not aggressive and should probably not be treated. At present, however, it is not possible to predict accurately which cancer needs treatment. A better understanding of the molecular mechanisms underlying prostate cancer may result in identification of superior prognostic factors. In addition, knowledge of molecular events in prostate cancer may lead to novel therapeutic regimens for metastatic prostate cancer, for which at present there is no adequate therapy.

Tumours are characterized by genomic instability, including gene defects and chromosomal alterations. Frequently deleted chromosomal regions are expected to harbour genes, which are involved in the inhibition of tumour development. Examples of tumour suppressor genes important in the progression of prostate cancer are *P53* on chromosome 17p and *PTEN/MMAC1* on chromosome 10q. In several studies aberrant expression of these genes was shown to correlate with tumour grading (Gleason score), pathological staging or clinical outcome [3-6].

Analyses of prostate cancer by comparative genomic hybridization (CGH) and by whole genome allelotyping have consistently indicated the frequent loss of (part of) chromosome arms 6q, 8p, 10q, 13q and 16q [7-9]. In these studies, deletion of 6q was found to be present in up to 39% of the tumours. Despite this high percentage of loss, chromosome arm 6q has attracted little attention in molecular genetic analysis of prostate cancer. In chromosome-focussed allelotyping, loss of 6q was described in radical prostatectomy specimens, with a minimal commonly deleted interval 6q14-21 [10]. Srikantan et al. defined a minimal deletion at 6q16.3-21 and a homozygous deletion (HD) at marker D6S314 at 6q24 [11]. Recently, Hyytinen et al. described loss of 6q16-22 in approximately 50% of primary prostate cancers, xenografts and cell lines [12].

Deletion of 6q is not only characteristic of prostate cancer, but has also been implicated in breast cancer [13, 14], ovarian cancer [15-17], urothelial cancer [18], melanoma [19], T cell leukemia [20], small cell lung cancer [21], mesothelioma [22], hepatocellular carcinoma [23] and pancreatic cancer [24]. Most of these studies showed evidence for multiple regions of loss at 6q.

As a first step to the identification of the genes on 6q involved in prostate cancer, we describe in the present study a comprehensive analysis of chromosome 6 aberrations in tumour cell lines, in xenografts which were generated in our laboratory [25-27] and in tumour specimens obtained by transurethral resection (TURP). The main focus of this study is on proximal 6q.

MATERIAL AND METHODS

Prostate cell lines, xenografts and tumours

The *in vitro* growing cell lines PC3, LNCaP, TSU and DU145 were cultured under standard conditions. The xenografts PCEW, PC82, PC133, PC135, PC295, PC310, PC324, PC329, PC339, PC346 and PC374 were grown by serial transplantation on male nude mice 27. TURP material and blood were obtained from nineteen prostate cancer patients after informed consent. The majority of these tumours were locally progressive under hormone treatment. The specimens were snap-frozen and stored in liquid nitrogen.

DNA isolation

Standard protocols were used to isolate genomic DNA from *in vitro* cultured cells and blood [28]. Genomic DNA from xenografts and TURPs was isolated from five consecutive 5 μm cryostat sections according to standard procedures, including overnight proteinase K incubation at 55 ºC, phenol extraction and ethanol precipitation. DNAs were dissolved in 10TE buffer (10 mM Tris HCl, pH 7.8; 1 mM EDTA). The TURP sections from which DNA was isolated contained at least 80% tumour. Genomic DNA for CGH was further purified by RNAse treatment.

Comparative genomic hybridization

CGH was performed essentially as described [29]. In brief, tumour and normal DNA, 200 ng each, were labelled by nick translation (nick translation system, Life Technologies, Rockville, MD) with biotin-16-dUTP (Roche Diagnostics, Indianapolis, IN) and digoxiginin-11-dUTP (Roche Diagnostics), respectively. Labelled DNA samples were mixed with 15 μg unlabeled Cot-1 DNA (Roche Diagnostics), and subsequently ethanol-precipitated and dissolved in 10 μl hybridization mix (50% formamide, 0.1% Tween-20 and 10% dextran sulfate in 2xSSC, pH 7.0). The probe mixture was denatured, and hybridized to normal male lymphocyte metaphase chromosomes (Vysis, Downers Grove, IL) for 72 h at 37ºC. After the slides were washed, detection of the biotin- and digoxigenin-labelled DNA probes was accomplished by staining with fluorescein isothiocyanate (FITC)-conjugated avidin (Vector Labs, Burlingame, CA) and anti-digoxigenin-rhodamin (Boehringer Mannheim). Samples were counterstained with DAPI (4’6’-diamino-2-phenylindole) (Sigma, St. Louis, MO) and mounted in Vectashield anti-fade solution (Vector Labs).

Slides were analysed on a Leica DM-RXA epifluorescent microscope equipped with a cooled CCD camera (Photometrics Inc., Tucson, AZ), triple-band pass beamsplitter emission filters (P-1 filter set, Chroma Technology, Brattleboro, VT), and a Quips XL image analysis system (version 3.0.1 Vysis). Chromosomal regions were scored as lost, if the mean green to red ratio was below 0.85 and gained, if the ratio was above 1.15.

Polymorphic markers and selection of ESTs

For allelotyping the following microsatellite markers were used: D6S1640, D6S260, D6S273, D6S1575, D6S438, D6S257, D6S1589, D6S286, D6S460, D6S1609, D6S1627, D6S1652, D6S1613, D6S462, D6S1570, D6S417, D6S275, D6S300, D6S1716, D6S1671, D6S1543, D6S1709, D6S407, D6S292, D6S1564, D6S1633, D6S1719, D6S281. D6S310, D6S314, D6S409, D6S308, D6S279, D6S1703 and D6S1637 were applied for screening for a HD at 6q23-24. All markers are described in the Genome Database (http://www.gdb.org). One hundred and twenty-four additional ESTs, genes and microsatellite markers were selected from GeneMap99 (http://www.ncbi.nlm.gov/) and from the UCSC (http://genome.ucsc.edu/) and Ensembl databases (http://www.ensembl.org/) for HD screening at 6q14-16. A list of PCR primers is available as supplementary material. The April 2002 freeze version of the UCSC database was used for physical mapping of STSs and genes (http://genome.ucsc.edu/).

Amplification of microsatellite loci and ESTs

For allelotyping, 100 ng tumour or normal DNA was amplified in a PCR using Taq polymerase (Promega, Madison, WI). Typical settings were 30 cycles of 1 min at 95 ºC, 1 min at 55 ºC and 1 min at 72 ºC in a total volume of 15 μ1 with 1 μCi [α^{32}P]dATP (Amersham, Buckinhamshire, UK). Products were separated on a denaturing polyacrylamide gel and visualized by exposure to X ray film overnight. For detection of HDs, unlabeled PCR was performed in a total volume of 50 μl. Amplified fragments were separated on a 2% agarose gel.

RESULTS

Chromosome 6 CGH of prostate cancer cell lines and xenografts

Figure 1a shows the results of chromosome 6 CGH of four prostate cancer cell lines and eleven xenografts. Seven of the fifteen DNA samples showed loss of chromosome arm 6q. The three DNAs with loss of the complete q arm, PC135, PC329 and DU145, all had gain of 6p (see PC 329 as an example; figure 1b). Four samples showed deletion of proximal 6q (PCEW, PC295, PC310 and PC3) (6q13-22, 6q14-21, 6q12-16 and 6q12-22, respectively) (see PC310 as an example; figure 1c). Three xenografts had gain of part of chromosome arm 6p (PCEW, PC324 and PC339). The LNCaP cell line demonstrated loss of 6p.

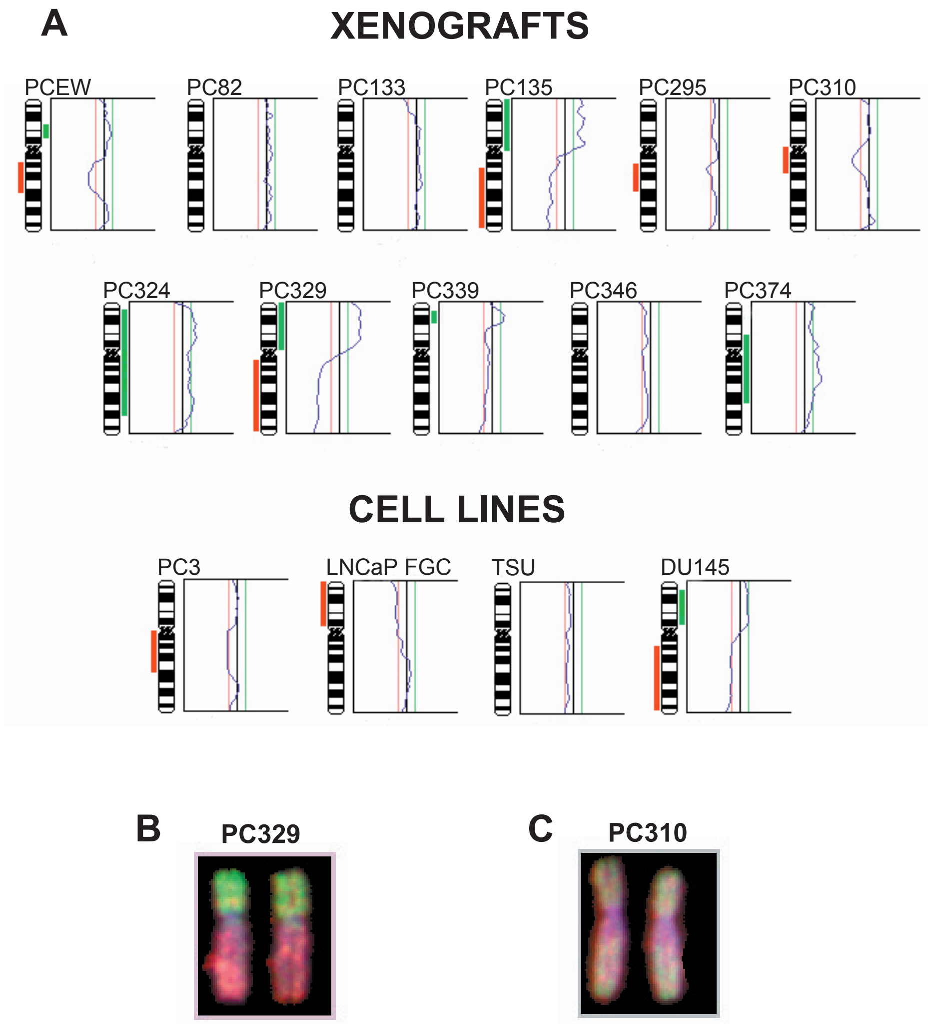

Figure 1: CGH results for chromosome 6 in prostate cancer xenografts and cell lines. (a) Red bar to the left of the chromosome 6 symbol indicates loss (mean green-to-red ratio < 0.85); green bar to the right of the symbol indicates gain (mean green-to-red ratio > 1.15). Loss of (part of) 6q is present in 7 of the 15 samples. (b) Example of chromosome 6 metaphase image from PC329 CGH experiment. (c) Example of PC310 CGH experiment. Loss is indicated by red and gain by green.

Allelotyping of chromosome 6 in prostate cancer cell lines and xenografts

Allelotyping of chromosome 6 in the prostate cancer xenografts and cell lines with twenty-eight highly polymorphic microsatellites is summarized in figure 2. Experiments were mainly focused on the region of proximal 6q loss as detected by CGH. Matched normal DNAs were not available for comparison. However, the detection of consecutive highly polymorphic markers with one allelic band, which can easily be scored because of the absence of contaminating DNA from normal human cells, is indicative for loss of the corresponding chromosomal region. Five samples had loss of the complete q arm (PC135, PC329, PC339, PC3 and TSU); three tumour DNAs showed loss of proximal 6q (PCEW, PC295 and PC310). The proximal 6q deletions in PC295 and PC310 were calculated to be less than 20 and 43 Mbp, respectively. The smallest overlapping interval of allelic loss was between D6S286 and D6S417, with a length of approximately 13.6 Mbp. PC339 and TSU demonstrated loss of (part of) chromosome arm 6p. Microsatellite instability (MSI) hampered interpretation of the results in PC346, PC374, LNCaP and DU145. However, deletion of large regions of 6q could be ruled out in these four DNAs, because of the presence of two allelic forms of several polymorphic markers that were not instable.

chromosome 6

			XENOGRAFTS											CELL LINES			
marker	map	Mbp from top	pc-ew	pc82	pc133	pc135	pc295	pc310	pc324	pc329	pc339	pc346	pc374	pc3	lncap	tsu	du145
d6s1640	6p24.3	8.0	I	I	I	II	I	II	II	II	II	II	msi	II	II	I	msi
d6s260	6p22.3	17,0-19,0	I	II	II	II	II	II	II	II	I	II	msi	I	msi	I	msi
d6s273	6p21.3	34.7	I	II	II	II	II	II	I	II	I	msi	msi	II	II	I	msi
d6s1575	6p21.1	45.9	II	II	I	I	II	I	II	II	I	msi	msi	I	II	I	I
d6s438	6p12.3	54.2	I	II	I	II	II	II	I	I	I	II	I	I	II	I	msi
d6s257	6p12.1	61.9	II	II	II	I	I	II	II	II	I	msi	msi	II	msi	I	msi
d6s1589	6q14.1	85.1	I	I	II	I	I	I	II	I	I	msi	II	I	II	I	I
d6s286	6q14.1	86.2	I	II	I	I	II	I	II	I	I	II	msi	I	I	I	I
d6s460	6q14.1	87.2	I	II	I	I	I	I	II	I	I	I	msi	I	msi	I	II
d6s1609	6q14.3	92.0	I	II	I	I	I	I	II	I	I	II	II	I	II	I	I
d6s1627	6q14.3	93.4	I	II	II	I	I	I	II	I	I	II	I	I	msi	I	II
d6s1652	6q15	92,8-94,0	I	I	II	I	I	I	II	I	I	I	II	I	I	I	msi
d6s1613	6q15	99.1	I	I	II	I	I	I	II	I	I	II	msi	I	msi	I	msi
d6s462	6q15	99.4	I	II	II	I	I	I	II	I	I	II	msi	I	II	I	msi
d6s1570	6q15	99.7	I	II	I	I	I	I	I	I	I	II	msi	I	II	I	II
d6s417	6q16.1	101.1	I	II	I	I	I	II	I	I	I	I	msi	I	II	I	msi
d6s300	6q16.1	103.7	I	II	II	I	I	II	II	I	I	I	msi	I	II	I	II
d6s275	6q16.1	105.6	I	II	II	I	I	II	II	I	I	II	II	I	msi	I	I
d6s1716	6q16.2	108.0	I	II	II	I	II	II	II	I	I	I	II	I	msi	I	II
d6s1671	6q16.3	109.9	I	II	II	I	II	II	I	I	I	II	msi	I	msi	I	msi
d6s1543	6q16.3	111.4	I	I	II	I	I	II	II	I	I	II	II	I	msi	I	msi
d6s1709	6q16.3	111.7	I	II	II	I	II	II	I	I	I	I	I	I	II	I	I
d6s407	6q22.3	139.6	I	II	I	I	II	II	II	I	I	II	msi	I	II	I	I
d6s292	6q23.3	147.5	II	II	II	I	II	II	II	I	I	msi	msi	I	msi	I	I
d6s1564	6q25.1	160.4	II	II	I	I	II	II	II	I	I	II	msi	I	msi	I	msi
d6s1633	6q25.3	166.8	II	II	I	I	II	I	II	I	I	msi	msi	I	msi	I	II
d6s1719	6q26	176.9	I	II	II	I	II	I	I	I	I	I	II	I	msi	I	msi
d6s281	6q27	180.9	II	II	II	I	I	II	I	I	I	msi	msi	I	msi	I	II

Figure 2: Allelotyping results of chromosome 6 markers in xenografts and cell lines. I, 1 allele; II, 2 alleles; grey zone indicates lost chromosomal region. The common region of loss is flanked by D6S286 and D6S417.

Comparison of CGH and allelotyping of chromosome 6

Results from CGH and allelotyping were consistent for 6q in eleven of fifteen samples. In PCEW, PC295 and PC310, CGH and allelotyping showed a perfect match pointing to loss of a proximal 6q region. In TSU, CGH failed to show loss of chromosome 6, despite the presence of only one allelic form for all polymorphic markers tested. In PC339, the most distal 6p marker analysed, D6S1640, showed two allelic forms, all other markers had one allelic band. However, CGH did not indicate chromosomal loss. In DU145 cells, CGH showed loss of 6q, whereas allelotyping pointed to the presence of two alleles. Similarly, in LNCaP cells, CGH showed loss of 6p, but allelotyping demonstrated two allelic forms for the majority of markers.

Allelotyping of TURP specimens

Five polymorphic markers, D6S1589, D6S1609, D6S1613, D6S1716 and D6S1709, in a 25 Mbp interval at proximal 6q were used to allelotype nineteen prostate tumour specimens obtained by TURP. The markers were selected to span the minimal commonly deleted region in the cell lines and xenografts. In figure 3 the results are summarized. With one exception, T-PC300, the clinical tumours showed alterations in the same region and at approximately the same frequency (45%) as the cell lines and xenografts. In T2-9, marker D6S1589 fixed the centromeric border of the deleted region; in T4-1, T5-8 and T10-7, this border was determined by D6S1609. D6S1709 was the telomeric border of the deleted region in T10-7. Combining the allelotyping of cell lines, xenografts and tumour tissues indicated a common region of loss between D6S1609 and D6S417, with a length of approximately 8.6 Mbp.

