CYTOGENETICS OF PROSTATE CANCER

CYTOGENETICS OF PROSTATE CANCER

CYTOGENETICA VAN PROSTAAT KANKER

Proefschrift

Ter verkrijging van de graad van doctor

aan de Erasmus Universiteit Rotterdam

Op gezag van de Rector Magnificus

Prof.dr. P.W.C.Akkermans, M.A.

en volgens besluit van het college voor promoties

De openbare verdediging zal plaatsvinden op

Woensdag 10 juni 1998 om 13.45 uur

door

Josée Jeannette König

geboren te 's Gravenhage

Promotie-commissie

Promotores:	Prof.dr. F.H.Schröder Prof.dr. A.M.M.J.Hagemeijer
Overige leden:	Prof.dr. J.W.Oosterhuis Prof.dr. A.H.M.Geurts van Kessel Prof.dr. F.C.S.Ramaekers

The investigations presented in this thesis were performed at the Urological Department (Division Urological Oncology), Medical faculty, Erasmus University Rotterdam.
The studies were supported by the Dutch Cancer Society (NKB), grants IKR 86-14 and IKR 90-12.

Het onderzoek dat in dit proefschrift wordt gepresenteerd, is uitgevoerd op de afdeling Urologie van de Erasmus Universiteit Rotterdam, bij de onderzoeksgroep Urologische Oncologie. Het onderzoek werd gefinancieerd door de Kankerbestrijding (NKB), projectnummers IKR 86-14 en IKR 90-12.

Het verschijnen van dit proefschrift wordt mede mogelijk gemaakt door de financiële bijdrage van de Stichting Urologisch Wetenschappelijk Onderzoek (SUWO) (Rotterdam) en Molecular Probes BV (Leiden).

Only two things in life are important, love and work.
Sigmund Freud

Voor Jan Willem, Thomas en Julia

CONTENTS 6

LIST OF ABBREVIATIONS

AgNOR	silver stainable nuclear organiser region
ANC	average number of copies
APC	adenomatous polyposis coli susceptibility gene
AR	androgen receptor gene
BCL-2	apoptosis suppressor gene
BPH	benign prostatic hyperplasia
BRCA1	breast cancer susceptibility gene 1
C-CAM	oncogene
C-MYC	oncogene
CDH1	e-cadherin gene
CDKN2	tumor suppressor gene
CGH	comparative genome hybridization
CRBC	chicken erythrocytes
CT	cholera toxin
CV	coefficient of variation
DAPI	4',6-diamidino-2-phenylindol
DCC	deleted in colorectal carcinoma, TSG colon cancer
DHT	dihydrotestosterone
DI	DNA index
DNA	deoxyribonucleic acid
e.g.	for instance
EGF	epidermal growth factor
EGFR	EGF receptor gene
FCM	flow cytometry
FCS	fetal calf serum
FGC	fast growing colony
FISH	fluorescence in situ hybridization
FITC	fluoresceine isothiocyanate
HC	hydrocortisone
HPC1	prostate cancer susceptibility gene 1
i.e.	that is
INS	insulin
ISH	in situ hybridization
KAI1	metastasis suppressor gene
LNCaP	lymph node carcinoma of the prostate
LNO	lymph node original
LOH	loss of heterozygosity
MSG	metastasis suppressor gene
MSI	microsatellite instability
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazoliumbromide
MXI1	candidate tumor suppressor gene
P53	tumor suppressor gene
PBP	promotor binding protein
PBS	phosphate buffered saline
PC	prostate cancer
PIN	prostatic intra-epithelial neoplasia
PRLTS	candidate tumor suppressor gene
PSA	prostate specific antigen
PSAP	prostate specific acid phosphatase
PTEN	candidate tumor suppressor gene
RB	retinoblastoma tumor suppressor gene
RFLP	restriction length polymorphism
T	testosterone
TNM	tumor node metastasis
TSG	tumor suppressor gene
TUR	transurethral resection
WCP	whole chromosome paint

CHAPTER 1

GENERAL INTRODUCTION

1.1. CLINICAL ASPECTS OF PROSTATE CANCER

EPIDEMIOLOGY AND RISK FACTORS

Incidence and mortality

In most Western countries, prostate cancer (PC) is a common malignancy. In the United States cancer statistics of 1994, PC has the highest incidence rate and is the third cause of cancer deaths [Boring et al '94]. In the Netherlands, which takes the ninth place on the list of PC cancer deaths by country, PC is currently the second cause of cancer morbidity and death behind lung cancer [Van der Gulden et al '94]. Over the last decades, the incidence and mortality rates of PC show a steady increase in most areas of the world. In the Netherlands the age adjusted mortality has risen 1.5-fold since 1950. Van der Gulden et al. have shown in their report on the PC related mortality trend in the Netherlands a continuous increase of risk in the period 1950-1989. There is evidence that this effect is partly based on the successive improvements in urological diagnostics, which caused an increase of the detection rate. However, better detection does not necessarily mean larger mortality figures. Early detection can cause a shift towards localized disease. For instance, in Norway, an increase of the 5-year survival rate was reported [Zaridze et al '84].

Study of the incidence and mortality trends can contribute to a clearer understanding of the factors predisposing for PC. Histologic PC -the term proposed by Carter [Carter et al '90A] to describe PC that is found in many older men at autopsy- has a similar prevalence throughout the world. The prevalence of clinical PC however, is greatly different among countries, from about 5/100,000 in Japan to 100/100,000 in the U.S. If only tumor growth would be required to cause a histological PC to progress to a clinical cancer, the incidence rates would be the same everywhere. Because they are not, it is clear that other crucially important factors are responsible.

Cancer promoting factors

The factors which possibly promote the transformation from histological PC to a clinical cancer can be classified as either environmental, endogenous or familial.

That environmental factors are indeed of importance, is illustrated by the fact that in people migrating from several countries with a low PC incidence to countries with a high incidence, an increase in PC incidence was found [Carter et al '90A]. Occupational exposure to cadmium or heavy metal oxides, and occupations such as sheet metal worker, mechanic and farmer have been mentioned as having an increased relative risk estimate [Carter et al '90A]. According to the review on risk factors for PC by Key, in most studies dietary factors like animal fat intake and consumption of dairy products are associated with an increased risk for PC [Key '95]. Carrots on the other hand seem to have a protective effect, which however cannot be contributed to the beta-carotene in them. Conclusions about consumption of green vegetables are inconsistent. A negative effect from smoking is not conclusively proven, while alcohol intake does not seem to increase the risk for PC. Some aspects of sexual activity, like age at first intercourse, multiple sexual contacts and a history of a sexually transmitted disease, increase the risk for PC. Key speculated that this might be due to a hormonal factor that increases both sexual activity and cancer risk, or to an infectious agent. HPV, as a sexually transmittable virus, is a candidate. In four of seven studies HPV was detected in PC tissues [Cuzick '95]. However, the rates in benign prostatic hyperplasia (BPH) are similar, which is not suggestive for a direct role of HPV in PC. Cusick has suggested that

the prostate may possibly serve as a reservoir for HPV, but more studies are needed to prove this. The suspected association of vasectomy with increased risk for PC has been the subject of extensive debates, mainly because the reported association was rather weak [Kawachi et al '94, Hsing et al '94] and could not be confirmed in other studies [John et al '95, Moller et al '94].

The endocrine dependence of the normal prostate and the ability of manipulating growth by hormonal therapy has suggested a hormonal etiology for PC. Levels of estrogens, prolactin and androgens have been studied in high and low risk populations and in PC patients vs. controls. The results were inconsistent, reporting higher as well as lower levels of testosterone (T), higher or the same levels of prolactin and lower or the same levels of dihydrotestosterone (DHT) in high versus low risk populations and in PC patients versus controls [Carter et al '90A].

A possible association between BPH and PC has been suggested, but since their anatomical localization in the prostate is not similar -BPH is localized in the central zone, while PC mostly occurs in the peripheral zone- direct transformation of BPH cells into PC cells is highly unlikely. It has been speculated that indirect effects, like the disruption of the prostatic architecture, or the stasis of luminal fluids, might foster a carcinogenic microenvironment.

A positive association between the risk of acquiring PC and family history has been reported in different studies [Carter et al '95]. Case-control studies showed that the risk increases with the number of first (father or brother) and second degree (grandfather or uncle) relatives affected [Carter et al '93]. Furthermore, the closer genetically a man is to an affected relative, the greater his risk becomes. So familial factors, which can be both environmental and genetic, also have a role in PC development. The possible environmental factors that predispose to PC have already been described. The importance of genetic factors has been established by epidemiologic studies that concentrated on PC occurrence in families, and twin studies. Segregation analysis showed that part of the familial cases must be of hereditary nature, because the distribution of PC in such families was consistent with a mendelian inheritance pattern [Carter et al '92]. Furthermore, the concordance rate of PC occurrence was considerably higher in monozygotic- than in dizygotic twins [Grönberg et al '94]. The mendelian inheritance pattern [Carter et al '93] can be explained by postulation of a single rare autosomally dominant susceptibility gene [frequency in the population 0.36%), that causes PC at an early age (55 years or less). This gene is highly penetrant: PC is projected to develop by the age of 85 years in 88%of the male carriers compared to only 5% of the noncarriers. The overall frequency of inherited PC, which is estimated at 9%, is similar to the calculated inherited proportion of two other common cancers that are well recognized to have a familial component, namely breast and colon cancer. According to the frequency of occurrence of the disease in other members of the family, PC patients can be divided into three groups: hereditary, familial and sporadic. The precise criteria for each of these groups have been described in detail in the review on hereditary PC by Carter et al from 1993.

No differences have been found in the clinical and pathological variables between hereditary and sporadic PC [Aprikian et al '95, Bastacky et al '95]. This is consistent with the model for hereditary cancers [Knudson '85], that is based on the assumption that hereditary and sporadic cancers have a similar genetic etiology.

The hereditary forms of breast and colon cancer are known to be genetically heterogeneous. This means that in different families mutations in different genes may cause the same disease [Easton '94]. It is legitimate to assume that the same is true for hereditary PC. The existence of genetically homogeneous subsets of hereditary PC, as were found for other hereditary cancers, are at present merely

suspected because only slight associations with breast and brain cancer were found [Tulinius et al '92, Carter et al '93].

CLINICAL MANAGEMENT OF PROSTATE CANCER

Localized disease

For localized and incidental PC radical surgery is presently the most frequently employed treatment, but also radiation therapy is often applied [Coffey '93]. There is still debate as to what is the best treatment at this disease stage. For incidental T1 disease, so-called histological PC, there seems to be little clinical justification for such radical approaches [Newling '95]. However, deferred treatment will always lead to progression in a certain group of patients. At present there are no markers available to identify this group up front. Recently developed powerful techniques for the early detection of PC, like serologic detection of prostate specific antigen (PSA) and endorectal ultrasonography, which have made screening for PC feasible [Schröder et al '96], will result in an increase of the number of organ confined tumors detected. By then it will be essential for the clinician to be able to discern the tumors requiring immediate therapy.

Advanced disease

The treatment of choice for locally advanced and metastatic PC has since long been, and at present is still, hormone ablation by castration [Schröder '91, Newling '95]. This treatment is effective, both subjectively, with regard to the quality of life, and objectively, with regard to tumor size and occurrence of distant metastases. The timing of androgen ablation, whether it needs to be done with or without delay, has been the subject of clinical and experimental studies [Isaacs, JT et al '81, Schröder '91, Newling '95]. It is now recognized that hormone therapy given early in the disease course may have preference, although this is still a matter of debate. In general, prognostic factors like tumor stage and grade strongly influence the outcome of treatment [Schröder '93]. Treatments with oestrogenic compounds as an alternative for castration have become impopular, because of the rather severe cardiovascular side effects. At present treatments with anti-androgens, that cause considerably less cardiovascular problems, are considered standard endocrine treatment next to castration.

The concept of total androgen blockade has received much attention. The issue was whether only the androgens synthesized in the testes, or also the adrenal androgens needed to be annihilated. Although in most clinical studies overall survival was not significantly improved, the time to progression was generally longer, and there are indications that patients with a relatively small tumor load might benefit more from complete androgen ablation [Newling '95].

Despite all efforts to refine hormone therapy, all advanced PC's will eventually become nonresponsive and show relapse. The regrown tumor consists of cells that are hormone independent. Several explanations to this phenomenon have been offered. The original tumor could be heterogeneous, consisting of both hormone dependent and -independent cells, changing its composition in answer to changing environmental conditions, like androgen ablation [Isaacs, JT et al '81]. Another option is that independent subpopulations are generated during hormone therapy [Isaacs, JT et al '82]. Once a tumor has become hormone refractory, in principle chemotherapy would be possible, but unfortunately at present no chemotherapeutic drugs are available that are sufficiently active [Newling '95].

Study of characteristic cellular abnormalities of hormone independent PC might provide clues to the underlying mechanisms that rule the escape from hormonal control. To that purpose impaired programmed cell death, altered growth factor

(receptor) expression and altered stromal-epithelial interactions are the subject of investigation. Ultimately, all these abnormalities are based on altered gene expression which in their turn result from the genetic instability inherent to cancer cells. This aspect will be discussed later.

PROGNOSTIC MARKERS FOR PROSTATE CANCER

Grade and stage, the classical markers

The pathologic stage (tumor extent in the prostate gland, capsular penetration, seminal vesicle- and/or pelvic lymph node involvement) and histologic grade currently are the most important prognostic markers for PC. For grading both the Gleason and the Mostofi system are used [Gleason '92, Mostofi et al '80]. Both suffer from a degree of subjectivity inherent to all grading systems. A major problem is the intrinsic heterogeneity of PC, which means that the histologic grade in a biopsy is not always representative for the whole tumor. The dominant staging system in the US is the one developed by Whitmore and modified by Jewett [Jewett '75]. In Europe the Tumor Node Metastasis (TNM) system [Schröder et al '92] is the most widely used.

Possible additional and alternative prognosticators

The above mentioned prognosticators indicate trends and the likelihood of outcome, but they cannot absolutely predict the outcome for individual patients. To make better risk estimates for progression of individual patients, more quantitative prognosticators are needed. Growth factors and hormones and their receptors, a variety of cytoplasmic proteins, cytokeratins, lectins, blood group antigens, mucins, stromal factors and extracellular matrix proteins, all these have been investigated on their potential usefulness as markers in PC [Bostwick et al '92, Humphrey et al '93, Noordzij et al '95B].

Potential future additional prognosticators are nuclear features, growth fraction, circulating prostate specific antigen (PSA) producing cells, neuroendocrine markers, adhesion molecules, deoxyribonucleic acid (DNA) ploidy and genetic markers.

Nuclear features that have been reported to be of prognostic value are nucleolar prominence [Tannenbaum et al '82], morphometry of nuclear shape [Blom et al '90] and the number of silver stained nuclear organiser regions (AgNOR's) [Mamaeva et al '91]. Unfortunately none of these can be easily used in routine practice.

The growth fraction of PC may be assessed by a variety of methods, including counting of mitoses, determination of the S-phase fraction by DNA flow cytometry (FCM) and immunohistochemical analysis of different nuclear antigens (*KI-67*, proliferating cell nuclear antigen (*PCNA*)). The best prognosticator in this group is the number of mitoses. When present (mitoses are relatively rare in histological sections of PC; they are present in only about 10% of the tumors), these signify a poor prognosis [Schröder et al '85]. The prognostic significance of the S-phase fraction is still controversial [Visakorpi et al '91, Foster et al '92], while the value of *KI-67* and *PCNA* seems limited [Oomens et al '91, Sadi et al '91]. The prognostic value of the MIB-1 antibody, that has an affinity for the *KI-67* antigen several times higher than the KI-67 antibody itself, is under investigation [Noordzij et al '95A].

The presence of PSA producing cells in the peripheral blood has shown independent prognostic value in predicting progression following radical prostatectomy [Wood et al, '94]. However, since this effect could be shown only with a short follow up time [Olsson et al, '96], the clinical value of this marker is still unsure.

Several neuroendocrine markers (e.g. chromogranin-A) and adhesion molecules (e.g. E-cadherin) and their potential prognostic significance are currently the subject of intensive research [Umbas et al '94, Noordzij et al '95B]. Some seem important,

but still much work needs to be done to come to definitive conclusions.

In a recent report [Chan et al, '98], IGF-I has been indicated as a new independent predictor for prostate cancer risk. High plasma IGF-I levels were strongly associated with an increased risk for PC.

DNA ploidy and genetic markers and their potential usefulness as prognosticators are discussed in more detail in the next paragraphs.

DNA flow cytometry, additional or independent prognosticator?

In the search for additional and/or alternative markers that may contribute to the prediction of the disease course of PC patients, DNA content analysis, using mostly flow cytometry and sometimes also image analysis, has been investigated by many groups. In most studies a strong relationship was demonstrated between changes in DNA content and increasing grade and stage. In general, low grade tumors were mostly diploid, whereas high grade tumors were more frequently aneuploid [Shankey et al '93, Adolfsson '94].

When multivariate analysis, with overall or disease-specific survival as endpoint, was applied, the DNA content was mostly shown as an additional, but not always independent, prognostic marker next to other prognostic variables like grade and stage [Stephenson et al '87, Montgomery et al '90,Visakorpi '92A, Forsslund et al '92, Song et al '92, Adolfsson '94, Carmichael et al '95]. In the majority of the studies where univariate analysis was applied, DNA diploidy was strongly associated with favorable outcome, whereas DNA aneuploidy predicted poor outcome, irrespective of stage or therapy [Tribukait '87, Song et al '92, Bazinet et al '92, Adolfsson '94, Carmichael et al '95]. When only patients with localized disease were studied, upon univariate analysis the DNA content showed prognostic value [Montgomery et al '90, Forsslund et al '92]. However, when only patients with metastatic disease were evaluated, the results of similar univariate analyses were completely discordant in different reports [Adolfsson '94].

With respect to response to (anti-androgen) therapy with progression as endpoint, DNA diploid tumors of patients with advanced disease were associated with significantly longer remission times than DNA aneuploid tumors [Lee et al '88, Nativ et al '89, Zincke et al '92]. For patients with early disease the results were inconclusive [Adolfsson et al '90, Mohler et al '92, Shankey et al '93].

DNA flow cytometry, more an experimental than a clinical marker

Despite the general predictive qualities of DNA ploidy assessment, the role of DNA content analysis for clinical management of individual patients remains controversial. Practical problems that arise with DNA content analysis in PC, like the presence of normal tissue and the intrinsic heterogeneity of the tumor, are difficult to account for. This makes sampling errors a significant problem, illustrated by the finding of both diploid and aneuploid populations in a substantial number of cases when multiple samples were studied [Greene et al '91]. However, for research purposes, DNA ploidy analysis provides global information on the genetic evolution and heterogeneity of prostate tumors. It therefore is a powerful marker to study both genetic stability and clonal divergence of tumor cell populations in PC.

Another clinical problem is that by DNA content analysis alone the biological behavior of individual tumors cannot be predicted with sufficient certainty. As an example, it has been documented that some early tumors with a diploid DNA content have already metastasized [Adolfsson et al '90, Mohler et al '92]. This means that, although the DNA content suggests otherwise, in these tumors substantial genetic rearrangements towards aggressive malignancy must have occurred. In these cases investigation of additional genetic parameters, like karyotype, chromosome copy

number and/or loss of heterozygosity (LOH) may discriminate such "pseudodiploid" tumors from the "real diploid" tumors without rearrangements.

1.2. PROSTATE CANCER GENETICS

THE DEVELOPMENT OF TUMOR CYTOGENETICS

General

As early as 1914 Boveri postulated that cancer was characterized by abnormalities in the hereditary material: the somatic mutation theory. The first visible evidence of this concept was shown by Nowell and Hungerford in 1960, who discovered that in chronic myeloid leukemia cells an abnormally small chromosome was consistently present. This chromosome was called the Philadelphia chromosome. Later, when banding analysis made identification of individual chromosomes possible [Caspersson et al '67, Wurster '72], it was demonstrated that the Philadelphia chromosome was an aberrant chromosome 22, the result of a balanced translocation between chromosomes 9 and 22 [Rowley '73]. Two years before this discovery, Knudson postulated his now famous "two-hit" theory for the development of hereditary retinoblastoma. According to this theory a minimum of two mutational events is needed to develop this cancer, whereby the first mutation is inherited via the germ line [Knudson '71]. The second mutation should be a second mutation in the other allele of the same gene, resulting in a complete knock out of the gene function. This view led to the concept that both dominantly acting genetic changes (determined by oncogenes), as well as recessively acting changes (determined by tumor suppressor genes (TSG's)) can lead to cancer. Combined with the general acceptance that the multistep process of carcinogenesis is associated with genetic instability, characterized by chromosomal abnormalities like deletions, translocations and duplications, this marks the beginning of tumor cytogenetics as a separate subspecialism of genetics.

During the first years, tumor cytogenetic research focussed almost entirely on hematologic neoplasms. It was established that certain chromosomal changes significantly influence the biologic behavior of these cancer cells, because specific changes were identified that were associated with certain defined subtypes of hematological cancers [Dewald et al '85]. An even more important finding was that in several of these diseases cytogenetic analysis proved to be of prognostic significance [Han et al '84, Bloomfield et al '86, Kristoffersson et al '87]. Subsequent molecular genetic analysis of the chromosomal regions involved showed that chromosomal aberrations in cancer are caused by different mechanisms and that these in turn can have a variety of consequences.

Oncogenes and tumor suppressor genes

The consequences of the development of chromosomal aberrations are illustrated by the mechanisms acting in the activation of oncogenes and the inactivation of TSG's.

In the case of activation of oncogenes the following mechanisms have been described:

1) the creation of a new, chimeric gene, like the *BCR-ABL* gene in the Philadelphia chromosome formation in chronic myeloid leukemia through translocation of *ABL* [Gale et al '85], 2) the activation of an oncogene by altering its regulation through translocation of the oncogene to another locus where, for instance, it is regulated by the promoter of a constitutionally active gene (*C-MYC* translocation in Burkitt lymphoma, [Adams et al '85, Showe et al '86]), 3) through a specific deletion (point

mutation) in the regulatory sequences of an oncogene which influences its activity (makes it constitutionally active, e.g.the *RAS* oncogene), or 4) through overexpression by gene amplification [Alitalo '85].

In the case of TSG's, whereby the loss of function of the TSG is the prerogative for the tumorigenic phenotype to develop, other mechanisms are operative. This can be illustrated by the way a hereditary tumor is thought to develop. Hereby a germline mutation of one allele occurs, followed by loss of the remaining allele. The loss of the retinoblastoma tumor suppressor (*RB*) gene (located on 13q), which predisposes to retinoblastoma and osteosarcoma, is the most well known [Sparkes et al '80, Sparkes et al '83, Knudson '85, Lee et al '87]. Most hereditary solid tumors are presently thought to develop through the same mechanism, each with its own specific (set of) TSG's. The mutations can vary from complete loss, via interstitial deletion, to just a frameshift or point mutation [Nowell et al '60]. In non-hereditary cancer the same mechanism is thought to act, whereby both alleles of the TSG(or 's) undergo somatic mutation.

THE DEVELOPMENT OF SOLID TUMOR CYTOGENETICS

General

With the success of the cytogenetic studies on hematological neoplasms in mind, around 1980 the first studies with solid tumors were launched [see Teyssier '89 for review]. However, very soon it became evident that such studies could not be performed in the same way as those on blood cancer. Several serious technical problems had to be solved, mostly relating to the preparation of chromosome suspensions from solid tumors.

The causes for the low yield of analyzable metaphases in most solid tumor samples were: low mitotic activity in vivo, cryptic sample infection, infiltration of tumor material with normal cells, low yield of viable cells and low proliferative activity in tissue culture. To circumvent these problems immortal cell lines, of which metaphases could be obtained easily and reproducibly, were extensively analysed. However, this approach suffered from the fact that the karyotypes of most solid tumor cell lines were shown to be of great complexity. The interpretation of these aberrations was also difficult since several of these abnormalities were likely to be generated during tissue culture rather than tumor derived. Therefore, further efforts were necessary to improve the procedures of solid tumor cell preparation and karyotyping.

Most investigators used a general approach for all solid tumors to solve the above mentioned problems [Wake et al '81,Trent et al '86, Limon et al '86, Tanaka et al '87], while others, like us, attempted to design a specialized approach, applicable to a specific tumor type.

PROSTATE CANCER CYTOGENETICS

Technical challenges

Prostate cancer generally has a low mitotic activity in vivo, so harvesting metaphases directly from tumor cell suspensions that had been briefly incubated with colcemid would potentially not be very successful. Nevertheless the first karyotypes that were obtained from specimens of PC patients were harvested by this method, because other methods were not yet available at that time [Atkin et al '85]. However, although aberrations could be identified in some cells, chromosome morphology was generally poor and, as expected, metaphase yield was very low. This method has further been used only occasionally, also by us (see chapter 3).

The next option was to isolate PC cells from the tumor tissue, put them into short term tissue culture, add colcemid when the cells proliferated sufficiently and

harvest metaphases. The general concepts of this technique could be applied to a wide variety of solid tumors [Trent et al '86, Limon et al '86, Tanaka et al '87], but specific modifications were necessary for each tumor type. For PC, there were two technical challenges: 1) The isolation of predominantly highly viable PC cells, and 2) The establishment of proliferation in tissue culture, preferably only of PC cells bot no normal and benign cells. This was necessary because PC nearly always grows next to- and infiltrates in normal and benign glands, so consequently a PC tissue specimen is commonly composed of normal, benign and malignant cells.

The disaggregation of PC tissue by mechanical means yielded few viable cells, that poorly attached to the culture vials and exhibited minimal growth [Tanaka et al '87]. Disaggregation of PC tissue by collagenase, followed by in vitro culture of the isolated cells for 4-12 days in a generally applied liquid culture medium gave better results with respect to cellular viability and in vitro proliferation rate [Stone et al '76, Limon et al '86]. However, the resulting karyotypes of such cultures were in most cases normal diploid ([Brothman et al '90, Brothman et al '91, Micale et al '92] and chapter 3). Since it had been shown by DNA-FCM that at least 50% of the PC tumors had cells with an abnormal DNA content (see also paragraph 2.3.2), it was clear that the results of karyotype analysis performed by the methods as described above in most cases did not reflect the cellular composition of the original tumor sample. Thus it was necessary to critically evaluate the different steps in the karyotyping procedure with respect to the presence and preservation of PC cells:

1) sampling -a histological check of the PC tissue sample to determine the percentage of tumor present (chapter 3),

2) isolation -which cells are isolated and which are lost by different disaggregation methods (chapter 4.1),

3) culture process -which cells proliferate and which do not under different culture conditions, i.e. addition of different nutritional supplements (hormones, growth factors, trace elements), different types of culture (suspension, monolayer or explants) and the suppression of proliferation of normal cells (chapters 3 and 4.2).

Identification and interpretation of chromosomal changes

Until 1989, when we published our investigations on cytogenetics in the LNCaP cell lines (chapter 7.1), few PC specimens had been karyotyped [Gibas et al '85, Atkin et al '86, Ahmann et al '86, Lundgren et al '88, Brothman et al '89, Limon et al '89] and most data were from cell lines [Nelson-Rees et al '78, Ohnuki et al '80, Gibas et al '84, Iizumi et al '87, Pittman et al '87], as was our very first report on the xenografted cell line PC82 [König et al '88]. The most common aberrations observed were deletions, which pointed to the involvement of TSG's in PC. Aberrations of chromosomes 1, 7 and 10 were reported for most tumors, although not consistently. Aberrations that occurred in more than one tumor involved chromosomes 2, 3, 6, 8, 13 and 15. While chromosome 1 aberrations already had been demonstrated in other solid tumors [Mitelman '85, Teyssier '87, Hecht '88], it was suggested that 7 and 10 were specifically involved in PC development [Atkin et al '85]. These aberrations were located in regions of the human genome that until then had not been indicated as important for tumor development. However, the aberration patterns found were not yet consistent, so many more PC karyotypes were needed to establish which aberrations were recurrent and thus important in development and progression of PC.

MOLECULAR (CYTO)GENETICS

LOH and CGH studies

In the mean time, other lines of PC genetics research began to generate results

[Isaacs, JT et al '91, Linehan '92, Bookstein '93, Bookstein '94, Rinker-Schaeffer et al '94, Isaacs, WB et al '94A, Isaacs, WB et al '94B]. In molecular genetics research the first step in localizing the genes important in PC development and progression were LOH studies. By LOH mutations can be detected in any chromosomal region of which DNA probes are available. Since TSG inactivation may be achieved through deletion, it is widely used to identify chromosomal regions that contain TSG's . In several studies, including an allelotyping study, where each chromosome arm was separately studied, the highest frequencies of LOH, given in descending order, were found on 8p, 16q, 10p, 10q, 18q, 13q and 17p [Carter et al '90B, Kunimi et al '91, Bergerheim et al '91]. Subsequently most molecular genetic research focussed on these regions. However, the potential importance of other regions indicated by cytogenetics, like chromosomes 1, 2, 3, 6, 7 and 15 could not be ruled out, since only a small part of each of these chromosomes had been investigated for LOH.

By comparative genome hybridization (CGH) loss or gain of chromosomal regions throughout the genome, with a resolution specificity of one or two chromosome bands, can be detected [Visakorpi et al '95, Joos et al '95, Cher et al '96]. These studies showed specific loss of 2q, 5q, 6q, 8p, 9p, 10q, 13q, 15q, 16q, 17p and 18q and specific gain of 1q, 2p, 3q, 7q, 8q, 9q, 11p, 16p, 20, 22 and X. These results confirmed the importance of 8p, 10, 13q, 16q, 17p and 18q and added 1q, 2, 3q, 5q, 6q, 7q, 8q, 9, 11p, 15q, 16p, 20, 22 and X as new regions of interest.

The possible localization of one or more TSG's on 8p was further investigated in a number of studies, by using several marker probes with a known localization on the 8p physical map. Two smallest regions of overlapping deletions, where a TSG is most likely to be situated, were identified on 8p22 [Bova et al '93] and 8p21 respectively [Trapman et al '94]. Recent investigations on other important regions that showed LOH will be discussed in chapter 8.

Other studies focussed on the loss of known TSG's, located in regions where relatively high percentages of LOH in PC were found, like *P53* on 17p [Visakorpi et al '92B, Bookstein et al '93, Navone et al '93, Dinjens et al '94], *RB* on 13q [Bookstein et al '90A, Bookstein et al '93, Brooks et al '94] and deleted in colorectal carcinoma (*DCC*), the TSG first identified in colon cancer on 18q [Carter et al '90B, Gao et al '93]. Both *P53* and *RB* were shown to be mutated in a subset of advanced PC's, but only rarely in early tumors. Mutations in the *RB* gene product were identified in several PC cell lines [Bookstein et al '90A, Huang et al '88]. Suppression of tumorigenicity of one PC cell line (DU145) was demonstrated when its mutated *RB* gene was complemented with the wild type allele [Bookstein et al '90B]. Later the same effect was demonstrated when wild type *P53* was introduced in Du145 and in two other PC cell lines [Isaacs, WB et al '91]. These experiments were strong indications that both *RB* and *P53* can function as a TSG in PC.

FISH studies, general

Fluorescence in situ hybridization (FISH) is a molecular cytogenetic technique that combines the advantages of DNA specificity with microscopic visualization [Manuelidis '85, Burns et al '85, Cremer et al '86]. This technique can be applied to metaphase spreads, but also to interphase nuclei in cytological preparations and tissue slides. The chromatin is denatured and hybridized in situ with the DNA probe(s) of choice that are tagged with non-isotopic fluorochromes [Cremer et al '88, Hopman et al '88, van der Ploeg et al '88, Poddighe et al '91]. When the same technique is applied with nonfluorescent haptenes, the technique is called ISH [Hopman et al '88, van der Ploeg et al '88, Poddighe et al '91]. Several types of probes are available, that hybridize with different targets on the human genome and thus can be applied for different goals: 1) repetitive probes, identifying for instance the chromosome

specific alphoid DNA sequences near each centromere [Cooke et al '79, Devilee et al '86, Waye et al '87, Devilee et al '88A]. These probes are useful for determining the copy number of a specific chromosome, especially in interphase nuclei [Hopman et al '88, Gray et al '92] ; 2) single copy probes, identifying a discrete and unique region of the genome. These probes are useful to map the chromosomal location of new genes and can detect translocations and microdeletions that cannot be seen with conventional cytogenetic analysis [Bhatt et al '88, Viegas-Pequignot et al '89A, Viegas-Pequignot et al '89B, Nederlof et al '89]; 3) chromosome specific paint probes, which are in fact pools of probes with homology to sequences along the entire length of a specific chromosome [Cremer et al '88, Pinkel et al '88, Deaven '91, Collins et al '91]. Paint probes are useful in determining the copy number of a specific chromosome, and can be instrumental in determining the chromosomal origin of parts of marker chromosomes [Weijerman et al '98].

Although FISH analysis could not provide a picture as detailed as can be achieved by banding analysis of metaphases, the application of the FISH technique to solid tumors made it possible that significant numbers of tumor specimens could be reliably and reproducibly evaluated. Especially for slow growing tumors like PC, that always needed tissue culture to provide enough metaphases for cytogenetic analysis, it was an important new technique. FISH analysis is performed on isolated tumor nuclei or on tissue slides, determining the copy number of the chromosomes of interest in interphase cells. With this technique isolation of viable cells and subsequent tissue culture are no longer obligatory. In this way the problem of selective tumor cell loss during tissue disaggregation (chapter 4.1) and tissue culture (chapter 4.2) could be circumvented.

The first reports on FISH analysis in solid tumors mainly focussed on numerical chromosomal aberrations [Devilee et al '88B, Hopman et al '89, Nederlof et al '89]. Occasionally chromosome specific library probes [Cremer et al '88] and a probe specific for a chromosome segment [Viegas-Pequignot et al '89] were used.

FISH studies, PC results

The first FISH study on PC was performed on in vitro cultured cells [Brothman et al '92] and the first ISH study was on tissue sections [Henke et al '93]. In both reports numerical abnormalities were reported for all investigated chromosomes. However, the impact of the results of both studies was still limited. With respect to the FISH study it was unknown whether the in vitro cultured cells that were analyzed were representative for the original tumor. In the ISH study no statistical evaluation of the hybridization results from the tumor cells and the surrounding normal tissue was given. However, such an evaluation is of critical importance, since a significant number of all nuclei will be truncated by sectioning [Arnoldus et al '91].

To my opinion the more reliable studies on PC that used FISH, are those in which the origin of the cells was histologically checked, like in our own studies (chapters 5 and 6).

LITERATURE

Adams JM, Harris AW, Pinkert CA, Corcoran LM, Alexander WS, Cory s, Palmiter RD, Brinster RL. The c-myc oncogene driven by immunoglobulin enhancers induces lymphoid malignancy in transgenic mice. Nature 318 :533-538, 1985.

Adolfsson J, Rönström L, Hedlund PO, Löwhagen T, Carstensen J, Tribukait B. The prognostic value of modal deoxyribonucleic acid in low grade, low stage untreated prostate cancer. J Urol 144 :1404-1407, 1990.

Adolfsson J. Prognostic value of deoxyribonucleic acid content in prostate cancer: a review of

current results. Int J Cancer 58: 211-216, 1994.

Ahmann FR, Woo L, Hendrix M, Trent JM. Growth in semisolid agar of prostate cancer cells obtained from bone marrow aspirates. Cancer Res 46 :3560-3564, 1986.

Alitalo K. Amplification of cellular oncogenes in cancer cells. Trends Biomed Sci 10: 194-197, 1985.

Aprikian AG, Bazinet M, Plante M, Meshref A, Trudel C, Aronson S, Nachabe M, Peloquin F, Dessureault J, Narod S, Begin L, Elhilali MM. Family history and the risk of prostatic carcinoma in a high risk group of urological patients. J Urol 154: 404-406, 1995.

Arnoldus EPJ, Dreef EJ, Noordermeer IA, Verheggen MM, Thierry RF, Peters ACB, Cornelisse CJ, van der Ploeg M, Raap AK. Feasibility of in situ hybridization with chromosome specific DNA probes on paraffin wax embedded tissue J Clin Pathol 44 :900-904, 1991.

Atkin NB, Baker MC. Chromosome study of five cancers of the prostate. Hum Genet 70 :359-364, 1985.

Bastacky SI, Wojno KJ, Walsh PC, Carmichael MJ, Epstein JI. Pathological features of hereditary prostate cancer. J Urol 153: 987-992, 1995.

Bazinet M, Hamdy SM, Begin LR, Stephenson RA, Fair WR. Prognostic significance of antigenic heterogeneity, Gleason grade, and ploidy of lymph node metastasis in patients with prostate cancer. Prostate 20 :311-326, 1992.

Bergerheim USR, Kunimi K, Collins VP, Ekman P. Deletion mapping of chromosomes 8, 10, and 16 in human prostatic carcinoma. Genes Chrom Cancer 3: 215-220, 1991.

Bhatt B, Burns J, Flannery D, McGee JOD. Direct visualization of single copy genes on banded metaphase chromosomes by nonisotopic in situ hybridization. Nucleic Acids Res 16: 3951-3961, 1988.

Blom JHM, Ten Kate FJW, Schröder FH, van der Heul RO.Morphometrically estimated variation in nuclear size: A useful toolin grading prostatic cancer. Urol Res 18 :93-99, 1990.

Bloomfield CD, Goldman AI, Alimena G, Berger R, Borgstrom GH, Brandt L, Catovsky, de la Chapelle A, Dewald GW, Garson OM, Garwitz S, Golomb HM, Hossfeld DK, Lawler SD, Mitelma F, Nilsson P, Pierre RV, Philip P, Prigogina E, Rowley JD, Sakurai M, Sandberg AA, Secker-Walker LM, Tricot G, van den Berghe H, van Orshoven A, Vuopio P, Whang-Peng J. Chromosomal abnormalities identify high-risk and low-risk patients with acute lymphoblastic leukemia. Blood 67 :415-420, 1986.

Bookstein R, Rio P, Madreperla S, Hong F, Allred C, Grizzle W, Lee WH. Promotor deletion and loss of retinoblastoma gene expression in human prostate carcinoma. Proc Natl Acad Sci 87 :7762-7766, 1990.(A)

Bookstein R, Shew JY, Chen PL, Scully P, Lee WH. Suppression of tumorigenicity of human prostate carcinoma cells by replacing a mutated RB gene. Science 247 :712-715, 1990.(B)

Bookstein R, Allred DC. Recessive oncogenes. Cancer 71 :1179-1186, 1993.

Bookstein R, MacGrogan D, Milsenbeck SG, Sharkey F, Allred D. P53 is mutated in a subset of advanced-stage prostate cancer. Cancer Res 53 : 3369-3373, 1993.

Bookstein R. Tumor Suppressor genes in prostatic oncogenesis. J Cell Biochem 19 (Suppl): 217-223, 1994.

Boring CC, Squires TS, Tong T, Montgomery S. Cancer statistics 1994. CA Cancer J Clin 44:

7-26, 1994.

Bostwick DG, Montironi R, Nagle R, Pretlow T, Miller G, WheelerT, Epstein J, Sakr W. Current and proposed biologic markers inprostate cancer. J Cell Biochem 16 (Suppl H): 65-67, 1992.

Bova GS, Carter BS, Bussemakers MJG, Emi M, Fujiwara Y, Kyprianou N, Jacobs SC, Robinson JC, Epstein JI, Walsh PC, Isaacs WB. Homozygous deletion and frequent allelic loss of chromosome 8p22 loci in human prostate cancer. Cancer Res 53: 3869-3873, 1993.

Boveri T. Zur frage der Entstehung maligner Tumoren. Jena, Germany: Gustav Fisher, 1914.

Brooks JD, Bova GS, Isaacs WB. Allelic loss of the retinoblastoma gene in prostate cancer. Prostate 26: 35-39, 1994.

Brothman AR, Lesho LJ, Somers KD, Schellhammer PF, Ladaga LE, Merchant DJ. Cytogenetic analysis of four primary prostatic cultures. Cancer Genet Cytogenet 37 :241-248, 1989.

Brothman AR, Peehl DM, Patel AM, McNeal JE. Frequency and pattern of karyotypic abnormalities in human prostate cancer. Cancer Res 50 :3795-3803, 1990.

Brothman AR, Peehl DM, Patel AM, MacDonald GR, McNeal JE, Ladaga LE, Schellhammer PF. Cytogenetic evaluation of 20 cultured primary prostatic tumors. Cancer Genet Cytogenet 55: 79-84, 1991.

Brothman AR, Patel AM, Peehl DM, Schelhammer PF. Analysis of prostatic tumor cultures using fluorescence in-situ hybridization (FISH). Cancer Genet Cytogenet 62 :180-185, 1992.

Burns J, Chan VTW, Jonasson JH, Fleming KA, Taylor S, McGee JOD. Sensitive system for visualizing biotynilated DNA probes hybridized in situ; rapid sex determination of intact cells. J Clin Pathol 38: 1085-1092, 1985.

Carmichael MJ, Veltri RW, Partin AW, Miller MC, Walsh PC, Epstein JI. Deoxyribonucleic acid ploidy analysis as a predictor of recurrence following radical prostatectomy for stage T2 disease. J Urol 153: 1015-1019, 1995.

Carter BS, Carter HB, Isaacs JT. Epidemiologic evidence regarding predisposing factors to prostate cancer. Prostate 16: 187-197, 1990.(A)

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

Carter BS, Beaty TH, Steinberg GD, Childs B, Walsh PC. Mendelian inheritance of prostate cancer. Proc Natl Acad Sci 89 :3367-3371, 1992.

Carter BS, Bova GS, Beaty TH, Steinberg GD, Childs B, Isaacs WB, Walsh PC. Hereditary prostate cancer: epidemiologic and clinical features. J Urol 150 :797-802, 1993.

Carter BS, Steinberg GD, Beaty TH, Childs B, Walsh PC. Familial risk factors for prostate cancer. Cancer Surveys 23 :5-13, 1995.

Caspersson T, Arber S, Foley GE, Kudynowski J, Modest EJ, Simonsson E, Wagh U, Zech L. Chemical differentiation along metaphase chromosomes. Exp Cell Res 49: 219-222, 1967.

Chan JM, Stampfer MJ, Giovanucci E, Gann PH, Ma J, Wilkinson P, Hennekens CH, Pollak M. Plasma Insulin-like growth factor-I and prostate cancer risk: a prospective study. Science 279: 563-565, 1998.

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

Coffey DS. Prostate cancer. An overview of an increasing dilemma. Cancer Suppl 71(3) :880-886, 1993.

Collins C, Kuo WL, Segraves R, Fuscoe J, Pinkel D, Gray JW. Construction and characterization of plasmid libraries enriched in sequences from single human chromosomes. Genomics 11: 997-1006, 1991.

Cooke HJ, Hindley J. Cloning of human satellite III DNA: different components are on different chromosomes. Nucl Acids Res 6:3177-3197, 1979.

Cremer T, Landegent J, Bruckner A, Scholl HP, Schardin M, Hager HD, Devilee P, Pearson P, van der Ploeg M. Detection of chromosome aberrations in the human interphase nucleus by visualization of specific target DNAs with radioactive and nonradioactive in situ hybridization techniques: diagnosis of trisomy 18 with probe L1.84. Human Genet 74: 346-352, 1986.

Cremer T, Lichter P, Borden J, Ward DC, Manuelidis L. Detection of chromosome aberrations in metaphase and interphase tumor cells by in situ hybridization using chromosome-specific library probes. Hum Genet 80 :235-246, 1988.

Cuzick J. Human papillomavirus infection of the prostate. Cancer Surveys 23 :91-95, 1995.

Deaven LL. Chromosome specific human gene libraries. In: Encyclopedia of human biology, ed.R Dulbecco. Academic Press Inc, Orlando, Fla.pp455-464, 1991.

Devilee P, Cremer T, Slagboom P, Bakker E, Scholl HP, Hager HD, Stevenson AFG, Cornelisse CJ, Pearson PL. Two subsets of human alphoid repetitive DNA show distinct preferential localization in the pericentric regions of chromosomes 13, 18 and 21. Cytogenet Cell Genet 41 :193-201, 1986.

Devilee P, Kievits T, Waye JS, Pearson PL, Willard HF. Chromosome-specific " satellite DNA : isolation and mapping of a polymorphic alphoid repeat from human chromosome 10. Genomics 3 :1-7, 1988.(A)

Devilee P, Thierry RF, Kievits T, Kolluri R, Hopman AHN, Willard HF, Pearson PL, Cornelisse CJ. Detection of chromosome aneuploidy in interphase nuclei from human primary breast tumors using chromosome-specific repetitive DNA probes. Cancer Res 48 :5825-5832, 1988.(B)

Dewald GW, Noel P, Dahl RJ, Spubreck JL. Chromosome abnormalities in malignant hematologic disorders. Mayo Clin Proc 60 :675-689, 1985.

Dinjens WN, van der Welden MM, Schröder FH, Bosman FT, Trapman J. Frequency and characterization of p53 mutations in primary and metastatic human prostate cancer. Int J Cancer 56 :630-633, 1994.
Easton DF. The inherited component of cancer. Brit Med Bull 50: 527-535, 1994.

Forsslund G, Esposti PL, Nilsson B, Zetterberg A. The prognostic significance of nuclear DNA content in prostatic carcinoma. Cancer Res 45: 1418-1439, 1992.

Foster CS, Abel PD. Clinical and molecular techniques fordiagnosis and monitoring of prostatic cancer. Hum Pathol 23 :395-401,1992.

Gale RP, Cannani E. The molecular biology of chronic myelogenous leukemia. Br J Haematol 60 :395-408, 1985.

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

Gibas Z, Becher R, Kawinski E, Horoszewicz J, Sandberg AA. A high-resolution study of chromosome changes in a human prostatic carcinoma cell line (LNCaP). Cancer Genet Cytogenet 11 :399-404, 1984.

Gibas Z, Pontes JE, Sandberg AA. Chromosome rearrangements in a metastatic adenocarcinoma of the prostate. Cancer Genet Cytogenet 16 :301-304, 1985.

Gleason DF. Histologic grading of prostate cancer: A perspective.Hum Pathol 23 :273-279, 1992.

Gray JW, Pinkel D. Molecular cytogenetics in human cancer diagnosis. Cancer Suppl 69 :1536-1542, 1992.

Greene DR, Taylor SR, Wheeler TM, Scardino PT. DNA ploidy by image analysis of individual foci of prostate cancer: A preliminary report. Cancer Res 51 :4084-4089, 1991.

Grönberg H, Damber L, Damber JE. Studies of genetic factors in prostate cancer in a twin population. J Urol 152: 1484-1489, 1994.

Han T, Ozer H, Sadamori n, Emrich L, Gomez GA, Henderson ES, Bloom ML, Sandberg AA. Prognostic importance of cytogenetic abnormalities in patients with chronic lymphocytic leukemia. N Engl J Med 15 :1146-1152, 1984.

Hecht F. Solid tumor breakpoint update. Cancer Genet Cytogenet 31 :129-131, 1988.

Henke RP, Kruger E, Ayhan N, Hubner D, Hammerer P. Numerical chromosomal aberrations in prostate cancer: correlation with morphology and cell kinetics. Virchows Archiv A Pathol Anat 422 :61-66, 1993.

Hopman AHN, Ramaekers FCS, Raap AK, Beck JLM, Devilee P, van der Ploeg M, Vooijs GP. In situ hybridization as a tool to study numerical chromosome aberrations in solid tumors. Histochemistry 89 :307-316, 1988.

Hopman AHN, Poddighe PJ, Smeets AWGB, Moesker O, Beck JLM, Vooijs GP, Ramaekers FCS. Detection of numerical chromosome aberrations in bladder cancer by in situ hybridization. Am J Pathol 135 :1105-1117, 1989.

Hsing AW, Wang RT, Gu FL, Lee M, Wang T, Leng TJ, Spitz M, Blot WJ. Vasectomy and prostate cancer risk in China. Cancer Epidemiol Biomarkers Prev 3 :285-288, 1994.

Huang HJS, Yee JK, Shew JY, Chen PL, Bookstein R, Friedmann T, Lee EYHP, Lee WH. Suppression of the neoplastic phenotype by replacement of the RB gene in human cancer cells. Science 242 :1563-1566, 1988.

Humphrey PA, Walther PJ. Adenocarcinoma of the prostate. Part II: Tissue prognosticators. Am J Clin Pathol 100 :256-269, 1993.

Iizumi T, Yakasi T, Kanoh S, Kondo I, Koiso K. Establishment of a new prostatic carcinoma cell line (TSU-PRI). J Urol 137 :1304-1306, 1987.

Isaacs JT, Coffey DS. Adaptation versus selection as the mechanism responsible for the relapse of prostatic cancer to androgen ablation therapy as studied in the Dunning R3327-H prostatic adenocarcinoma. Cancer Res 41: 5070-5075, 1981.

Isaacs JT, Wake N, Coffey DS, Sandberg AA. Genetic instability coupled to clonal selection as a mechanism for tumor progression in the Dunning R-3327 rat prostate adenocarcinoma system. Cancer Res 42: 2353-2361, 1982.

Isaacs WB, Carter BS, Ewing CM. Wild-type p53 suppresses growth of human prostate cancer cells containing mutant p53 alleles. Cancer Res 51 :4716-4720, 1991.

Isaacs WB, Carter BS. Genetic changes associated with prostate cancer in humans. Cancer Surveys 11: 15-24, 1991.

Isaacs WB, Bova GS, Morton RA, Bussemakers MJG, Brooks JD, Ewing CM. Genetic alterations in prostate cancer. CSH Symp Quant Biol LIX :653-659, 1994.(A)

Isaacs WB, Bova GS, Morton RA, Bussemakers MJG, Brooks JD, Ewing CM. Molecular biology of prostate cancer. Sem Oncol 21 :514-521, 1994.(B)

Jewett HJ. The present status of radical prostatectomy forstages A and B prostatic cancer. Urol Clin North Am 2 ;105-124, 1975.

John EM, Whittemore AS, Wu AH, Kolonel LN, Hislop TG, Howe GR, West DW, Hankin J, Dreon DM, Teh CZ, Burgh JD, Paffenbarger RS. Vasectomy and prostate cancer: results from a multiethnic case-control study. J Natl Cancer Inst 87 :662-669, 1995.

Joos S, Bergerhelm USR, Pan Y, Matsuyama H, Bentz M, Manoir S du, Lichter P. Mapping of chromosomal gains and losses in prostate cancer by comparative genomic hybridization. Genes Chromos Cancer 14 :267-276, 1995.

Kawachi I, Colditz GA, Hankinson S. Long-term benefits and risks of alternative methods of fertility control in the United States. Contraception 50 :1-16, 1994.

Key T. Risk factors for prostate cancer. Cancer Surveys 23: 63-77, 1995.

Knudson AG. Mutation and cancer: statistical study of retinoblastoma. Proc Nat Acad Sci 68 :820-823, 1971.

Knudson AG. Hereditary cancer, oncogenes, and antioncogenes. Cancer Res 45: 1437-1443, 1985.

König JJ, Hagemeijer A, Smit B, Kamst E, Romijn JC and Schröder FH. Cytogenetic characterization of an established xenografted prostatic adenocarcinoma cell line (PC-82). Cancer Genet Cytogenet 34: 91-99, 1988.

Kristoffersson U, Heim S, Mandahl N, Hakan O, Ranstam J, Akerman M, Mitelman F. Prognostic implication of cytogenetic findings in 106 patients with non Hodgkin lymphoma. Cancer Genet Cytogenet 25 :55-64, 1987.

Kunimi K, Bergerheim USR, Larsson IL, Ekman P, Collins VP. Allelotyping of human prostatic adenocarcinoma. Genomics 11: 530-536, 1991.

Lee WH, Bookstein R, Hong F, Young LJ, Shew JY, Lee EY. Human retinoblastoma susceptibility gene: cloning, identification, and sequence. Science 236 : 1657-1661, 1987.

Lee SE, Currin SM, Paulson DF, Walther PJ. Flow cytometric determination of ploidy in prostatic adenocarcinoma: a comparison with seminal vesicle involvement and histopathological grading as a predictor of clinical recurrence. J Urol 140 :769-774, 1988.

Limon J, Dal Cin P, Sandberg AA. Application of long-term collagenase disaggregation for the cytogenetic analysis of human solid tumors. Cancer Genet Cytogenet 23 :305-313, 1986.

Limon J, Lundgren R, Elfving P, Helm S, Kristoffersson U, Mandahl N, Mitelman F. Double minutes in two primary adenocarcinomas of the prostate. Cancer Genet Cytogenet 39 :191-194, 1989.

Linehan WM. Molecular genetics of tumor suppressor genes in prostate carcinoma: the challenge and the promise ahead. J Urol 147 :808-809, 1992.

Lundgren R, Kristofferson U, Helm S, Mandahl N, Mitelman F. Multiple structural chromosome rearrangements, including del(7q) and del(10q), in an adenocarcinoma of the prostate. Cancer Genet Cytogenet 35 :103-108, 1988.

Mamaeva S, Lundgren R, Elfving P, Limon J, Mandahl N, Mamaev N,Henrikson H, Helm S, Mitelman F. AgNOR staining in benign hyperplasiaand carcinoma of the prostate. Prostate 18 :155-162, 1991.

Manuelidis L. Individual interphase chromosome domains revealed by in situ hybridization. Human Genet 91: 28-38, 1985.

Micale MA, Mohamed A, Sakr W, Powell IJ, Wolman SR. Cytogenetics of primary prostatic adenocarcinoma. Clonality and chromosome instability. Cancer Genet Cytogenet 61: 165-173, 1992.

Mitelman F. Catalog of chromosomal aberrations in cancer. AA Sandberg ed, 2nd ed, Alan R Liss, New York, pp.16-28, 1985.