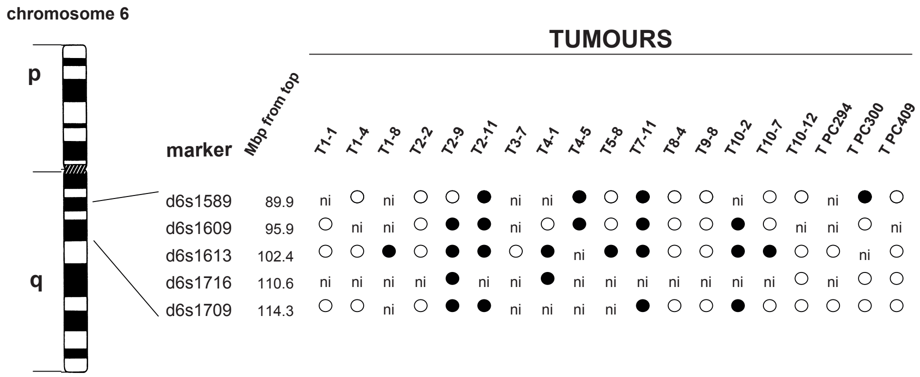

Figure 3: Allelotyping results of TURP specimens. Open circles, retention of heterozygosity; closed circles, loss of heterozygosity; ni, not informative.

Search for homozygous deletions

In the 14 Mbp interval between markers D6S286 and D6S417, 89 ESTs, and microsatellite markers were tested by PCR in the panel of xenografts and cell lines, in search of HDs. However, no HDs were found for these sequences. Additionally, 34 genes and candidate genes mapping in the 8.6 Mbp interval between D8S1609 and D6S417 were tested. Again, HDs were not detected.

DISCUSSION

Xenografts, transplantable in nude mice, and *in vitro* cell lines are powerful tools in the analysis of human cancer, because they are available in unlimited quantity, and can easily be manipulated by modification of growth conditions. The absence of normal human cells simplifies the detection of chromosomal alterations and small deletions, insertions and point mutations in individual genes. In general, the presence of contaminating normal mouse cells in xenograft tissues does not cause a major problem in genetic studies. An obvious drawback of cell lines is that during *in vitro* culturing they can acquire additional genetic alterations, which is probably less the case for slowly growing xenografts. In this study we examined chromosome 6 alteration in prostate cancer xenografts and cell lines, and analysed the commonly deleted region in prostate cancer tissues. Chromosome 6 alterations were studied by CGH and by allelotyping. Each of these two methodologies has its specific advances and limitations. Alterations detected by CGH are indicative for larger deletions and gains of chromosomal regions; allelotyping allows more focused studies of small genetic intervals. Allelotyping also discriminates between the presence of one or two of the original chromosome copies, and allows identification of HDs.

The xenografts have not been genetically characterized before. Previously, chromosome 6 alterations in cell lines PC3, DU145, LNCaP and TSU have been documented by karyotyping [30-34] and CGH [35]. More recently, PC3, DU145 and LNCaP have also been studied by spectral karyotyping (SKY) [36]. The xenograft DNAs show a perfect match between chromosome 6 alterations as found by CGH and allelotyping, with the exception of PC339. Concomitant loss of 6q and gain of 6p (PC135, PC329) might indicate isochromosome 6p. However, as proven for DU145 [36], it can also be explained by chromosome 6 translocation, followed by loss or gain of translocated chromosome regions.

The frequency of 6q loss in xenografts (54%) and in local progressive tumours obtained by TUR (47%) is comparable. These percentages are slightly higher than previously reported by CGH and allelotyping in primary tumours obtained by radical prostatectomy, 22-33% [7,10,11] and in local progressive tumours and metastases, 37-39% [9,35]. Frequent chromosome 6p gain, as found in 5 of 11 xenografts, has not been documented previously. The relevance of the latter finding remains to be studied in more detail in patient tissues.

Differences for LNCaP and DU145 between our CGH experiments and previous CGH studies by Nupponen et al. (1998), 6p deletion in LNCaP versus 6cen-6q16 deletion, and 6p gain versus absence of 6p gain, respectively, might be due to the analysis of different subclones. The allelotyping provided important complementary information. PC3 contained one allelic copy of all 6q markers. In LNCaP and DU145 two bands of 6p and 6q markers, respectively, were found, indicative of the presence of two allelic forms, and contradicting loss of genetic information as detected by CGH. Discrepancies between CGH and allelotyping might be explained by aneuploidy of the mainly tetraploid LNCaP cells and the partially triploid DU145 cell line. In TSU, CGH does not indicate loss of chromosome 6. The finding that all polymorphic markers on chromosome 6 are represented by one allelic band strongly indicates that one chromosome 6 copy has been lost, and the second copy was amplified. Interestingly, the same observation was made for chromosomes 8 and 10 (data not shown), suggesting a defect in chromosome segregation in this cell line.

The minimal overlapping region of loss has a length of approximately 8.6 Mbp, and is flanked by D6S1609 and D6S417. In three previous papers 6q loss in prostate cancer was studied by allelotyping [10-12]. Cooney et al. (1996) described allelic loss in 17 of 52 tumours and Srikantan et al. (1999) found allelic loss in 11 out of 38 samples. In these studies the tumours were obtained from radical prostatectomy specimens. Hyytinen et al. found 6q loss in 21 of 44 primary prostate cancers and in 12 of 23 xenografts/cell lines which is similar to the percentage of loss detected in our series (45%). The more frequent loss reported by Hyytinen et al. and in this study is most likely due to more advanced stage of the tumour samples analysed. The D6S1609-D6S417 interval lies within the 20.8 Mbp deletion, between D6S251 and D6S283, reported by Cooney et al. [10] and overlaps with the common region of loss reported by Srikantan et al. [11]. This interval also overlaps with the common region of loss found by Hyytinen et al. in the clinical specimens [12]. Another region of loss found by de analysis of xenografts and cell lines is more distal, indicating a complex pattern of 6q alteration in prostate cancer.

Deletion of 6q is not unique for prostate cancer. Many other tumours show a variety of deleted regions, which most commonly map in the distal part of 6q. Small interstitial deletions of proximal 6q as identified by allelotyping are only found in T cell leukaemia, between D6S1652 and D6S1644 (approximately 4 Mbp) 20 (Figure 4). This deletion overlaps with the deletion found in our experiments. The pattern of overlapping deleted regions, as depicted in figure 4, suggests the involvement of one or more classical tumour suppressor gene. Alternatively, diminished expression of several genes, due to haploinsufficiency might play a role in tumour growth.

So far, our search for a HD in prostate cancer xenograft DNAs, which was very successful for *PTEN/MMAC1* on 10q [37], was negative for proximal 6q. Because Srikantan et al. found in one of their tumour DNAs evidence for a HD at marker D6S314 [11] that maps at 6q23-24 we tested D6S314 and six polymorphic markers mapping close to D6S314 in our material. However, a HD was not found (data not shown). Our failure to detect a HD within the D6S1609-D6S417 region would be in favour of the haploinsufficiency hypothesis. However, we cannot exclude the possibility that we have missed a small HD. It might also be that HDs are rare, and that complete gene inactivation of the second allele of a tumour suppressor gene at 6q14-16 is by point mutation.

Thirty-four bona fide genes and candidate genes have been mapped within the 6q14-16 region (UCSC and ensemble databases). Methodical study of the expression pattern and structure of these individual genes will be necessary to clarify the role of proximal 6q in prostate cancer. RNA and DNA from prostate cancer xenografts are excellent starting materials for such analyses.

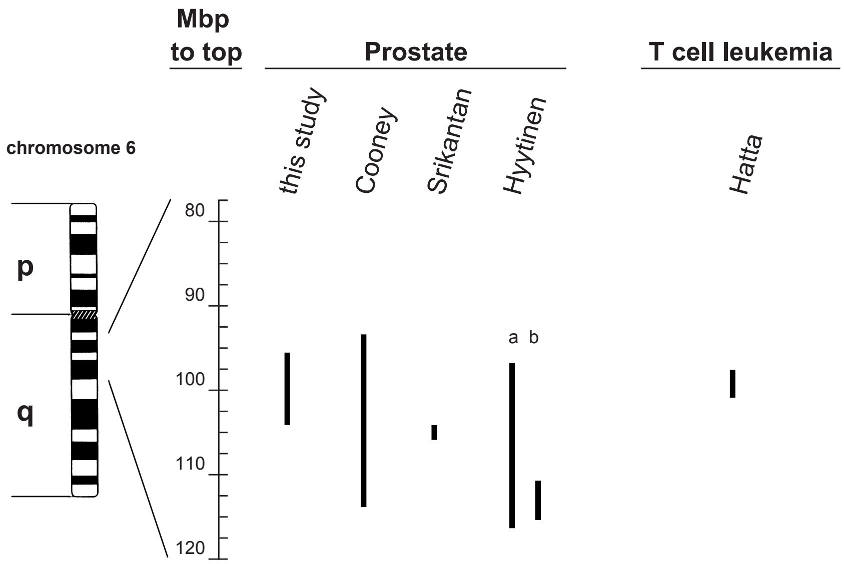

Figure 4: Allelotyping studies with reported proximal 6q loss. Bars indicate region of common loss. The common region of loss found in our study overlaps with the regions reported in previous prostate cancer studies [10–12]. Hyytinen et al. [12] found loss in the same region in clinical specimens (a). More distal regions were documented in xenografts and cell lines; the most proximal is depicted (b) [12]. In T-cell leukaemia, loss has also been reported at proximal 6q [20].

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Chapter 5

THE *PTEN* GENE IN CLINICAL PROSTATE CANCER IS PREFERENTIALLY INACTIVATED BY BIALLELIC GENE DELETION

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ABSTRACT

PTEN is frequently inactivated during the development of many cancers, including prostate cancer. Not only bi-allelic but also mono-allelic *PTEN* inactivation might contribute to tumorigenesis. *PTEN* mutations in clinical cancer specimens can easily be recorded, however, mono- or bi-allelic gene deletions are often difficult to assess. We performed a comprehensive study to detect *PTEN* inactivation in 40 locally progressive clinical prostate cancer specimens obtained by transurethral resection of the prostate, utilizing a variety of complementary technical approaches. The methods to detect *PTEN* deletion included allelotype analysis, dual colour FISH and array-based CGH. We also applied a novel semi-quantitative high-throughput approach, assessing the *PTEN* wild-type to *PTEN*-Ψ (pseudo-gene) ratio (WPR). Structural analysis of *PTEN* was done by single-strand conformational polymorphism (PCR-SSCP) and sequencing. PTEN protein expression was assessed by immunohistochemistry. Our data predict complete *PTEN* inactivation in 12 samples (30%), of which 9 by bi-allelic deletion. Loss of 1 *PTEN* copy was also detected by several methodologies, however, the number could not be accurately assessed. Immunohistochemistry indicated absence of PTEN protein in 15 samples, and heterogeneous expression of the protein in 8 tumours. Taken together, the data show that bi-allelic deletion is a major mechanism of *PTEN* inactivation in locally progressive prostate cancer.

INTRODUCTION

Prostate cancer is the most common cancer in men in the Western world and the second leading cause of male cancer death [1]. Effective treatments exist for localized prostate cancer, but when metastases develop no curative therapy is available. Hormonal therapy gives a response in most patients with bone metastases, but after an average period of 18 months hormone refractory disease develops. Then, only palliative procedures remain. A better understanding of the molecular mechanisms underlying prostate cancer is important for development of new, targeted therapies. In addition, there is an urgent need for improved prediction of the clinical course of prostate cancer to better select patients for treatment options [2].

Germ-line mutations of *PTEN* are the cause of Cowden disease, characterized by hamartomas at multiple sites and a predisposition to various tumour types [3]. Many sporadic tumours contain aberrant *PTEN*, in particular neuroblastomas, endometrial cancer and prostate cancer [4, 5]. PTEN dephosphorylates inositol phospholipids (PtdIns-3-P) by removal of the D3 phosphate from the inositol ring in this way counteracting phosphoinositide 3-kinase (PI3K). Absence of PTEN leads to increased phosphorylation and thereby activation of the serine/threonine kinase phospho-Akt, a downstream target of phosphorylated inositols, which connects PTEN to cell cycle regulation, cell survival, cell size and cell polarity [6]. Prostate-targeted *PTEN* knockout mice develop prostate hyperplasia, high grade prostatic intraepithelial neoplasia (PIN) and invasive cancer [7-9].

The frequency and mode of *PTEN* inactivation reported at various stages of clinical prostate cancer are variable. Homozygous deletions of *PTEN* have been detected in up to 10 percent of locally confined cancers and metastases [10-14], and in 30 percent of prostate cancer cell lines and xenografts [15]. *PTEN* point mutations have been described in 2 to 15 percent of primary prostate cancers [12, 16, 17], 20 to 30 percent of metastases [14, 17] and 25 percent of xenografts and cell lines [15]. Immunohistochemical studies showed absence of PTEN expression in 20 to 25 percent of primary tumours [18, 19]. No data are available on PTEN inactivation in locally progressive prostate cancer.

Although the high frequency of *PTEN* inactivation by bi-allelic deletion in prostate cancer xenografts and cell lines as compared to clinical tumour specimens might be due to selection, an alternative explanation is the underestimation of the frequency of gene deletions in clinical samples. The finding that loss at 10q23 might concern only *PTEN* and a few directly flanking genes indicates that sensitive methods are needed to assess the *PTEN* copy number [20]

Gene deletions are frequently difficult to determine in DNA from clinical tumour samples. In case of bi-allelic deletion, standard allelotype analysis might suggest apparent retention of two copies of a deleted gene, due to the presence of DNA from normal cells in a tumour DNA sample, and loss of neighbouring markers [10]. In multiplex PCR [17] experimental conditions may favour one of the amplification reactions. Dual colour FISH is technically challenging, laborious and results might be affected by sample selection. Recently developed array-based CGH is reliable, however, it is time consuming and needs relatively large amounts of DNA.

To obtain information on *PTEN* alterations in locally progressive prostate cancer tissues collected by transurethral resection (TURP), we used several complementary techniques, including classical allelotype analysis, dual colour FISH, and a novel high-throughput PCR approach, denoted WPR (wild-type to pseudogene ratio analysis), which is based on the simultaneous amplification of a fragment of *PTEN* and the highly homologous processed *PTEN* pseudogene *PTENΨ*. In WPR a single nucleotide mismatch between *PTEN* and *PTENΨ* discriminates between the respective gene copy numbers. Selected samples were assayed by array-CGH. Mutation analysis by PCR-SSCP and sequencing, and PTEN expression studies by immunohistochemistry completed the study. Our data show preferential bi-allelic deletion of *PTEN* in locally progressive prostate cancer. They also indicate frequent loss of one *PTEN* gene copy in these tumour specimens.

MATERIALS AND METHODS

Tumour samples

The study was approved by the institutions ethical committee. Tumour tissues collected by TURP were obtained from 40 prostate cancer patients after informed consent. The majority of the tumours were locally progressive under hormone treatment. From approximately half of the patients DNA from blood cells was available. The tissue specimens were snap-frozen and stored in liquid nitrogen until use. In addition paraffin-embedded tissue sections were available from all patients.

DNA isolation

Standard protocols were used to isolate genomic DNA from TURPs and blood. Tumour DNA was isolated from five consecutive 5 μm cryostat tissue sections containing at least 80% tumour according to standard procedures, including overnight proteinase K incubation at 55ºC, phenol extraction and ethanol precipitation. DNAs were dissolved in TE buffer (10 mM Tris HCl, pH 7.8; 1 mM EDTA). For array-CGH DNA was further purified by RNAse treatment.

Allelotype analysis

For allelotype analysis, 100 ng tumour DNA and normal DNA were analyzed. Typical PCR settings were 30 cycles of 1 min at 95 ºC, 1 min at 55 ºC and 1 min at 72 ºC in 15 μ1 standard reaction buffer, supplemented with 1 μCi [α^{32}P]dATP (Amersham Biosciences, Buckinhamshire, UK). Four polymorphic markers in or flanking the *PTEN* gene were used: *D10S1687, D10S1765, D10S541* and *D10S583* (see Figure 1 for map positions). Products were separated on a denaturing polyacrylamide gel and visualized by exposure to X ray film.

Wild-type to pseudogene ratio analysis (WPR)

WPR is based on the simultaneous PCR amplification of a *PTEN* fragment that maps on chromosome 10q23 and the highly homologous *PTEN* pseudogene on 9p. A single mismatch (T/C) between the exon 9 sequence of *PTEN* (T) and the corresponding sequence of *PTENΨ* (C) was used to discriminate between wild-type *PTEN* and the pseudogene.

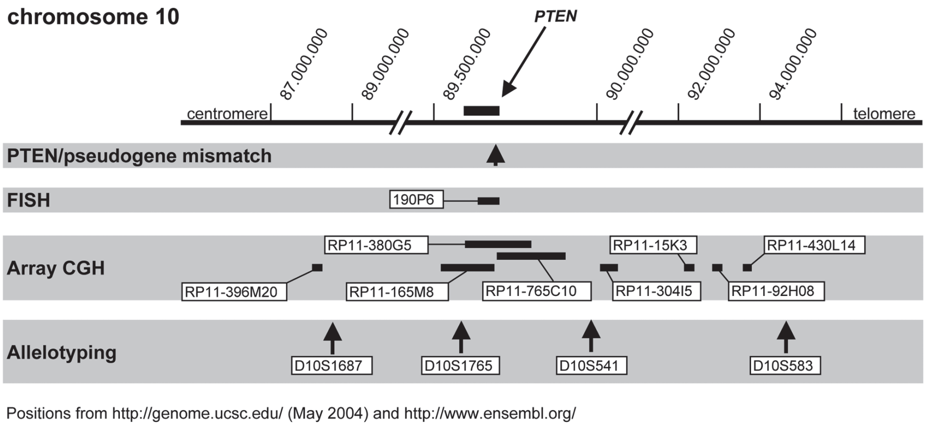

Figure 1: Overview of the *PTEN* region on chromosome 10q23.3. The upper part of the figure shows the position of *PTEN* in Mb from the top of chromosome arm 10p. Below are indicated the positions of the PAC (190P6) utilized for FISH, the BAC clones encompassing and flanking *PTEN* in the BAC array, and the positions of the markers utilized in allelotype analyses.

Because the primer sequences used for amplification were completely identical for *PTEN* and the pseudogene, amplification of fragments was essentially proportional to the gene copy numbers present in the tumour DNA sample. Importantly, in most prostate cancers the chromosome 9p copy number is normal allowing the use of *PTENΨ* as internal control in this method [21].