Mohler JL, Partin AW, Epstein JI, Becker RL, Mikel UV, Sesterhenn IA, Mostofi FK, Gleason DF, Sharief Y, Coffey DS. Prediction of prognosis in untreated stage A2 prostatic carcinoma. Cancer 69 :511-519, 1992.

Moller H, Knudsen LB, Lynge E. Risk of testicular cancer after vasectomy: cohort study of over 73,000 men. Brit Med J 309 :295-309, 1994.

Montgomery BT, Nativ O, Blute ML, Farrow GM, Myers RP, Zincke H, Therneau TM, Lieber MM. Stage B prostate adenocarcinoma. Arch Surg 125 :327-331, 1990.

Mostofi FK, Sesterhenn IA, Sobin LH. International histologicalclassification of prostate tumors. Geneva: World Health Organization,1980.

Nativ O, Winkler HZ, Raz Y, Therneau TM, Farrow GM, Myers RP, Zincke H, Lieber MM. Stage C prostatic adenocarcinoma: flow cytometric nuclear DNA ploidy analysis. Mayo Clin Proc 64 :911-919, 1989.

Navone NM, Troncoso P, Pisters LL, Goodrow TL, Palmer JL, Nichols WW, von Eschenbach AC, Conti CJ. P53 protein accumulation and gene mutation in the progression of human prostate carcinoma. J Natl Cancer Inst 85 :1657-1669, 1993.

Nederlof PM, van der Flier S, Raap AK, Tanke HJ, van der Ploeg M, Kornips F, Geraedts JPM. Detection of chromosome aberrations in interphase tumor nuclei by nonradioactive in situ hybridization. Cancer Genet Cytogenet 42: 87-98, 1989.

Nelson-Rees WA, Flandermeyer RR. Letter to the editor: DU145 cell line karyotype. Int J Cancer 21 :796-797, 1978.

Newling DWW. The geography of prostate cancer and its treatment in Europe. Cancer Surveys 23 :289-296, 1995.

Noordzij MA, van der Kwast TH, van Steenbrugge GJ, vanWeerden WM, Oomen MHA, Schröder FH. Determination of Ki-67 defined growthfraction by monoclonal antibody MIB-1 in

formalin-fixedparaffin-embedded prostatic cancer tissues. Prostate 27 :154-159,1995.(A)

Noordzij MA, van SteenbruggeGJ, van der Kwast TH, Schröder FH.Neuroendocrine cells in the normal, hyperplastic and neoplasticprostate. Urol Res 22 :333-341, 1995.(B)

Nowell PC, Hungerford DA. Chromosome studies on normal and leukemic human leukocytes. J Natl Cancer Inst 25: 85-109, 1960.

Ohnuki Y, Marnell MM, Babcock MS, Lechner JF, Kaighn ME. Chromosomal analysis of human prostatic adenocarcinoma cell lines. Cancer Res 40 :52-534, 1980.
Olsson CA, de Vries GM, Raffo AJ, Benson MC, O'Toole K, Cao Y, Buttyan RE, Katz AE. Preoperative reverse transcriptase polymerase chain reaction for prostate specific antigen products predicts treatment failure following radical prostatectomy. J Urol 155: 1557-1562, 1996.

Oomens EHGM, van Steenbrugge GJ, van der Kwast THH, Schröder FH.Application of the monoclonal antibody Ki-67 on prostate biopsies toassess the proliferative cell fraction of human prostatic carcinoma.J Urol 145 :81-85, 1991.

Pinkel D, Landegent J, Collins C, Fuscoe J, Segraves R, Lucas J, Gray J. Fluorescence in situ hybridization with human chromosome-specific libraries: Detection of trisomy 21 and translocations of chromosome 4. Proc Natl Acad Sci 85: 9138-9142, 1988.

Pittman S, Russell PJ, Jelbart ME, Wass J, Raghavan D. Flow cytometric and karyotypic analysis of a primary small cell carcinoma of the prostate : a xenografted cell line. Cancer Genet Cytogenet 26 :165-169, 1987.

Poddighe PJ, Ramaekers FCS, Hopman AHN Interphase cytogenetics of tumours. J Pathol 166 :215-224, 1991.

Rinker-Schaeffer CW, Partin AW, Isaacs WB, Coffey DS, Isaacs JT. Molecular and cellular changes associated with the acquisition of metastatic ability by prostatic cancer cells. Prostate 25 :249-265, 1994.

Rowley JD. A new consistent chromosomal abnormality in chronic myelogenous leukemia identified by quinacrine fluorescence and Giemsa staining. Nature 243 :290-293, 1973.

Sadi MV, Barrack ER. Determination of growth fraction inadvanced prostate cancer by Ki-67 immunostaining and its relationshipto the time to tumor progression after hormonal therapy. Cancer 67:3065-3071, 1991.

Schröder FH, Blom JHM, Hop WCJ, Mostofi FK. Grading of prostaticcancer:I. An analysis of the prognostic significance of singlecharacteristics. Prostate 6 :81-100, 1985.

Schröder FH. Endocrine therapy: where do we stand and where are we going? Cancer Surveys 11 :177-194, 1991.

Schröder FH, Hermanek P, Denis L, Fair WR, Gospodarowicz MK, Pavone-Macaluso M. The TNM classification of prostate cancer. Prostate Suppl 4:129-138, 1992.

Schröder FH. Adjuvant endocrine treatment of prostatic cancer. Eur Urol Update Series 2: 186-191, 1993.

Schröder FH, Damhuis RAM, Kirkels WJ, de Koning H, Kranse R, Nijs HGT, Blijenberg B. European randomized study of screening for prostate cancer. The Rotterdam pilot studies. Int J Cancer 65: 145-151, 1996.

Shankey TV, Kallioniemi OP, Koslowski JM, Lieber ML, Mayall BH, Miller G, Smith GJ. Consensus review of the clinical utility of DNA content cytometry in prostate cancer. Cytometry

14 :497-500, 1993.

Showe LC, Croce CM. Chromosome translocations in B and T cell neoplasia. Sem Hematol 23 :237-244, 1986.

Song J, Cheng WS, Cupps RE, Earle JD, Farrow GM, Lieber MM. Nuclear deoxyribonucleic acid content measured by static cytometry: Important prognostic association for pateints with clinically localized prostate carcinoma treated by external beam radiotherapy. J Urol 147: 794-797, 1992.

Sparkes RS, Sparkes MC, Wilson MG, Towner JW, Benedict W, Murphree AL, Yunis JJ. Regional assignment for human esterase D and retinoblastoma to chromosome band 13q14. Science 208 : 1042-1044, 1980.

Sparkes RS, Murphree AL, Lingua RW, Sparkes MC, Field LL, Funderbunk SJ, Benedict WF. Gene for hereditary retinoblastoma assigned to human chromosome 13 by linkage to esterase D. Science 219 :971-973, 1983.

Stephenson RA, James BC, Gay H, Fair WR, Whitmore WF, Melamed MM. Flow cytometry of prostate cancer :relationship of DNA content to survival. Cancer Res 47: 2504-2509, 1987.

Stone KR, Stone MP, Paulson DF. In vitro cultivation of prostatic epithelium. Invest Urol 14 :79-82, 1976.
Tanaka K, Testa JR. Assessment of methods for the cytogenetic analysis of human solid tumors. J Natl Cancer Inst 79 :1287-1293, 1987.

Tannenbaum M, Tannenbaum S, DeSanctis PN, Olsson CA. Prognosticsignificance of nucleolar surface area in prostate cancer. Urology 19:546-551, 1982.

Teyssler JR. Nonrandom chromosomal changes in human solid tumors: application of an improved culture method. J Natl Cancer Inst 79 :1189-1198, 1987.

Teyssier JR. The chromosomal analysis of human solid tumors. Cancer Genet Cytogenet 37 :103-125, 1989.

Trapman J, Sleddens HFBM, van der Weiden MM, Dinjens WNM, König JJ, Schröder FH, Faber PW, Bosman FT. Loss of heterozygosity of chromosome 8 microsatellite loci implicates a candidate tumor suppressor gene between the loci D8S87 and D8S133 in human prostate cancer. Cancer Res 54: 6061-6064, 1994.

Trent J, Crickard K, Gibas Z, Goodacre A, Pathak S, Sandberg AA, Thompson F, Whang-Peng J, Wolman S. Methodologic advances in the cytogenetic analysis of human solid tumors. Cancer Genet Cytogenet 19 :57-66, 1986.

Tribukait B. Flow cytometry in assessing the clinical aggresiveness of genito-urinary neoplasms. Wid J Urol 5 :108-122, 1987.

Tulinius H, Egilsson V, Olafsdottir GH, Sigvaldason H. Risk of prostate, ovarian, and endometrial cancer among relatives of women with breast cancer. Brit Med J 305 :855-857, 1992.

Umbas R, Isaacs WB, Bringuier PP, Schaafsma HE, Karthaus HFM, Oosterhof GON, Debruyne FMJ, Schalken JA. Decreased e-cadherin expression is associated with poor prognosis in patients with prostate cancer. Cancer Res 54: 3929-3933, 1994.

Van der Gulden JWJ, Klemeney LALM, Verbeek ALM, Straatman H. Mortality trend from prostate cancer in the Netherlands. Prostate 24: 33-38, 1994.

Van der Ploeg M, Raap AK. In situ hybridization: an overview. In: New frontiers in cytology,

eds.K.Goerttler, GE Feichter, S Witte. Springer Verlag, New York. pp13-21, 1988.

Viegas-Pequignot E, Dutrillaux B, Magdelenat H, Coppey-Moisan M. Mapping of single-copy DNA sequences on human chromosomes by in situ hybridization with biotinylated probes : enhancement of detection sensitivity by intensified-fluorescence digital-imaging microscopy. Proc Natl Acad Sci 86 :582-586, 1989.(A)

Viegas-Pequignot E, Jeanpierre M, Dutrillaux AM, Gerbault-Seureau M, Muteris M, Dutrillaux B. Detection of 1q polysomy in interphase nuclei of human solid tumors with a biotinylated probe. Hum Genet 81: 311-314, 1989.(B)

Visakorpi T, Kallioniemi OP, Paronen IYi, Isola JJ, HeikkinenAI, Koivula TA. Flow cytometric analysis of DNA ploidy and S-phasefraction from prostatic carcinomas: implications for prognosis andresponse to endocrine therapy. Br J Cancer 64: 578-582, 1991.

Visakorpi T. Proliferative activity determined by DNA flowcytometry and proliferating cell nuclear antigen (PCNA)immunohistochemistry as a prognostic factor in prostatic carcinoma. JPathol 168 :7-13, 1992.(A)

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

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

Wake N, Slocum HK, Rustum YM, Matsui S, Sandberg AA. Chromosomes and causation of human cancer and leukemia. XLIV. A method for chromosome analysis of solid tumors. Cancer Genet Cytogenet 3 :1-10, 1981.
Waye S, England SB, Willard HF. Genomic organization of " satellite DNA on human chromosome 7 : evidence for two distinct alphoid domains on a single chromosome. Mol Cell Biol 7 :349-456, 1987.

Weijerman PC, van Drunen E, König JJ, Teubel W, Romijn JC, Schröder FH, Hagemeijer A. Specific cytogenetic aberrations in two novel human prostatic cell lines immortalized by human papillomavirus type 18 DNA. Cancer Genet Cytogenet 99: 108-115, 1997.

Wood DP, Banks ER, Humphreys S, McRoberts WJ, Rangnekar VM. Identification of bone marrow micrometastases in patients with prostate cancer. Cancer 74: 2533-2540, 1994.

Wurster DH. Mouse chromosomes identified by Trypsin-Giemsa (T-G) banding. Cytogenetics 11 :379-387, 1972.

Zaridze DG, Boyle P, Smans M. International trends in prostatic cancer. Int J Cancer 33 :223-230, 1984.

Zincke H, Bergstralh EJ, Larson-Keller JJ, Farrow GM, Myers RP, Lieber MM, Barrett DM, Rife CC, Gonchoroff NJ. Stage D1 prostate cancer treated by radical prostatectomy and adjuvant hormonal treatment. Evidence for favorable survival in patients with DNA diploid tumors. Cancer 70 (Suppl1): 311-323, 1992.

CHAPTER 2

SCOPE

SCOPE

This thesis describes the developments in prostate cancer cytogenetics. In seven papers our contributions to the knowledge about what areas in the human genome are important in the development and progression of PC, as well as contributions to technical developments in the reseach field are presented.

We investigated the value of different in vitro culture- and karyotyping techniques and karyotyped cultured cells from PC patient tumors (chapter 3). Our efforts to optimize the culture of primary tumor cells and to karyotype as many PC's as possible gave results that were comparable to those obtained by others, but nevertheless they were not entirely satisfactory. A lower percentage of aberrant karyotypes than expected was found, so there was a need to investigate the reasons for this.

We performed two studies, investigating the effects of tissue digestion by collagenase (chapter 4.1) and of short term tissue culture (chapter 4.2) on the cellular composition of the tissue samples we used. In this way the impact of subsequent preparation techniques used to obtain metaphases was evaluated separately. In these studies we used techniques like DNA flow cytometry (FCM) and fluorescence in situ hybridization (FISH). As a spin off we demonstrated the feasibility to use FISH analysis on PC to evaluate ain and loss of whole chromosomes.

Subsequently FISH was used to assess the copy number of chromosomes 1, 7, 8, 10, 18 and Y in PC patient tumors (chapters 5 and 6). These six chromosomes were chosen based on either cytogenetic or molecular genetic evidence that they are connected with PC. Our aim was to identify chromosomal abnormalities involved in initiation and/or progression of PC.

Several cell lines, all originating from the same precursor PC material, but showing different biological behaviour with respect to androgen sensitivity, were cytogenetically characterized (chapter 7.1). With this study we made a first attempt to unravel the biological evolution of PC, by comparing the cytogenetic abnormalities found and trying to relate some of these to a significant hallmark in PC development, in this case androgen insensitivity.

Finally, we used FISH technology for the investigation of deletion patterns of 8p (chapter 7.2). This study was performed on the same set of sublines karyotyped earlier, displaying different androgen sensitivities. We used different cosmids mapping to 8p, detected by FISH analysis to gain more insight into the chromosomal region that is possibly involved in the development of androgen insensitivity.

CHAPTER 3

CYTOGENETIC ANALYSIS OF 39 PROSTATE CARCINOMAS AND EVALUATION OF SHORT TERM TISSUE CULTURE TECHNIQUES

Josée J.König, Wilma Teubel, Eric Kamst, Johannes C.Romijn, Fritz H.Schröder and Anne Hagemeijer

Published in : Cancer Genetics and Cytogenetics 101: 116-122, 1998

ABSTRACT

Karyotypic analysis was performed on 102 prostate cancer specimens, which were obtained at radical prostatectomy, transurethral resection or regional lymph node dissection. Short term tissue culture was applied in all cases. Of the media and growth factors evaluated, F12/DMEM, supplemented with 2% fetal calf serum, insulin, epidermal growth factor, hydrocortisone and cholera toxin produced the largest increase of in vitro proliferation. Such in vitro cultured cells were all phenotypically acinar epithelial cells, the supposed targets for neoplastic transformation. Stromal cell growth appeared to be completely suppressed.

Of the three culture techniques investigated, the method developed in Lund, Sweden, was the most successful: 11/15 cultures yielded metaphases and in three of these clonal aberrations were identified. All 39 karyotypes obtained essentially had a 46,XY karyotype with clonal aberrations (8 cases) and/or nonclonal aberrations (30 cases). Clonal structural aberrations involved 2p, 3q, 11p, 17p and 21q. The clonal numerical aberrations found were: +8, +dmin and -Y. The most frequently observed nonclonal aberrations were 8p deletions (5 cases) and loss of 6, 7, 8, 15, 17, 18, 21 and/or Y (>5 cases). In summary, clonal aberrations were observed in 20% of the evaluable PC cell cultures, and nonclonal aberrations in 77%. So, although diploid cells without clonal abnormalities still had a growth advantage, under optimal conditions PC cells were able to proliferate in primary in vitro culture.

INTRODUCTION

Cytogenetic analyses of prostatic carcinoma are characterized by a surprising lack of aberrant karyotypes in a large part of the investigated tumors [1-3]. In a minority of cases tumors with very complex karyotypes were described whereby aberrations of chromosomes 1, 7, 8, 10, 16 and Y were indicated to be of potential importance in the origin and progression of PC [1, 3, 4].

From 1987 until 1992 we performed karyotypic analysis on 102 prostate cancer specimens, using different tissue culture procedures. With the aim to optimize the procedure of PC karyotyping, several different tissue culture media and short term culture techniques were developed and/or evaluated. Culture media that differed with respect to their basic composition, fetal calf serum (FCS) and growth factor content [5, 6] were investigated for their ability to establish maximal proliferation of prostate epithelium with minimal contamination by stromal cells. The cell type composition of such cultures was determined by morphological and immunohistochemical analysis [7, 8].

Three different culture methods were applied: a monolayer culture method developed at our institute, a suspension culture method that was developed for cytogenetic analysis of breast cancer [9] and a monolayer culture method that was developed for cytogenetic analysis of prostate cancer in Sweden [10].

The culture success of each culture method used and the karyotypes that were obtained are discussed.

MATERIALS AND METHODS

Tissue sources

Tissue specimens from 102 PC specimens, obtained sterile at radical prostatectomy (73 cases), pelvic lymph node dissection (14 cases) or TUR (15 cases) were used for short term tissue culture. Tissue specimens from the xenografted PC-82 human prostate tumor cell line [11] were used for the proliferation experiments.

Media and growth factors

DMEM, RPMI 1640 and F12/DMEM were obtained ready-for-use from Gibco. PFMR4A was

prepared according to the specifications given by the authors [5]. FCS was obtained from Boehringer and Flow. Insulin (INS), hydrocortisone (HC) and cholera toxin (CT) were obtained from Sigma. Epidermal growth factor (EGF) was obtained from Collaborative Research. All media contained penicillin/streptomycin and glutamine.

Proliferation experiments

To monitor the growth rate of prostate cancer cells in the presence of different growth factors, a three day MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazoliumbromide) test was performed [12]. The xenograft PC82 cell line, that has a finite life span when grown in vitro, was used as a model to determine the optimal culture medium composition to support in vitro growth of prostate cancer cells. In short, 10^4 PC-82 cells, obtained after collagenase digestion of xenografted human tumor tissue [11] were inoculated in 200 µl test medium per well in a 96 well microtiter plate. For each medium eight wells were inoculated. After three days 30 µl MTT (5 mg/ml) was added per well. After incubation for four hours at 37°C the medium was sucked off, 100 µl dimethylsulfoxide was added and the absorption at 515nm was determined as a measure of the amount of viable cells present. For statistical evaluation of the results the Kruskal-Wallis test was used. A value of $P \leq 0.05$ was considered significant.

Preparation of cultures

1. Rotterdam method.

Tumor tissue was minced with scissors, incubated overnight at 37°C with 200 U/ml collagenase (CLS III, Worthington) in DMEM and 10% FCS and then further disaggregated by pipetting. The resulting cell suspension was washed in phosphate buffered saline (PBS) and plated in 25 cm^2 tissue culture flasks in HEPES buffered RPMI + 10% FCS, PFMR4A or F12/DMEM 1:1 + 2%FCS with 10 ng/ml EGF, 10 ng/ml CT, 1 µg/ml HC and 20 µg/ml INS (F12/DMEM +GF). After two days of culture at 37°C and 5% CO_2 floating debris was washed away with PBS and attached cells were given fresh medium. The cultures were inspected every day and when growth was observed (usually within 2-7 days after initiation of cultures), colcemid (2 µg/ml) was added. Metaphase preparation was done as described below.

2. Paris method

PC tissue treatment, culture of cell suspensions and preparation of metaphases was performed essentially as described by Gerbault-Seureau et al.[9]. In short, PC tissue was thoroughly minced with a scalpel. The resulting cell suspension was mixed with RPMI + 6.25% human serum and cultured for 2-3 days. Then colcemid was added for 2 hrs and metaphase preparation was done as described below.

3. Lund method

PC tissue was treated and cultures were prepared essentially as described by Limon et al.[10], with minor modifications. In short, after disaggregation of the tissue by collagenase A (250 U/ml, Boehringer), remaining cell clumps were further dissociated by pulling the tissue suspension through a syringe. The resulting cell suspension was washed and resuspended in 3 ml of Lund medium, which was, like the Rotterdam medium, prepared from F12/DMEM. In the Lund medium however, being a chemically defined medium, no FCS was present. In stead bovine serum albumine and more than 15 different growth factors, hormones, trace elements and minerals were added. Subsequently, through sequential sedimentation at unit gravity, three size fractions of cell clumps were collected. The fractions were plated each in a 25 cm2 culture flask (Nunc, Vitrogen coated, or Falcon Primaria). Inspection for growth was done as described for the Rotterdam method and metaphase preparation was done as described below.

Metaphase preparation and analysis

After overnight incubation with colcemid (2µg/ml), the culture medium was removed, the cells were washed with PBS and, when needed, detached from the plastic with 0.05% trypsin + 0.02% EDTA. Subsequently the cell suspension was incubated in hypotonic solution (KCl 3g/l,+EGTA 0.2 g/l,+HEPES 4.8 g/l [13]) for 20-30 min at 37°C and gently fixed in methanol/ acetic acid as described before [11]. Metaphase spreads were stained for R, Q and/or G bands. Chromosomes were identified according to the ISCN of 1995.

DNA Flow cytometry
The DNA of PC cells was stained with propidium iodide according to the method described by Vindelöv et al.[14] and the relative DNA content was measured using a FACS-II (Becton-Dickinson). Resulting histograms were evaluated as described [15].

Immunohistochemistry
Antibody binding was made visible with an indirect conjugated peroxidase method on cytospin preparations. The nuclei were counterstained with hematoxylin. The monoclonal anti-(α-)keratin antibody RGE53 (α-keratin 18, luminal cell specific) was obtained from Euro-Diagnostics; RCK103 (α-keratin 5, basal cell specific) was kindly provided by Dr.F.C.S.Ramaekers (University of Maastricht, The Netherlands). PSAP and PSA antibodies, respectively polyclonal and monoclonal, were both generated at the Pathology department of the Erasmus University [16]. The monoclonal 1A4 antibody, directed against α-smooth muscle actin, was obtained from Sigma.

RESULTS

Proliferation experiments

Figure 1 shows that growth of PC82 cells was minimal with all three media tested, unless FCS was added. However, the increase in cell number observed with FCS was partly caused by proliferation of non-epithelial (murine stromal) cells (not shown). Several combinations of growth factors (INS + HC, INS + HC + CT and INS + EGF + HC + CT) demonstrated a significant growth promoting effect, while when added alone, no significant increase of proliferation was seen (Figure 2). Moreover, in the medium with all four growth factors added (F12/DMEM+GF) stromal cell growth appeared to be completely suppressed.

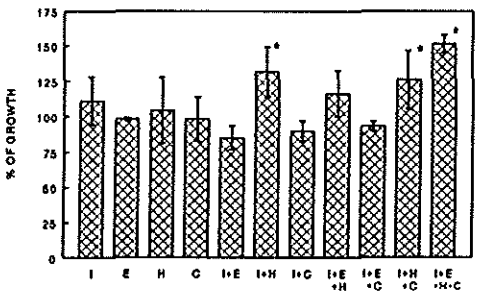


Figure 1. In vitro growth of PC-82 cells with different culture media. The proliferation in RPMI without FCS was taken as 100%. Bars represent averaged data from three experiments, ±S.D.. X-axis: numbers under the bars are percentages of FCS.

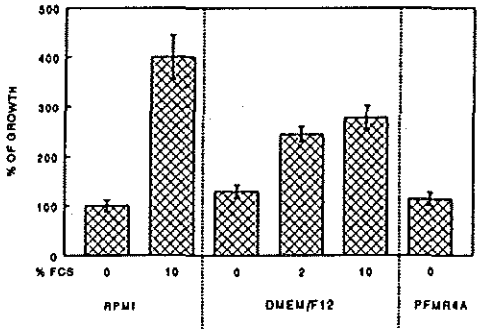


Figure 2. Stimulation of primary in vitro growth of PC-82 by addition of growth factors. Bars represent averaged data from three experiments, expressed as percentage of growth, ±S.D.. Growth in F12/DMEM +2%FCS, which was set at 100%, was taken as a control. I= insulin, 20 Fg/ml; E= epidermal growth factor, 10 ng/ml; H= hydrocortisone, 1 Fg/ml ; C= cholera toxin, 10 ng/ml. * Statistically significant stimulation (P≤0.05, Kruskal-Wallis test).

Characterization of epithelial cells

The cell composition in uncultured cell suspensions, isolated by collagenase digestion of five PC tissue specimens was compared with that of the same cells after culture in F12/DMEM+GF. In uncultured cell suspensions about 40% of the cells were of basal origin (Figure 3A), and about 25% stained with the luminal cell specific α-keratin antibody RGE 53 (Figure 3B). The remaining 35% consisted of stromal cells (not shown).

After in vitro culture all cells reacted positively with the basal cell specific α-keratin antibody (Figure 3C). About 10% of these cells coexpressed the luminal keratin (Figure 3D). While PSAP and PSA are both abundantly present in the luminal epithelium of prostate tissue in vivo, PSAP continued to be expressed in cultured cells, at a frequency comparable with the in vitro expression of luminal keratin (Figure 3E), but PSA expression was not detectable (Figure 3F). Stromal cells could not be demonstrated in samples cultured in F12/DMEM+GF (not shown).

Culture methods and success

Metaphases were obtained from 39 of the 102 tumor samples investigated. The clinical data of these tumors and details on the methods used for in vitro culture are listed in Appendix 1. In Table 1, the success rates for in vitro growth, metaphase yield and occurrence of chromosomal aberrations are given for each of the culture methods tested. It was inherent to the culture method that growth could not be monitored in Paris' cultures. From 5/19 Rotterdam-, 5/9 Paris' and 3/11 Lund cultures less than 5 metaphases could be karyotyped. In 7/19 Rotterdam-, 3/9 Paris' and 9/11 Lund cultures one or more metaphases were found that could not be completely analyzed due to poor quality. In 6/19 Rotterdam and in 6/11 Lund cultures (incomplete) tetraploid cells were observed (Appendix 2). The Lund culture method scored highest in culture success (15/16) as well as in metaphase yield (11/15). Clonal chromosomal aberrations were also most frequent in Lund cultures (3/11). Nonclonal aberrations were about equally frequent with all culture methods (in total 29 cases).

Table 1. Success rates of different culture methods

	R'DAM	PARIS	LUND	ALL
SPECIMENS	60	26	16	102
CULTURES GROWING[1]	44	NE[2]	15	59
CULTURES WITH METAPHASES[3]	19	9	11	39
CULTURES WITH CLONAL ABERRATIONS	3	2	3	8
CULTURES WITH NONCLONAL ABERRATIONS	15	7	8	30

Notes
[1] A culture was classified as growing when on daily microscopic examination of the culture flask an attached colony clearly had increased in size over three consecutive days. [2] NE= not evaluated. In Paris cultures growth, as defined before, was not evaluated, because cells remained floating in de medium. [3] A culture was classified as having yielded metaphases, when more than one analyzable metaphase was obtained.