Fifty ng DNA was amplified using Taq polymerase (Promega, Madison, WI). Amplification settings were 35 cycles of 1 min at 95ºC, 1 min at 50ºC and 1 min at 72ºC. *PTEN* exon 9 primers: PTENex9F2: 5'- TAGTGACAATGAACCTGATCA – 3' and PTENex9R:5'-GGTAATCTGACACAATGTCCTA–3'. The PCR products were loaded on a 1 % agarose gel, isolated from gel and purified using Qiaquick gel extraction columns according to the manufacturer's description (Qiagen, Hilden, Germany). Fragments were sequenced using the ABI Prism BigDye terminator v3.1 ready reaction cycle sequencing kit (Applied Biosystems, Foster City, CA) and primer PTENex9F2 [25 cycles of 96°C for 10 sec, 50°C for 5 sec, 60°C for 4 min (ramping 1°C/sec)]. Sequence reactions were run on the ABI PRISM 3100 Genetic Analyzer (Applied Biosystems). To determine the *PTEN* copy number the T/C ratio at the mismatch position in the amplified fragments was corrected for differences in T and C signal intensity, based on the average signal of flanking T and C sequences. T/C< 0.4 was indicative of bi-allelic *PTEN* loss, 0.4 ≤ T/C < 0.8 as mono-allelic *PTEN* loss, and T/C ≥ 0.8 as allele retention.

Array-based CGH

The human 3600 BAC/PAC genomic clone set, covering the full genome at 1 Mb-spacing for array production was obtained from the Sanger Institute (see Figure 1 for BACs mapping in and close to *PTEN*). Degenerated oligonucleotide PCR-products were prepared for spotting on CodeLink® slides (Amersham Biosciences) according to Fiegler [22] with some modifications [23]. DNA labelling and hybridization was performed essentially as described [22]. Briefly, 450 ng of test or reference DNA, 60 µl 1x Random primer solution (Invitrogen) and water was heated for 10 min at 100°C, and subsequently cooled on ice. After the addition of 15 µl dNTP mix (1 mM dCTP, 2 mM dATP, 2 mM dGTP and 2 mM dTTP), 2µl of 1mM Cy3- or Cy5-dCTP (test and reference respectively) (Amersham Biosciences) and 120 U Klenow fragment (Invitrogen), the mixture was gently mixed and incubated overnight at 37°C. In one tube the Cy3-test sample and Cy5-reference sample were mixed with 125 µg Cot-I DNA (Invitrogen), 42.5 µl 3 M NaAc. Next, 1 ml cold 100% ethanol was added. In a second tube, 40 µg denatured herring sperm was mixed with 125 µg human Cot-I DNA, 16.5 µl 3 M NaAc and 400 µl cold 100% ethanol. After gentle mixing, the mixes were precipitated for 3 h at –20°C. Hybridizations of arrays were performed as described [22]. Following hybridization, slides were washed for 15 min at 48°C in 50% formamide/2x SSC, for 30 min at 48°C in 2 x SSC/0.1% SDS, for10-15 min at room temperature in 0.1 M sodium phosphate, 0,1% NP40, and dipped in ddH2O, before drying by centrifugation for 3 min at 750g. Arrays were scanned in a ScanArray Express HT (Perkin Elmer). The resulting TIFF images were analyzed with GenePix Pro 5.0 software (Axon Instruments) and subsequently analyzed and visualized with an excel macro [23].

Dual colour fluorescent in situ hybridization

Four µm frozen tissue sections were fixed on microscope slides treated with 3-aminopropyl-triethoxysilane (Sigma, St. Louis, MO). Slides were air-dried at room temperature for 1 h, next the slides were submerged in methanol-acetone (1:1, v/v) at -20°C for 20 min, washed twice in PBS containing 0.5% (v/v) Tween 20 (Sigma). Subsequently, tissue sections were treated with 0.0005% pepsin (Sigma) in 0.01 M HCl for 3 min at 37°C, washed 5 times in water for 1 min and 5 times in PBS for 1 min. Slides were postfixated in 1% formaldehyde in PBS for 10 min, washed 5 times in PBS for 1 min and 5 times in water for 1 min, dehydrated in an increasing ethanol series (70%, 80%, 90% and 100%, 5 min each) and air dried. Probes used were labelled by nick translation and hybridized essentially as described [24]. Chromosome 10 centromere probe pα10 RR8 was lissamin-11-dUTP (Roche, Mannheim, Germany) labelled and PAC 190P6, containing *PTEN* exons 3 to 9 inclusive, (Figure 1) (Genome Systems, St Louis, MO) was digoxigenin-11-dUTP labelled and visualized with FITC-conjugated sheep antidigoxigenin (Roche). To verify the results, the labels of the probes were interchanged. One hundred ng of both labelled probes and 0.5 µg Cot1 DNA were dissolved in 10 µl hybridization mixture containing 50% formamide/2xSCC/10% dextransulfate. Results were visualized using a Leica DM fluorescent microscope with a DAPI/FITC/Cy3 triple band-pass filter.

PTEN mutation analysis by single-strand conformation polymorphism and sequencing

Fragments for PCR-SSCP analysis were obtained for all *PTEN* exons using primer sets and PCR conditions as described previously [15]. Amplification reactions were in a 15 μ1 volume in the presence of 1 μCi [α^{32}P]dATP. Products were separated over a 6% non-denaturing polyacrylamide gel containing 5 or 10% glycerol at 7 W overnight at room temperature. Sequencing of samples containing an aberrant band was performed as described above (WPR analysis).

Immunohistochemistry

The mouse monoclonal anti-human PTEN antibody (Clone 6H2.1, Cascade Bioscience, Winchester, Ms) was used for immunohistochemistry on formalin-fixed, paraffin- embedded tissue sections. Sections were pretreated by a modified heat-induced antigen-retrieval method [25] in 10 mM citrate buffer, pH 6.0. PTEN antibody (1:200) incubation was overnight at room temperature. As secondary antibody biotinylated rabbit-anti-mouse was used (DAKO, Glostrup, Denmark). Signals were visualized using the avidin-biotin-Horse Radish Peroxidase complex (DAKO). PTEN staining was independently scored by 2 observers (ThHvdK, PCMSV). The cytoplasmic staining intensity was estimated as absent (0), weak (1), normal (2) or strong (3). In case of heterogeneity, the range was indicated.

RESULTS

Because *PTEN* inactivation is the most frequent genetic alteration in late stage prostate cancer, PTEN downstream effectors are important candidates for therapeutic targeting of prostate cancer. In order to select patients for targeted therapies, knowledge of the status of the *PTEN* gene is of utmost importance. Exon sequencing can easily assess the structure of *PTEN* in tumour DNA. However, detection of *PTEN* deletions is more challenging. Loss of *PTEN* may concern a small genomic fragment [15] [20]. We applied a variety of complementary methods to investigate *PTEN* inactivation in locally progressive prostate cancer obtained by TURP.

Structural analysis of *PTEN*

The structure of *PTEN* was assessed in 40 TUR DNA's by PCR-SSCP analysis of all exons. PCR-SSCP was abnormal in 3 DNA's (T1-8, T2-2 and T10-7). In all 3 samples inactivating mutations of *PTEN* were found by subsequent sequence analysis (summarized in Figure 2). In T1-8 a C deletion in exon 7 gave rise to a frame shift and a truncated protein. In T2-2 a TTAC deletion in exon 8 also introduced a frame shift and a shorter protein. T10-7 contained a nonsense mutation (CAG>TAG) in exon 7.

Allelotype analysis

Allelotype analysis was performed with 4 highly polymorphic markers (*D10S1687, D10S1765,*

TUMOUR	Horm Ther *	Mutation	LOH predicted	Ratio PTEN/pseud.	FISH	Immuno PTEN
T1-1	no	nl	○◒●	●	●	0
T1-2	yes	nl	-	○	●	2
T1-4	no	nl	○	○	○	3
T1-7	yes	nl	-	●	●	0-3
T1-8	yes	mutation	◒	◒	◒	0-3
T1-14	yes	nl	-	◒	◒	0
T2-2	yes	mutation	◒	◒	◒	0
T2-4	yes	nl	-	-	○	1
T2-9	yes	nl	○◒●	◒	○	0-3
T2-11	yes	nl	◒	○	◒	0
T2-14	yes	nl	-	-	◒	0-3
T3-1	no	nl	-	○	○	0-3
T3-7	yes	nl	○◒●	●	●	0
T4-1	yes	nl	◒	○	○	0
T4-5	yes	nl	○	◒	○	2
T4-10	no	nl	-	◒	○	3
T5-5	yes	nl	-	○	○	2
T5-8	yes	nl	○	○	○	-
T6-1	no	nl	-	○	○	0-2
T6-9	yes	nl	-	◒	●	0
T6-14	yes	nl	-	●	○	0
T7-1	yes	nl	-	○	○	1
T7-8	no	nl	-	●	○	0
T7-11	yes	nl	○	○	○	0-2
T8-4	no	nl	●	●	●	0
T8-9	no	nl	-	○	○	2
T8-13	yes	nl	-	◒	○	0
T8-14	yes	nl	-	◒	○	3
T9-4	yes	nl	-	◒	◒	1
T9-8	?	nl	-	◒	○	0
T9-11	yes	nl	○	○	○	2
T10-2	no	nl	○◒●	○	○	1
T10-3	yes	nl	-	●	●	0
T10-4	yes	nl	-	◒	○	0-1
T10-7	no	mutation	◒	◒	◒	0
T10-12	no	nl	●	●	●	0
T10-15	yes	nl	-	◒	○	2
T29-4	yes	nl	○	◒	-	3
T30-0	no	nl	○	○	-	1
T40-9	yes	nl	○	○	-	-

○	2 alleles
◒	1 allele
●	homozygous deletion
nl	normal
-	not done
*	at time of TUR

immunohistochemistry
0 absent
1 weak
2 normal
3 strong

Figure 2: Overview alterations of *PTEN* structure as obtained by mutation analyses, allelotype analyses (LOH), WPR analyses and FISH and of PTEN expression by immunohistochemistry of prostate tumours obtained by TUR.

D10S541 and *D10S583*), mapping directly upstream or downstream of *PTEN* (Figure 1). Nineteen DNA's from tumours obtained by TUR for which corresponding normal blood DNA was available, were investigated. Eleven samples showed loss of at least one of the markers in the *PTEN* region (55%), indicating loss of one *PTEN* copy (Figures 2 and 3). Among these are T1-8, T2-2 and T10-7, which contain an inactivating mutation in the second *PTEN* copy (see above). In 2 DNA's (T8-4, T10-12) markers directly adjacent to *PTEN* showed the presence of 2 alleles, whereas more distal markers showed loss of 10q, indicative of a homozygous deletion of *PTEN*. In T1-1, T3-7 and T10-2 markers directly flanking *PTEN* were not lost, but a more proximal or distal marker showed allelic imbalance, indicating bi-allelic *PTEN* loss, or no loss of *PTEN* at all.

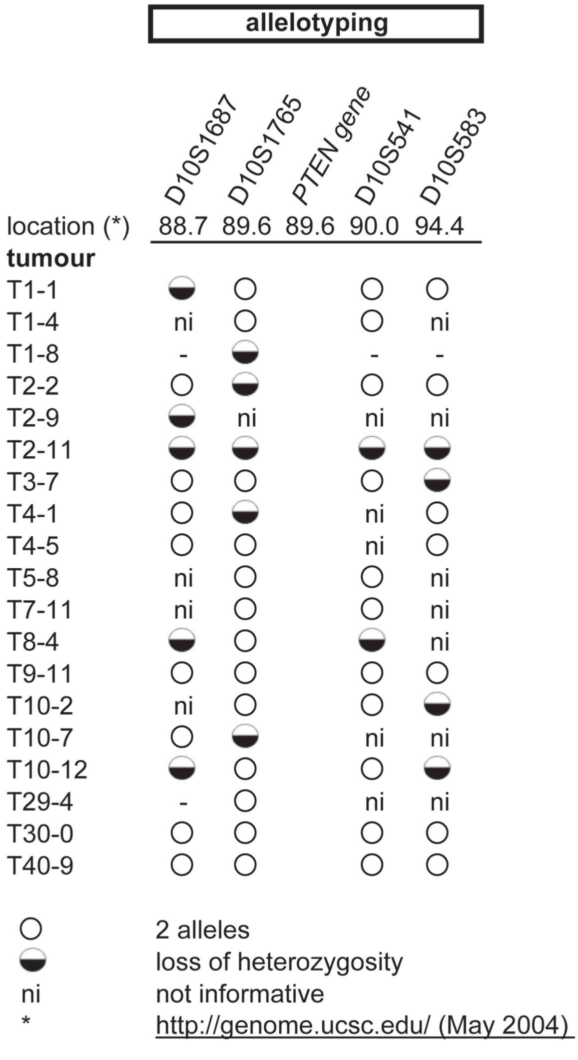

Figure 3: Allelotype analysis of the *PTEN* region of DNA from locally progressive prostate cancers. See Figure 1 for positions of markers.

PTEN analysis by dual colour FISH

In 37 cases, frozen tissue sections with representative tumour were available for dual colour FISH. Results are summarized in Figure 2. Dual colour FISH of T10-12 is shown as a *PTEN* negative example (Figure 4). In 22 samples a normal *PTEN* signal was obtained. Eight cases showed complete absence of *PTEN* signals in cancer cells, in contrast to neighbouring stromal cells, indicative of complete *PTEN* deletion (T1-1, T1-2, T1-7, T3-7, T6-9, T8-4, T10-3, T10-12). Seven tumour sections indicated loss of one *PTEN* copy.

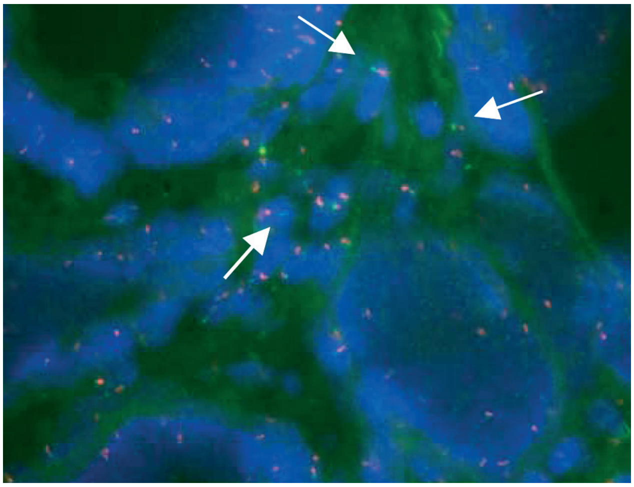

Figure 4: Dual colour FISH in situ hybridization of the T10-12 prostate tumour. Red spots represent chromosome 10 centromeres; green spots represent *PTEN*. *PTEN* is absent from the luminal epithelial cells. Arrows indicate the presence of *PTEN* in the stromal cells.

PTEN analysis by WPR assay

WPR is a semi-quantitative high-throughput method to assess the *PTEN* gene copy number. In the WPR assay the *PTEN* pseudogene (*PTENψ)* is used as an internal control to assess the wild-type *PTEN* gene copy number (see Materials and Methods). A fragment of *PTEN* exon 9 was amplified using primers that are 100 % complementary to both *PTEN* and *PTENΨ*. In the amplified fragment the T/C sequence difference between *PTEN* (T) and *PTENΨ* (C) resulted in a double peak in the sequence plot at the mismatch position. The ratio between the two peaks at this position is indicative of the *PTEN* copy number. In theory, in DNA samples containing 2 copies of both *PTEN* and *PTENΨ*, the *PTEN/PTENΨ* ratio is 1; if *PTEN* is completely deleted only *PTENΨ* will be amplified; a WPR value of 0.5 is indicative of mono-allelic loss of *PTEN*. In practice, values deviate from the theoretical numbers, because tumour DNA contains DNA from normal cells and sequence peaks are not completely quantitative. The cut-off WPR values for complete absence of *PTEN* or loss of one *PTEN* copy were accordingly adjusted.

WPR was applied on thirty-eight TUR DNA's. Examples of relevant parts of sequence plots from tumour DNA's with either 2, 1 or 0 wild-type *PTEN* copies, as confirmed by other methods, are shown in Figure 5. The results of all TUR DNA's are summarized in Figure 2. In 15/38 tumour DNA samples two *PTEN* alleles were detected; WPR indicated also loss of one allele in 15 samples. WPR predicted homozygous deletions of *PTEN* in 8 tumour DNA's (T1-1, T1-7, T3-7, T6-14, T7-8, T8-4, T10-3 and T10-12).

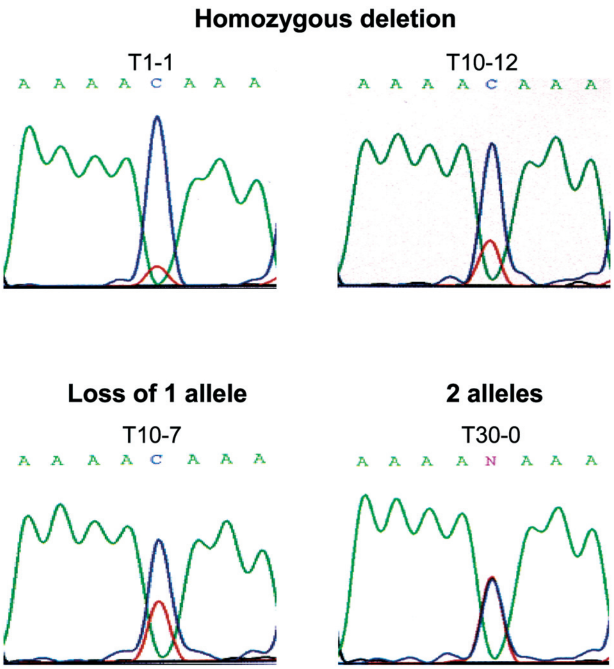

Figure 5: Examples of *PTEN* exon 9 analyzed by WPR. T1-1 and T10-12 show a homozygous deletion of *PTEN*. T10-7 contains one *PTEN* allele. T30-0 shows the presence of two *PTEN* alleles.