Karyotypes

Metaphases were obtained from 39 of the 102 tumor samples investigated. Thirty-two of these tumor cultures showed an essentially normal diploid karyotype (not shown). Eight tumor cultures showed clonal chromosomal aberrations in a minority of cells against a diploid background (Table 2). In three of these tumors (PC329, PC389 and PC411, Figure 4) rearranged chromosomes were observed that involved 3q (2x), 2p, 11q, 17p (2x) and 21q. One tumor showed gain of chromosome 8 and one showed loss of Y (resp. PC395 and PC216, not shown). Two tumors had double minutes in several cells (PC210, see Figure 5 and PC307, not shown). PC210 also showed an additional small marker as well as decondensed chromatin in different cells (Figure 5).

Nonclonal loss was most frequently observed for chromosomes 6, 7, 8, 15, 17, 18, 21 and Y. Nonclonal gain was restricted to chromosomes 6, 7, 20 and 22 (Figure 6). Nonclonal structural aberrations were observed for all chromosomes except 4, 19, 20 and Y (Figure 7). The most frequent nonclonal structural aberrations observed (in 5 cases) were different p-arm deletions of chromosome 8. The frequency of nonclonal aberrations observed per tumor specimen varied from one cell to more than 75% of the karyotyped cells.

Table 2. Prostate carcinoma cultures with clonal aberrations

PC NR	SITE[1]	MET HOD[2]	TNM[3]	KARYOTYPE
207	P	R	T3G2N0	46,XY [8]/ 46,XY, **del(17)(p11)** [1]/ 46,XY, **add(3)(qter)**, **del(11)(q13)**, **der(12)**, **del(17)(p11)** [1]/ 46,XY, **t(2;7)(q11;p11)**, **t(11;17)(q23;p11)** [1]/ 46,XY, Ncl.(S) [7]/ 45,XY, Ncl.(N,S) [2]/ 47,XY, Ncl.(N,S) [2].
210	LM	R	T3G2N2	46,XY [4]/ 42-47,XY, Ncl.(N,S), additional fragments, dmin, decondensed chromatin.[25]/ 47,XY,**+marG** [3].
216	LM	R	T3G3Nx	46,XY [7]/ 45,X,-Y [1]/ 44,X,-Y, 3p-,-13 [1]/ 45,XY,-13 [1]/ 47,X,-Y,+12,+18 [1].
307	P	P	T2G2N0	46,XY [6]/ 46,XY, **+dmin** [1]/ 40-42,XY, Ncl.(N) [2]/ 46, **+dmin**, NA [1]/42-46, NA [7].
329	P	P	T3G3N0	44,XY, **dic(3)**,-8,-11 [1]/ 86,XXYY,-1, **dic(3)**, **dic(3)**, -5, -11,-11,-19 [1]/ 48, **dic(3)** (NA) [1]/ 33, **dic(3)** (NA) [1]. **dic(3)= t(3;11)(q21;p11)**
389	P	L	T3G2N0	46,XY [9]/ 42,XY,-15, **del(17)(p12)**,-18,-19,-22,-22, **+marE** [1]/ 36,XY, **del(3)(q13)**, -6,-8,-9,-13,-13, **del(17)(p12)**, -18,-20,-20,-21,-22 [1]/ 41,-X-Y, **del(3)(q13)**,-8,-19,-19 [1]/40-45,XY, Ncl.(N,S) [8]/ 22-38, Ncl.(N) [11]/ 74-92, Incompl.tetra.[3].
395	P	L	T2G2N0	46,XY [6]/ 47,XY,**+8** [2]/ 43-46, NA [4].
411	LM	L	T4G2Nx	46,XY [8]/ 46,XY, **del(21)** [3]/ 45,XY,-15, **del(21)** [1]/ 46,XY, **del(2)**, **del(21)** [1]/ 46,XY, **del(2)** [1]/ 46,XY, **t(1;5)(q11;p11)**, **del(2)**, **18q-** [1]/ 43-46, Ncl.(N,S) [6]/ 86-91, Incompl.tetra.[2] **del(2)= del(2)(p?)** **del(21)= del(21)(q11.2q21.2)**

Notes
[1] P= primary nondisseminated tumor; LM= lymph node metastasis. [2] culture methods: R= Rotterdam; P= Paris'; L= Lund. [3] TNM system according to TNM system for PC of 1992 [31]. Numbers between brackets are the numbers of metaphases found with a given karyotype. Clonal aberrations are in bold type. Ncl.= nonclonal aberrations. N= numerical aberrations. S= structural aberrations. NA= not completely analyzable metaphases. Incompl.tetra.= incomplete tetraploid.

DNA-FCM

55 specimens were analyzed by DNA-FCM to determine their ploidy. 23 tumors were diploid, 14 tumors had an additional tetraploid stem line and 18 tumors showed one or more non-tetraploid aneuploid stem lines (see also Appendix 2).

Tumor site, grade and stage

No correlations were found between obtainment of a karyotype or the presence of nonclonal aberrations and tumor site or stage. However, most karyotypes (23/ 39) were obtained from moderately differentiated (G2) tumors. Also, specimens

with clonal aberrations were unequally distributed: five were primary tumors, six were graded G2 and five were staged T3.

DISCUSSION

The main purpose of the present study was to perform karyotype analysis on a large series of prostate cancer specimens. As part of this study, the results obtained by different culture conditions were evaluated. The Lund method, using collagenase A digestion, size fractionation and a specialized medium for short term culture, yielded the highest percentage of growing cultures, of analyzable metaphases and of clonal chromosomal aberrations. Therefore, at least in our hands, it was the most suitable method for PC cytogenetic analysis.

The clonal numerical changes found in our study were loss of the Y chromosome and gain of chromosome 8. Loss of Y has been described in many PC's [1-4], but also in other tumors and in nonneoplastic tissue [see 4 for ref.]. The significance of -Y as a tumor related aberration is therefore dubious. Numerical aberrations of chromosome 8 have been detected by cytogenetic analysis [4] and by FISH studies [16-22].

Breakpoints at 3q13, 3q21, 11p11, 17p11, 17p12, 21q11 and 21q22 were observed in tumors with clonal structural aberrations. One not further determined deletion involved 2p. Most of the breakpoints have been found in other PC karyotypes [1, 3], except the one at 3q13. Double minutes and an additional small undefined marker chromosome were found in two, respectively one tumor. Such aberrations have also been previously observed in PC [3, 4, 23]. On the other hand, more common aberrations in PC, like +7 and rearrangements of chromosomes 1, 7, 10 and 16 were not found as a clonal change in our series.

Nonclonal aberrations have received much attention in PC cytogenetics. This is partly due to their high frequency but undoubtedly also caused by the absence of clonal aberrations in most PC karyotypes. In our study nonclonal chromosomal aberrations occurred frequently and with all culture methods and media. The majority of these (58%) were random whole chromosome losses, which may represent chromosome instability, as was suggested by Micale et al.[2]. Lundgren et al.[4] described clustering of breakpoints from nonclonal structural changes to the same regions as those of clonal abnormalities and therefore concluded that some of these changes may in fact reflect aberrations in the tumor. In our study structural aberrations comprised 34% of the nonclonal abnormalities, whereby deletion of the p-arm of chromosome 8 was the most frequently observed change. It has already been discussed that this is an important clonal change in PC. Gain of chromosome 7, which has been indicated as a marker for poor prognosis [24], was also observed as a nonclonal abnormality in our study.

The fact that, in agreement with the literature, a large proportion of PC karyotypes analysed was found to be normal, may be explained by the preferential isolation [15] and/or growth [25] of diploid (normal) epithelial cells. Here we have shown that PC cells cultured in F12/DMEM+GF are phenotypically acinar epithelial cells, which in vitro retain some differentiated functions, like PSAP expression. These cells coëxpressed basal and luminal cytokeratins. Cells with the same characteristics have been identified in prostate cancer sections and were postulated to be the target for neoplastic transformation [8].

The finding that the tumors that showed clonal aberrations were mostly advanced (T3-T4) and moderately or poorly differentiated (G2-G3) is in concordance with other reports [4, 23].

DNA-FCM analysis of PC has shown that more than 50% of all PC's are aneuploid

[26, 27]. Fluorescence in situ hybridization (FISH) analyses of PC describe aneusomies for all sixteen different chromosomes investigated until now in 52-97% of the tumors [18-20, 22, 28, 29]. In the present study we found clonal aberrations in only 20% of the evaluable PC cell cultures, and nonclonal aberrations in 77%. We have shown that, under optimal conditions, PC cells will proliferate in vitro, but that apparently the diploid cells without clonal abnormalities have a growth advantage. Therefore, the application of cytogenetic techniques to PC which do not require tissue culture, like FISH and comparative genome hybridization [20, 30], will be essential to the further understanding of the chromosomal rearrangements that are important in the initiation and progression of this common malignancy.

Acknowledgements

We like to thank Bep Smit and Ellen Schoenmaker for their expert technical assistance with karyotyping.

We like to thank Peter Elfving and Rolf Lundgren, working at the institute of prof.Mitelman in Lund, Sweden, for their willingness to teach Wilma Teubel everything about the prostate culturing method that was developed in Lund. We gratefully acknowledge the financial support from the EC Biomed I concerted action program, which made the exchange between Rotterdam and Lund possible. This work was supported by grants IKR 86-14 and IKR 90-12 from the Dutch Cancer Society.

LITERATURE

1. Sandberg AA (1992): Chromosomal abnormalities and related events in prostate cancer. Hum Pathol 23:368-380.

2. Micale MA, Mohamed A, Sakr W, Powell IJ, Wolman SR (1992): Cytogenetics of primary prostatic adenocarcinoma. Clonality and chromosome instability. Cancer Genet Cytogenet 61:165-173.

3. Arps S, Rodewald A, Schmalenberger B, Carl P, Bressel M, Kastendieck H (1993): Cytogenetic survey of 32 cancers of the prostate. Cancer Genet Cytogenet 66:93-99.

4. Lundgren R, Mandahl N, Heim S, Limon J, Hendrikson H, Mitelman F (1992): Cytogenetic analysis of 57 primary prostatic adenocarcinomas. Genes Chromos Cancer 4:16-24.

5. Peehl DM, Stamey TA (1985): Growth responses of normal, benign hyperplastic, and malignant human prostatic epithelial cells in vitro to cholera toxin, pituitary extract, and hydrocortisone. Prostate 8:51-61.

6. McKeehan WL, Adams PS, Fast D (1987): Different hormonal requirements for androgen-independent growth of normal and tumor epithelial cells from rat prostate. In Vitro 23:147-152.

7. Svanholm H, Nielsen B, Starklint H (1988): Keratin patterns in prostatic hyperplasia and adenocarcinoma. APMIS suppl 4:100-108.

8. Verhagen APM, Ramaekers FCS, Aalders TW, Schaafsma HE, Debruyne FMJ, Schalken JA (1992): Colocalization of basal and luminal cell-type cytokeratins in human prostate cancer. Cancer Res 52:6182-6187.

9. Gerbault-Seureau M, Vielh P, Dutrillaux B (1987): Recurrent HSR in the centromeric region of chromosome 8 in breast cancer. Ann Genet 30:146-151.

10. Limon J, Lundgren R, Elving P, Heim S, Kristofferson U, Mandahl N, Mitelman F (1990): An improved technique for short-term culturing of human prostatic adenocarcinoma tissue for cytogenetic analysis. Cancer Genet Cytogenet 46:191-199.

11. König JJ, Hagemeijer A, Smit B, Kamst E, Romijn JC, Schröder FH (1988): Cytogenetic characterization of an established xenografted prostatic carcinoma cell line (PC-82). Cancer Genet Cytogenet 34:91-99.

12. Romijn JC, Verkoelen CF, Schröder FH (1988): Application of the MTT assay to human prostate cancer cell lines in vitro :establishment of test conditions and assessment of hormone-stimulated growth and drug-induced cytostatic and cytotoxic effects. Prostate 12:99-110.

13. Trent J, Crickard K, Gibas Z, Goodacre A, Pathak S, Sandberg AA, Thompson F,

Whang-Peng J, Wolman S (1986): Methodologic advances in the cytogenetic analysis of human solid tumors. Cancer Genet Cytogenet 19:57-66.
14. Vindelöv LL, Christensen IJ, Nissen NI (1983): A detergent-trypsin method for the preparation of nuclei for flow cytometric DNA analysis. Cytometry 3:323-327.
15. König JJ, Dongen van JJW, Schröder (1993): Preferential loss of abnormal prostate carcinoma cells by collagenase treatment. Cytometry 14: 805-810.
16. Gallee MP, Visser-de Jong E, van der Korput JA, van der Kwast TH, ten Kate FJ, Schröder FH, Trapman J (1990): Variation of prostate-specific antigen expression in different tumour growth patterns present in prostatectomy specimens. Urol Res 18:181-187.
17. Baretton GB, Vallna C, Vogt T, Schnelderbanger K, Diebold J, Lohrs U (1994): Interphase cytogenetics of prostatic carcinomas by use of nonisotopic in situ hybridization. Cancer Res 54:4472-4480.
18. Visakorpi T, Hyytinen E, Kallioniemi A, Isola J, Kallioniemi OP (1994): Sensitive detection of chromosome copy number aberrations in prostate cancer by fluorescence in situ hybridization. Am J Pathol 145:624-630.
19. Alers JC, Krijtenburg PJ, Vissers KJ, Bosman FT, Kwast van der TH, Dekken van H (1995): Interphase cytogenetics of prostatic adenocarcinoma and precursor lesions: Analysis of 25 radical prostatectomies and 17 adjacent prostatic intraepithelial neoplasias. Genes Chromos Cancer 12:241-250.
20. Cher ML, MacGrogan D, Bookstein R, Brown JA, Jenkins RB, Jensen RH (1994): Comparative genomic hybridization, allelic imbalance, and fluorescence in situ hybridization on chromosome 8 in prostate cancer. Genes Chromos Cancer 11:153-162.
21. Macoska JA, Trybus TM, Sakr WA, Wolf MC, Benson MC, Powell IJ, Pontes JE (1994): Fluorescence in situ hybridization analysis of 8p allelic loss and chromosome 8 instability in human prostate cancer. Cancer Res 54:3824-3830.
22. König JJ, Teubel W, Romijn JC, Schröder FH, Hagemeijer A (1996): Gain and loss of chromosomes 1, 7, 8, 10, 18, and Y in 46 prostate cancers. Hum Pathol 27: 720-727, 1996.
23. Brothman AR, Peehl DM, Patel AM, McNeal JE (1990): Frequency and pattern of karyotypic abnormalities in human prostate cancer. Cancer Res 50:3795-3803.
24. Bandyk MG, Zhao L, Troncoso P, Pisters LL, Palmer JL, Eschenbach AC von, Chung LWK, Liang JC (1994): Trisomy 7: a potential cytogenetic marker for human prostate cancer progression. Genes Chromos Cancer 9:19-27.
25. König JJ, Teubel W, Dongen JJW van, Hagemeijer A, Romijn JC, Schröder FH (1993): Tissue culture loss of aneuploid cells from carcinomas of the prostate. Genes Chromos Cancer 8:22-27.
26. Nativ O, Myers RP, Farrow GM, Therneau TM, Zincke H, Lieber MM (1990): Nuclear deoxyribonucleic acid ploidy and serum prostate specific antigen in operable prostatic adenocarcinoma. J Urol 144:303-306.
27. Miller J, Horsfall DJ, Marshall VR, Rao DM, Leong ASY (1991): The prognostic value of deoxyribonucleic acid flow cytometric analysis in stage D2 prostatic carcinoma. J Urol 145:1192-1196.
28. König JJ, Teubel W, Dongen JW van, Romijn JC, Hagemeijer A, Schröder FH (1994): Loss and gain of chromosomes 1, 18 and Y in prostate cancer. Prostate 25:281-291.
29. Brown JA, Alcaraz A, Takahashi S, Persons DL, Lieber MM, Jenkins RB (1994): Chromosomal aneusomies detected by fluorescence in situ hybridization analysis in clinically localized prostate carcinoma. J Urol 152:1157-1162.
30. Visakorpi T, Kallioniemi AH, Syvanen AC, Hyytinen ER, Karhu R, Tammela T, Isola JJ, Kallioniemi OP (1995): Genetic changes in primary and recurrent prostate cancer by comparative genomic hybridization. Cancer Res 55:342-347.
31. Schröder FH, Hermanek P, Denis L, Fair WR, Gospodarowicz MK, Pavone-Macaluso M (1992): The TNM classification of prostate cancer. Prostate Suppl 4:129-138.

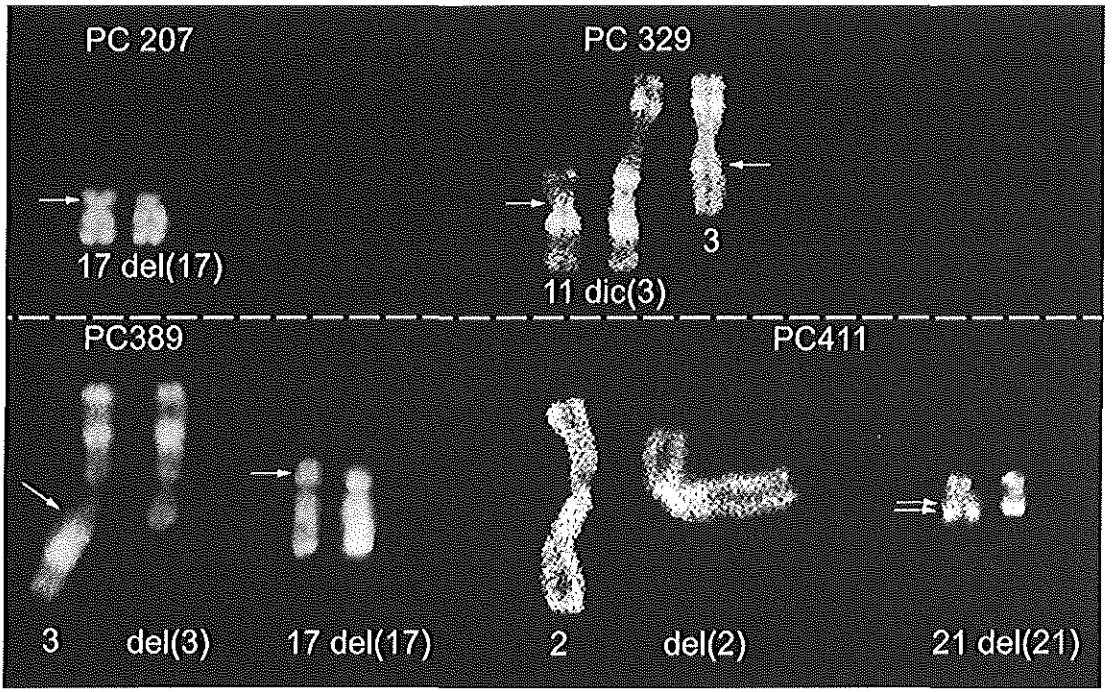


Figure 4. Clonal structural aberrations from five PC cultures. Normal chromosomes are placed left of the marker chromosomes, with identified breakpoints indicated by arrows. R-banding analysis.
Del(17)(p11), observed in PC207.
Dic(3), observed in PC329, resulting from an unbalanced translocation between chromosomes 3 and 11: t(3;11)(q21;p11).
Del(3)(q13) and del(17)(p12), observed in PC389.
Del(2)(p?p?) and del(21)(q11q22), observed in PC411.

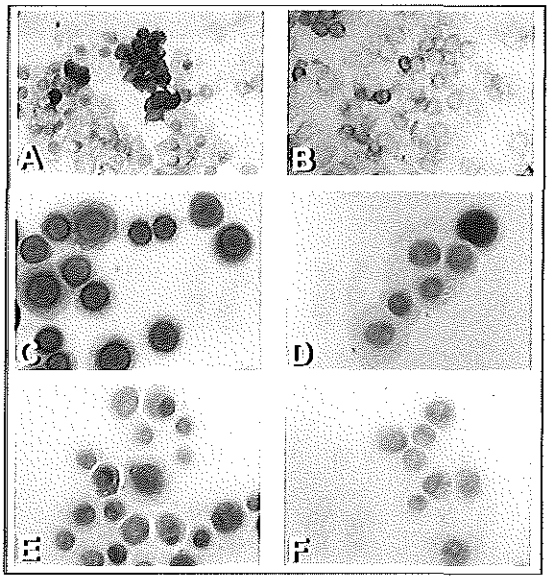


Figure 3. Photomicrographs of cytospin preparations from PC305. Immunoperoxidase staining. Magnification under microscope for all photographs: 125x. A and B: uncultured samples, C-F: cultured samples. Antibodies: A and C. RCK103; B and D. RGE 53; E. -PSAP; F. - PSA.

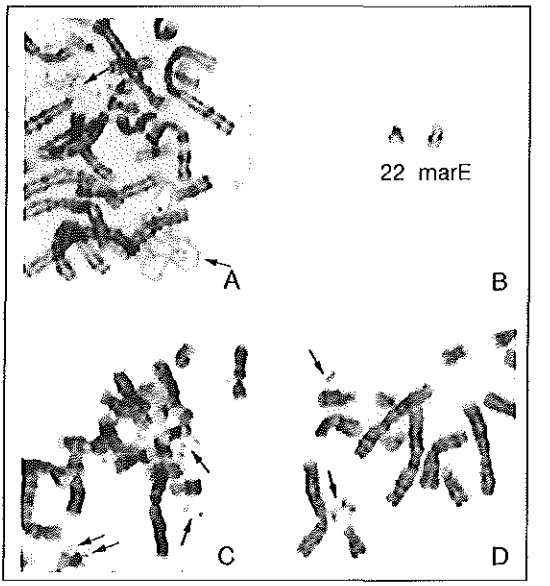


Figure 5. Partial metaphases of PC210.
A. Decondensed chromatin. B. Marker 22 like chromosome. C and D. Double minutes.
G-banding analysis.

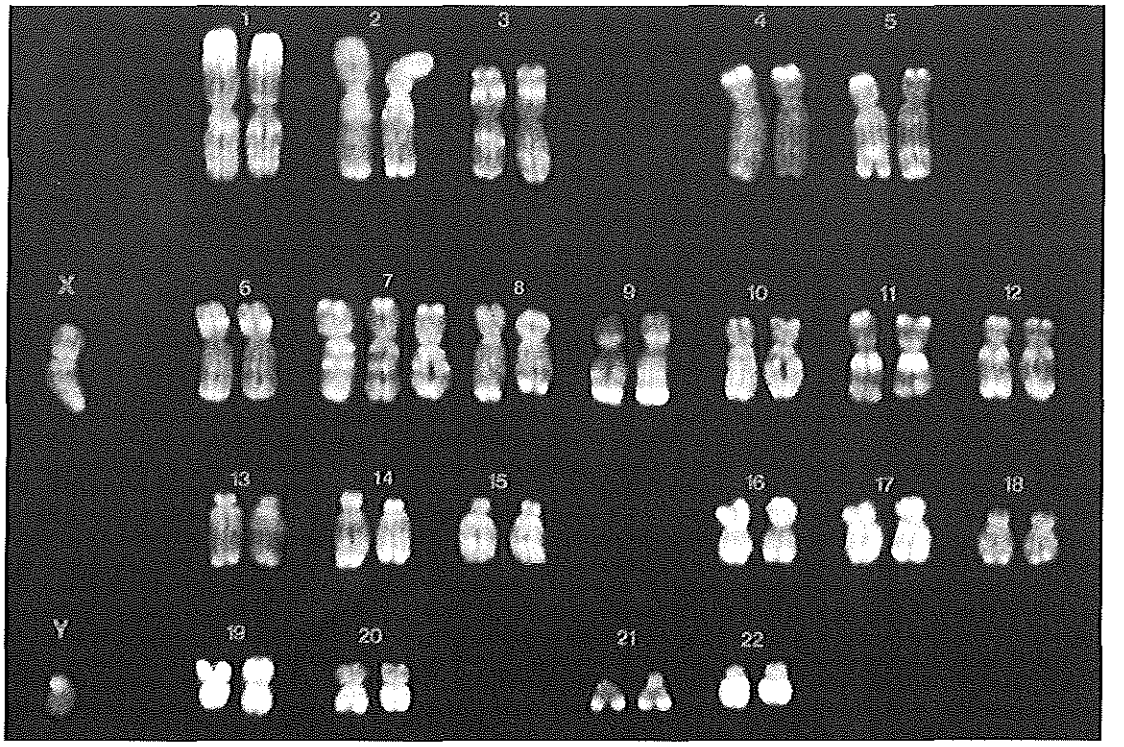


Figure 6. Karyotype of a metaphase from PC207, R-banding: 47, XY, der(3q), +7.

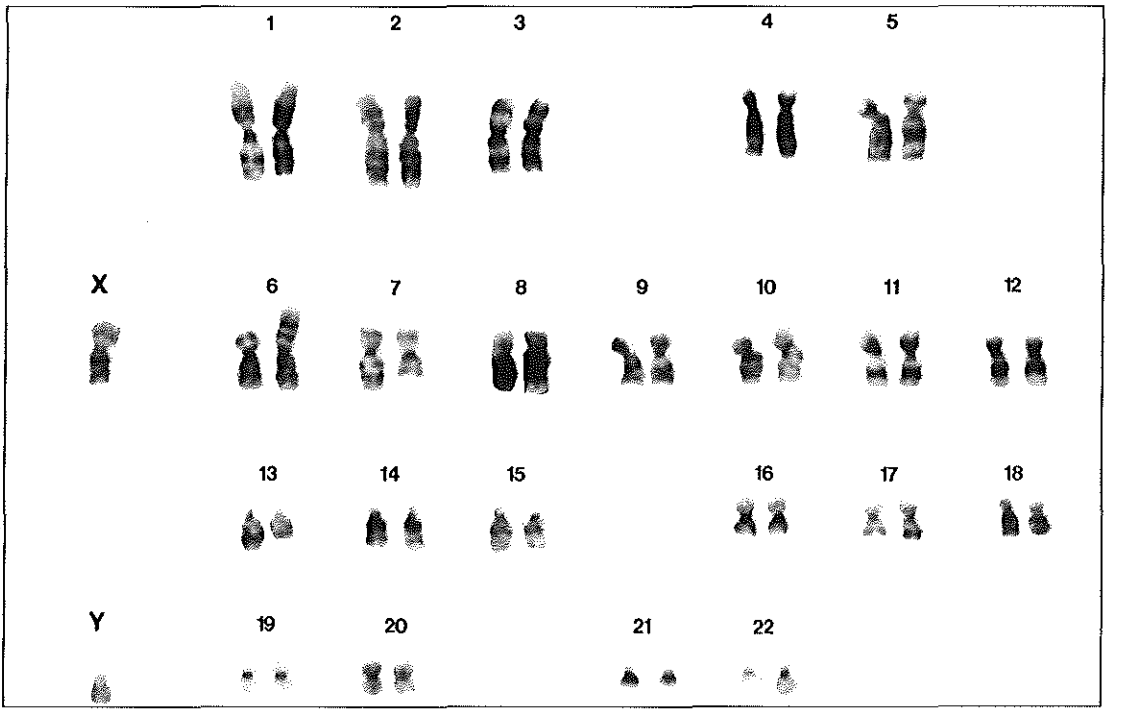


Figure 7. Karyotype of a metaphase from PC207, G-banding: 46, XY, t(6p11;7q11).

Appendix 1
Clinical and culture parameters of PC cultures

PC NR	TISSUE CULTURE			TNM AND GRADE[4]	P/LM/ TUR[5]
	GROWTH RATE[1]	MEDIUM[2]	CULTURE TIME[3] (DAYS)		
ROTTERDAM AND EXPLANT METHOD					
159	±	1	7	T3G2N0M0	P
164	±	2	2	T3G2N0M0	P
171	±	2	1	T4G3N0M0	P
172	±	2	4	T3G3N0M0	P
179	+	2	7	T3G2N0M0	P
194	±	2	3	T3G2N0M0	P
198	±	3	6	T3G2N0M0	P
202	±	3	14	T4G1N3M0	P
207	+	3	5	T3G2N0M0	P
210	+	3	5	T3G2N2M0	LM
212	±	3	5	T3G3N0M0	P
213	+	3	4	T3G3N0M0	P
215	+	3	3	T3G3NxMx	P
216	±	3	6	T3G3NxM0	LM
227	±	3	4	T2G2N0M0	P
242[6]	+	3	9	T3G3N1M0	P
246	+	3	4	T3G2N0M0	P
252	+	3	7	T2G2N0M0	P
253	ND	3	7	T4G2N2M1	LM

Appendix 1, continued

PC NR	TISSUE CULTURE			TNM AND GRADE[4]	P/LM/ TUR[5]
	GROWTH RATE[1]	MEDIUM[2]	CULTURE TIME[3] (DAYS)		
PARIS METHOD					
293	ND	4	3	T2G2N0M0	P
294	ND	4	3	T4G3NxMx	TUR
295	ND	4	3	T1G3NxM0	LM
297	ND	4	3	T3G2NxM0	TUR
305	ND	4	3	T2G1N0M0	P
307	ND	4	3	T2G2N0M0	P
319	ND	4	3	T2G2N0M0	P
324	ND	4	3	T4G3NxMx	TUR
329	ND	4	3	T3G3N0M0	P
LUND METHOD					
373	+	5	8	T3G3N0M0	P
376	++	5	12	T4G3N0M0	P
377	+	5	12	T3G3N0M0	P
378	+	5	12	T1G2N0N0	P
379	++	5	9	T3G2N0M0	P
382	++	5	6	T2G1N0M0	P
383	++	5	7	T3G3N0M0	P
385	+	5	5	T3G2N0M0	P
387	+	5	6/18	T4G2N0M0	P
389	++	5	5	T3G2N0M0	P
394	+	5	6/7	T3G3N0M0	P
395	+	5	4	T2G2N0M0	P
396	+	5	5	T2G2N0M0	P
404	±	5	8	T4G2N1M0	LM
411	±	5	11	TxG2NxM0	LM

Notes

[1]Growth rates: ± = moderate growth; + = good growth; ++ = abundant growth; ND= not determined. [2] Media: 1= PFMR4A; 2= RPMI + 10%FCS; 3= F12/DMEM + GF; 4= RPMI + 10% human serum; 5= Lund medium. [3] Culture time from initiation until harvest of metaphases. [4] TNM and grade : Pathological staging was done according to the TNM system for PC of 1992 [31]. Histological grading: G1= well differentiated, G2= moderately differentiated, G3= poorly differentiated. [5] P= primary tumor; LM= regional lymph node metastasis; TUR= tumor tissue obtained at palliative transurethral resection. [6] explant culture.

Appendix 2
FCM and cytogenetics results of PC cultures

PC NR	PLOIDY[1]	DI TUMOR (%)[2]	KARYOTYPE
ROTTERDAM AND EXPLANT METHOD			
159	ND	-	46,XY,-7,+marD [1].
164	ND	-	46,XY [2].
171	ND	-	46,XY [14]/ 45-47, Ncl. (N,S) [4]/ 90, Incompl.tetra.[1].
172	ND	-	46,XY [9]/ 45,XY,-10 [1]/ 47,XY,+mar [1]/ 88, Incompl.tetra.[1]/ 40-46, NA [5].
179	ND	-	45,XY,-3 [1].
194	ND	-	46,XY [1].
198	ND	-	46,XY [2]/ 44-49, NA [6].
202	A	1.6 (20)	46,XY [23]/ 46,XY, t(3;7)(q27;q33) [1]/ 92,XXYY, t(3;3)(q;q) [1].
207	ND	-	46,XY [8]/ 46,XY, del(17)(p11) [1]/ 46,XY, add(3)(qter), del(11)(q13), der(12), del(17)(p11) [1]/ 46,XY, t(2;7)(q11;p11), t(11;17)(q23;p11) [1]/ 46,XY, Ncl.(S) [7]/ 45,XY, Ncl.(N,S) [2]/ 47,XY, Ncl.(N,S) [2].
210	T	2(15)	46,XY [4]/ 42-47,XY, Ncl.(N,S), additional fragments, dmin, decondensed chromatin [25]/ 47,XY,+marG [3].
212	A	1.85 (11)	46,XY [15]/ 44-47,XY, Ncl.(N,S) [2].
213	ND	-	41-46, NA [5].
215	D	-	38-47,XY, Ncl.(N) [6]/ 43-46, NA [7]/ 87, NA [1].
216	ND	-	46,XY [7]/ 45,X,-Y [1]/ 44,X,-Y, 3p-,-13 [1]/ 45,XY,-13 [1]/ 47,X,-Y,+12,+18 [1].
227	ND	-	46,XY [2]/ 45,XY,-5 [1]/ 39-46, NA [8].
242	D	-	46,XY [17]/ 45-46,XY, Ncl.(N,S) [2].
246	ND	-	45, Ncl.(N) [2].
252	A	1.75 (10)	46,XY [2]/ 41-47,XY, Ncl.(N) [3]/ 92, NA [1].
253	A	1.85 (13)	46,XY [8]/ 45-47, Ncl.(N) [2]/ 84, NA [1].

Appendix 2, continued

PC NR	PLOIDY[1]	DI TUMOR (%)[2]	KARYOTYPE
PARIS METHOD			
293	A	0.85 (95) 1.6 (5)	46,XY [1]/ 44,XY,-10,-12,-15,+mar [1]/ 46,XY,-4,+marC [1].
294	T	2(62) 3.1(8)	46,XY [1].
295	A	0.7(38) 1.85 (13)	46,XY [4].
297	A	0.65	46,XY [4]/ 43,X,-Y,-14,-22 [1]/ 44,XY,-14,-21 [1]/ 45,XY,-21 [1].
305	D	-	46,XY [7]/ 41-46,XY, Ncl.(N) [4]/ 40-46, NA [6].
307	D	-	46,XY [6]/ 46,XY, +dmin [1]/ 40-42,XY, Ncl.(N) [2]/ 46, +dmin, NA [1]/42-46, NA [7].
319	D	-	46,XY [8]/ 46,XY,-13,-14,+15,-20,+21,+marE [1].
324	D	-	46,XY [1]/ 44,X,-Y,-17 [1]/ 45,XY,-16 [1].
329	D	-	44,XY, dic(3),-8,-11 [1]/ 86,XXYY,-1, dic(3), dic(3), -5, -11,-11,-19 [1]/ 48, dic(3) (NA) [1]/ 33, dic(3) (NA) [1]. dic(3)= t(3;11)(q21;p11)

Appendix 2, continued

PC NR	PLOIDY[1]	DI TUMOR (%)[2]	KARYOTYPE
LUND METHOD			
377	D	-	46,XY [5]/ 44-46, Ncl.(N) [14]/ 40,XY,-2,-3,-6,-1,-8,-9,+11,-12,-13,+21 [1].
378	A	0.85 (95)	46,XY [28]/ 47,XY,+mar [1]/ 46,X,-Y,+marC [1]/ 45,X,-Y [1]/ 41-45, Ncl.(N) [4]/ 58, NA [1]/ 92,XXYY [1].
379	T	2(15)	46,XY [27]/ 45,XY,-17 [1]/ 41,X,-Y,-11,-17,-20,-22 [1]/ 46, NA [4]/ 61, NA [1]/ 85, NA [1].
382	D	-	46,XY [6]/ 44-46, Ncl. (N) [5]/ 62, NA [1]/ 46, NA [1].
385	T	2(35)	46,XY [1]/ 51, NA [1].
387	ND	-	46,XY [1]/ 47, NA [1].
389	D	-	46,XY [9]/ 42,XY,-15, del(17)(p12),-18,-19,-22,-22,+marE [1]/ 36,XY, del(3)(q13), -6,-8,-9,-13,-13, del(17)(p12),-18,-20,-20,-21,-22 [1]/ 41,-X-Y, del(3)(q13),-8,-19,-19 [1]/ 40-45,XY, Ncl.(N,S) [8]/ 22-38, Ncl.(N) [11]/ 74-92, Incompl.tetra.[3].
394	A	1.1(79)	46,XY [12]/ 46,XY,10p-,13p+ [1]/ 44,Y,-X,-16 [1]/ 44-46, NA [6]/ 88-92, NA [2].
395	D	-	46,XY [6]/ 47,XY,+8 [2]/ 43-46, NA [4].
396	D/A3	0.7-1 (94)	46,XY [2]/ 46, NA [1]
404	ND	-	46,XY [2]/ 44-47, Ncl.(N) [3]/ 43-89, NA [5].
411	ND	-	46,XY [8]/ 46,XY, del(21) [3]/ 45,XY,-15, del(21) [1]/ 46,XY, del(2), del(21) [1]/ 46,XY, del(2) [1]/ 46,XY, t(1;5)(q11;p11), del(2), 18q- [1]/ 43-46, Ncl.(N,S) [8]/ 86-91, Incompl.tetra.[2] del(2)= del(2)(p?) del(21)= del(21)(q11.2q21.2)

Notes

[1] Ploidy: D= diploid; T= tetraploid; A= aneuploid; ND= not determined. [2] DI tumor= DNA Index of the tumor cells, i.e. position of the major tumor cell peak(s) in relation to the normal diploid peak in the DNA histogram; percentage of these cells between parentheses. [3] Hypodiploid peak and diploid peak were merged into one, nonseparable peak. Karyotype analysis: - = no metaphases; NA= not completely analyzable; Ncl.= nonclonal aberrations; N= numerical;S= structural; Incompl.tetra.= incomplete tetraploid. The number of analyzed metaphases with a given karyotype is between brackets.

CHAPTER 4.1

PREFERENTIAL LOSS OF ABNORMAL PROSTATE CARCINOMA CELLS BY COLLAGENASE TREATMENT

Josée J.König, Jan Willem van Dongen and Fritz H.Schröder

Published in : Cytometry 14: 805-810, 1993

ABSTRACT

Effects of two different methods of tumor disaggregation on flow cytometric ploidy distribution and intact cell yield were investigated. Either mechanical disaggregation or collagenase digestion was applied to 35 prostate tumor specimens. Seven collagenase treated samples failed to yield any intact cells, whereas with mechanical disaggregation in all cases a sufficient number of intact cells were obtained. No differences in the FCM ploidy distribution of tumors with a DNA diploid stemline were observed comparing both techniques. In DNA aneuploid tumors however, collagenase treatment had an adverse effect on the abnormal cell populations. In 14/17 of such tumors the abnormal cell populations were significantly reduced; in 8 of these the percentage of DNA aneuploid cells declined even below the minimum percentage (10%) that was defined for DNA aneuploidy. Since collagenase is a widely used enzyme for tissue disaggregation, especially in tumor cytogenetics, the presented data will have consequences for the interpretation of results obtained by methods involving the use of this enzyme.

INTRODUCTION

Solid tumor cytogenetics comprises a small but rapidly expanding field of research. A number of reports on the cytogenetics of prostate carcinoma (PC) have indicated several structural chromosome rearrangements, which are thought to be possibly tumor specific (1, 7, 8, 14). However, the majority of the investigated PC specimens (73%) demonstrate a surprising lack of both structural and numerical chromosomal changes, resulting in essentially normal diploid karyotypes (3, 8, 14). In contrast, using DNA flow cytometry (FCM) more than 50% of the PC's were shown to have one or more DNA aneuploid cell populations (unpublished results and 11, 15, 16, 22). The causes for this observed discrepancy between cytogenetic and FCM results have not been identified until now.

In general, the preparation of tumor tissue samples for cytogenetic analysis can be divided into two stages:

a) disaggregation of the tissue into a cell suspension and

b) short term tissue culture to generate an adequate number of proliferating cells.

Tissue disaggregation can be established by mechanical or enzymatic treatment or by a combination of these. Trypsin, collagenase and DNAseI, alone or in combination, are most commonly used for this purpose. For the collagen-rich prostate tissue collagenase disaggregation has long since been the method of choice to obtain highly viable cell suspensions that are capable to proliferate in vitro (5, 18, 21). This enzyme has therefore also been used to prepare prostate tumor cell suspensions for the initiation of short term tissue cultures for cytogenetic analysis (3, 13). Collagenase is currently the most frequently used enzyme for preparing cell suspensions for cytogenetic analysis of solid tumors (12, 19). Some reports on solid tumors however, mention negative effects of collagenase treatment on FCM results, ranging from a poor yield (17) to a larger percentage of DNA diploid histograms (2, 4, 17). Here we describe our experiences with collagenase digestion and its effects on the ploidy of prostate tumor cells, investigated by FCM. The effects of tissue culture on the ploidy of PC are described in another paper (9).

MATERIAL AND METHODS

Thirty-five prostate adenocarcinoma specimens were studied, obtained from radical prostatectomy (N=26) pelvic lymphadenectomy (N=5) and palliative transurethral resection

(N=4). Suspected carcinoma tissue was excised. For each specimen, tumor grade and stage were assessed from paraffin sections of adjacent tissue. The specimen was cut into small fragments (2 by 1 mm) with scissors under sterile conditions and divided in two parts (See Fig.1). In this way a total number of 70 samples was obtained for FCM analysis.
To the first part approximately 0.5 ml of phosphate buffered saline (PBS) was added and the tissue pieces were further minced by a scalpel into a suspension of small cell clumps and single cells. The clumps were allowed to sediment for 3-5 min in 5 ml of PBS and then discarded. The supernatant was centrifuged, the pellet was washed with PBS, resuspended in citrate buffer and stored in liquid nitrogen until use.

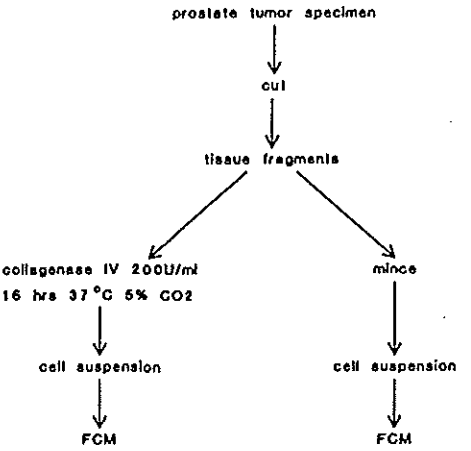


Figure 1. Schematic presentation of sample preparation.

The second part was transferred to a 25 cm^2 culture flask. 5 ml of RPMI (GIBCO) supplemented with 10% fetal calf serum and 200U/ml collagenase IV (CLS III, Worthington; collagenase activity 118U/mg; low proteolytic activity) was added, and the sample was incubated at 37°C and 5% CO_2 for 16 hrs. After incubation most tissue pieces were still intact and pipetting was needed to disperse these into small cell clumps and single cells. The resulting cell suspension was washed two times in PBS and treated further as described above.
Thawed suspensions were processed for FCM by the Vindelov method (20). Nuclear DNA content was measured in a FACS II flow cytometer equipped with a Argon Ion laser (Spectra Physics model 2016) set at 200mW and used at a wavelength of 514 mm. Suspensions of prepared nuclei contained less than 7% doublets, irrespective of the isolation method. They were passed through a 20µ nylon filter prior to FACS analysis. A 50µ nozzle was used. Before and after each run a sample of cultured normal prostate fibroblasts (10) was measured to determine the position of the normal G0/G1 (DI (DNA Index)=1) and G2M (DI=2) peaks as an external control. Chicken erythrocytes (CRBC), added to each sample, served as an internal control. Of each sample a dot plot of 11,000 events was recorded. The dot plots were evaluated with an especially designed FCM analysis computer program. Low fluorescent debris, present at the low left part of the dot plot and thus collected in the first two or three channels, was eliminated from the dot plot data by excluding these channels.
The mean value of the G2M fraction in DNA diploid cells, determined in 34 mechanically disaggregated BPH (benign prostatic hyperplasia) specimens, was 5.2 $\pm$ 2.1 % (N=34). Using this average, the cut off percentage for tumor nuclei with a DI of 1.9-2.1 was defined at 10% (mean value + two times SD: 5.2 + 2x2.1 = 9.4).

RESULTS

Ploidy measurements on mechanically treated samples

Thirteen tumors (37.1%) had only a DNA diploid DNA stemline (DI 0.9-1.1; not shown). All other DNA histograms from mechanically disaggregated tumor material showed one or more DNA aneuploid stemlines in addition to a DNA diploid stemline. Fifteen of these tumors (42.9%) had one additional DNA aneuploid stemline in the range between DI 1.9 and 2.1 (>10% of the nuclei; see materials and methods). Four tumors had an DNA aneuploid stemline in the DI range between DI 1.6 and 1.9 (nrs. 1, 2, 11 and 15), two had a DI< 0.9 (nrs. 17 and 36) and one had two DNA aneuploid stemlines (nr. 35) (see also Table 2).

Effect of collagenase incubation on sample quality

Collagenase treatment failed to yield intact cells from 5/15 DNA aneuploid and 2/13 DNA diploid tumor samples. In contrast, using mechanical disaggregation, always a sufficient number of cells was obtained for FCM analysis.

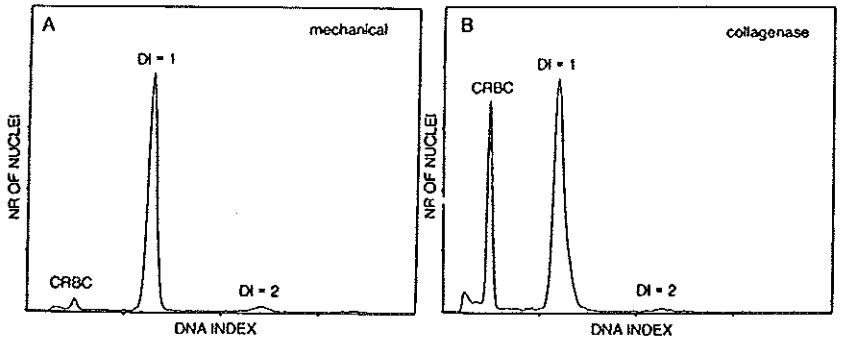
In general there was more cell debris present in the samples obtained with collagenase treatment, mainly indicated by a higher percentage of small material with low fluorescence, which was collected in the low left part of the dot plots. This debris had no effect on any further calculations, however (see materials and methods section).

The mean CV (coefficient of variation) for tumors with only a DNA diploid stemline was 5.4±1.4% with mechanical disaggregation. Although not dramatically increased, the mean CV was significantly higher with collagenase treatment (7.4±2.0%, $p<0.05$). In one case the CV was lower with collagenase treatment (nr.19, respectively 7.9% and 6.2%).

For DNA aneuploid tumors the CV's were also higher, but not significantly different whether mechanical or enzymatic disaggregation was applied. In these cases the mean CV's for the DNA diploid stemline were respectively 6.4±1.5% and 7.4±3%. For the DNA aneuploid stemline(s) the mean CV's were 5.9±1.2% and 6.7±2.6%. Two other cases were characterized by high CV's (>10%) for all stemlines, occurring in either the mechanically (nr.36) or in the collagenase treated samples (nr.8).

Collagenase effect on ploidy

In the 11 evaluable tumors with a DNA diploid stemline no difference in the ploidy distribution of either mechanical- or collagenase treated samples could be observed (see Figures 2A and 2B).



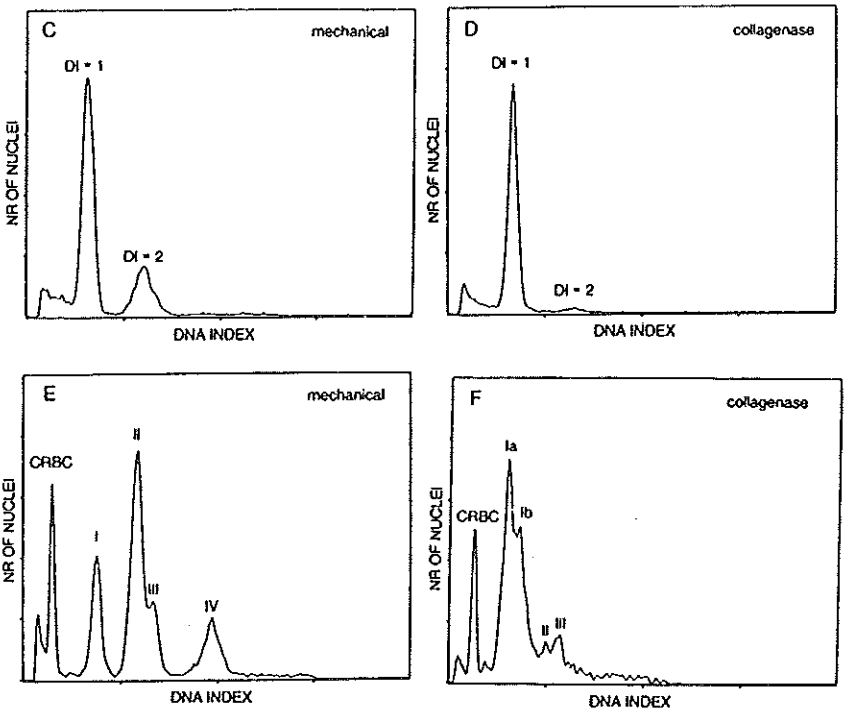


Figure 2. A, C and E are DNA histograms of mechanically treated samples. B, D and F are DNA histograms of collagenase treated samples. A and B. Representative tumor with one DNA diploid stemline; C and D. tumor with one stemline with DI=2 (nr.6); E and F. tumor with multiple DNA aneuploid stemlines (nr.35). E. DI of peaks I-IV are respectively 0.95, 1.5, 1.7 and 2.5; F. DI of peaks Ia-III are respectively 0.85 (Ia), 1.05 (Ib), 1.45 and 1.65. Peak I has apparently split into two peaks, Ia (DI=0.85) and Ib (DI=1.1). This was probably caused by the low number of intact nuclei (3,000) that could be measured in this sample.

The percentage of G2M cells (DI .2) was respectively 6.3 $\pm$ 2.3% and 6.0 $\pm$ 3.1%.

In Table 1 the FCM results of samples obtained with mechanical- or collagenase disaggregation are compared for the 10 evaluable tumors with a DNA aneuploid stemline between DI 1.9 and 2.1. Representative histograms are shown in Figures 2C and 2D. In nearly all collagenase treated samples the percentage of DNA aneuploid cells was much lower compared to that in mechanically treated tumor samples. This is illustrated in the last column were the percentage of DNA aneuploid cell loss is stated.

Table 1. Effect of collagenase treatment on percentage of prostate nuclei with an aneuploid DNA content: FCM of tumors with DI 1.9-2.1

NR	MECHANICAL DISAGGREGATION (%)		COLLAGENASE TREATMENT (%)		LOSS
	DI 0.9-1.1	DI 1.9-2.1	DI 0.9-1.1	DI 1.9-2.1	
3	85	15	96	4	73
4[1]	85	12	100	0	100
5	89	11	95	5	55
6	76	24	95	5	79
7[2]	77	15	96	4	73
9[1]	77	19	85	10	47
10	83	17	90	10	42
22[2]	80	15	82	16	0
28	89	11	95	5	55
31	89	11	87	13	0

Notes
DI 0.9-1.1 = G1 nuclei of DNA diploid cells (G1N). DI 1.9-2.1= G2 of DNA diploid cells and G1 of the tumor cells (G2N + G1T). Numbers in columns 2-5 are percentages of prostate nuclei.
1) In nrs.4 and 9 respectively 3 and 4% S-phase nuclei (between DI 1.1 and 1.9) were present. In tumor nr.9 also a peak with 5% G2T nuclei was detected in the collagenase treated sample.
2) In nrs.7 and 22 respectively 8 and 5% S-phase nuclei were present in the mechanically disaggregated sample. 2% S-phase nuclei remained in tumor nr.22 in the collagenase treated sample; all S-phase nuclei were lost in nr.7.

In Table 2 the FCM results are compared of mechanically treated and collagenase treated samples from the tumors with other DNA aneuploid stemlines. In all these tumors except for one (nr.2) there was major loss of DNA aneuploid cells in collagenase treated samples. Figures 2E and 2F show the histograms of tumor nr.35, obtained respectively with mechanical disaggregation and collagenase treatment. In the collagenase treated sample peak IV (=G2T, see also Table 2) was absent and peak II had decreased.

Table 2. Effect of collagenase treatment on percentage of prostate nuclei with an DNA aneuploid DNA content: FCM of tumors with DI P 2 and with more than two stemlines

NR	DI	MECHANICAL DISAGGREGATION				COLLAGENASE TREATMENT		LOSS
		G1N	G2N	G1T	G2T	G1N	G1T	
1	1.6	75	4	20	<1	85	11	45
2	1.7	73	*	22	<1	71	14.5	34
11	1.7	83	*	17	<1	93	7	59
15	1.8	4	0	89	7	10	88	8
17	0.85	76	**	24	<1	89	11	54
35	1.5	22	*	45	18	74	6	74
	1.7			15			14	
36	0.85	22	**	69	9	96	4	95

Notes
Column 2 (headed DI) gives the DI of the tumor peak. Numbers in columns 3-8 are percentages of prostate nuclei. * G2N peak not separable from G1T. ** G2N peak not separable from G2T. No G2T nuclei were detected in either of these tumors in collagenase treated samples.

DISCUSSION

In the present paper we describe the effect of collagenase pretreatment on the FCM ploidy of prostate tumor cell samples. One stock tissue sample was used for each tumor that was processed to a cell suspension with different techniques. In this way we ruled out that sampling errors, caused by the heterogeneity of the tissue, would influence the results of this comparative study. The possibility remains that due to limited sampling not all tumor stem lines were identified, but this would not affect our conclusions. We found in most of the investigated tumor samples that about 50% of the DNA aneuploid cells were lost in collagenase treated samples, but not in mechanical disaggregated samples. Twenty-one (60%) of the investigated 35 tumors showed a DNA diploid histogram in collagenase treated samples, against 13/35 (37%) tumors showing a DNA diploid stemline in mechanically disaggregated samples.