PTEN copy number analysis by array-CGH

For comparison with allelotype analysis, FISH and WPR, two tumour samples for which sufficient DNA was available (T1-1, T10-12) were analyzed by array-CGH. FISH and WPR predicted bi-allelic *PTEN* loss for T1-1; in case of T10-12 all three assays indicated complete *PTEN* loss (Figure 2). The array-CGH pattern of T1-1 clearly showed losses of many chromosome regions (Figure 6A). Such a pattern is representative for DNA from a homogeneous high-grade tumour, containing a low percentage of normal cells. Tumour T10-12 contained less chromosomal alterations; one of the most prominent changes was loss of chromosome 10 (Figure 6B). In both tumour DNAs, but most clearly in T1-1, the chromosomal region with the lowest T/R ratio is 10q23, where *PTEN* is located, indicative of bi-allelic *PTEN* deletion. In T1-1 the predicted homozygous deletion (T/R ration approx. –2) is located in a small 1 Mbp region of 10q23 loss (see Figure 6C). T10-12 has lost one copy of chromosome 10 and contains a small deletion in the second copy (Figure 6D). T10-12 seems to contain a considerable number of cells without chromosome 10 losses, because the log2 T/R ratio of chromosome 10 is approx. -0.5; the log2 T/R ratio over the homozygous deletion is approx. -1 (Figure 6D). Even in the presence of DNA from cells with a normal chromosome 10 copy number (non-cancer cells) the deletion can be visualized.

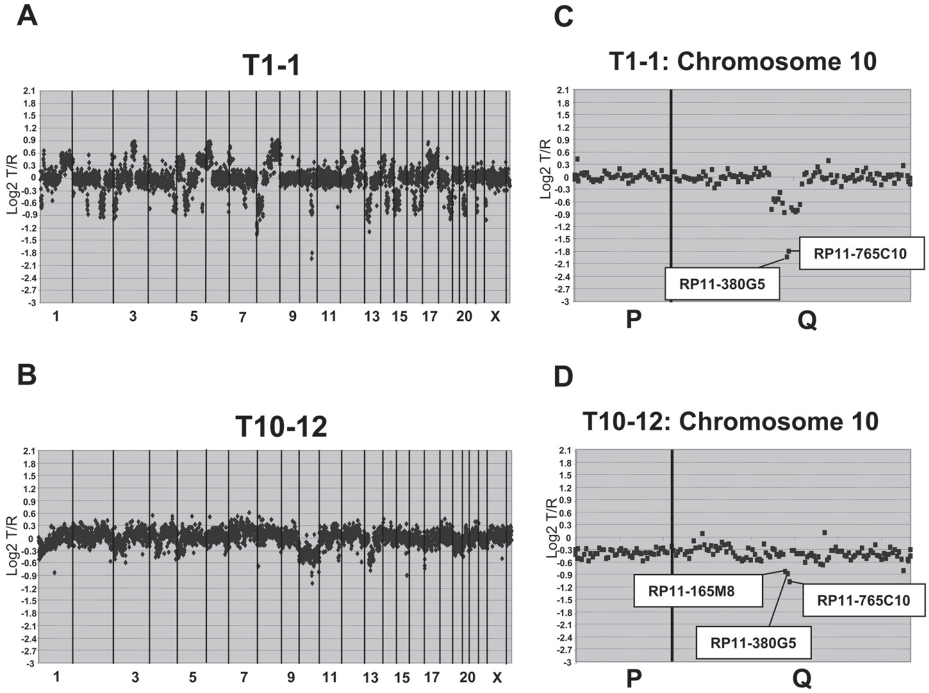

Figure 6: Array CGH patterns of T1-1 and T10-12. A and B: whole genome array CGH. C and D: chromosome 10 profiles. Both tumours contain a homozygous deletion of *PTEN*. In T10-12 one complete copy of chromosome 10 is lost. In T1-1 the *PTEN* homozygous deletion is in a small region of 10q loss.

Expression of PTEN in locally progressive prostate cancer

Immunohistochemistry showed cytoplasmic and nuclear PTEN staining in normal prostatic epithelium and stromal cells (Figure 7A). Intraprostatic nerves and urothelial cells showed strong cytoplasmic staining. As summarized in Figure 2, 15 out of 38 evaluable tumours were completely negative for PTEN (39%) (see T10-12 as an example, Figure 7B). Heterogeneous cytoplasmic staining with locally complete absence of staining was noted in 8 tumours (7C).

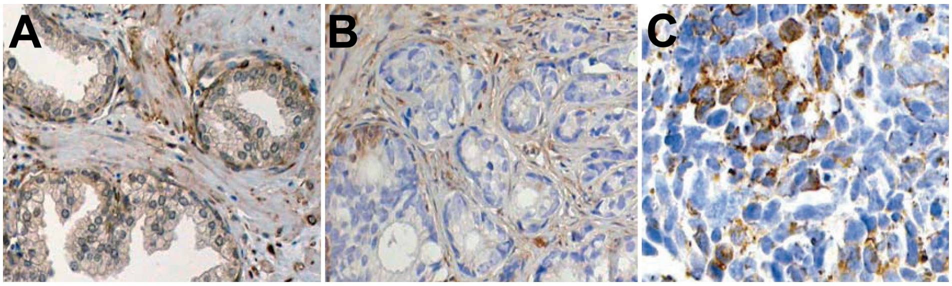

Figure 7: Immunohistochemical staining of PTEN (brown) in a positive control prostate (A), absence of staining indicates absence of PTEN expression in the tumour cells in T10-12 (B), heterogeneous cytoplasmic staining with locally complete absence of staining in T1-8 (C).

DISCUSSION

PTEN inactivation is the most frequent genetic alteration in prostate cancer. Previously we have shown that *PTEN* can be inactivated by bi-allelic deletion or mutation in over 60% of prostate cancer xenografts and cell lines, derived from prostate tumours of different clinical background [15]. It has also been shown that *PTEN* can be inactivated in clinical prostate cancer, however, mostly at a lower frequency [10, 12, 13, 16, 26]. In the most complete clinical study [10] loss of (part of) chromosome 10q was reported in 23 out of 80 prostate cancer specimens obtained by radical prostatectomy (60) or from regional lymph node metastases (20). In 10 of these samples, 7 lymph node metastases and 3 primary tumours, *PTEN* was found completely inactivated by bi-allelic deletion or by point mutation. *PTEN* was most frequently inactivated in metastases of patients who died from prostate cancer (60% [14]). Recently we observed that *PTEN* inactivation by deletion frequently occurs by loss of a small chromosomal region [20]. Such small deletions might be overlooked in clinical prostate samples. This prompted us to further investigate the *PTEN* status in such samples.

In the present study we analyzed *PTEN* alterations in 40 locally progressive prostate cancers obtained by TUR. In three samples we detected nonsense mutations or frame shifts in exons 7 or 8, causing the synthesis of a truncated protein. These results extend previous observations [15, 27] that missense mutations in *PTEN* hardly occur in prostate cancer. The low frequency of *PTEN* inactivation in TUR by small mutations was in agreement with the data of Gray et al. (1998) who described 4 mutations in 37 TUR specimens [26]. Others found 1 stage B prostate cancer with a *PTEN* mutation out of 10 studied [11]. Dong et al (1998) detected 1 point mutation in 40 pT2-pT3 clinical prostate tumours (radical prostatectomy) [12]. In contrast, a later report from the same institute reported on a series of 32 primary prostate cancers from Chinese patients, with 5 mutations detected [17].

In the present study we utilized a variety of complementary methodologies, including allelic analyses, dual colour FISH and the high-throughput WPR method to assess the *PTEN* status in clinical prostate cancer samples. Overall the various genetic approaches gave consistent results (Figure 2). In 6 samples WPR and FISH data were consistent. The 2 homozygous deletions detected by allelotype analysis, and the 2 homozygous deletions found by array-CGH were observed by both WPR analysis and FISH (see Figure 2). In the 3 tumours containing inactivating point mutations (T1-8, T2-2 and T10-7) both allelotype analysis and WPR experiments indicated loss of the second allele. In 2 cases, FISH indicated homozygous deletions that were not found by WPR (T1-2 and T6-9), also in 2 cases results were the opposite (T6-14 and T7-8). In 3 out of 4 discrepant cases immunohistochemistry confirmed absence of PTEN expression, indicating a likely total number of 9 specimens containing homozygous *PTEN* deletion. Discrepancies might be due to small deletions, precluding detection by FISH or array-CGH (see Figure 1). Further, tumours might be heterogeneous, affecting results of FISH experiments. Both tumour heterogeneity and presence of normal cell DNA can affect WPR data, array-CGH and allelotype analysis. The number of 6 to 10 homozygous deletions in 40 clinical tumour specimens (15-25%) is lower than reported in xenografts and cell lines (35%), but higher than previous reports in clinical samples (0-15%), utilizing different methodologies [10, 14].

Fifteen samples (40%) were completely negative for PTEN expression by immunostaining, among those are 10 showing complete genetic inactivation of *PTEN* by 1 or more genetical analyses. Eight samples (20%) stained heterogeneously with fields of complete absence of PTEN, of which 6 cannot be explained by genetic defects. So, our data indicate, in addition to genetic alterations, down-regulation of PTEN expression. Down-regulation might be by promoter hypermethylation, other epigenetic mechanisms or by mutations in regulatory gene sequences. Evidence for promoter hypermethylation has been reported in prostate cancer xenografts [28], however, others observed that *PTEN* promoter hypermethylation is rare or absent in clinical prostate cancer [15, 29]. McMenamin et al. (1999) and Halvorsen et al (2003) applied immunostaining and showed absence of PTEN expression in approx. 25% of radical prostatectomies [18, 19]. However, genetic or epigenetic molecular mechanisms underlying PTEN down-regulation have not been investigated in these studies.

The higher frequency of loss of 10q23, as compared to complete *PTEN* inactivation gave rise to the speculation that 10q23 might contain a second tumour suppressor gene or that mono-allelic *PTEN* inactivation might contribute to tumorigenesis. The latter hypothesis is supported by studies in mouse models of prostate cancer, although we have recently shown that in a prostate targeted *PTEN* knock-out model, monoallelic *PTEN* inactivation by itself is insufficient for tumorigenesis [8]. However, there is ample evidence that a *PTEN+/-* genetic background can induce tumorigenesis or accelerate tumour progression in compound mouse prostate cancer models including *Nkx3.1*, *Ink4a/Arf* or *Cdkn1b* inactivation, and in TRAMP transgenic mice [30-33]. In the present study we postulate many tumour specimens with mono-allelic loss of *PTEN* (Figure 2). However, the limited accuracy of our data shows the complexity in discriminating between 2 copies and 1 copy of *PTEN*. Further, more quantitative experiments are essential to assess the frequency of mono-allelic *PTEN* inactivation in prostate cancer. We hypothesize that mono-allelic *PTEN* inactivation in late stage prostate cancer will synergistically cooperate with genetic or epigenetic alterations in other signalling pathways. Complete *PTEN* inactivation might further accelerate tumour progression.

In the *PTEN* expression study of McMenamin (1999) absence of PTEN was associated with high Gleason grade and advanced tumour stage [18]. In the study of Halvorsen (2003) absence of PTEN expression was associated with increased tumour diameter and seminal vesicle invasion. In the latter study, combined loss of PTEN and p27kip1 expression was associated with increased tumour diameter, capsular penetration, seminal vesicle invasion and pathological stage. In multivariate analysis, combined loss of PTEN and p27kip1 predicted biochemical recurrence [19]. In our study, aberrant PTEN expression appeared not to be predictive of survival, however, the sample size is limited. The TURP specimens were generally taken from patients progressive under hormonal therapy. Even if absence of PTEN expression would be predictive of a more aggressive course of the disease, this might not have become apparent because only aggressive cancers were included in this study.

In conclusion, we show aberrant PTEN expression in up to 58% of locally progressive prostate cancers, with bi-allelic deletion being the predominant mechanism of genetic inactivation.

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Chapter 6

PTEN INACTIVATION IN RADICAL PROSTATECTOMY SPECIMENS: FREQUENCY, GENETICS AND PROGNOSTIC VALUE

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ABSTRACT

PTEN is the most frequently inactivated tumour suppressor gene in prostate cancer. In radical prostatectomy specimens knowledge of the mechanisms, frequency and prognostic value of *PTEN* inactivation is limited. We performed a comprehensive analysis of PTEN expression in combination with genetic studies. The prognostic value of PTEN inactivation was evaluated based on PSA recurrence data.

PTEN expression was assessed by immunohistochemistry applied to a tissue microarray containing cores from 90 tumours collected by radical prostatectomy from prostate cancer patients with known clinical outcome. From 62 samples adequate tumour DNA was available and used for array-based CGH analysis. Tumours showing loss of one *PTEN* allele were screened for mutations. PTEN expression was correlated to array-CGH data and clinical follow up (PSA recurrence).

By immunostaining 35 out of 90 cases were partially or completely negative for PTEN. In 9 out of 62 cases, array-CGH pointed to a homozygous deletion of *PTEN*. In 6 samples array-CGH indicated loss of one copy of *PTEN*. Sequencing of all *PTEN* exons in the latter group did not reveal mutations. A strong inverse correlation was not only found between bi-allelic loss of *PTEN* and PTEN expression, but also between mono-allelic *PTEN* loss and PTEN expression. Absence of PTEN expression was associated with PSA recurrence. In multivariate analysis, however, in contrast to pT stage and Gleason sum score, PTEN was not an independent predictor of PSA progression

In summary, both mono- and bi-allelic loss contribute to *PTEN* inactivation, which is frequently present in radical prostatectomy specimens. Absence of PTEN expression occurred more frequently in tumours showing PSA progression, although this correlation was not statistically significant.

INTRODUCTION

Prostate cancer is the most frequently diagnosed cancer in Western countries and the second leading cause of male cancer death [1]. Localized prostate cancer can be treated effectively, but when metastases have developed there is no curative therapy possible. PSA based screening leads to diagnosis of a considerable number of prostate cancer cases that do not need radical therapy. Currently, there is a great need for accurate prognostic markers to better predict the clinical course of prostate cancer. Furthermore, it is important to get more insight in the mechanisms underlying prostate cancer in order to develop more targeted treatments of advanced disease.

The *PTEN* tumour suppressor gene that maps to chromosome 10q23 encodes a dual specific phosphatase. The PTEN protein negatively regulates cell migration, cell survival and inhibits cell proliferation. Germ line mutations of *PTEN* lead to the development of the related hereditary cancer predisposition syndromes Cowden disease and Bannayan-Zonana syndrome [2]. The *PTEN* gene is a frequently inactivated gene in many tumour types. In prostate cancer, *PTEN* is even the most common altered gene. *PTEN* inactivation is thought to be a late event in prostate cancer [3, 4]. Mechanisms of *PTEN* inactivation are homozygous deletion [5-8], mutation [3, 4, 9] and promoter hypermethylation [10], although evidence for promoter hypermethylation in clinical prostate cancer specimens is lacking [11].

Several studies showed a prognostic value of PTEN down-regulation in different tumour types, including hepatocellular carcinoma and gastrointestinal tumours [12, 13]. In breast cancer loss of PTEN expression was associated with invasiveness and lymph node metastasis [14]. In prostate cancer loss of PTEN expression has been reported to correlate with tumour stage and grade [15, 16]. Fenic et al. (2004), however, did not find a clear association between decreased PTEN expression and prostate cancer stage [17]. Halvorsen et al. (2003) showed that PTEN protein expression was lost in 27 percent of radical prostatectomy specimens. Combined loss of PTEN and $p27^{kip1}$ expression was detected in 18 percent of the samples, and was an independent predictor of biochemical recurrence after radical prostatectomy [18]. All studies on prognostic value of PTEN protein expression in prostate cancer were solely based on immunohistochemistry.

Currently no information is available on the frequency of aberrant PTEN expression in combination with analyses of genetic mechanism and prognostic value in radical prostatectomy specimens. Genetic analysis can support immunohistochemical results and in addition can unravel the inactivation mechanisms. In the present study we performed such a combined analysis, including assessment of PTEN expression, mono- or bi-allelic loss and mutations in radical prostatectomy specimens. Furthermore, the prognostic significance of these results was investigated.

MATERIAL AND METHODS

Patients

The study was approved by the institutional ethical committee. Tumours from prostatectomy patients were retrospectively selected for inclusion in the tissue microarray. Following surgery, serum PSA levels were monitored every 3 months during the first year, bi-annually in the second year and yearly thereafter. PSA progression was defined according the following criteria: (1) the PSA levels should be ≥ 0.2 ng/ml for at least two successive times, with a minimal interval of three months and the levels should remain high (≥ 0.2 ng/ml) (unless hormonal treatment was initiated). (2) a single observation of PSA > 1 ng/ml followed by an elevated PSA (≥ 0.2 ng/ml). PSA levels ≥ 0.2 ng/ml occurring in the first 3 months after radical prostatectomy were not considered a biochemical relapse if followed by undetectable PSA (< 0.1 ng/ml). The progression-free survival was defined as the interval between the time of surgery and the first elevated PSA level (≥ 0.2 ng/ml).

Construction of the tissue microarray

A tissue microarray of formalin-fixed, paraffin-embedded radical prostatectomy specimens from 90 patients (Gleason score 5-8) was generated. Ten benign prostate specimens were added as normal controls. Construction of the tissue microarray was essentially according to Kononen et al (1998) [19]. Of each tumour, four tissue cores, two cores per Gleason pattern, were included in the array. A sample near the tissue array cores was taken for subsequent DNA extraction.

Immunohistochemistry

Microwave pre-treatment of tissue arrays for antigen unmasking was applied in 10 mM sodium citrate buffer (pH 6.0) for 15 min and cooling at room temperature for 30 min. The primary monoclonal antibody against PTEN (Clone 6H2.1 1:200; Cascade Biosciences, Winchester, MA) was incubated overnight at 4º C, and biotin labelled secondary rabbit-anti-mouse E0413 antibody (1:400; DAKO A/S, Glostrup; Denmark) was incubated at room temperature for 1 h. Immunoreactivity was visualized by streptavidin-peroxidase incubation (1:50, Biogenex, San Ramon, CA). PTEN reactivity was independently assessed by two observers (H. Korsten, P. Verhagen). Cytoplasmic staining of the luminal epithelial cells was scored as absent (0), weak (1), moderate (2) and strong (3). If PTEN expression was completely absent in at least one core, the tumour was considered negative.