Also in other tumors the DNA aneuploid cell population was reported to decline following collagenase treatment (2, 4, 17).

The collagenase preparation we used in our experiments was chosen for its low proteolytic activities, as most commercial collagenase preparations generally contain not only several types of collagenase, but also relatively high proportions of proteases. Such components could potentially cause the tumor cell loss observed. Any trypsin activity that was possibly present was inactivated by incubation in the presence of serum. Crude collagenase is not toxic to normal epithelial cells, but it inhibits fibroblast growth and even shows cytotoxicity for fibroblasts (21). At our laboratory it was established that the collagenase preparation we used was not toxic for cells from a PC tumor cell line. These cells were incubated with 200 and 400 U/ml for 16 hours and remained >96% viable, at both collagenase concentrations used.

The relatively long overnight incubation period could also be an important factor in the loss of (tumor) cells. We determined the total cell yield of several tissue samples from a heterotransplanted PC cell line and from BPH after a 16 hour sham incubation in complete medium without collagenase, parallel to a collagenase incubated sample and mechanical disaggregation at t=0. All 16 hour incubated samples yielded 3-4 times more cells than mechanical disaggregation at t=0. After collagenase digestion both the total cell yield and the cell viability were slightly higher than in the sham incubated samples. The total cell yield was not affected by the 16 hour incubation or the collagenase digestion, as both methods yield similar results.

Interestingly, during mechanical disaggregation of tumor tissue a selection in favor of the abnormal cells is conceivable. Possibly due to changes in their adhesive properties, tumor cells could be more readily released from the tissue matrix by this treatment than normal cells. This effect will be favorable when the analysis of mainly tumor cells is preferable, as in tumor cytogenetics. Unfortunately, mechanically disaggregated PC tumor tissue is not accessible for classical cytogenetic studies, which use short term cultured cells. These cells will not grow in tissue culture, although they look physically undamaged (5). Furthermore, due to the generally low mitotic index of PC tissue, uncultured, so called "direct" preparations seldom yield enough metaphases. However, with the development of FISH (fluorescence in situ hybridization) (6) it has become possible to investigate interphase nuclei cytogenetically. Using a DNA probe for the centromere of chromosome 1 we compared the ploidy distribution of this chromosome in mechanically treated preparations, and collagenase treated- and cultured samples from the same tumor specimens. In agreement with the results reported here we found that the percentage of cells which showed trisomy and/or tetrasomy for chromosome 1 in mechanically treated samples, declined in collagenase treated samples. After tissue culture this percentage was reduced even further (9).

These findings, in combination with the negative collagenase effect described here, could well account for the high percentage of diploid karyotypes found until now in cytogenetic studies of PC specimens (3, 8, 14). In a study of renal carcinomas comparable results were obtained (23). The authors attributed their findings of diploid karyotypes in originally abnormal tumors (by DNA FCM) solely to the preferential growth of diploid cells in culture, but they ignored the possible effect of their collagenase treatment. In another study of breast carcinomas only 3/44 specimens showed clonal abnormalities after short term tissue culture (24). These authors concluded that neither by tissue disaggregation, nor by culture any abnormal cells were eliminated. However, their conclusion was based on the phenotypic properties of the cultured cells and the ploidy of the original tissue was not checked by DNA FCM, unfortunately.

Our conclusion is that the collagenase treatment procedure as a whole causes the tumor cell loss observed by us and by others. Regrettably, at present no alternative for collagenase treatment of PC and also other solid tumor tissues is available. Maybe the use of more refined culture methods (13) in combination with purified collagenase preparations, or the introduction of a totally different new disaggregation method will allow more reliable tumor karyotyping studies than is presently possible. Meanwhile, decrease of the number of abnormal cells is a factor one should take into account and control of the ploidy of the original tissue by DNA FCM or FISH remains necessary.

Acknowledgements

We are grateful to Ton Verkerk for his expert assistance with the use of the

FACS II machine and the FCM computer program.

We thank Hans Romijn for critical reading of the manuscript and helpful suggestions.

The financial support from The Dutch Cancer Society is gratefully acknowledged.

LITERATURE

1. Atkin NB, Baker MC: Chromosome study of five cancers of the prostate. Hum Genet 70: 359-364, 1985.
2. Bijman JT, Wagener DJT, van Rennes H, Wessels JMC, van den Broek P: Flow cytometric evaluation of cell dispersion from human head and neck tumors. Cytometry 6: 334-341, 1985.
3. Brothman AR, Peehl DM, Patel AM, MacDonald GR, McNeal JE, Ladaga LE, Schellhammer PF: Cytogenetic evaluation of 20 cultured primary prostatic tumors. Cancer Genet Cytogenet 55 79-84, 1991.
4. Frankfurt OS, Slocum HK, Rustum YM, ARbuck SG, Pavelic ZP, Petrelli N, Huben RP, Pontes EJ, Greco WR: Flow cytometric analysis of DNA aneuploidy in primary and metastatic human solid tumors. Cytometry 5: 71-80, 1984.
5. Franks LM, Riddle PN, Carbonell AW, Gey GO: A comparative study of the ultrastructure and lack of growth capacity of adult human prostate epithelium mechanically separated from its stroma. J Pathol 100: 113-119, 1969.
6. Gray JW, Pinkel D: Molecular cytogenetics in human cancer diagnosis. Cancer 69: 1536-1542, 1992.
7. König JJ, Kamst E, Hagemeijer A, Romijn JC, Horoszewicz J, Schröder FH: Cytogenetic characterization of several androgen responsive and unresponsive sublines of the human prostatic carcinoma cell line LNCaP. Urol Res 17: 79-86, 1989.
8. König JJ: Genetic changes associated with progression to endocrine independence in carcinoma of the breast and prostate. In: Mechanisms of progression to hormone-independent growth of breast and prostatic cancer, PMJJ Berns, JC Romijn and FH Schröder (eds) Parthenon Publishing, New Jersey. pp 123-134, 1991.
9. König JJ, Teubel W, Dongen JJW van, Hagemeijer A, Romijn JC, Schröder FH: Tissue culture correlated loss of aneuploid cells in prostate carcinoma, measured by fluorescence in situ hybridization analysis of the ploidy of chromosome 1. Genes Chrom Cancer :in press, 1993.
10. Kooistra A, König JJ, Romijn JC, Schröder FH: Negative control of epithelial cell proliferation by prostatic stroma. Anticancer Res 11: 1495-1500, 1991.
11. Lee SE, Currin SM, Paulson DF, Walther PJ: Flow cytometric determination of ploidy in prostatic adenocarcinoma: a comparison with seminal vesicle involvement and histopathological grading as a predictor of clinical recurrence. J Urol 140: 769-774, 1988.
12. Limon J, Dal Cin P, Sandberg AA: Application of long-term collagenase disaggregation for the cytogenetic analysis of human solid tumors. Cancer Genet Cytogenet 23: 305-313, 1986.
13. Limon J, Lundgren R, Elving P, Heim S, Kristofferson U, Mandahl N, Mitelman F: An improved technique for short-term culturing of human prostatic adenocarcinoma tissue for cytogenetic analysis. Cancer Genet Cytogenet 46: 191-199, 1990.
14. Lundgren R, Mandahl N, Heim S, Limon J, Hendrikson H, Mitelman F: Cytogenetic analysis of 57 primary prostatic adenocarcinomas. Genes Chrom Cancer 4: 16-24, 1992.
15. Miller J, Horsfall DJ, Marshall VR, Rao DM, Leong ASY: The prognostic value of deoxyribonucleic acid flow cytometric analysis in stage D2 prostatic carcinoma. J Urol 145: 1192-1196, 1991.
16. Nativ O, Myers RP, Farrow GM, Therneau TM, Zincke H, Lieber MM: Nuclear deoxyribonucleic acid ploidy and serum prostate specific antigen in operable prostatic adenocarcinoma. J Urol 144: 303-306, 1990.
17. Smeets AWGB, Pauwels RPE, Beck HLM, Feitz WFJ, Geraedts JPM, Debruyne FMJ, Laarakkers L, Vooijs GP, Ramaekers FCS: Comparison of tissue disaggregation techniques of transitional cell bladder carcinomas for flow cytometry and chromosomal analysis. Cytometry 8: 14-19, 1987.
18. Stone KR, Stone MP, Paulson DF: In vitro cultivation of prostatic epithelium. Inv Urol 14: 79-82, 1976.

19. Tanaka K, Testa JR: Assessment of methods for the cytogenetic analysis of human solid tumors. J Natl Cancer Inst 79: 1287-1293, 1987.
20. Vindelov LL, Christensen IJ, Nissen NI: A detergent-trypsin method for the preparation of nuclei for flow cytometric DNA analysis. Cytometry 3: 323-327, 1983.
21. Webber MM: Normal and benign human prostatic epithelium in culture. In Vitro 15 967-982, 1979.
22. Winkler HZ, Rainwater LM, Myers RP, Farrow GM, Therneau TM, Zincke H, Lieber MM: Stage D1 prostatic adenocarcinoma : significance of nuclear DNA ploidy patterns studied by flow cytometry. Mayo Clin Proc 63: 103-112, 1988.
23. Wolman SR, Camuto PM, Golimbu M, Schinella R.: Cytogenetic, flow cytometric, and ultrastructural studies of twenty-nine nonfamilial human renal cell carcinomas. Cancer Res 48 :2890-2897., 1988.
24. Zhang R, Wiley J, Howard SP, Meisner LF, Gould MN.: Rare clonal karyotypic variants in primary cultures of human breast carcinoma cells. Cancer Res 49 :444-449., 1989.

CHAPTER 4.2

TISSUE CULTURE LOSS OF ANEUPLOID CELLS FROM CARCINOMAS OF THE PROSTATE

Josée J.König, Wilma Teubel, Jan Willem van Dongen, Anne Hagemeijer, Johannes C. Romijn, and Fritz H.Schröder.

Published in: Genes Chromosomes and Cancer 8: 22-27, 1993

ABSTRACT

The frequency of aneuploid cells in cultured prostate carcinoma specimens was investigated. Ploidy distribution of the original tissue was established by flow cytometry (FCM). Fluorescence in situ hybridization (FISH) of chromosome 1 was applied to directly isolated and cultured cells to investigate whether any modifications in the ploidy distribution of chromosome 1 took place during tissue culture. In six tumor specimens that were diploid by FCM and FISH, no differences were found in the ploidy distribution of chromosome 1 before and after tissue culture. In eight tumors that were aneuploid by FISH, the percentage of aneuploid nuclei was significantly reduced from 28.0 ± 15.0 (range 13-59%) in uncultured cells to 9.1 ± 4.4% (range 4-18%) after tissue culture. The reduction of aneuploid nuclei ranged from 44 to 85%, which means that the majority of the aneuploid cell populations that were observed in the original specimens were undetectable in cultured samples. This suggests a preferential growth of normal epithelial cells. The presented data can explain the high percentage of diploid karyotypes usually found in short-term cultured prostate carcinoma specimens.

INTRODUCTION

Several reports on the cytogenetics of prostate carcinoma (PC) have suggested that structural as well as numerical aberrations of chromosomes 1, 7, 8, 10 and Y might be of importance in the origin and progression of this type of cancer (Atkin and Baker, 1985; König et al., 1989; Brothman et al., 1990, 1991; König, 1991; Lundgren et al., 1991). The majority of the investigated PC specimens (73%) have shown a normal diploid karyotype (Brothman et al., 1991, Lundgren et al., 1991; unpublished data). Results obtained by DNA flow cytometry (FCM) of PC tissues, on the other hand, have shown that more than 50% of PC's have one or more aneuploid cell populations (Lee et al., 1988; Winkler et al., 1988; Nativ et al., 1990; König 1991; Miller et al., 1991; König et al., 1993).

The apparent discrepancy between the results obtained by FCM and karyotype analysis may be explained, at least in part, by differences in the sample preparation protocols. Whereas FCM requires only mechanical disaggregation, karyotyping usually involves enzymatic tissue digestion followed by short-term in vitro culture. A previous study (König et al., 1993) has shown that enzymatic digestion with collagenase, the most effective enzyme for disaggregation of PC tissue, may result in the preferential loss of aneuploid tumor cells. In the present study we investigated whether tissue culture could selectively affect the proliferation of certain cell populations and thus be an additional factor accounting for the observed enrichment of diploid cells in samples prepared for karyotype analysis (Wolman et al., 1988).

MATERIALS AND METHODS

Fifteen prostatic adenocarcinoma specimens were studied, all obtained at radical prostatectomy. Suspected carcinoma tissue was excised, cut into small fragments (2x2 mm) with scissors under sterile conditions and divided into three parts (See Table 1). For each tumor, grade and stage were assessed from paraffin sections of adjacent tissue.

To one part approximately 0.5 ml of phosphate buffered saline (PBS) was added and the tissue pieces were further minced by a scalpel into a suspension of small cell clumps and single cells. The clumps were discarded after sedimentation for 3-5 min in 5 ml of PBS. The supernatant was centrifuged and the resulting pellet washed and resuspended in PBS. From this cell suspension a sample was stored for FCM. The rest of the cell suspension was incubated with hypotonic solution (0.075M KCl) for 10 min at 37°C and fixed in methanol/ acetic acid. Fixed nuclei were stored in methanol at -20°C until used (sample FISH-A).

Another part was transferred to a 25 cm^2 culture flask and incubated overnight with 200U/ml

collagenase (Worthington CLSIII). After washing to eliminate the collagenase and cell debris, the resulting cell suspension was plated in a 25 cm^2 culture flask in prostate culture medium (for composition, see below) and cultured for 7 days. Medium was changed on the 2nd and 5th day. On day 7, outgrown cells were harvested by trypsinization, treated with hypotonic solution, and fixed as described above (sample FISH-B).

Table 1. Nomenclature of samples for FISH analysis

SAMPLE	COLLAGENASE TREATMENT	TISSUE CULTURE
FISH-A	-	-
FISH-B	+	+
FISH-C	-	+

Undigested tissue fragments formed the third sample. These were transferred to several 60 cm^2 dishes without further treatment (5 cubes per dish) with 3 ml of prostate culture medium. When a sufficient number of cells had grown out from the explants (usually after about 14 days), cells were harvested by trypsinization and treated with hypotonic solution and fixative as described above (sample FISH-C).

Prostate culture medium
RPMI 1640 and F12/MEM 1:1 were obtained ready-for-use from Gibco. The growth factor concentrations used were 20 µg/ml for insulin, 1 µg/ml for hydrocortisone, 10 ng/ml for epidermal growth factor, and 10 ng/ml for cholera toxin. All growth factors were obtained from Sigma. Penicillin/streptomycin, glutamine and 2% fetal calf serum (Boehringer) were added at preparation of the culture medium.

FCM procedure
Stored samples were processed for FCM as described (Vindelov et al., 1983; König et al., 1992). The ploidy of the different peaks in histograms from tumor samples was calculated from their relative position to the G0/G1 peak (DNA Index (DI)=1) in a histogram of cultured normal diploid prostate fibroblasts. Diploid: DI 0.9-1.1; hypodiploid DI<0.9; triploid: DI 1.4-1.6; tetraploid: DI 1.8-2.2.

FISH procedure
PUC 1.77, hybridizing to SatIII repeats on the pericentromeric heterochromatin region of chromosome 1 (Cooke and Hindley, 1979), was used as a chromosome 1 - specific probe. Hybridization and detection were performed as described by Pinkel et al. (1986), with modifications. In short, the probe was labeled with Bio-11-dUTP by nick translation. Slides with fixed nuclei were pretreated with RNase and postfixed with formaldehyde. Hybridization of the probe to the nuclei (15 ng probe per slide) occurred during overnight incubation at 37°C in a moist chamber in 65% formamide. Slides were subsequently washed in 60% formamide/ 2xSSC (3x 5 min), 2xSSC (3x 5 min) both at 40°C, followed by 3x 5 min in 0.1xSSC at 60°C, 5 min 2xSSC and 5 min 4xSSC/0.05% Tween at 20°C. Detection of hybridized sequences was with 0.56µg Avidin-fluoresceine isothiocyanate (FITC) per slide (incubation: 20 min at 37°C), followed by biotinylated goat "-Avidin (incubation: 30 min at 37°C) and another Avidin-FITC incubation. Nuclei were counterstained with propidium iodide in antifade solution. Spots representing the number of centromeric regions of chromosome 1 per nucleus were scored at 1250x magnification on a Zeiss Axiophot fluorescence microscope equipped with a FITC filter combination.
For the evaluation of FISH signals we used the criteria of Hopman et al. (1988). Especially for the interpretation of difficult spot patterns, like fuzzy spots in nuclei from cultured samples

(FISH-B and C), these criteria were useful. When the criteria could not be met, such nuclei were excluded from counting. When there were more than 10% of these nuclei on a slide, the hybridization was repeated. The percentage of nuclei with one spot was generally below 5%. These nuclei were considered disomic with overlapping signals. When there were more than 5% nuclei with one spot on a slide, the hybridization was repeated. At least 300 nuclei were scored per sample.
The mean percentage of aneuploid nuclei in diploid tumors, determined in 48 tumor samples with ≤10% nuclei with 3 and 4 spots, was 3.4 ± 3.0. Using this average, the cut off percentage for aneuploid tumors was defined at 10% (mean value + two times SD: 3.4 + 3.0 + 3.0 = 9.4).

RESULTS

Based on the number of chromosomes 1 detected by FISH in untreated tumor tissue samples (FISH-A), tumors were divided into two groups, representing diploid and aneuploid tumors. Table 2 lists the data obtained by FISH and FCM for seven diploid tumors and compares the chromosome 1 distribution as observed after immediate processing (FISH-A) to that after tissue culture (FISH-C).

Table 2. Distribution of chromosome 1 in diploid tumors before and after short term tissue culture

PC NO.	SAMPLE[b]	FISH			FCM[a]			
		% 2 SPOTS	% 3 SPOTS	% 4 SPOTS	% DIPLOID		% ANEU-PLOID	
288[c]	A	90	9	1	94		6	
	B	94	5	1	-		-	
	C	96	4	0	-		-	
304	A	94	3	3	95		5	
	C	93	3	4	76		24	
305	A	99.5	0.5	0	97		3	
	C	97	1	2	75		25	
306	A	96	3.5	0.5	96		4	
	C	95.5	2	2.5	77		23	
307	A	98	2	0	96		4	
	C	94	3	3	75		25	
308[d]	A	92	5	3	66	30	3	1
	C	98	1	1	0	66	0	34
309	A	96	3	1	92		8	
	C	97.5	1.5	1	70		30	

Notes
[a] The ploidy of the different peaks in histograms from tumor samples was calculated from their relative position to the G0/G1 peak (DI=1) in a histogram of cultured normal diploid prostate fibroblasts. Diploid: DI 0.9-1.1; aneuploid: DI 1.6-2.2. [b] Samples FISH-A , FISH-B and FISH-C correspond with those in Table 1. [c] The number of cells obtained after tissue culture of PC288 was insufficient to perform DNA FCM analysis. [d] DNA FCM of PC308 revealed a hypodiploid (DI 0.85) and a diploid (DI 1.05) population. After tissue culture only one peak (DI 1.09) was present.

There was no significant difference between the mean percentages of diploid nuclei in FISH-A samples (95.1 ± 3.3%) and in FISH-C samples (95.9 ± 1.8%). The mean percentage of aneuploid nuclei in FISH-A samples of diploid tumors was 4.8 ± 3.2 and ranged from 0.5% (PC305) to 10% (PC288). After tissue culture the mean percentage was 4.1 ± 1.8 (range: 2 to 7%), which is not different from the mean of FISH-A. Except for one tumor (PC308), the results obtained by FCM of uncultured cells were in agreement with the FISH-A and FISH-C data. FCM of FISH-C samples, however, showed a 4 to 8 fold rise of the aneuploid fraction (DI 1.6-2.2, see Table 2) compared with the number of aneuploid nuclei detected by FCM in FISH-A samples. This probably reflected proliferating cells in the S- and G2M-phase. Such cells were recognized by FISH analysis as disomic. In Figure 1, the FCM analysis of the FISH-C sample (FCM-C) shows 25% nuclei in the triploid to tetraploid range, whereas FISH-C as well as FISH-A and FCM-A show that this tumor is disomic for chromosome 1 and diploid.

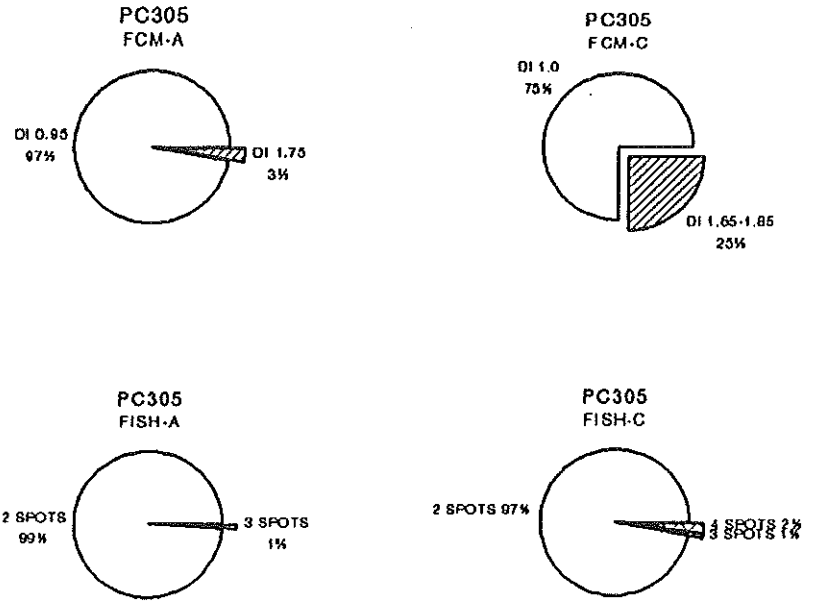


Figure 1. Comparison of FISH and FCM of samples FISH-A (=FCM-A) and FISH-C (=FCM-C) from PC305. DI= DNA index; DI=1= diploid etc. Diploid FCM; 2 spots with FISH. Triploid FCM; 3 spots with FISH. Tetraploid FCM; 4 spots with FISH. % in FCM distribution (pie) figures are the percentages of the total number of prostate nuclei analyzed. % in FISH pie figures are the percentages of the total number of counted nuclei.

In uncultured cells of PC308, two stem lines (hypodiploid and diploid) were detected by FCM. The hypodiploid population appeared to be absent after tissue culture. FISH showed disomy for chromosome 1 in PC308, indicating that chromosomes other than chromosome 1 were lost in these hypodiploid cells.

Nuclei from PC288, isolated from tissue culture after collagenase treatment (FISH-B), had the same ploidy distribution as those in FISH-C (see Table 2). Due to the limited quantity of tumor tissue available, all other tumors (diploid and aneuploid) were analyzed after either explant culture or culture of collagenase treated cells.

In Table 3, eight tumors that were aneuploid for chromosome 1 by FISH are listed. The percentage of aneuploid nuclei in uncultured cells from this group was 28.0 ± 15.0 and ranged from 13% (PC279) to 59% (PC281). After tissue culture (samples FISH-B or C) the mean percentage of aneuploid nuclei was significantly ($p<0.005$) reduced (9.1 ± 4.4%). This reduction ranged from 44% (PC292) to 85% (PC281) of the original percentage of aneuploid cells (mean: 64.5 ± 16.3%).

In Table 4, the number of hybridization signals found on metaphases in cultured samples of one diploid (PC288) and four aneuploid tumors are listed. No metaphases were found on the rest of the FISH-B and FISH-C slides. All metaphases obtained from PC280 and PC288 were disomic, whereas PC279, PC281 and PC298 showed a small number of monosomic and tetrasomic metaphases. Trisomy for chromosome 1 was not seen.

Table 3. Chromosome 1 distribution by FISH in aneuploid tumors before and after tissue culture.

		FISH			FCM[a]				
PC NO	SAMPLE[b]	% 2 SPOTS	% 3 SPOTS	% ≥4 SPOTS	% LOSS[c]	% HYPODIPL	% DI-PL.	% TRI-PL.	% TE-TRA-PL.
279	A	87	8	5		0	89	0	11
	B	93	5	3	38	-	-	-	-
280	A	77	17	8		-	-	-	-
	B	88	7	5	52	-	-	-	-
281	A	41	22	37		-	-	-	-
	B	91	7	2	85	-	-	-	-
289	A	76	11	13		0	89	0	11
	B	95	3	2	79	-	-	-	-
290	A	65	23	12		0	22	60	18
	C	93	5	2	80	-	-	-	-
292	A	82	15	3		69	22	9	0
	C	90	9	1	44	-	-	-	-
298	A	85	3	12		0	92	0	8
	C	96	1	3	73	-	-	-	-
303	A	65	6	29		0	90	0	10
	C	82	7	11	49	-	-	-	-

Notes

- = not done. [a] Hypodipl.= hypodiploid; dipl.= diploid. tripl.= triploid; tetrapl.= tetraploid.
[b] Samples FISH-A, -B and -C correspond with those in Table 1. [c] % loss indicates the relative reduction of the number of aneuploid nuclei observed upon tissue culture. It was calculated from the total percentage of aneuploid nuclei (3 spots + >4 spots) according to the formula:

$$\text{percentage of loss} = \frac{\%\ \text{FISH-A} - \%\ \text{FISH-B or -C}}{\%\ \text{FISH-A}} \times 100$$

Table 4. Numbers of hybridization signals on chromosome 1 in metaphases of cultured cells.

PC NO.	N	1 SPOT	2 SPOTS	4 SPOTS
279	13	3	8	2
280	17	0	17	0
281	11	2	8	1
288	15	0	15	0
298	23	2	21	0

Figure 2 shows FCM and FISH analysis of different samples of PC290. FCM of uncultured cells demonstrated that this tumor had multiple stem lines. FISH (sample FISH-A) showed that this tumor was heterogeneous for chromosome 1. The ploidy distributions of total DNA and of chromosome 1 did not correspond, however. After collagenase treatment, 67% of the triploid and hypertetraploid cells were lost. Of the remaining aneuploid cells, another 73% were lost during subsequent tissue culture.