Array-CGH

Tissue blocks were counterstained in 4,6-dimidino-2-phenylindole (DAPI) and placed under a fluorescence microscope, enabling a precise selection of the tumour area. Microdissection of the tumour area was performed using a hollow bore needle coupled to the microscope. Lower boundaries were checked for the presence of tumour on 4 μm H&E-stained tissue sections. DNA was extracted using the Puregene DNA isolation kit (Gentra Systems, Minneapolis, MN).

The chromosome 10 BACs were part of the human version 2.0 whole genome BAC array as provided by the UCSF Array Core. Each array consisted of 2460 BAC clones spotted in triplicate on chromium slides. The resolution of the array is ~1.4 Mb. The array-CGH protocol and details regarding the imaging system and software were described previously [20]. In clinical prostate cancer specimens, DNA from normal cells causes an increase of the theoretical log2 T/N ratios indicative for mono- and bi-allelic gene losses. Also heterogeneity within a tumour can result in an increase of the expected log2 T/N ratio. Based on observations of the individual whole genome arrays and the specific chromosome 10 profiles, in this study a log2 ratio cut-off of ≤ -0.7 was chosen as indicative of homozygous deletion. A log2 ratio ≥ -0.7 and < -0.3 was regarded as loss of one allele.

Mutation analysis

For mutational analysis of tumour DNA, all nine *PTEN* exons were individually amplified by PCR for 35 cycles at 55 ˚C. Two overlapping fragments were generated for the exons 5, 8 and 9 to cover the whole coding region. The PCR products were analyzed on 1% agarose gel and purified using Qiaquick gel extraction columns according to the manufacturer's description (Qiagen, Hilden, Germany). Purified fragments were cycle-sequenced using the ABI BigDye Terminator kit v3.1 ready reaction cycle sequencing kit (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. Identical primers were used for both amplification and sequencing. Sequence reactions were run on the ABI 3100 Genetic Analyzer (Applied Biosystems).

Primer pairs used:

Exon 1	PTENE1-F 5' CAAGTCCAGAGCCATTTCCATC 3'
	PTENE1-R 5' CTAAGAGAGTGACAGAAAGGTA 3'
Exon 2	PTENE2-F 5' TTAGTTTGATTGCTGCATATTTC 3'
	PTENE2-R 5' GTCCATTAGGTACGGTAAGC 3'
Exon 3	PTENE3-F 5' CTGTCTTTTGGTTTTTCTTG 3'
	PTENE3-R 5' TACCTCACTCTAACAAGCAG 3'
Exon 4	PTENE4-F 5' CACAGCATAATATGTGTCAC 3'
	PTENE4-R 5' TACAGTCTATCGGGTTTAAGT 3'
Exon 5	PTENE5-FI 5' GCAACATTTCTAAAGTTACCTA 3'
	PTENE5-RI 5' TCATTACACCAGTTCGTCC 3'
	PTENE5-FII 5' TCATGTTGCAGCAATTCAC 3'
	PTENE5-RII 5' CTGTTTTCCAATAAATTCTCA 3'
Exon 6	PTENE6-F 5' GAAATAACTATAATGGAACA 3'
	PTENE6-R 5' ATGGAAGGATGAGAATTTCAAGC 3'
Exon 7	PTENE7-F 5' ATCGTTTTTGACAGTTTG 3'

	PTENE7-R 5’ TCCCAATGAAAGTAAAGTACA 3’
Exon 8	PTENE8-FI 5’ TGCAAATGTTTAACATAGGTGA 3’
	PTENE8-RI 5’ CTTGTCATTATCTGCACGCT 3’
	PTENE8-FII 5’ GAAAATGGAAGTCTATGTG 3’
	PTENE8-RII 5’ CAGCTGTACTCCTAGAATTA 3’
Exon 9	PTENE9-FI 5’ GTTCATCTGCAAAATGGA 3’
	PTENE9-RI-R 5’ GATCAGAGTCAGTGGTGT 3’
	PTENEx9-FII 5’ TAGTGACAATGAACCTGATCA 3’
	PTENE9-RII 5’ GGTAATCTGACACAATGTCCTA 3

Statistical analysis

Multivariate logistic regression analysis (with the backward method) was performed linking the following variables to PSA progression: pre-treatment PSA, pT stage, Gleason sum score and PTEN expression. PSA progression free survival was calculated using the Kaplan-Meier method with the log rank significance test. The correlation between array-CGH results and PTEN expression was calculated by Spearman rank analysis.

RESULTS

Immunohistochemical analysis of PTEN expression

A tissue microarray, composed of radical prostatectomy specimens from 90 prostate cancer patients and 10 benign prostate tissues, was stained for PTEN expression. The prostate samples from patients with benign disease all showed homogeneous, moderate PTEN expression (score 1-2). The expression levels of the tumour tissue cores varied from absent (score 0) to strong (score 3) (Figures 1 A-D). In 35 cases (39 %) at least one out of four cores taken from each tumour tissue was completely negative for PTEN. From 13 patients (14%) all available tumour cores were completely PTEN negative. More frequently, a heterogeneous staining pattern was observed with absence of PTEN in part of the cores.

Array-CGH and mutation analysis

DNA from paraffin-embedded tumour tissues from 62 of the 90 patients was available to analyze by array-CGH. BAC RP11-129G17 on the array was closest to *PTEN,* mapping approximately 200 kbp distal of *PTEN.* Loss of this BAC was taken as indicative of loss of *PTEN*. In 15 tumour samples (24 %), loss of RP11-129G17 was found. In 9 tumour DNAs (15 %) array-CGH data indicated deletion of both copies of *PTEN*, and in 6 samples array results showed loss of one copy. Examples of the chromosome 10 array-CGH profiles of tumour DNAs, showing mono- or bi-allelic *PTEN* loss are depicted in Figure 2.

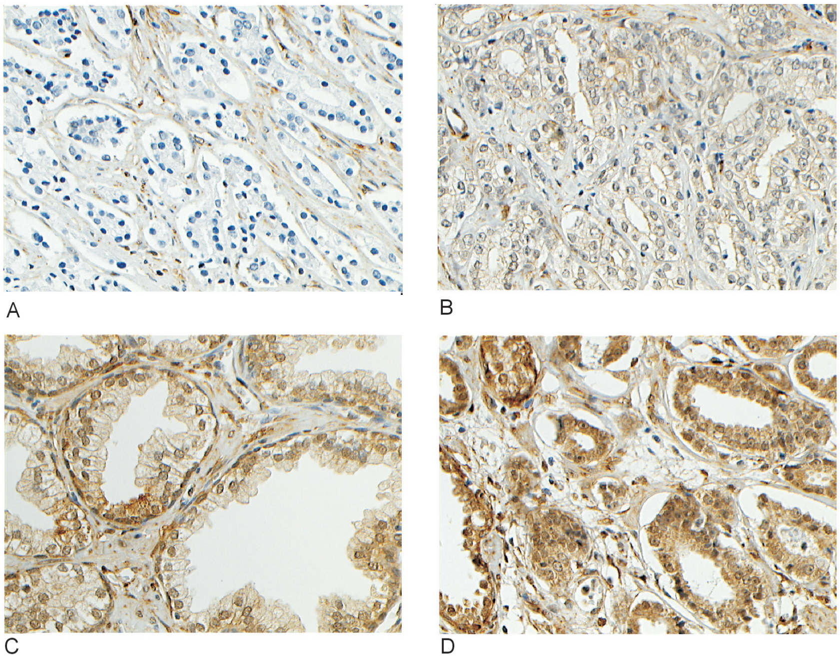

Figure 1. Examples of immunohistochemical staining for PTEN expression in radical prostatectomy samples. A, score 0: no PTEN expression. B, score 1: weak PTEN expression. C, score 2: moderate expression. D, score 3: strong expression. Magnification: 200x.

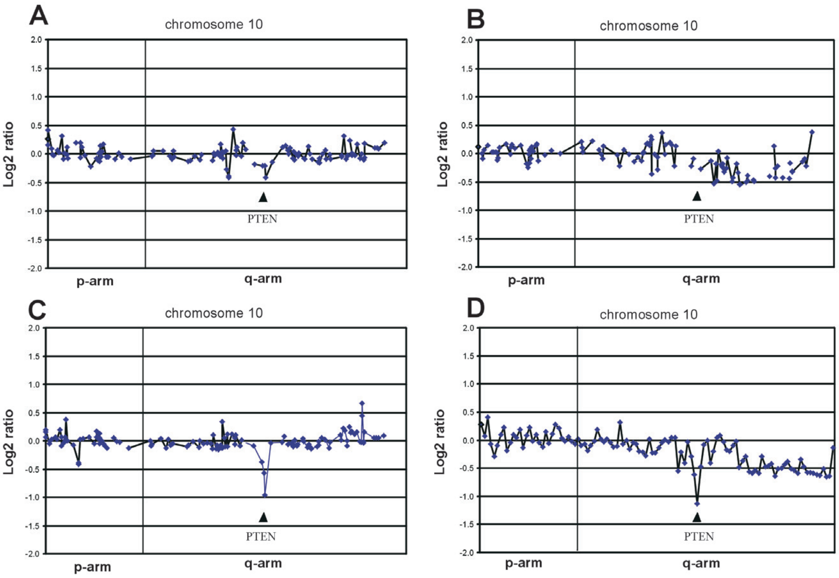

Figure 2. Examples of chromosome 10 array CGH profiles of DNAs from prostate tumours obtained by radical prostatectomy. The arrow head points to the *PTEN* locus. A: tumour with a small region of mono-allelic *PTEN* loss. B: tumour with loss of the major part of 10q, including *PTEN*. C: tumour with a small region of bi-allelic *PTEN* loss. D: tumour with a homozygous deletion of *PTEN* in a large region of 10q loss.

In Figures 2A and 2C, the array-CGH profiles indicate small regions of loss of one or two copies of 10q23.3, including *PTEN*, respectively. Figure 2B shows the chromosome 10 profile of a tumour with a large region of 10q loss, including *PTEN*. Figure 2D depicts an example of a large region of 10q loss, including a *PTEN* homozygous deletion. However, the majority of homozygous deletions of *PTEN* (7 out of 9) were in a small region of 10q loss (see Figure 2C).

We screened the *PTEN* exons of all 6 DNAs showing loss of one *PTEN* allele for mutations in the second allele. However, in none of the samples a *PTEN* mutation was found.

Correlation between array-CGH data and PTEN expression data

Twenty-three out of the 62 tumours analyzed by array-CGH were PTEN negative. As expected, a strong inverse correlation was observed between bi-allelic loss of *PTEN* and PTEN expression (Figure 3; p: 0.006). Only in two cases immunohistochemical staining indicated PTEN expression, whereas genetic studies showed deletion of *PTEN*.

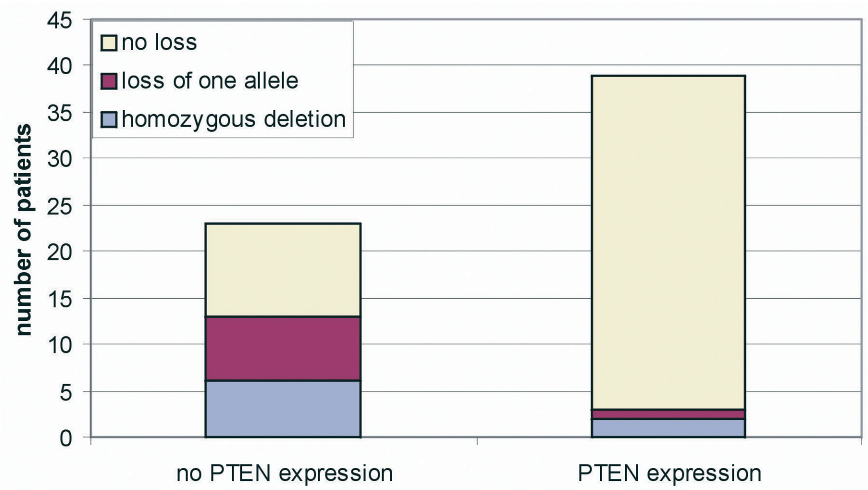

Figure 3. An inverse correlation between PTEN expression and loss of the *PTEN* locus in radical prostatectomy specimens from prostate cancer patients. Note that tumours without PTEN expression showed loss of 10q23 in 52 % of the cases, in contrast to patient samples positive for PTEN (8%).

Not only tumour samples with bi-allelic loss of *PTEN* showed absence of PTEN protein expression, but also those with loss of one *PTEN* copy showed a significant correlation with absence of PTEN expression (Figure 3, p: 0.013). Because the second *PTEN* allele in these samples was intact, non-structural mechanisms should account for the lack of PTEN protein expression. Eleven tumour samples stained negative for PTEN expression, although we did not have evidence that the *PTEN* gene was altered.

Correlation between PTEN protein expression and PSA recurrence

Long-term clinical follow-up data were available for all 90 patients. In total 59 of the 90 patients (65%) had PSA recurrence during the follow-up period. Absence of PTEN expression in at least one core in the tissue microarray was associated with an increased risk for PSA progression, although the difference was not statistically significant (Figure 4; logrank p: 0.058).

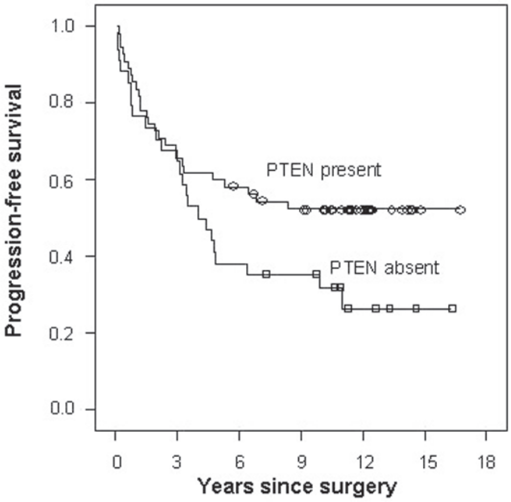

Figure 4. Progression free survival of prostate cancer patients after radical prostatectomy. More frequent biochemical recurrence is seen in the patient group negative for PTEN protein expression (p value: 0.056, logrank). Squares: no PTEN protein expression; circles: PTEN protein expression.

In multivariate analysis pre-treatment PSA, Gleason sum score, pathological stage and PTEN protein expression were correlated to PSA recurrence. Gleason sum score and pathological stage were independent predictors of PSA recurrence, in contrast to pre-treatment PSA and PTEN expression.

DISCUSSION

The PI3K/PTEN signalling pathway has drawn attention as promising target for prostate cancer therapy. In addition, absence of PTEN is a potential prognostic factor, which might be important because of the growing detection of early and potentially non-aggressive prostate cancer cases. Currently, it is very difficult to select patients with early cancers for radical treatment. In radical prostatectomy specimens there is only limited information available on PTEN changes and its prognostic value.

The present study was undertaken to assess the frequency and prognostic value of *PTEN* inactivation in a set of 90 radical prostatectomy specimens with long- term follow-up information available. Moreover, the underlying genetic changes of *PTEN* in the tumours were assessed.

PTEN expression was found to be absent in 39% of the radical prostatectomy specimens. However, many tumours showed absence of PTEN expression in part of the cores, whereas in other cores PTEN could clearly be detected. In our analyses, we scored a tumour negative for PTEN if the protein was absent in at least one tissue core. The percentage tumours, which were PTEN negative is higher (39%) than that reported by Halvorsen et al. (2003) (27%)[18]. McMenamin et al. (1999) found 20% of the prostate tumour tissues totally negative for PTEN and 64% with a mixed staining pattern of negative and positive glands [16]. The differences in percentages PTEN negative tumours between these studies and ours might be explained by the criteria used for PTEN protein expression scoring and the heterogeneity of a tumour.

To get insight in the underlying genetic changes of *PTEN* inactivation the tumours were screened by array-CGH for chromosome 10q losses, including mono-allelic and bi-allelic loss of *PTEN*. Array-CGH showed bi-allelic loss of *PTEN* in 9 (15 %) and mono-allelic loss in 6 (10 %) out of 62 cases, respectively. Mutations in *PTEN* were not detected. A strong correlation was seen between absence of PTEN expression and loss of one or two alleles (Figure 3).

The frequency and mode of *PTEN* inactivation reported so far at various stages of clinical prostate cancer are variable. Homozygous deletions of *PTEN* have been detected in up to 10 percent of locally confined cancers and metastases [3, 7, 8, 21] and in 30 percent of prostate cancer cell lines and xenografts [5]. The frequency of homozygous deletions in radical prostatectomies found in this study (15 %) is considerably higher than previously described in two other studies of primary tumours obtained by radical prostatectomy by Cairns et al. (1997) [8] and Dong et al. (1998) [3], but comparable with the study of Wang et al. (1998) [7]. We propose that the higher frequency observed in our study and in the study of Wang et al. (1998) is the result of the higher sensitivity of the technical approach applied (array-CGH and Southern blotting, respectively). The array-CGH data of clinical prostate cancer extend our previous observations in xenografts [6], that loss of a small chromosomal region, encompassing *PTEN* is a major mechanism of *PTEN* inactivation.

We did not find point mutations in *PTEN*. Other studies reported loss of one *PTEN* allele combined with mutation of the second allele at varying frequency. In agreement with our data, low frequency of point mutations in primary tumours has been described by others [3, 4, 9]. Point mutations in *PTEN* were most frequently found (15 %) in primary tumours from Chinese patients [22]. The reported frequency of point mutations in *PTEN* in metastases is 20 to 30 percent [21, 22] and 25 percent of xenografts and cell lines [5]. In summary we propose that deletion of *PTEN* might be an early event in prostate cancer development, whereas mutation of *PTEN* is a later step in tumour progression.

There are several explanations for the lack of PTEN expression in the samples with loss of one *PTEN* allele, and in samples without genetic alteration of *PTEN*. First, although hypermethylation of the *PTEN* promoter seems to be a rare event in clinical prostate cancer [11], it cannot be completely excluded. *PTEN* expression might also be down-regulated by other not yet identified epigenetic events.