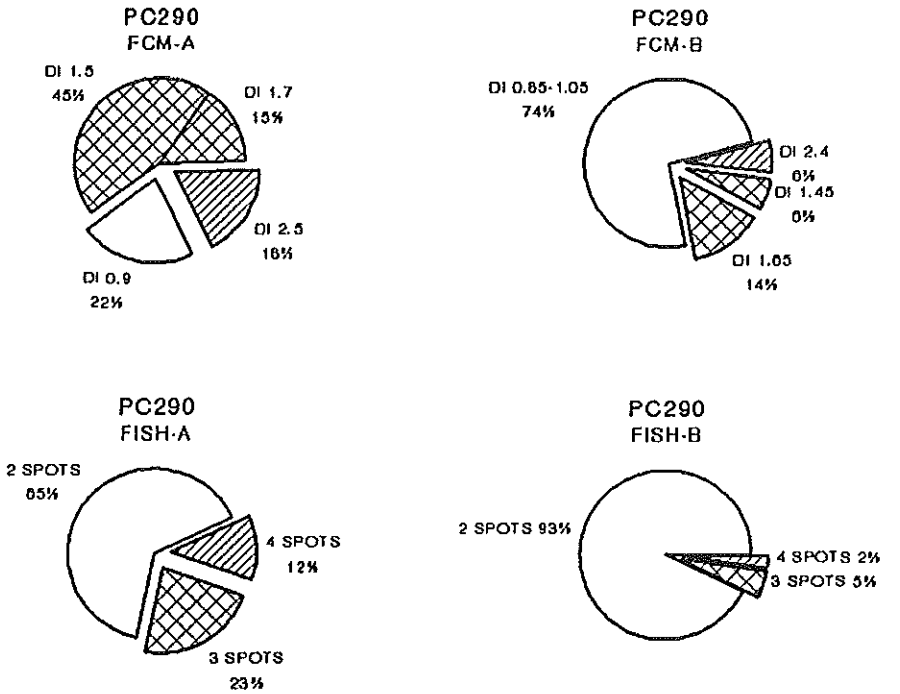


Figure 2. Samples FISH-A (=FCM-A) and FISH-B from PC290. FCM-B is from a sample that was collected after collagenase digestion, but before tissue culture. DI= DNA index; DI=1= diploid etc. Diploid FCM; 2 spots with FISH. Triploid FCM; 3 spots with FISH. Tetraploid FCM; 4 spots with FISH. % in FCM distribution (pie) figures are the percentages of the total number of prostate nuclei analyzed. % in FISH pie figures are the percentages of the total number of counted nuclei.

DISCUSSION

In this study we show that FISH with a repetitive DNA probe for the pericentromeric region of chromosome 1 can detect the copy number of that chromosome in freshly prepared nuclear suspensions from prostatic carcinoma cells. This is in line with similar findings in studies of other solid tumors (Devilee et al., 1988; Hopman et al., 1988, 1991; Gray and Pinkel, 1992; Poddighe et al., 1992).

Our most important observation was that the majority of the aneuploid cell populations were undetectable in cultured samples from tumors that were clearly aneuploid by FISH as well as FCM in the original (fresh) preparations. The apparent negative selection for aneuploid cells occurred irrespective of the culture method. This effect could not be demonstrated by FCM, because in cultured samples about 25% of the cells were in S and G2M phase (see Table 2: the percentage of aneuploid nuclei in FCM of FISH-C samples and Figure 1: the FCM-C pie). Diploid cells in G2M had a DNA content equal to that of (near)tetraploid cells in G1. In contrast, the number of FISH signals did not change during the cell cycle, probably due to the pericentromeric localization of the target DNA repeat sequences (Devilee et al. 1988; Hopman et al. 1989).

Several results emerged from the present study that could be common to aneuploid PC. In tumors judged to be tetraploid by DNA-FCM, the FISH results always showed a substantial number of trisomic cells in addition to tetrasomic cells (PC279, PC289 and PC303). Secondly, no loss of chromosome 1 was detected in hypodiploid tumors (PC292 and PC308). Thirdly, in aneuploid tumors always far more disomic cells for chromosome 1 were observed by FISH than was to be expected based on the total DNA distribution (see Table 3).

While proliferation after collagenase digestion started after one or two days in culture, only after about one week did cells begin to grow out from explants. The relatively long culture time of explant cultures increases the possibility of overgrowth by normal epithelial or stromal cells, and also collagenase digestion leads to the elimination of many aneuploid cells, as we have shown before (König et al., 1993). Both these effects result in loss of aneuploid cells and/or enrichment of diploid cells after culture. Negative selective effects of trypsinization have not been described for cultured prostatic cells. Overgrowth of fibroblasts was not likely to occur in our cultures, because the chosen combination of growth factors is known to restrict the growth of stromal cells (Peehl et al., 1988). Interestingly, the cultured cells had epithelial morphology and showed antigen expression patterns characteristic for prostate epithelium (manuscript in preparation). However, preferential growth of normal (diploid) cells cannot be ruled out. Unfortunately there are no markers available which can distinguish between normal prostatic epithelium and prostate carcinoma cells. Peehl et al (1988) concluded that their tissue culture procedure resulted in the culture of tumor cells, because proliferation of only a small fraction of normal cells could never account for the plating efficiencies they observed. However, the recurring observation of mainly disomic cells after tissue culture of PC specimens with large aneusomic cell populations originally suggests a selection in favor of disomic cells. Whether these cells are normal or tumor cells, cannot be verified, because of the lack of markers to discriminate between these cell types. The results of the present study indicate that during tissue culture important aneuploid cell populations are lost, because they are not stimulated to proliferate in vitro. Consequently, metaphase preparations of such cultures will consist mainly, if not exclusively, of diploid cells (see Table 4). Karyotyping studies of PC reported until now are characterized by a high percentage (73%) of diploid karyotypes (Brothman et al., 1991; Lundgren et al., 1991). Based on FCM observations where around 50% of the cases showed aneuploid cells (Lee et al., 1988; Winkler et al., 1988;

Miller et al., 1991) it may be assumed that in at least 25% of the tumors with diploid karyotypes aneuploid cell populations were missed because they did not proliferate in tissue culture.

For FISH analysis tissue culture is not necessary, since this technique can be performed on interphase nuclei. We have shown in the present study that FISH with a centromere specific probe on PC specimens provides information on the ploidy distribution of the tested chromosome which cannot otherwise be obtained. We intend to expand the number of analyzed chromosomes per specimen in order to obtain a specific ploidy distribution pattern for each investigated tumor. The results of this ongoing study will hopefully lead to a better understanding of the importance of chromosomal loss and gain in the development of PC.

LITERATURE

Atkin NB, Baker MC (1985) Chromosome study of five cancers of the prostate. Hum Genet 70:359-364.

Brothman AR, Peehl DM, Patel AM, McNeal JE (1990) Frequency and pattern of karyotypic abnormalities in human prostate cancer. Cancer Res 50:3795-3803.

Brothman AR, Peehl DM, Patel AM, MacDonald GR, McNeal JE, Ladaga LE, Schellhammer PF (1991) Cytogenetic evaluation of 20 cultured primary prostatic tumors. Cancer Genet Cytogenet 55:79-84.

Cooke HJ, Hindley J (1979) Cloning of human satellite III DNA: different components are on different chromosomes. Nucl Acids Res 6:3177-3197.

Devilee P, Thierry RF, Kievits T, Kolluri R, Hopman AHN, Willard HF, Pearson PL, Cornelisse CJ (1988) Detection of chromosome aneuploidy in interphase nuclei from human primary breast tumors using chromosome-specific repetitive DNA probes. Cancer Res 48:5825-5832.

Gray JW, Pinkel D (1992) Molecular cytogenetics in human cancer diagnosis. Cancer Suppl 69:1536-1542.

Hopman AHN, Ramaekers FCS, Raap AK, Beck JLM, Devilee P, van der Ploeg M, Vooijs GP (1988) In situ hybridization as a tool to study numerical chromosome aberrations in solid tumors. Histochemistry 89:307-316.

Hopman AHN, Poddighe PJ, Smeets AWGB, Moesker O, Beck JLM, Vooijs GP, Ramaekers FCS (1989) Detection of numerical chromosome aberrations in bladder cancer by in situ hybridization. Am J Pathol 135:1105-1117.

Hopman AHN, Moesker O, Smeets AWGB, Pauwels RPE, Vooijs GP, Ramaekers FCS (1991) Numerical chromosome 1, 7, 9, and 11 aberrations in bladder cancer detected by in situ hybridization. Cancer Res 51:644-651.

König JJ, Kamst E, Hagemeijer A, Romijn JC, Horoszewicz J, Schröder FH (1989) Cytogenetic characterization of several androgen responsive and unresponsive sublines of the human prostatic carcinoma cell line LNCaP. Urol Res 17:79-86.

König JJ (1991) Genetic changes associated with progression to endocrine independence in carcinoma of the breast and prostate. In PMJJ Berns, JC Romijn and FH Schröder (eds): Mechanisms of progression to hormone-independent growth of breast and prostatic cancer. New Jersey: Parthenon Publishing, pp 123-134.

König JJ, Dongen van JJW, Schröder FH (1993) Preferential loss of abnormal prostate carcinoma cells by collagenase treatment. Cytometry : (in press).

Lee SE, Currin SM, Paulson DF, Walther PJ (1988) Flow cytometric determination of ploidy in

prostatic adenocarcinoma: a comparison with seminal vesicle involvement and histopathological grading as a predictor of clinical recurrence. J Urol 140:769-774.

Lundgren R, Mandahl N, Heim S, Limon J, Hendrikson H, Mitelman F (1992) Cytogenetic analysis of 57 primary prostatic adenocarcinomas. Genes Chrom Cancer 4:16-24.

Miller J, Horsfall DJ, Marshall VR, Rao DM, Leong ASY (1991) The prognostic value of deoxyribonucleic acid flow cytometric analysis in stage D2 prostatic carcinoma. J Urol 145:1192-1196.

Nativ O, Myers RP, Farrow GM, Therneau TM, Zincke H, Lieber MM (1990) Nuclear deoxyribonucleic acid ploidy and serum prostate specific antigen in operable prostatic adenocarcinoma. J Urol 144:303-306.

Peehl DM, Wong ST, Stamey TA (1988) Clonal growth characteristics of adult human prostatic epithelial cells. In Vitro Cell Dev Biol 24:530-536.

Pinkel D, Gray J, Trask B, Van den Engh G, Fuscoe J, Van Dekken H (1986) Cytogenetic analysis by in situ hybridization with fluorescently labeled nucleic acid probes. Cold Spring Harbor Symp Quant Biol L1:151-157.

Poddighe PJ, Ramaekers FCS, Hopman AHN (1992) Interphase cytogenetics of tumors. J Pathol 166:215-224.

Vindelov LL, Christensen IJ, Nissen NI (1983) A detergent-trypsin method for the preparation of nuclei for flow cytometric DNA analysis. Cytometry 3:323-327.

Winkler HZ, Rainwater LM, Myers RP, Farrow GM, Therneau TM, Zincke H, Lieber MM (1988) Stage D1 prostatic adenocarcinoma : significance of nuclear DNA ploidy patterns studied by flow cytometry. Mayo Clin Proc 63:103-112.

Wolman SR, Camuto PM, Golimbu M, Schinella R (1988) Cytogenetic, flow cytometric, and ultrastructural studies of twenty-nine nonfamilial human renal cell carcinomas. Cancer Res 48:2890-2897.

CHAPTER 5

LOSS AND GAIN OF CHROMOSOMES 1, 18 AND Y IN PROSTATE CANCER

Josée J.König, Wilma Teubel, Jan Willem van Dongen, Johannes C.Romijn, Anne Hagemeijer and Fritz H.Schröder

Published in: The Prostate 25: 281-291, 1994

ABSTRACT

Nuclear suspensions of 42 prostate carcinoma specimens obtained at surgery were used to investigate loss and gain of chromosomes 1, 18 and Y by fluorescence in situ hybridization (FISH) with centromere specific probes. The outcome of FISH analysis was correlated with clinical parameters and the relationship between DNA-FCM (ploidy at cellular level) and FISH (ploidy of individual chromosomes) was assessed.

Significant loss of chromosomes 1 and 18 was infrequent (respectively 3 and 5 cases), but 53% of the tested specimens showed loss of Y. Loss was not correlated with DNA ploidy.

Significant gain occurred in 36% (#1), 63% (#18) and 28% (Y) of the specimens. Gain of chromosome 18 was shown in DNA diploid (7/14) and aneuploid tumors (18/26), while gain of chromosome 1 and Y was nearly restricted to DNA aneuploid specimens. Significant unbalance between these chromosomes occurred in 11 cases. Most cases which had significant gain of #1 or #18 showed trisomic as well as tetrasomic cells. Simultaneous loss of some, and gain of other investigated chromosomes is suggestive of clonal heterogeneity and/or multiclonality. This was observed in 8 tumors.

Correlation between DNA-FCM and FISH was best for the Y chromosome. DNA-FCM showed more aberrant histograms with increasing stage and grade of tumors. The presence of numerical aberrations of the investigated chromosomes however, seemed independent of clinical grade or stage.

INTRODUCTION

In prostate cancer (PC), investigation of chromosomal aberrations might provide clues to the changes that play an important role in the origin and progression of this tumor. Classical cytogenetic studies, which mostly made use of short term tissue culture to obtain an adequate number of metaphase spreads have shown chromosomal aberrations in only a minority of cases (1-5). Recurrent structural changes were reported for chromosomes 1, 2 and 7 (p and q arm), 3p, 6p, 8p, 10q, 13q, 15q and 16q. Whole chromosome gain of #7, #14, #20 and #22 and loss of one homolog of #1, #2, #4, #5 and Y were most common (for review, see ref 6). However, most cases showed normal diploid karyotypes, probably due to selective isolation and preferential growth of normal epithelial cells (7, 8).

An alternative method to study chromosomal abnormalities is by measurement of the total DNA content of individual cells using DNA flow cytometry (FCM). Unlike cytogenetic analyses, such studies showed that most prostate tumors had one or more aneuploid cell populations (9-12), which was associated with an unfavorable outcome of the disease. The cytogenetic sensitivity of DNA-FCM is limited however: numerical or structural changes of individual chromosomes remain undetected.

In the present study we used a third technique for the investigation of chromosomal changes, namely fluorescence in situ hybridization (FISH) with chromosome specific DNA probes (13). FISH has the advantage over classical cytogenetics that tissue culture is not needed, because signals can be detected in interphase nuclei as well as in metaphases. Furthermore, the larger number of cells that can be analyzed, allows the discrimination between random abnormalities (which are frequently observed in karyotyping studies of PC (1, 2, 4)) and clonal changes. We have shown before that FISH on fresh nuclear suspensions of PC specimens is feasible (8). FISH studies with centromere specific probes investigate the copy number of particular chromosomes, to establish whether the chromosome copy number is simply a reflection of the total ploidy (14), or an indication for gene

loss, regardless whether a chromosome is lost (loss of one homolog), or gained (chromosomal duplication driven by gene loss on one homolog).

We report here the frequencies of loss and gain of chromosomes 1, 18 and Y. The rationale for studying these chromosomes was: 1) for #1 not only structural abnormalities, but also whole chromosome loss and gain have been reported in PC (for review, see ref.6), 2)in two separate studies loss of heterozygosity (LOH) was reported on the long arm of #18 (15, 16), 3) loss of Y was frequently found in PC (1-3, 5, 17), sometimes even as the only aberration. The results of these studies were correlated with tumor grade and stage and DNA-FCM.

MATERIALS AND METHODS

Tissue processing

Forty-two prostatic adenocarcinoma specimens were studied, obtained at radical prostatectomy (N=25), transurethral resection (TUR, N=13), or pelvic lymph node dissection (N=4). Suspected carcinoma tissue was excised and, whenever the amount of tissue allowed, cut with scissors into two parts. One part was cut into several smaller fragments, snap frozen in liquid nitrogen and stored at -80°C for later use. To the other part approximately 0.5 ml of phosphate buffered saline (PBS) was added and the tissue pieces were further minced with a scalpel into a suspension of small cell clumps and single cells. The clumps were discarded after sedimentation for 3-5 min in 5 ml of PBS. The supernatant was centrifuged and the resulting pellet washed and resuspended in PBS. From this cell suspension a sample was stored for DNA-FCM. The rest of the cell suspension was incubated with hypotonic solution (0.075M KCl) for 10 min at 37°C and fixed in methanol/acetic acid (3:1). Fixed cells were stored in methanol at -20°C until used for FISH. The above described procedure for tissue processing and fixation of nuclei gave good results with both primary tumor tissue and lymph node metastases. As nuclei from TUR tissue tended to coagulate in suspension, an additional sedimentation step at unit gravity was performed with these preparations.

Histology was taken from adjacent tissue, in between the immediately processed sample and the sample for storage. From a paraffin section of each tumor, grade, stage and percentage of tumor cells were assessed. The mean percentage of tumor cells in the sections was 56.3 ± 22.1% (range 25-95%). Specimens with less than 25% tumor cells were not included in the study.

For control purposes, leucocytes were obtained from each patient. A sample of peripheral blood in heparin was mixed with a 3% solution of high molecular weight dextran in saline (1 part on 2-3 parts of blood). The erythrocytes were allowed to sediment at unit gravity for 15-30 min, and the leucocytes in the supernatant were washed once with RPMI medium by centrifugation. The enriched leucocytes were then suspended in fetal calf serum with 12% dimethylsulfoxide and stored in liquid nitrogen.

DNA-FCM procedure

Stored samples were processed for DNA-FCM as described (7). The ploidy of the different peaks in histograms from tumor samples was calculated from their position, relative to the G0/G1 peak (C-value = 2) in a histogram of cultured normal diploid prostate fibroblasts. Diploid: C= 1.9-2.2; hypodiploid C≤ 1.8; hyperdiploid C= 2.3-2.7; triploid: C= 2.8-3.4; tetraploid: C= 3.5-4.2. Tumors showing more than 10% of the nuclei to be in the tetraploid range, representing diploid G2M phase cells as well as tetraploid tumor cells, were considered to comprise a significant population of tetraploid tumor cells (7).

FISH procedure

The chromosome specific probes used were: PUC 1.77 (18), hybridizing to SATIII repeats on the pericentromeric heterochromatin region of chromosome 1; L1.84 (19), hybridizing to alphoid repeats on the centromere of chromosome 18 and DYZ3 (20), hybridizing to SATIII repeats on the centromere of the Y chromosome.

Hybridization and detection were performed as described by Pinkel et al. (21), with modifications. In short, the probe was labeled with Bio-11-dUTP by nick translation. Slides with fixed nuclei were pretreated with RNase and postfixed with formaldehyde. Hybridization of the probe to the nuclei (15 ng probe per slide) occurred during overnight incubation at 37°C

in a moist chamber in 65% formamide. Slides were subsequently washed in 60% formamide/ 2xSSC (3x 5 min), 2xSSC (3x 5 min) both at 40°C, followed by 3x 5 min in 0.1xSSC at 60°C, 5 min 2xSSC and 5 min 4xSSC/0.05% Tween at 20°C. Detection of hybridized sequences was with Avidin-FITC (incubation: 20 min at 37°C), followed by biotinylated goat "-Avidin (incubation: 30 min at 37°C) and a second Avidin-FITC incubation. Nuclei were counterstained with propidium iodide in antifade solution. The number of spots per nucleus, representing the number of centromeric regions of a respective chromosome, was counted at 1250x magnification on a Zeiss Axiophot fluorescence microscope equipped with a FITC filter combination.

Evaluation and statistics

Due to the limited amount of tissue available, hybridization with the probe for Y was not possible with 10/42 specimens. In two specimens the hybridization with the probe for chromosome 18 was not evaluable.

For the evaluation of FISH signals we used the criteria of Hopman et al. (22): a) nuclei should be intact and should not overlap; b) FISH signals within one nucleus should be completely separated (split or paired spots should be counted as one) and of the same intensity. When these criteria could not be met, such nuclei were excluded from counting. When more than 10% of the nuclei on a slide had to be excluded, the hybridization was repeated. When there were more than 5% nuclei with one spot on a slide, the hybridization was also repeated. At least 300 nuclei were scored per sample and per probe.

Aneusomy was defined to be significant when the percentage of nuclei with numerical changes differed from the average percentage in benign prostatic hyperplasia (BPH) specimens by at least 2 times S.D. (see also Table I).

Dependent on the number of nuclei counted, the Kolmogorov-Smirnov (KS) test (23) gives a minimally required discrepancy percentage for two observations. When two observations (in this study counts of spots from probes of two different chromosomes) differed more than the discrepancy percentage, these observations were considered statistically significant.

Table I. Values of BPH specimens obtained by FISH analysis

CHR	N	LOSS[1]	CUT-OFF % LOSS[2]	NORMAL COPY NUMBER[1]	GAIN[1]	CUT-OFF % GAIN[2]
1	15	3.3±2.5	8.3	93.7±3.5	2.8±3.3	9.4
18	12	2.4±2.3	7.0	96.0±2.6	1.7±1.0	3.7
Y	11	0.9±1.2	3.3	94.2±1.9	2.6±2.8	8.2

Notes

At least 300 nuclei were scored per chromosome and per sample. [1] All numbers are percentages ±S.D. Loss: % 0+1 spot for #1 and #18, 0 spots for Y; Normal copy number: 2 spots for #1 and #18, 1 spot for Y; Gain: >2 spots for #1 and #18, >1 spot for Y. [2] average + 2x S.D.

RESULTS

Frequency of chromosomal aberrations in BPH specimens

The frequency of numerical aberrations of chromosomes 1, 18 and Y was determined in BPH specimens in order to gain insight in: 1) the sensitivity of the hybridization reaction for each probe and 2) to assess cut-off percentages for significant gain and loss of each investigated chromosome.

Sensitivity was considered good for #1 and #18, and very high for Y (see Table I, column headed loss). Extra spots (see Table I, column headed gain) were scored with an acceptable frequency for #18 (range 0.3-3.5%), but the frequencies for #1 (range 0.8-12.7%) and Y (range 2.5-7.2%) were higher.

The distribution of hybridization spots in nuclei from BPH specimens was used to calculate the cut-off values for significant gain or loss of each of the three chromosomes investigated in PC specimens (see Table I).

Frequency of chromosomal loss and gain in PC specimens

Figure 1A and Table II show the percentages of nuclei with less than two copies (for #1 and #18), respectively less than 1 copy (Y), scored for each tumor individually. The average percentages were 3.1 ± 2.9 (#1), 3.6 ± 3.2 (#18) and 7.9 ± 17.6 (Y). Note that in one tumor the Y chromosome was entirely lost (PC320, see also Table II). Since no loss of Y was observed in cultured peripheral lymphocytes of this patient, this loss was considered tumor specific.

Chromosome loss above cut-off level was infrequent for chromosomes #1 and #18, as it occurred in only 3/42 tumors for chromosome 1, and in 5/40 tumors for chromosome 18 (see Table II). Significant loss of the Y chromosome however, occurred in 17/32 tested specimens (53%) (see Table II). Unbalanced loss, as determined by the KS test, occurred in 4 samples. In each of these cases the Y chromosome was involved. In one case (PC320) also chromosome 18 was underrepresented. Hypodiploid populations (by DNA-FCM) were not detected in either of these tumors.

Table II. Loss of chromosomes 1, 18 and Y using FISH

PC NR.	% OF NULLI- MONOSOMIC NUCLEI FOR TARGET CHROMOSOME		
	#1	#18	Y
202	8.3	4.8	11.2
288[1]	0.0	4.2	11.3
289	0.6	0.0	6.4
290[1]	0.4	0.6	15.5
295	0.7	14.2	ND
296	3.4	6.4	4.9
301	13.3	9.4	ND
303	1.3	NE	11.2
308	0.8	0.7	3.4
320[1]	6.2	9.5	100
329	2.4	4.4	9.2
342	2.0	2.9	3.7
343	9.0	3.8	4.1
353	2.7	8.6	ND
359[1]	5.4	NE	15.2
360	3.5	3.2	4.6
371	6.5	2.8	5.7
384	7.0	1.4	7.2
395	6.3	7.3	7.2
396	3.0	1.6	4.9

Notes

[1] Unbalanced chromosome loss. Underlined percentages are above cut-off value. ND= not determined. NE= not evaluable. For DNA-FCM results of these samples, see Table III.

Table III. Gain of chromosomes 1, 18 and Yand FCM results

	% OF POLYSOMIC NUCLEI[1]			FCM			
PC NR.	#1	#18	Y	MAJOR ANEUPLOID POPUL.[2]		OTHER ANEUPLOID POPUL.[3]	
				%	C	%	C
285	7.0	11.0	6.9	7	3.9	0	-
288[4]	10.0	2.3	4.8	6	3.5	0	-
301[4,5]	2.8	9.3	ND	8	3.8	0	-
323	10.0	9.3	9.5	7	3.5	0	-
329	3.9	4.4	5.1	9	3.0	0	-
340	1.0	1.4	ND	7	3.1	0	-
341	0.1	0.4	1.0	2	3.4	0	-
352	1.1	2.0	1.8	6	2.9	0	-
353	7.3	5.7	ND	6	3.3	0	-
357	4.2	0.4	ND	7	3.0	0	-
360	0.3	3.4	8.7	3	3.6	0	-
382	1.5	4.0	4.2	0	-	0	-
389	8.3	13.5	0.0	9	3.9	0	-
395	2.0	0.9	4.4	4	3.8	0	-
256[5]	6.6	6.8	5.6	15	4.0	0	-
270	23.6	27.0	25.6	70	3.8	10	4.8
286	10.0	14.0	ND	11	3.6	0	-
289	19.9	18.7	11.3	8	3.6	3	4.4
303	33.6	NE	5.1	10	3.8	0	-
355	24.2	21.2	4.6	37	3.6	0	-
362[4]	28.4	6.6	5.3	9	3.6	5	>4
384[5]	7.8	9.9	5.3	14	3.8	0	-
202	9.6	9.5	8.1	25	3.2	0	-
290[4]	33.0	20.4	16.3	45	3.2	15	3.6
						18	5.3
291[4,5]	32.0	33.3	2.8	38	3.1	29	5.4
296	3.4	6.8	9.1	8	2.9	6	3.8
318	5.8	3.2	ND	17	3.3	9	>4.2
320[5]	2.3	2.3	0.0	15	3.0	0	-
342[4]	23.8	7.4	24.7	81	3.0	10	4.7
343	1.8	2.0	0.7	22	2.9	0	-

Notes

PC numbers are listed chronologically, but are grouped according to the main aneuploid population detected by FCM. ND= not determined. NE= not evaluable. Underlined percentages are above cut-off values. [1] For #1 and #18: percentage of nuclei >2 spots; for Y: percentage of nuclei >1 spot. [2] and [3] Aneuploid P diploid (1.9-2.1 C); major population: with highest percentage. [4] Unbalanced chromosome gain. [5] PC236 and PC301, respectively PC256 and PC384, and PC295 and PC320 are tissue samples from the same patients, sequentially obtained (see text).