PTEN expression was reported to correlate inversely with tumour grade and stage [16] and biochemical progression if combined with p27kip1 expression [18]. Although in our study, both in multivariate and univariate analysis, absence of PTEN expression correlated to PSA progression, this correlation was not statistically significant. Ideally, to assess prognostic value a collection of untreated tumours should be studied. In clinical cancers, however, only highly selected patients are left without any treatment. In these subjects only biopsy material is available, with the problems of sampling error and limited material for repeated analyses. A cohort of radical prostatectomies as investigated in this study is the second best population to assess prognostic significance of a potential marker

In conclusion, the present study extends previous observations that PTEN is frequently not expressed in radical prostatectomy specimens. An important mechanism of genetic inactivation is by small homozygous deletion. Also, in most tumours showing mono-allelic loss no PTEN expression was detected. Cases with loss of PTEN expression were at a moderately increased risk for biochemical progression after radical prostatectomy, although PTEN expression was not a statistical significant predictor of PSA recurrence.

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Chapter 7

GENERAL DISCUSSION

MOLECULAR ANALYSES OF PROSTATE CANCER

Prostate cancer is an important health problem and the second cause of male cancer death in Western countries. A better understanding of the molecular processes underlying prostate cancer is important to develop new therapies for metastatic disease, for which at present no curative therapy is available. Prostate cancer incidence has increased in the past decade due to the use of the PSA serum test. Not every patient has an aggressive cancer, but at present it is not possible to accurately distinguish those cancers that need immediate treatment and those that may be managed expectantly. It is important to identify prognostic markers that can predict the course of the disease.

The hallmark of the cancer cell is abnormal DNA content and abnormal growth. In prostate cancer DNA abnormalities especially involve deletions of small or large parts of the genome. Amplifications of DNA also occur, but less frequently. The Knudson 2 hit hypothesis assumes inactivation of a tumour suppressor gene by a genetic alteration in both DNA copies of the gene. This can be an inactivating mutation in one allele and a deletion of the second allele, or a deletion of both copies (homozygous deletion). Knowledge of the precise location of commonly deleted regions can thus point to important tumour suppressor genes, both in hereditary and sporadic prostate cancer. When activated, both copies of most autosomal genes are transcribed and contribute to mRNA formation. Deletion of one copy of the gene may lead to lower mRNA and protein levels and thus cause a gene dosage effect, which may contribute to tumour formation (haploinsufficiency).

Apart from genetic changes other processes can influence expression of genes in a cancer cell. An example is silencing of a gene by promoter hypermethylation. The studies in this thesis focussed on genetic changes in cancer cells. DNA changes are the hallmark of a cancer cell. There are many indications that genetic changes are a crucial and early step in the development of cancer. While epigenetic changes are reversible in certain conditions, DNA changes are passed on to daughter cells. DNA is a very stable molecule and changes in DNA can be assessed in tissues that have been stored for many years. An advantage is that a long follow-up is available and that the prognostic value of such changes can be studied.

High-throughput molecular techniques that became available in the past decade made it possible to assess copy number changes of many locations throughout the genome in one experiment. Allelotype analysis, an assessment of relative copy numbers of alleles in a precise genomic location, was followed by CGH (genome wide loss or gain at chromosome band level), genome wide SNP analysis and array-CGH (copy numbers at BAC level). The studies described in this thesis all look at DNA changes in specified prostate tumour collections. In Chapter 3 CGH was used in a selection of familial prostate cancers, identified by the Utah Population Database. In Chapter 4 a combination of allelotype analysis and CGH was used in a panel of xenografts and cell lines and in a set of clinical specimens. This study focused on changes at chromosome 6. The studies described in Chapters 5 and 6 focused on the *PTEN* tumour suppressor gene. This gene was identified in 1997, and is to date the most frequently inactivated gene in prostate cancer. In addition to array-CGH, complementary approaches were used to get a complete view of the genomic changes in the *PTEN* region.

LASER MICRODISSECTION, DOP-PCR AND CGH IN FAMILIAL PROSTATE CANCER

A positive family history is among the strongest epidemiological risk factors for prostate cancer. In 1992, Carter et al. estimated that approximately 10% of all prostate cancers are hereditary with an autosomal dominant pattern of inheritance [1]. More recently, three candidate susceptibility genes have been described, namely *ELAC2* (chromosome 17p11) [2], *2'-5'-oligoadenylate-dependent ribonuclease L* (*RNASEL*) (chromosome 1q24-25) [3], and *Macrophage Scavenger Receptor 1 (MSR1)* (chromosome 8p22-23) [4]. *BRCA2*, one of the breast cancer susceptibility genes is also associated with increased prostate cancer risk (odds ratio, 4.78) whereas the risk in *BRCA1* mutation carriers is not significantly increased [5]. These genes appear responsible for only part of familial prostate cancer cases and appear not to play an important role in sporadic cases [6].

At the time that the study described in Chapter 3 was undertaken no susceptibility genes were identified yet. Linkage analysis suggested several regions to be linked to hereditary prostate cancer: 1q24-25 [7], Xq27-28 [8], and 1q42.2-43 [9]. More recently, a joined analysis by several groups (426 families from 4 study populations) identified additional regions with evidence for linkage, namely: 17q22, 2q32, 15q11, Xq25 and 6q22 [10].

Our hypothesis was that familial prostate cancer harboured unique genetic changes compared to sporadic cases. We used CGH to obtain genome wide information on DNA copy number changes. Current technology allows array-CGH analysis, which gives much more detailed information on copy number changes in precise locations. This technique was not available at the time of the study. Heterogeneity of prostate tumours is a problem in molecular analyses. Presence of non-malignant cells (stromal cells, leukocytes, vessels) is another problem that can influence results from molecular analysis. Laser capture microdissection was chosen in this study because it has the advantage of obtaining a tumour sample without the interfering non-malignant cells. The sample is too small, however, to obtain sufficient DNA for CGH. DOP-PCR was used to obtain an adequate amount for this test. A risk of DOP-PCR is that certain regions are better amplified than others, which could influence the results. By applying the same procedure to the reference DNA, this risk was minimized.

The finding of abnormalities documented in previous studies on sporadic prostate cancer (loss of 7q, 10q, 16q and gain of 8q) illustrates that the combination of DOP-PCR and CGH is feasible in paraffin embedded materials. These changes were present in both familial and sporadic tumours. Distinctive abnormalities included loss of 3p12-3p22 in three of six HPC-1 linked cases and in two of three X-linked cases and gain of 6q11-6q21 in two each of HPC-1 and X-linked tumours. Chang et al. investigated 188 families with increased prostate cancer risk which also included other cancers. Interestingly, a significant evidence for linkage was observed between susceptibility to all cancers (putatively general cancer susceptibility locus) and markers at 3p24, close to the 3p region found in our study [11]. Rokman et al. describe a locus at 3p25-26 with linkage to prostate cancer in 16 Finnish families [12]. Larson et al. conducted a linkage analysis of 80 candidate genes in 201 brother pairs affected with prostate cancer. A high LOD score was reported for 3p14.2. Involvement of germ line variations of *FHIT* at 3p14.2 was reported to be associated with increased prostate cancer risk [13]. So, although several reports, including the study described in Chapter 3, indicate chromosome arm 3p to harbour gene(s) involved in hereditary prostate cancer, there appear to be multiple loci and currently definite conclusions cannot be drawn. A positive conclusion from this study is that DOP-PCR in combination with CGH is feasible on DNA from formalin fixed, paraffin embedded materials. In addition, the results suggest that familial prostate cancer carries unique DNA changes.

Current experimental evidence supports the hypothesis that familial risk is due to inheritance of multiple moderate-risk genetic variants. This is unlike hereditary breast cancer where the majority of cases are accounted for by BRCA1 or BRCA2 germ line mutations. It remains uncertain why the techniques that identified those genes in breast cancer fail to identify the counterparts for prostate cancer. Apart from being caused by multiple genes, different models of Mendelian inheritance, incomplete penetrance and varying population ethnicity frequencies may contribute to the difficulties in identifying genes involved in prostate cancer predisposition.

DELETION OF CHROMOSOMAL REGION 6q14-16 IN PROSTATE CANCER

Regions of chromosomal loss are thought to contain recessive tumour suppressor genes. Regions with frequent loss in prostate cancer include (part of) chromosome arm 6q, 8p, 10q, 13q and 16q. In Chapter 4, a detailed analysis of chromosome 6 in DNAs from prostate cancers was performed, in order to define a region for subsequent search for cancer genes. The study was done on DNA from four prostate cancer cell lines and eleven xenografts. The absence of interfering normal cells make these materials ideal for molecular analysis, especially for the detection of homozygous deletions. A possible disadvantage is that these tumour models may have acquired genetic changes during the process of their establishment.

We used comparative genomic hybridization and allelotype analysis with polymorphic microsatellite markers. Loss of proximal 6q was studied in more detail by high-density allelotype analysis of xenografts, cell lines and nineteen prostate tumour specimens from transurethral resections (TURP). Loss of heterozygosity was detected in nine out of nineteen TURP specimens, indicating that the results found in the xenografts and cell lines also applied to clinical tumours.

Homozygous deletions are usually small, because cells cannot survive if essential genes are missing. They have lead to the identification of several important tumour suppressor genes, both in hereditary and sporadic disease (*BRCA2, Rb, WT1, PTEN, DPC4* [14-16]). In our panel of 15 xenografts and cell lines, 5 showed homozygous deletions in the *PTEN* region on chromosome 10 [17]. We screened chromosome arm 6q for homozygous deletions in order to define a region for subsequent search for tumour suppressor genes.

A common minimal region of loss was found at 6q14-16 with a length of 8.6 Mbp flanked by the markers D6S1609 and D6S417. One hundred and twenty-three Sequenced Tag Sites (STSs), genes and candidate genes mapping in this interval were used to screen the xenografts and cell lines for homozygous deletions, but none were detected. The use of array-CGH or SNP analysis, not available at the time of the study, would have been a much faster technique to screen the samples for homozygous deletions. Now, it has been performed on the panel of 15 xenografts and cell lines, but no homozygous deletions were found at chromosome arm 6q (Hermans, unpublished). Also in the literature, no homozygous deletions at chromosome 6q have been reported to date, not in prostate cancer nor in any other cancer. From the fact that homozygous deletions were not found, one cannot conclude that they were not there. Small homozygous deletions may have been missed. It is also possible that genes crucial for cell survival are in the direct vicinity of a tumour suppressor gene, which may cause a strong selection against cells with a homozygous deletion. Cells with homozygous deletions extending into these neighbouring genes will not be able to survive.

Loss of chromosome arm 6q appeared to be of no prognostic value in the TURP samples. However, this group was mainly composed of tumours progressive under hormonal therapy, which is not the optimal group to assess prognostic value. Chromosome region 6q14-16 was deleted in approximately 50% of the prostate cancer specimens analyzed. The high percentage of loss underscores the importance of genes within this region in prostate cancer growth. Apart from the possibility that this region contains a classical tumour suppressor gene, characterized by genetic inactivation of both alleles in accordance with Knudson's two hit hypothesis, loss of one chromosome arm may induce a gene dosage effect (haploinsufficiency), which implies that the lower concentration of a gene product from this region results in a cell survival advantage.

THE *PTEN* GENE IN CLINICAL PROSTATE CANCER IS PREFERENTIALLY INACTIVATED BY BIALLELIC GENE DELETION

PTEN is currently the most frequently inactivated gene in prostate cancer. The study in Chapter 5 describes the findings of a comprehensive analysis of *PTEN* status in 40 locally progressive prostate cancer specimens. Knowledge of *PTEN* aberrations may become important as new therapies targeting the PTEN phospho-Akt pathway are being tested in a clinical setting.

Previous studies have shown that homozygous deletion is a frequent mechanism of *PTEN* inactivation in xenografts and cell lines (5 out of 15) [17, 18]. Homozygous deletions are difficult to detect in clinical specimens. In allelotype analysis, absence of a signal, characteristic for homozygous deletion, is obscured by low numbers of non-malignant cells present in the material. FISH is possible but it is technically challenging. Array-CGH is a rapid method that can detect homozygous deletions but it has only recently become available, is expensive and requires a relatively large amount of DNA or an amplification step. The *PTEN* wild-type to pseudogene ratio assessment (WPR) was used as a complementary method to the above mentioned techniques. An advantage is that it can be performed on a small DNA sample. In addition it is fast and cheap. Disadvantages are that chromosome 9 copy number changes influence the result. Although in prostate cancer chromosome 9 changes are not very frequent [19], in some cases the ratio may not reflect the *PTEN* copy number changes. The problem of interspersed normal cells in tumours that may obscure presence of a homozygous deletion is not solved with the *PTEN* wild-type to pseudogene ratio assessment. If considerable numbers of normal cells are present in the sample the ratio will be influenced. The cut off levels for assessment of homozygous deletion and loss are therefore difficult to define. *PTEN* wild-type to pseudogene ratio assessment can very well be performed on microdissected tissues.

PTEN wild-type to pseudogene ratio assessment makes use of the sequencing reaction to obtain a semi-quantitative estimate of DNA copy numbers. Other techniques may be more suitable to obtain a quantitative result, which may improve the accuracy of the method. Ultimately, *PTEN* wild-type to pseudogene ratio assessment may be developed into a new, high-throughput, clinical tool.

Genetic *PTEN* inactivation nor absence of PTEN protein expression appeared to have prognostic value in the investigated series of TURP specimens. The majority of the tumours investigated were progressive under hormonal therapy and therefore cases with an indolent nature were not present in the sample. The question of prognostic value in radical prostatectomy cases was addressed in Chapter 6. Absence of PTEN expression occurs frequently in TURP specimens (39%), and this may imply sensitivity to agents interfering with the PTEN phospho-Akt pathway.

PROGNOSTIC VALUE OF *PTEN* INACTIVATION IN RADICAL PROSTATECTOMY SPECIMENS

PSA based screening and case finding lead to diagnosis of a considerable number of early prostate cancer cases that do not need aggressive therapy. There is a great need for accurate prognostic factors. In prostate cancer *PTEN* is the most frequently inactivated tumour suppressor. In Chapter 6, a tissue microarray containing radical prostatectomy samples was used to assess PTEN expression. Immunohistochemical scoring for PTEN was possible in 90 cases. Thirty-five cases were partially or completely negative for PTEN. In 9 out of 62 cases, array-CGH cases pointed to homozygous deletion in *PTEN* and in 6 additional cases loss of 1 allele was found. The samples with loss of 1 allele were sequenced but no mutations were found. There was a good correlation between presence of homozygous deletion or loss of 1 allele and absence of PTEN expression. Promotor hypermethylation may play a role in the samples with loss of 1 allele. PTEN haploinsufficiency has also been suggested as mechanism for tumour formation, at least in animal models [20, 21]. However, this does not clarify complete absence of the protein.

PTEN expression was a predictor of PSA recurrence in this series, however in multivariate analysis only pT stage and Gleason sum score were independent predictors of biochemical progression. Absence of PTEN expression has only limited value in predicting PSA recurrence after radical prostatectomy. Although not a very good prognostic factor, absence of PTEN expression occurs frequently in radical prostatectomy specimens (39%), and this may imply sensitivity to agents interfering with the PTEN phospho-Akt pathway. A clinical trial testing these agents should focus on such cases, which can be readily identified by genetic analysis or immunohistochemistry.

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Chapter 8

EPILOGUE

ABERRANT TRANSDIFFERENTIATION OF BONE MARROW-DERIVED CELLS AS THE BASIS OF CANCER

A hypothesis based on observations in prostate cancer

Paul C.M.S. Verhagen

SUMMARY

The conventional concept of cancer development presupposes a monoclonal origin by dedifferentiation of a single cell. Recently, it has been postulated that stem cells are the target susceptible to malignant transformation. In this article, the hypothesis is put forward that the stem cells at stake are in fact bone marrow-derived. Cancer is presumed to arise from a distorted transdifferentiation process.

Transdifferentiation is the infrequent phenomenon that in adults bone marrow-derived cells transform into epithelial or mesenchymal cells. It has been shown to occur in various tissues including liver, muscle, brain and skin. The factors that direct bone marrow-derived cells through the transdifferentiation process are unknown. The presented hypothesis assumes that a disruption of this process results in cells with intermediate phenotypes. They thrive in the circulation, lymphatic system and bone marrow and in addition have epithelial characteristics. Genetic changes are acquired during the disrupted transdifferentiation process. This leads to a heterogeneous, polyclonal collection of cells with similar, but not identical genetic changes. The continuing generation of cells with genetic abnormalities will in time lead to aggressive phenotypes, which will dominate the population and determine the clinical course of the disease. Importantly, a direct link between transdifferentiation and the development of gastric cancer has recently been shown in a mouse model.

In this hypothesis prostate cancer has two compartments: one, a site in the prostate where new bone marrow-derived cells are recruited and two, the collection of derailed, partially differentiated cells present both in the prostate and in the metastases. Removal or irradiation of the primary tumour leaves one compartment which may then be susceptible to palliative therapies, an effect which is diminished when the primary tumour is left untreated and continues to generate derailed cells. Precisely this phenomenon is observed in prostate cancer, where a substantial survival benefit from early hormonal therapy can only be shown when the primary tumour is irradiated or surgically removed.

Aberrant transdifferentiation of bone marrow-derived cells is presented as mechanism for prostate cancer development. This hypothesis explains several observations that were thus far not understood. Importantly, acceptance of this hypothesis will directly affect the interpretation of apparently conflicting results from clinical trials and thus, the clinical management of patients with prostate cancer.

INTRODUCTION

Breast cancer has been described as 'a poorly controlled attempt to produce a breast by different organs'[1]. It is clear that a tumour is not merely an accumulation of aberrant cells, but rather a distorted developmental process. Tumour cells interact with normal counterparts to form a tumour resembling a tissue. Stromal cells and vessels within a tumour are genetically normal, but have developed in cooperation with the aberrant cancer cells.

Stem cells are clonogenic cells capable of self-renewal and generation of intermediate cells (transiently proliferating/amplifying cells). They are capable to differentiate into multiple distinct cell types. Stem cells or intermediate cells are postulated to be the target cells susceptible to malignant transformation because of their longevity and proliferative potential. Several features, including keratin patterns, androgen independence and expression of c-met are present both in intermediate cells and prostate tumour cells [2]. For several malignancies (e.g. myeloid leukemias) so-called 'cancer stem cells' have been identified [3]. In this article the stem cell hypothesis is taken one step further, namely that the target for malignant transformation in the prostate is a stem cell which is bone marrow-derived.

For a long time, the view that tissues developed early in embryogenesis and that cells had limited plasticity thereafter has dominated our thinking. In recent years, numerous reports have shown that certain adult cells possess more plasticity than was previously appreciated. The process of transdifferentiation is still poorly understood and subject to debate. Regardless of the specific mechanism and the rate at which circulating progenitor cells change into differentiated cells, there is little doubt that transdifferentiation does occur [4].

In this article the connection between transdifferentiation and cancer is discussed. Clinical observations are presented that support the hypothesis that transdifferentiation from bone marrow-derived cells might form the basis of prostate cancer. Furthermore, the observations in prostate cancer may reflect a universal connection of transdifferentiation to the development of cancer in general.

TRANSDIFFERENTIATION

In recent years, evidence has been presented that in adults, bone marrow-derived cells can undergo phenotypic changes and become liver, muscle, brain, heart, kidney, skin, and gastrointestinal cells [5-7]. An example of transdifferentiation observed in humans was presented by Theise et al. They studied liver biopsies from 2 female recipients of therapeutic bone marrow transplantations with male donors and from 4 male recipients of orthotopic liver transplantations from female donors. Using FISH for X and Y chromosomes they identified Y-positive hepatocytes and cholangiocytes in all subjects [8]. To rigorously test the phenomenon of transdifferentiation Krause et al. transplanted lethally irradiated mice with a single, marked bone marrow-derived stem cell. They found transdifferentiation to occur into stomach, oesophagus, small intestine, large intestine, liver, kidney, lung and skin [9]. It is uncertain if there is a universal circulating progenitor cell, or if certain progenitor cells exist that are predestined to transdifferentiate into specific tissues [7]. Fusion is proposed as an alternative mechanism to explain some of the results presented in this field [10], although there is firm evidence for true transdifferentiation, without fusion, to occur in lung, liver and skin [11]. Thus, although many questions remain open at this moment, there is evidence that bone marrow-derived cells exist that possess plasticity and can transform or fuse and beget another phenotype, including the epithelial phenotype.

TRANSDIFFERENTIATION AND CANCER

The most direct evidence that connects transdifferentiation to cancer comes from the group of Houghton et al. [12]. They induced metaplasia, dysplasia, intraepithelial neoplasia and invasive gastric cancer in mice by chronic infection with Helicobacter, a known carcinogen. Lethally irradiated mice were transplanted with ROSA26 labelled bone marrow. Chronic Helicobacter infection induced repopulation of the stomach with bone marrow-derived cells. In the precancerous and invasive lesions beta-galactosidase could be demonstrated, which showed that the transdifferentiation process was responsible for the development of cancer. The experiments were repeated in female animals which were lethally irradiated and transplanted with bone marrow from male transgenic mice expressing chicken beta-actin-EGFP (enhanced green fluorescent protein). Again, disrupted transdifferentiation was shown to be responsible for tumour formation by demonstration of EGFP and Y chromosomes in the precancerous and invasive lesions [12].

The literature was searched for reports on patients who developed a cancer in a transplanted organ or patients with a secondary cancer after bone marrow transplantation. Most reports on cancers in transplanted organs (kidney, liver) found them to be of donor origin. However, development of tumours from donor origin in transplanted organs does not exclude the mechanism of transdifferentiation. Examples of epithelial tumours with a bone marrow genotype on the other hand, seriously indicate a role for transdifferentiation in cancer development. Interestingly, 2 reports were identified describing this situation, which is in direct support of transdifferentiation events.

Al-Joundi et al. reported on a 41 year old man who underwent liver transplantation (from a female donor) for chronic hepatitis C and who presented 26 months later with hepatocellular carcinoma [13]. No evidence of hepatocellular carcinoma was found in the native liver. The patient had recurrent hepatitis C. Chromosomal analysis using in situ hybridization with a Y chromosome specific probe showed the tumours to be Y chromosome positive and thus, from acceptor origin. An alternative explanation for these findings is suggested by the authors who interpret the tumours as metastases from a unrecognized hepatocellular carcinoma in the native liver, despite the fact that these seldom develop beyond 24 months and that no other evidence for metastases was found.

The second case was presented by Chakraborty et al. [14]. They described a child who, after allogeneic liver and bone marrow transplants, developed renal cell carcinoma and then metastases. A right caval lymph node was investigated. The renal tumour was not available for analysis. Both the patient and his liver donor were immunotyped as O+; the bone marrow transplant donor was A+. The histology report of the lymph node mentioned presence of a renal cancer metastasis. Twenty-one tumour DNA samples from diverse areas from the lymph node were taken. Sixteen yielded primer products. Of these, 16 of 16 contained the A allele, while 14 of 16 contained the O allele as well. The donor A allele was present throughout the metastasis.

Further evidence for the role of transdifferentiation in the development of malignancy was presented by Labat et al. [15]. This group performed in vitro experiments by culturing mononucleated blood cells taken from a patient with chondrosarcoma. After one and a half months of culture, nodules and multilayered areas composed of fibroblasts and chondrocyte-like cells were observed. The cells were embedded in an abundant extracellular matrix. Immunohistochemistry showed that the cells resulted from transdifferentiation of monocytes. The authors suggested a retroviral origin of the transdifferentiation process, although this could not be substantiated by direct evidence.

THE ISSUE OF CLONALITY

The monoclonal concept of cancer

The traditional concept of development of cancer requires the accumulation of genetic abnormalities in a more or less differentiated cell. From this single cell, daughter cells develop which in their turn acquire more genetic changes. By inactivation of tumour suppressor genes and activation of oncogenes, ultimately the cells obtain an aggressive phenotype. Clonal expansion is held responsible for the heterogeneous phenotype observed in many tumours including prostate cancer [16]. Novelli et al. reported an observation that casts doubt on the assumption that clonal expansion alone is responsible for tumour heterogeneity. In a X0/XY mosaic individual with familial adenomatous polyposis the majority of microadenomas were polyclonal in origin [17].

Clonality of prostate cancer

Prostate cancer often is very heterogeneous and multifocal, with multiple separated foci of cancer within one prostate. The number of tumour foci in radical prostatectomy specimens ranges from 1 to 6, on average more than 2 [18, 19]. Cheng et al. studied the pattern of allelic loss in separate cancer foci and concluded that in 15 out of 18 patients separate tumour foci arose independently [20]. Bostwick et al. studied multiple foci of PIN (prostatic intraepithelial neoplasia) in a similar manner. Also in PIN, multiple foci appear to arise independently within the same prostate [21]. These observations may be explained by assuming a 'field effect' or 'field cancerization'. The concept of 'field cancerization' was first introduced by Slaughter et al [22]. The assumption is that a patch of tissue is formed by one stem cell which has acquired a first molecular step to the development of cancer. From this field, more cells can independently progress to a malignant phenotype [23].

The transdifferentiation model provides an alternative explanation for the heterogeneous and multifocal nature of prostate cancer. According to this hypothesis mobile stem cells are recruited in the primary tumour region. These cells may transform into epithelial cells close to this region or on a more remote place, within the prostate (multifocality) or beyond (lymph nodes, bone). The multifocal nature of prostate cancer reflects that the recruitment is a continuous process, with involvement of multiple mobile stem cells.

BONE METASTASES

Prostate cancer metastases preferentially develop in lymph nodes and bone. Prostate cancer cells are thought to move to bone by haematogenous spreading. However, not all parts of the skeleton are equally affected. In prostate cancer, there is a predominance of bone metastases in the pelvic bone and lumbar spine (Figure 1). In breast cancer conversely, there is a predominance in the ribs, thoracic spine and shoulder region [24, 25]. In head and neck tumours, a predominance is reported in the skull and cervical spine [26]. Evidently, there is a preference for bone metastases to develop in the vicinity of the primary tumour. If hematogenous spreading was the only route to bone metastases, this observation remains unexplained. Except for the portal systems in liver and pituitary gland, veins and lymph vessels transport directly to the right atrium. There is no anatomical explanation for the preferential site of bone metastases. The transdifferentiation hypothesis provides an explanation for this phenomenon. In this view, tumour cells are expected to carry characteristics of there previous phenotype, which includes active movement towards the bone, a feature of leukocytes [27]. The preferential site of metastases reflects active movement of cells towards the bone, in addition to the random distribution that is expected from the blood stream drag. The mobility of partially differentiated cells is expected to subside when the transdifferentiation process proceeds and epithelial characteristics attach cells to their neighbours.

Figure 1 Bone scan showing prostate cancer metastases. Example showing preferential site of metastases in vicinity of primary tumour, illustrating that the distribution of bone metastases is not merely the result of passive transportation by the blood or lymphatic system. Image provided courtesy of Dr. Sam Gambhir (UCLA/Stanford).

ABNORMAL CELLS IN THE CIRCULATION OF THE PROSTATE CANCER PATIENT

Epithelial cells do not circulate. They may be passively transported by the blood stream, but are expected to strand in the first capillary bed they encounter, usually in the lungs. Experiments to assess the fate of tumour cells in the circulation were reported by Fidler, in 1970. He intravenously injected 200.000 radioactive labelled malignant melanoma cells in syngeneic mice. Already after 1 minute, over 68% of the cells were captured by the lungs, leaving only 1.9 % in the circulation [28]. These findings are to be expected, given the large size and firm cytoskeleton of epithelial cells compared to blood cells. But if epithelial cells cannot circulate, what than is the nature of the aberrant cells populating the blood of the cancer patient?

I Cells with chromosomal abnormalities

Cytogenetic studies have revealed the presence of chromosomal changes in a subset of peripheral blood mononuclear cells in patients with lung, renal, colon, breast and prostate cancer [29, 30, 31, 32, 33]. These are non-random changes, the chromosomal regions involved differing among the various tumour types. As an example, the aberrations frequently effect chromosome 5 in patients with colon cancer or adenomatous polyps of the colon [31], chromosome arm 3p in renal cell cancer [30], 1q in breast cancer and 10q in prostate cancer [33]. The frequency of cells with chromosomal aberrations is referred to as aneuploidy index in blood (number of cells with abnormal karyotype / total number analyzed X 100) and ranges from 0 to over 20%. A high aneuploidy index in prostate cancer patients is associated with higher PSAs and advanced disease. Moreover, aneuploid cells were found in the majority of the patients (over 94%) [32]. One way to explain the phenomenon of non-random chromosomal changes in blood cells is by assuming that certain chromosomal regions are susceptible to breakage and translocations, which predisposes the patient to the development of a cancer. In this view these changes occur in all organs and ultimately in one organ a tumour is formed. However, if this is so, why is the number of aberrant cells directly associated with the severity of the disease? In the transdifferentiation hypothesis the abnormal blood cells are descended from incompletely transdifferentiated mobile precursor cells. In this view it is obvious that the longer the tumour has been in place, the more affected descendants there will be.

II Altered mode of allelic replication

During cell division, the temporal order of allelic replication is strictly regulated. Biallelically expressed genes replicate synchronously and monoallelically expressed genes replicate asynchronously. Application of fluorescence in situ hybridization for a biallelically expressed gene to peripheral blood lymphocytes gives 2 single dots (singlets; SS) for a normal resting cell and 2 double dots (doublets; DD) for a normal replicating cell. In prostate cancer patients the mode of allelic replication in peripheral blood lymphocytes appears to be changed. In non-cancer patients the number of SD cells for biallelically expressed genes is low (approximately

10-15%). This number increases to over 30% in prostate cancer patients. An altered mode of replication appeared associated with aneuploidy and was superior to PSA in distinguishing prostate cancer patients from patients with benign disease [34]. In the view of incomplete transdifferentiation, the cells with an altered mode of allelic replication represent descendants of a partially differentiated mobile precursor cell. It demonstrates that a substantial number of peripheral blood cells in a cancer patient is genetically damaged. The strict association with malignant disease suggests it is a result from this disease and not an associated abnormality, merely reflecting susceptibility to cancer.

III PSA positive cells

In an attempt to isolate circulating prostate cells Hamdy et al. applied flow cytometry to peripheral blood mononuclear cells of prostate cancer patients [35]. There appeared to be numerous PSA positive cells, up to 50% of the total number. Later stages of the disease were associated with a higher percentage of PSA positive cells. The cells were isolated and investigated by electron microscopy. They resembled monocytes and did not show epithelial characteristics. In a later publication on this topic isolated cells were investigated by RT-PCR to detect PSA-mRNA. In 1 out of 9 cases PSA-mRNA could be detected in these cells. The authors concluded that most likely the cells belong to the immune system (monocytes) but that definite conclusions could not be drawn [36].

Flow cytometry of peripheral blood cells can identify substantial numbers of PSA/CD14 double positive cells in prostate cancer patients. Possibly, they represent macrophages after phagocytosis of cancer cells. Presence of more than 2% of these double positive cells was associated with prostate cancer and was superior to PSA in distinguishing cancer patients from patients with benign disease [37].

RT-PCR directed to PSA-mRNA isolated from peripheral blood has been used for molecular staging of prostate cancer. The prognostic value of this procedure is controversial. There is no doubt however, that PSA mRNA can be found regularly in the blood of prostate cancer patients [38]. Bringing back to mind the experiments by Fidler (epithelial cells do not circulate) the question remains: what is the source of PSA mRNA in the peripheral blood?

In summary, various techniques have identified substantial numbers of abnormal cells in the circulation of prostate cancer patients. There are cells with (I) chromosomal changes, (II) altered replication or (III) presence of the PSA protein. These changes are strongly associated with cancer. PSA positive cells and cells with chromosomal aberrations are more numerous in advanced disease and better predict tumour stage than serum PSA, the best clinical marker at present. It is uncertain if these cells represent different populations or if the various techniques have looked at the same population: cells with an intermediate phenotype with characteristics of blood and prostate cells and in addition chromosomal changes acquired in the derailed process of transdifferentiation.

HORMONAL TREATMENT AND SURVIVAL: ROLE OF PRIMARY TUMOUR

Early versus late hormonal therapy

The optimal timing of hormonal treatment (early or late) has been subject to debate for decades. Early hormonal treatment is initiated immediately after diagnosis of a non-organ confined cancer, while late treatment is started on a specified moment, e.g. when symptoms develop. In spite of several large trials which directly or indirectly addressed this issue, no specific subgroup could be identified for which a substantial survival advantage of early treatment was demonstrated [39-41] for review see [42]. This was remarkable because in breast cancer, a tumour that is also influenced by hormonal manipulation, the advantages of immediate hormonal treatment have repeatedly been shown [43]. Unlike in prostate cancer, the primary breast tumour is usually treated (surgery or radiotherapy), irrespective of lymph node status.

In 1997 Bolla et al. published the results of EORTC trial 22863 comparing pelvic radiotherapy alone versus pelvic radiotherapy plus immediate hormonal treatment during 3 years in patients with prostate cancer at high risk for metastasis. Although the study looked at adjuvant hormonal therapy, for the 3 year survival results this was actually identical to an early versus late design. A clear benefit in local control and overall survival was reported after 3 years [44]. This attracted a lot of attention and comments because it was totally unexpected by the medical community [45-48]. The main points of criticism were that patients were not precisely staged (no lymph node dissection) and that a third treatment arm (only hormonal treatment) was missing. In the final evaluation of this study the survival advantage for early treatment remained large [49]. As discussed above, patients in this trial had locally advanced disease, and were at high risk to develop metastases. Lymph node dissections were not performed. The majority of patients were clinical T3 (82%), a large proportion had Gleason score 7-10 (35%) and a pretreatment serum PSA above 40 ng/ml (33%)[44]. According to the 1997 Partin tables, the risk for lymph node metastases in this population is 20-58% [50]. A survival advantage was seen after 3 years, which implies that by that time, enough men died from prostate cancer to find a statistically significant difference. One may assume that the patients with metastases at entry were at the highest risk to die from prostate cancer within 3 years. Accordingly, metastatic patients appear to benefit from the combined treatment. In a randomized study by Granfors et al. (1998) comparing orchiectomy and external radiotherapy to the prostate to radiotherapy alone in T1-4 pN0-3 prostate cancer, this was in fact demonstrated. Patients with lymph node metastases benefited from immediate hormonal therapy after definitive external beam radiation to the prostate [51]. More recently, a survival advantage was shown in a randomized study looking at 6 months of hormonal therapy adjuvant to external radiotherapy for localized prostate cancer [52].

Another important study on the topic of early versus deferred hormonal therapy was reported by Messing et al. Here, patients with positive lymph nodes underwent a radical prostatectomy and were then randomized to early or deferred hormonal treatment. This is again an example of a study with systematic treatment of the primary tumour. Also Messing et al. reported a markedly improved survival in patients receiving early treatment [53]. The main problem of this study was its inadequate sample size, which attracted critisism. The necessary sample size to detect a survival advantage was calculated to be 220 and the number actually enrolled in the study was only 100 [48]. Clearly, the survival advantage was unexpectedly large and in conflict with previous results, when not taking the treatment of the primary tumour into account.

Introduction of PSA testing and mortality changes

Introduction of the PSA serum test lead to an increase of prostate cancer incidence and to a decrease of prostate cancer mortality figures. The PSA serum test was introduced in 1986. In the USA a mortality decline was first shown after 1991 (white men) and 1992 (black men) [54]. An even more rapid drop in mortality was reported by Bartsch at al. after introduction of free PSA testing in Tyrol in 1993. Mortality rates were constant between 1993 and 1995 and subsequently fell [55]. The mortality changes following the introduction of PSA are too prompt to be attributable to screening, where a lead time of over 7 years is to be expected. It is therefore likely that a different effect is responsible for the changes observed, presumably the effect of early hormonal treatment in patients with an operated or irradiated prostate, as described above.

In conclusion, early hormonal treatment has been suggested to improve survival in prostate cancer patients, provided the primary tumour is irradiated or removed. This points to a distinctive feature present in the primary tumour that 1) does not respond to hormonal therapy and 2) adversely affects survival. If irradiated or removed, early hormonal treatment is superior. The apparently conflicting results illustrate that there is a qualitative difference between the primary tumour and the metastases. In the transdifferentiation hypothesis, recruitment of bone marrow-derived cells takes place only in the primary tumour. The collection of derailed, partially differentiated cells present both in the primary tumour and in the metastases forms the heterogeneous population resulting from this process. These cells may be susceptible to palliative hormonal therapy. The ultimate effect of the hormonal treatment upon survival is counteracted by the continuing recruitment of new derailed cells, when the primary tumour is left untreated.

IS CANCER TRANSFERABLE?

The transdifferentiation hypothesis assumes a molecular factor in the tissues that is responsible for the start of the transdifferentiation process. When a susceptible bone marrow-derived cell comes into contact with this factor, the cell changes phenotype. In a cancer, in the primary tumour, an erroneous factor initiates phenotypical changes, but they will be incomplete. Linked to the derailed process, the cell will acquire genetic changes. The molecular culprit must be present in the primary tumour and since it is a molecule, it must be transferable to others. Obviously, a factor responsible for transdifferentiation is expected to act within the direct vicinity of its production, because otherwise tissue boundaries would not exist. This implies contagiousness of cancer, but only when a very close contact between primary site and susceptible cells is possible. Two examples were found that fulfil this requirement and that indeed imply that cancer is to some extent contagious.

The first example comes from malignant melanoma, a skin cancer where direct contact with the primary tumour is possible. A number of case reports mention the development of malignant melanoma in husbands and wives [56]. It may of course be expected that couples are exposed to similar risk factors, such as UV light, but the incidence of malignant melanoma is low and the chances for simultaneous occurrence of malignant melanoma in couples is therefore very low. Importantly, three couples were reported with synchronous malignant melanoma with only axillary metastases and no primary tumour in one of the spouses. Sutherland and Wha Chu comment on this in the New England Journal of Medicine in 1985: "The unusual frequency with which cases with an axillary metastasis from an unknown primary lesion appear in these cases in spouses far exceeds that expected in the general population of patients with melanoma, and probably the frequency of cases occurring in spouses exceeds national incidence figures for melanoma. The reasons for these unusual findings are not apparent and need further investigation." [56]. In the transdifferentiation hypotheses, the continuing production of the molecular factor responsible for the derailed transdifferentiation process is the cause of a primary tumour, but temporary exposition to this factor will cause susceptible cells to form metastases without a primary tumour.

The second example demonstrating transferability of malignancy comes from the observation that donor cells are regularly involved in recurrent leukaemia following bone marrow transplantation [57, 58]. Out of 54 relapses in patients who received a marrow graft from a donor of the opposite sex, 3 relapses were in donor cells (6%) [57]. Similar to malignant melanoma, a direct contact is possible between susceptible cells and the primary tumour site, a prerequisite for transferability of malignancy according to the transdifferentiation hypothesis.

DISCUSSION

In this paper aberrant transdifferentiation of bone marrow-derived cells is proposed as an alternative model of prostate cancer development, in an attempt to clarify a number of remarkable observations in prostate cancer. Aberrant transdifferentiation of bone marrow-derived cells is not necessarily the only explanation for the presented observations. However, it has recently been shown to occur in a well controlled animal model for gastric cancer, which makes the hypothesis extraordinary, but not impossible.

An assumption based on the traditional, monoclonal concept of cancer development is that it is of no use to treat the primary tumour when metastases are present. Evidently, a patient will not be cured by this treatment, but it is very well possible that outcome is influenced. The transdifferentiation hypothesis changes the interpretation of trials addressing the issue of early versus late hormonal therapy. The apparently contradicting results regarding the timing of hormonal therapy are clarified. Trials on this topic should be stratified according to the treatment of the primary tumour. When the primary tumour is irradiated or surgically removed, early hormonal therapy leads to a better survival. This effect is not present with the primary tumour in place, because the beneficial effect of early treatment is counteracted by continuing recruitment of bone marrow-derived cells. Consequently, according to the transdifferentiation hypothesis, treatment of the primary tumour by radiotherapy or surgery must be considered, also in patients with lymph node metastases. This may even hold true for patients with bone metastases. A randomized trial to address this latter issue is currently being performed in the Netherlands (randomized comparison of hormonal therapy alone versus hormonal therapy plus external radiotherapy to the prostate in patients with metastatic prostate cancer).

CONCLUSION

In conclusion, observations in prostate cancer were presented to put forward transdifferentiation of bone marrow-derived cells as the underlying mechanism of cancer formation. This mechanism was recently demonstrated to occur in gastric cancer in a mouse model. Application of this paradigm clarifies apparently conflicting results from clinical prostate cancer trials. Acceptance of the transdifferentiation hypothesis would influence the clinical management of certain prostate cancer patients. An example is that treatment of the primary tumour must be considered, also when metastases are present. Taken together, there is a case for transdifferentiation of bone marrow-derived cells to be the universal basis of cancer development.

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Chapter 9

SUMMARY

SAMENVATTING

SUMMARY

Prostate cancer is an important health problem and in Western countries the second cause of male cancer death. Knowledge of the molecular changes underlying prostate cancer may lead to novel treatments or better prognostic factors.

In Chapter 1 an overview is presented on molecular techniques that can be used to obtain information on DNA copy number changes in cancer cells. Studies using these techniques are collectively called genomics.

In Chapter 2 a review is presented on prognostic factors in localized prostate cancer, with an emphasis on molecular factors. At present, molecular factors play no role in clinical decisions. Several promising factors are discussed and attention is given to the reasons why their role in a clinical setting is limited. It is expected that with time, molecular factors will become important to select patients for curative treatment options and to select patients with advanced disease for targeted therapy.

In Chapter 3 CGH was applied to 13 familial prostate cancers cases, identified by use of the Utah Population Database. DNAs from the paraffin embedded tissues were DOP amplified and used for CGH. Apart from losses known to occur frequently in sporadic tumours (7q, 10q, 16q) in familial cases distinctive abnormalities were found (loss of 3p12-3p22 and gain of 6q11-6q21). The results suggest that familial prostate cancer carries unique DNA changes. Current experimental evidence supports the hypothesis that familial risk is due to inheritance of multiple moderate risk genetic variants.

In Chapter 4, a detailed analysis of chromosome 6 changes in sporadic prostate cancer was performed, in order to define a region for subsequent search for cancer genes. Loss of heterozygosity was detected in nine out of nineteen TURP specimens. A common minimal region of loss at 6q14-16 with a length of 8.6 Mbp flanked by the markers D6S1609 and D6S417 was found. A search for homozygous deletions with one hundred and twenty-three Sequenced Tag Sites (STSs), genes and candidate genes mapping in this interval, was not successful. The high percentage of loss underscores the importance of genes within this region in prostate cancer growth.

Chapters 5 and 6 focus on the tumour suppressor gene *PTEN*, frequently altered during the development of many cancers, including prostate cancer. In Chapter 5, a study on 40 locally progressive clinical prostate cancer specimens obtained by transurethral resection of the prostate was reported. A variety of technical approaches was utilized including allelotype analysis, SSCP and sequencing, FISH, array-CGH, and immunohistochemistry. WPR, a variant to multiplex PCR, utilizing a single nucleotide mismatch between *PTEN* and its pseudogene located at chromosome 9p13 was also applied. Taken together, the data showed complete inactivation of *PTEN* in 30 percent of locally progressive prostate cancer, preferentially by homozygous deletion. Absence of PTEN protein expression was found in 15 tissue samples, heterogeneous PTEN expression was observed in 8 tumour samples. The study confirmed that

PTEN is frequently inactivated in clinical prostate cancer. Within the set of 40 TURP specimens, absence of PTEN expression appeared to have no prognostic significance.

In Chapter 6, PTEN expression was assessed by immunohistochemistry applied to a tissue microarray containing cores from 90 radical prostatectomies with known outcome. From 62 samples adequate tumour DNA was available which was used for array-CGH analysis. Thirty-five cases were partially or completely negative for PTEN. In 9 out of 62 cases, array-CGH cases pointed to homozygous deletion in *PTEN*. There was a good correlation between presence of a homozygous deletion and absence of PTEN expression. Absence of PTEN expression was associated with PSA recurrence. In multivariate analysis, however, only pT stage and Gleason sum score were independent predictors of PSA progression.

The results of the previous Chapters are summarized and discussed in Chapter 7.

In Chapter 8, the hypothesis is presented that aberrant transdifferentiation is the basis of (prostate) cancer. Transdifferentiation is the infrequent phenomenon that bone marrow-derived stem cells change into mesenchymal or epithelial cells. The connection between transdifferentiation and the development of cancer was recently shown in an animal model for gastric cancer. Observations that are consistent with the transdifferentiation hypothesis include: the presence of abnormal blood cells (with DNA abnormalities or altered allelic replication) in prostate cancer patients, the pattern of bone metastases and the observation that the results of randomized trials addressing the issue of early versus late hormonal therapy are dependent on the presence of the primary tumour. The transdifferentiation hypothesis presupposes the presence of an epigenetic factor in the primary tumour to be responsible for the continuing recruitment of bone marrow-derived cells. Examples of epigenetic transfer of malignancy ('horizontal oncogenesis') were presented to illustrate that this can indeed occur in special circumstances.

Confirmation of the transdifferentiation hypothesis would have a big impact on the clinical management of cancer patients. An example is that it should be considered to treat a primary tumour by surgery or radiotherapy, irrespective of the presence of (lymph node) metastases in prostate cancer patients.

SAMENVATTING

Prostaatkanker vormt een belangrijk gezondheidsprobleem. In Westerse landen is het de tweede doodsoorzaak door kanker bij mannen. Kennis van de moleculaire veranderingen die ten grondslag liggen aan prostaatkanker kan bijdragen aan nieuwe therapieën of aan een betere voorspelling hoe een bepaalde tumor zich zal gedragen.

In hoofdstuk 1 wordt een overzicht gegeven van moleculaire technieken die kunnen worden gebruikt om DNA veranderingen in kankercellen te detecteren. Dergelijke studies vallen onder de term: *genomics*.

Hoofdstuk 2 geeft een overzicht van prognostische factoren bij gelokaliseerd prostaatkanker, met de nadruk op moleculaire factoren. Op dit moment spelen moleculaire factoren geen rol bij klinische beslissingen. Een aantal veelbelovende factoren wordt besproken en redenen waarom de rol van deze factoren in de kliniek nog beperkt is worden aangegeven. De verwachting is dat in de toekomst moleculaire factoren gebruikt zullen worden om patiënten te selecteren voor curatieve behandelingsopties en om patiënten met gevorderd prostaatkanker te selecteren voor zogenaamde *targeted therapy*.

In hoofdstuk 3 wordt een studie beschreven uitgevoerd op 13 familiaire prostaattumoren die werden geïdentificeerd in de Utah Population Database. DNA uit het paraffine materiaal werd vermenigvuldigd door middel van DOP-PCR, en hierna gebruikt voor CGH. Naast deleties, waarvan bekend is dat ze vaak optreden bij sporadische prostaattumoren (chromosoom regio's op 7q, 10q, 16q) werden ook deleties gevonden die vooral bij de familiaire tumoren bleken voor te komen (verlies van 3p12-3p22 and amplificatie van 6q11-21). De resultaten suggereren dat familiaire prostaattumoren worden gekenmerkt door specifieke DNA afwijkingen. Recent bewijs op dit gebied ondersteunt de hypothese dat het familiaire risico op prostaatkanker wordt bepaald door overerving van multipele genen, die elk bijdragen aan het risico.

In hoofdstuk 4 wordt een gedetailleerde analyse van chromosoom 6 veranderingen in prostaattumoren beschreven, met het doel om de chromosomale regio te bepalen waarbinnen een tumor suppressor gen kan worden gezocht. LOH werd gevonden bij 9 van de 19 onderzochte tumoren (TURP). De gemeenschappelijke, minimale regio van verlies had een lengte van 8.6 Mpb en werd geflankeerd door de markers D6S1609 en D6S417. Binnen dit gebied werd gezocht naar een homozygote deletie met behulp van 123 STSs, genen en kandidaat genen, maar er werd geen homozygote deletie gevonden. Het hoge percentage verlies van 6q14-16 onderstreept het belang van genen in deze regio bij de ontwikkeling van prostaattumoren.

In de hoofdstukken 5 en 6 wordt ingegaan op de rol van het tumor suppressor gen *PTEN*, dat belangrijk is bij veel epitheliale tumoren en ook prostaattumoren. De studie beschreven in hoofdstuk 5 richtte zich op veranderingen van het *PTEN* gen in 40 lokaal progressieve tumoren (TURP). Er werden een aantal verschillende, complementaire technieken toegepast, waar-onder allelotypering, SSCP en sequencing, FISH, array-CGH en immunohistochemie. Ook WPR, een multiplex-PCR variant die gebruik maakt van een verschil van een enkel nucleotide tussen *PTEN* en het *PTEN* pseudogen op chromosoom 9p13 werd toegepast. Samengevat bleek *PTEN* bij 30% van de gevallen genetisch geïnactiveerd, meestal door homozygote deletie. Bij 15 gevallen was er een volledige afwezigheid van expressie van het PTEN eiwit en bij 8 gevallen werd een heterogeen patroon vastgesteld. Binnen deze serie van 40 lokaal progressieve tumoren bleek afwezigheid van PTEN expressie geen prognostische waarde te hebben.

In hoofdstuk 6 werd PTEN expressie bepaald door toepassing van immunohistochemie op een tissue microarray met monsters van 90 radicale prostatectomie patiënten waarvan de klinische follow-up bekend was. Van 62 gevallen was tumor DNA beschikbaar wat werd gebruikt voor array-CGH. Bij 35 tumoren was het PTEN eiwit geheel of gedeeltelijk afwezig. In 9 van de 62 gevallen wees array-CGH op aanwezigheid van een homozygote deletie in *PTEN*. Er was een goede correlatie tussen aanwezigheid van een homozygote deletie en afwezigheid van het PTEN eiwit. Afwezigheid van het PTEN eiwit was geassocieerd met PSA progressie. Echter, in multivariate analyse bleken alleen pT stadium en Gleason sum score onafhankelijke voorspellers van PSA progressie.

In hoofdstuk 7 worden de resultaten uit de voorgaande hoofdstukken samengevat en bediscussieerd.

Hoofdstuk 8 bevat de epiloog, waarin de hypothese wordt gepresenteerd dat een ontregeling van transdifferentiatie de basis vormt van het ontstaan van prostaatkanker. Transdifferentiatie is het fenomeen dat cellen uit het beenmerg van fenotype veranderen en mesenchymale of epitheliale cel worden. Dat transdifferentiatie gekoppeld kan zijn aan tumorontwikkeling werd recent aangetoond in een muizenmodel voor maagkanker. Observaties bij prostaatkanker die consistent zijn met het transdifferentiatiemodel zijn: de aanwezigheid van bloedcellen met chromosomale afwijkingen bij prostaatkankerpatiënten, het patroon van botmetastasering en de observatie dat de uitkomsten van trials die de rol van vroege versus uitgestelde hormonale behandeling bestuderen afhankelijk zijn van de aanwezigheid van de primaire tumor. De transdifferentiatie hypothese veronderstelt dat er een epigenetische factor aanwezig is in de primaire tumor die verantwoordelijk is voor een voortgaande rekrutering van cellen uit het bloed die onvolledig transdifferentiëren. Er worden voorbeelden van epigenetische overdracht van maligniteit (*horizontal oncogenesis*) gegeven om te illustreren dat dit inderdaad beschreven is in bijzondere omstandigheden.

Bevestiging van de transdifferentiatie hypothese zou een grote invloed hebben op het klinische beleid bij oncologische patiënten. Een voorbeeld is dat men zou moeten overwegen om de primaire prostaattumor te behandelen door middel van chirurgie of radiotherapie, ook als er (lymfklier) metastasen aanwezig zijn.

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CURRICULUM VITAE

Paul Verhagen werd geboren op 17 december 1961 in Boxtel. In 1980 behaalde hij het diploma Gymnasium ß aan het Jacob Roelandslyceum te Boxtel. Van 1980 tot 1981 studeerde hij biologie aan Hamline University, St Paul, Minnesota, USA, in het kader van een uitwisselingsprogramma van de NACEE (Netherlands American Commission for Educational Exchange). Hij volgde de opleiding geneeskunde aan de Katholieke Universiteit Nijmegen. Het kandidaatsexamen behaalde hij in 1983 cum laude. In 1989 behaalde hij het artsexamen. Van 1989 tot 1991 was hij in het kader van de militaire dienstplicht werkzaam als assistent urologie in het Militair Hospitaal dr. A. Mathijssen te Utrecht. Van 1992 tot en met 1993 volgde hij de opleiding algemene chirurgie in het Medisch Spectrum Twente te Enschede (opleider: dr. J. Hoogendam). Hierna volgde de opleiding urologie in het Bosch Medicentrum te Den Bosch (1994, opleider: drs. L.M.H. Schreinemachers) en in het Academisch Ziekenhuis Utrecht (1995 tot en met 1997, opleider: prof.dr. T.A. Boon). In 1998 en 1999 was hij klinisch research fellow van KWF Kankerbestrijding (supervisie: prof.dr. F.H. Schröder) waarmee de basis voor dit proefschrift werd gelegd. Tijdens dit fellowship werkte hij op de afdeling Pathologie van het Erasmus MC te Rotterdam onder leiding van prof.dr. J. Trapman en op de afdeling Cytogenetica van de University of Utah, Salt Lake City, Utah, USA, onder leiding van prof.dr. A.R. Brothman. Hierna startte hij in zijn huidige functie als staflid bij de afdeling urologie van het Erasmus Medisch Centrum te Rotterdam (hoofd: prof.dr. C.H. Bangma). In 2005 werd hij waarnemend afdelingshoofd. Hij is getrouwd met Marjo Ramakers en heeft 3 kinderen, Iris, Mathijs en Eline.