Table III, continued

PC NR.	% OF POLYSOMIC NUCLEI[1]			FCM			
	#1	#18	Y	MAJOR ANEUPLOID POPUL.[2]		OTHER ANEUPLOID POPUL.[3]	
				%	C	%	C
351	12.9	10.6	13.0	34	3.1	0	-
354[4]	2.3	22.9	4.4	46	2.9	7	4.4
359	10.3	NE	5.7	29	3.4	3	>4
371	4.9	1.7	3.5	12	3.4	4	4.0
269[5]	19.1	23.0	10.8	59	2.2	24	1.7
						8	3.6
						9	5.8
292[4]	17.6	8.7	ND	69	1.8	9	3.5
295[5]	0.9	1.4	ND	38	1.5	10	3.9
						3	3.2
306	6.8	9.3	ND	77	1.8	3	3.5
308	7.2	3.7	4.7	66	1.7	4	3.1
344[4]	4.0	17.9	ND	54	2.6	9	3.4
396	0.4	0.4	1.8	94	1.6-2.0	6	3.6

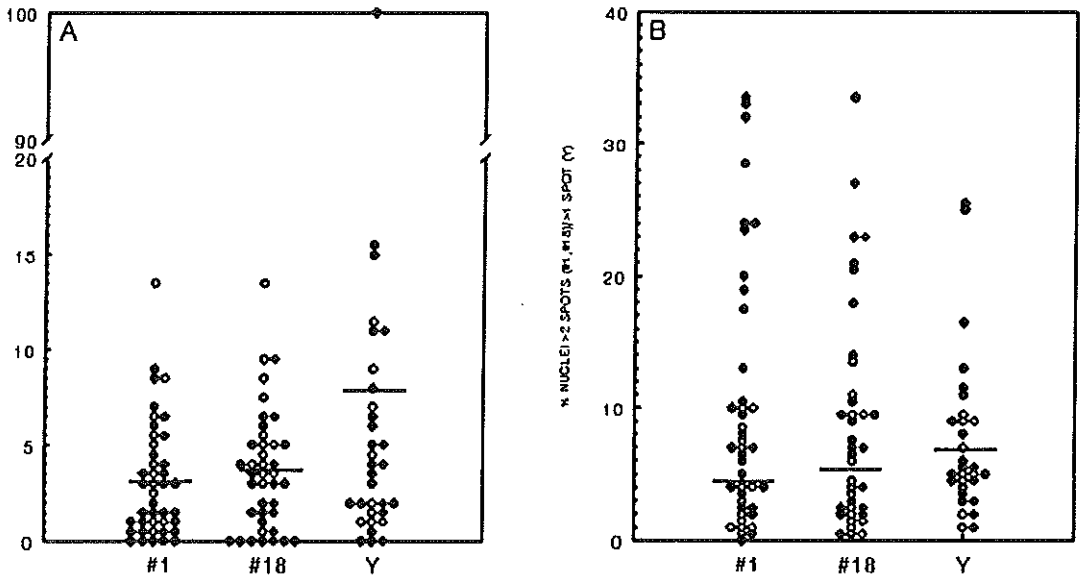


Figure 1. A. Distribution of percentages of mono- respectively nullisomic nuclei for chromosomes 1, 18 and Y. B. Distribution of percentages of polysomic nuclei for chromosomes 1, 18 and Y. Open symbols: DNA diploid, closed symbols: DNA aneuploid. Solid line: average percentage.

Figure 1B and Table III show the frequencies of polysomy of #1, #18 and Y in the investigated tumors. Table III also shows the percentages and C-values of the aneuploid cell populations, determined by DNA-FCM. The average percentages were respectively 4.0 ± 3.6, 4.6 ±4.2 and 5.6 ±3.0. Using the BPH values as a reference (see above), we observed significant gain in 16/42 (38%) cases for chromosome 1 and in 25/40 (63%) cases for chromosome 18. Significant gain of Y was less common: 9/32 tumors (28%). Two diploid tumors each showed gain of #1 (PC288 and PC323) and Y (PC323 and PC360), while gain of #18 occurred in 7/14 diploid tumors. In 7/32 tumors gain was homogeneous, i.e. all three chromosomes showed significant polysomic populations (PC269, PC270, PC289, PC290, PC323, PC342 and PC351). Significant unbalance between #1, #18 and Y, as determined by the KS test, occurred in nine tumors (PC288, PC290, PC291, PC292, PC301, PC342, PC344, PC354 and PC362). Populations of penta- and/or hexasomic nuclei of >1% were found in PC269, PC289, PC290 and PC291 for #1, PC269 and PC354 for #18 and PC269 and PC290 for Y (see also Fig.2D). In PC301 and PC353 (both DNA diploid), loss as well as gain of #18 was found. Simultaneous loss and gain of chromosome 1 was found in PC202, whereas simultaneous loss and gain of Y was found in PC289, PC290, PC296, PC342 and PC360.

From four patients two tissue samples were obtained. From one of these the samples were obtained from the primary tumor (PC256) and from a local recurrence, 34 months after radical prostatectomy (PC384). FCM and FISH results of these samples were comparable; in both samples an equal percentage of aneuploid cells was present and chromosome 18 showed a population of polysomic cells. From three other patients a lymph node metastasis (LM) and a sample from the primary tumor at palliative surgery (TUR) were available. One of these patients showed a high percentage of polysomy for chromosome 18 in the TUR material (PC301), but not in the LM (PC236), while FCM results were comparable in both samples. Another patient showed no difference with FISH, while FCM was different (PC295 and PC320). The third patient showed complex, but different FCM in both samples and a profound dissimilarity with FISH (PC269 and PC291).

Correlation between DNA-FCM and FISH results

In 10/14 tumors which showed a diploid DNA-FCM histogram, significant chromosomal aberrations were found (see Tables II and III). One specimen (PC323) showed polysomy for all three chromosomes. Significant chromosomal aberrations were detected in 26/28 tumors that were aneuploid by DNA-FCM. In most aneuploid tumors the percentage of aneuploidy, determined by DNA-FCM, was higher than the percentage of aneusomic nuclei. This was true for all three chromosomes. Only in two cases (PC362 and PC292) the percentage of polysomy found for #1 was two times higher than the percentage of aneuploid cells, as determined by FCM. This was not found for either #18 or Y.

When polysomy of #1, #18 and/or Y was detected, in nearly all cases trisomic as well as tetrasomic nuclei for #1 and #18, and di- and trisomic nuclei for Y were found, irrespective whether the total DNA content was DNA-tetraploid (PC236-384 in Table III), DNA-triploid (PC202-371), or otherwise aneuploid (PC269-396). Figures 2A-D show the spot distributions of #1, #18 and Y for representative tumors with different ploidy patterns. Six DNA histograms of PC specimens showed populations of hypodiploid cells (PC269, PC292, PC295, PC306, PC308 and PC396; see Table III). PC295, PC308 and PC396 showed loss of a chromosome (see Table II).

Figure 3 shows the relationship between the percentage of aneuploid nuclei found with DNA-FCM and the percentage of polysomic nuclei found with FISH. The correlation between DNA-FCM and FISH of #1 and #18 was about equal

(respectively 0.531 and 0.529), but the correlation between DNA-FCM and polysomy for Y was better (r = 0.78). All correlations were highly significant (p<0.0005).

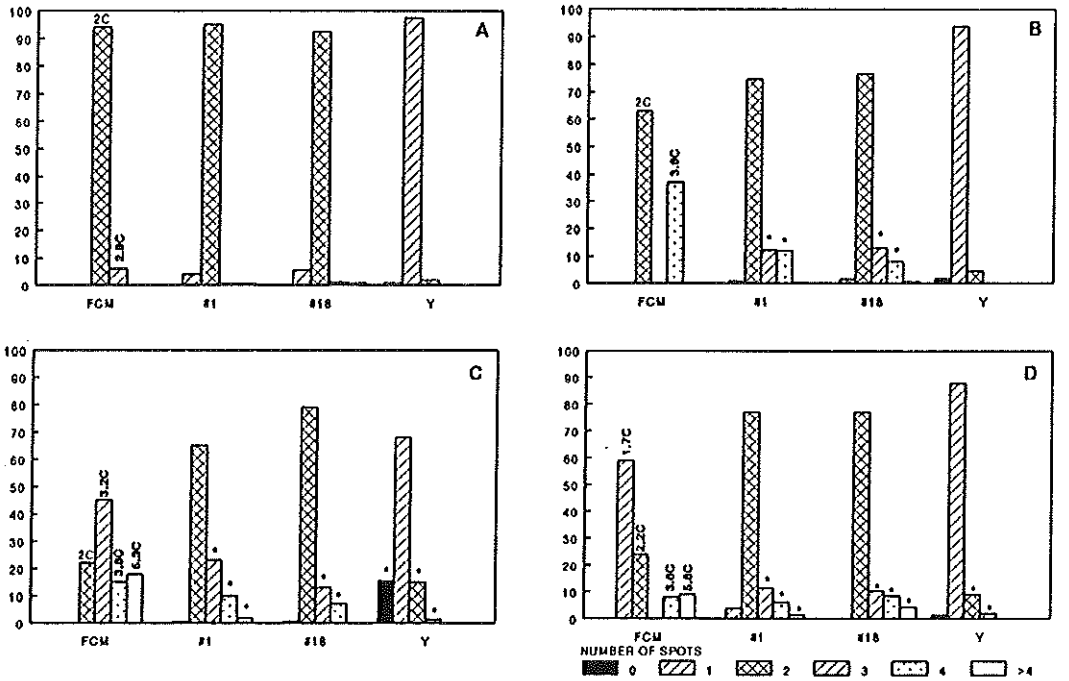


Figure 2. Examples of spot distribution of #1, #18 and Y and FCM analysis in: A. a DNA diploid tumor (PC352); B. a DNA tetraploid tumor (PC355); C. a tumor with multiple aneuploid stemlines (PC290); a DNA hypodiploid tumor (PC269). *= above cut-off value.

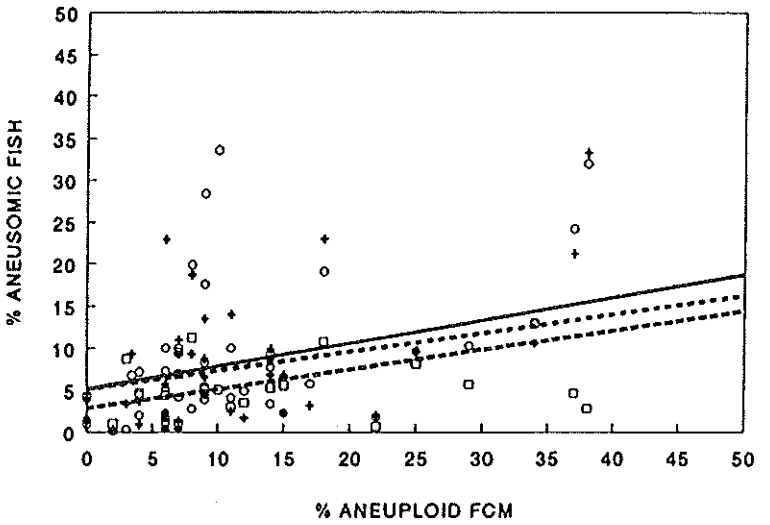


Figure 3. Correlation between percentage aneusomy by FISH and percentage aneuploidy by FCM. Regression analysis. chromosome 1, + chromosome 18, ~ Y. Regression lines: solid line: chromosome 1, hatched line: chromosome 18, dotted line: Y.

Relationship of stage and grade with numerical aberrations and ploidy

Aberrations of chromosomes 1, 18 and/or Y were equally distributed over all stages and grades, while with increasing grade or stage FCM showed more aberrant histograms (see Tables IV and V).

Table IV. Relationship between stage, numerical aberrations and ploidy

STAGE	N	% ABERRATION FISH	% ABERRATION FCM
T1	0	-	-
T2	7	86	43
T3	16	88	63
T4	19	84	79

Table V. Relationship between grade, numerical aberrations and ploidy

GRADE	N	% ABERRATION FISH	% ABERRATION FCM
1	5	80	60
2	16	88	50
3	21	86	81

DISCUSSION

We investigated the frequencies of loss and gain of chromosomes 1, 18 and Y by FISH and the total ploidy by DNA-FCM in the same nuclear suspension, obtained from tissue of patients with clinically evident PC. These three chromosomes are each, in one way or another, involved in the genetics of PC (see below).

Significant loss of chromosomes 1 and 18 was infrequent, and was not correlated with detectable loss of total DNA. Due to the high sensitivity of hybridization of Y in BPH specimens, the cut-off value was very low, so 17/32 PC specimens showed significant loss of Y. However, six of these tumors showed a loss percentage below 5%. The biological significance of such small subpopulations (≤5%) is presently unknown; clonal progression is a possibility, but technical artefacts cannot be completely excluded.

Significant gain of chromosomes 1 and Y was largely restricted to DNA aneuploid tumors. Around one-third of the DNA aneuploid tumors showed gain of chromosome 1 or Y. Gain of chromosome 18 occurred in more than half of the cases, but the cut-off percentage set through the results obtained with BPH specimens, was lowest for #18. Furthermore, the presence of trisomic cells together with tetrasomy for chromosomes 1 and 18, was a common finding. Both the finding of minor polysomic cell populations and the observation of trisomic and tetrasomic cells together in the same tumor specimens, are in agreement with the generally accepted theory that the genetic evolution of solid tumors which converges on repeated rounds of tetraploidization and subsequent chromosome loss (24). Following this concept, minor fractions of cells which show gain of hybridization signals, are thought to be a manifestation of the onset of tetraploidization of a tumor (25).

An explanation for the lower frequency of gain for Y could be that relative loss of

one Y chromosome in a tetraploid tumor cell will result in monosomy for Y, thereby making this cell indistinguishable from a diploid cell.

Simultaneous loss and gain of a chromosome in the same specimen, indicative for heterogeneous changes, was rare for chromosomes 1 and 18 (respectively one and two cases), and occurred in 16% of the cases investigated with the Y probe.

The correlation between DNA-FCM and FISH was less strong than expected. Surprisingly, most DNA diploid tumors showed significant gain of one or more of the investigated chromosomes. Apparently, already in diploid tumors additional copies of especially chromosome 18 could be present, whithout being detected by DNA-FCM. On the other hand, an aneuploid DNA histogram did not imply the presence of numerical aberrations for all three investigated chromosomes. However, as expected, with an increasingly aberrant and complex histogram, more of the investigated chromosomes became aberrant.

The occurrence of numerical aberrations in the three investigated chromosomes was independent of tumor grade or stage, nor was there a correlation between the presence of aberrations in each individual chromosome and grade or stage (not shown). However, as expected, the DNA-FCM of a tumor was more likely to be abnormal with increasing grade and stage.

At present two other studies report the detection of numerical chromosomal aberrations with centromere specific probes in PC (26, 27). The first study investigated chromosomes 7, 17, X and Y on paraffin sections. Although this method will lead to an underestimation of the number of in situ signals, due to the loss of nuclear material (28), aberrations were found in 5/11 investigated tumors. These tumors were all of advanced stage or grade, with a large tumor volume. No details were presented on specific chromosomal aberrations found, but some heterogeneity with respect to different regions in the tumor area was reported. The other study used cells obtained from tissue cultures of 10 PC specimens. The copy numbers of chromosomes 7, 8, 10, 16, 17 and 18 were assessed by FISH. Frequent losses and gains were observed for all chromosomes investigated. However, culturing artifacts, caused by preferential growth of subgroups of tumor cells, or even normal cells (8), cannot be ruled out. Unfortunately, as demonstrated by the high percentages of nuclei with 0 and 1 spot, the specificity of the hybridizations was rather low, making it difficult to draw any definite conclusions from these data.

In bladder carcinoma, FISH studies (25, 29) have shown numerical aberrations for chromosome 1 in 20-25% of the cases, and also in breast cancer aberrations of chromosome 1 were shown to occur (30). Cytogenetic studies on PC have shown that chromosome 1, when aberrant, is most frequently structurally rearranged (3, 4, 31). In PC cell lines, gain of chromosome 1 is also common (32-36). The relatively common gain of chromosome 1 we report here, is in agreement with these results.

Cytogenetically, chromosome 18 was not shown to be nonrandomly or specifically affected in PC, but in two LOH studies loss of loci on the long arm of chromosome 18 was reported in 3/7 (15) and 2/12 (16) informative cases. Our data showed that complete loss of one homolog was infrequent, but that gain was common. Chromosomal duplication, driven by gene loss could be a possible mechanism behind this observation.

Loss of Y is a recurrent chromosomal aberration in PC (1-3, 5, 17), sometimes found as the sole anomaly. Also with in situ hybridization on paraffin sections some PC's were shown to have loss of Y (27, 37). The frequency of 53% we found in the present study is quite high, but could be biased by the low cut-off percentage derived from the studies in BPH specimens, which served as a control. The loss of Y has been described, not only in other solid tumors, but also in benign and normal tissues. So, on the other hand, loss of the Y chromosome may also reflect a general state of

tissue hyperproliferation and as such may not exclusively be related to neoplastic processes.

The finding that gain of chromosomes 1 and Y was restricted to DNA aneuploid tumors is not surprising. However, we have noexplanation for the almost consistent finding of a higher percentage of disomic (monosomic for Y) cells in aneuploid tumors than was to be expected on the basis of DNA ploidy. Although applied on the same isolated cells, the sensitivity of the techniques appears to be different. By FISH the percentage of aneusomic nuclei seldom reached 30%, while with DNA-FCM the percentage of aneuploid nuclei could be over 90%. This phenomenon was observed before in PC and bladder carcinoma in comparative studies using FISH and DNA-FCM (8, 22, 29). A similar effect has been described recently in a comparative study of cytogenetic and DNA-FCM data on bone and soft-tissue tumors (38). In karyotypically abnormal tumors which were aneuploid, a tendency towards DNA indices higher than the chromosomal index was reported. This was partly explained by the assumption that in these tumors, which often had complex chromosomal rearrangements, the size of some marker chromosomes probably exceeded the size of normal homologs. This could also be the case for PC's which show moderate differences, but still cannot explain the large discrepancies found in some highly aneuploid tumors.

The correlation between tumor ploidy and tumor aggressiveness has been well established (9-12). Recently, Lundgren et al (31) showed the association of the presence of clonal chromosomal aberrations with an unfavorable outcome in PC patients. In the present study we found that the presence of numerical aberrations of the chromosomes we investigated was unrelated to tumor grade or stage.

In conclusion, the results obtained with FISH of only three centromere specific probes, show significant aneuploidy in small subpopulations in a total of about 90% of the investigated tumors. This is in contrast with FCM where 63% of the tumors show an aneuploid peak. Both the results obtained with FISH and with FCM indicate that cytogenetic studies of PC after short term tissue culture selectively have produced karyotypic data on the normal cell component of the tumor. Therefore, to gain more insight into the cytogenetic composition of PC, FISH studies of all relevant (parts of) chromosomes appears for the time being the technology of choice.

Acknowledgements

We wish to thank Herman van Dekken for providing us with the DNA probes. The financial support of the Dutch Cancer Society (project number IKR 90-12) is gratefully acknowledged.

LITERATURE

1.Lundgren R, Mandahl N, Heim S, Limon J, Hendrikson H, Mitelman F: Cytogenetic analysis of 57 primary prostatic adenocarcinomas. Genes Chrom Cancer 4:16-24, 1992.

2.Micale MA, Mohamed A, Sakr W, Powell IJ, Wolman SR: Cytogenetics of primary prostatic adenocarcinoma. Clonality and chromosome instability. Cancer Genet Cytogenet 61:165-173, 1992.

3. Arps S, Rodewald A, Schmalenberger B, Carl P, Bressel M, Kastendieck H: Cytogenetic survey of 32 cancers of the prostate. Cancer Genet Cytogenet 66:93-99, 1993.

4. Brothman AR, Peehl DM, Patel AM, McNeal JE: Frequency and pattern of karyotypic abnormalities in human prostate cancer. Cancer Res 50:3795-3803, 1990.

5. Brothman AR, Peehl DM, Patel AM, MacDonald GR, McNeal JE, Ladaga LE, Schellhammer PF: Cytogenetic evaluation of 20 cultured primary prostatic tumors. Cancer Genet Cytogenet 55:79-84, 1991.

6.Sandberg AA: Chromosomal abnormalities and related events in prostate cancer. Hum Pathol 23 :368-380, 1992.

7. König JJ, Dongen van JJW, Schröder: Preferential loss of abnormal prostate carcinoma

cells by collagenase treatment Cytometry :in press, 1993.
8. König JJ, Teubel W, Dongen JJW van, Hagemeijer A, Romijn JC, Schröder FH: Tissue culture loss of aneuploid cells from carcinomas of the prostate. Genes Chrom Cancer 8:22-27, 1993.
9. Lee SE, Currin SM, Paulson DF, Walther PJ: Flow cytometric determination of ploidy in prostatic adenocarcinoma: a comparison with seminal vesicle involvement and histopathological grading as a predictor of clinical recurrence. J Urol 140:769-774, 1988.
10. Winkler HZ, Rainwater LM, Myers RP, Farrow GM, Therneau TM, Zincke H, Lieber MM: Stage D1 prostatic adenocarcinoma : significance of nuclear DNA ploidy patterns studied by flow cytometry. Mayo Clin Proc 63:103-112, 1988.
11.Miller J, Horsfall DJ, Marshall VR, Rao DM, Leong ASY: The prognostic value of deoxyribonucleic acid flow cytometric analysis in stage D2 prostatic carcinoma. J Urol 145:1192-1196, 1991.
12.Nativ O, Myers RP, Farrow GM, Therneau TM, Zincke H, Lieber MM: Nuclear deoxyribonucleic acid ploidy and serum prostate specific antigen in operable prostatic adenocarcinoma. J Urol 144:303-306, 1990.
13. Poddighe PJ, Ramaekers FCS, Hopman AHN: Interphase cytogenetics of tumors. J Pathol 166:215-224, 1992
14. Persons DL, Gibney DJ, Katzmann JA, Lieber MM, Farrow GM, Jenkins RB: Use of fluorescent in situ hybridization for deoxyribonucleic acid ploidy analysis of prostatic adenocarcinoma. J Urol 150 :120-125, 1993.
15. Kunimi K, Bergerheim USR, Larsson IL, Ekman P, Collins VP: Allelotyping of human prostatic adenocarcinoma. Genomics 11:530-536, 1991.
16. Carter BS, Ewing CM, Ward WS, Treiger BF, Aalders TW, Schalken JA, Epstein JI, Isaacs WB: Allelic loss of chromosomes 16q and 10q in human prostate cancer. Proc Natl Acad Sci 87:8751-8755, 1990.
17. Konig JJ: Genetic changes associated with progression to endocrine independence in carcinoma of the breast and prostate. In: Mechanisms of progression to hormone-independent growth of breast and prostatic cancer, PMJJ Berns, JC Romijn and FH Schroder (eds) Parthenon Publishing, New Jersey. pp123-134, 1991.
18. Cooke HJ, Hindley J: Cloning of human satellite III DNA: different components are on different chromosomes. Nucl Acids Res 6:3177-3197, 1979.
19. Devilee P, Cremer T, Slagboom P, Bakker E, Scholl H, Hager H, Stevenson A, Cornelisse C, Pearson P: Two studies of human alphoid repetitive DNA show distinct preferential localization in the pericentromeric regions of chromosomes 13, 18 and 21 Cytogenet Cell Genet 41:193-202, 1986.
20. Cooke HJ, Schmidke J, Gosden JR: Characterization of a human Y chromosome repeated sequence and related sequences in higher primates Chromosoma 87:491-502, 1982.
21. Pinkel D, Gray J, Trask B, Van den Engh G, Fuscoe J, Van Dekken H: Cytogenetic analysis by in situ hybridization with fluorescently labeled nucleic acid probes. Cold Spring Harbor Symp Quant Biol L1:151-157, 1986.
22. Hopman AHN, Ramaekers FCS, Raap AK, Beck JLM, Devilee P, van der Ploeg M, Vooijs GP: In situ hybridization as a tool to study numerical chromosome aberrations in solid tumors. Histochemistry 89:307-316, 1988.
23. Dekken van H, Rotterdam van A, Jonker R, Voort van der HTM, Brakenhoff GJ, Bauman JGJ: Confocal microscopy as a tool for the study of the intranuclear topography of chromosomes. J Microscopy 158:207-214, 1989.
24. Shackney SE, Smith CA, Miller BW, Burholt DR, Murtha K, Giles HR, Ketterer DM, Pollice AA: Model for the genetic evolution of human solid tumors. Cancer Res 49:3344-3354, 1989.
25. Hopman AHN, Moesker O, Smeets AWGB, Pauwels RPE, Vooijs GP, Ramaekers FCS: Numerical chromosome 1, 7, 9, and 11 aberrations in bladder cancer detected by in situ hybridization. Cancer Res 51:644-651, 1991.
26. Brothman AR, Patel AM, Peehl DM, Schelhammer PF: Analysis of prostatic tumor cultures using fluorescence in-situ hybridization (FISH). Cancer Genet Cytogenet 62:180-185, 1992.
27. Henke RP, Kruger E, Ayhan N, Hubner D, Hammerer P: Numerical chromosomal aberrations in prostate cancer: correlation with morphology and cell kinetics. Virchows Archiv A Pathol Anat 422:61-66, 1993.
28. Arnoldus EPJ, Dreef EJ, Noordermeer IA, Verheggen MM, Thierry RF, Peters ACB,

Cornelisse CJ, Van der Ploeg M, Raap AK: Feasibility of in situ hybridization with chromosome specific DNA probes on paraffin wax embedded tissue. J Clin Pathol 44:900-904, 1991.
29. Hopman AHN, Poddighe PJ, Smeets AWGB, Moesker O, Beck JLM, Vooijs GP, Ramaekers FCS: Detection of numerical chromosome aberrations in bladder cancer by in situ hybridization. Am J Pathol 135:1105-1117, 1989.
30. Devilee P, Thierry RF, Kievits T, Kolluri R, Hopman AHN, Willard HF, Pearson PL, Cornelisse CJ: Detection of chromosome aneuploidy in interphase nuclei from human primary breast tumors using chromosome-specific repetitive DNA probes. Cancer Res 48:5825-5832, 1988.
31. Lundgren R, Heim S, Mandahl N, Anderson H, Mitelman F: Chromosome abnormalities are associated with unfavorable outcome in prostate cancer patients. J Urol 147:784-788, 1992.
32. König JJ, Kamst E, Hagemeijer A, Romijn JC, Horoszewicz J, Schröder FH: Cytogenetic characterization of several androgen responsive and unresponsive sublines of the human prostatic carcinoma cell line LNCaP. Urol Res 17:79-86, 1989.
33. Pittman S, Russell PJ, Jelbart ME, Wass J, Raghavan D: Flow cytometric and karyotypic analysis of a primary small cell carcinoma of the prostate : a xenografted cell line. Cancer Genet Cytogenet 26:165-169, 1987.
34. Iizumi T, Yakasi T, Kanoh S, Kondo I, Kolso K: Establishment of a new prostatic carcinoma cell line (TSU-PRI). J Urol 137:1304-1306, 1987.
35. König JJ, Hagemeijer A, Smit B, Kamst E, Romijn JC, Schröder FH: Cytogenetic characterization of an established xenografted prostatic carcinoma cell line (PC-82). Cancer Genet Cytogenet 34:91-99, 1988.
36. Stone KR, Mickey DD, Wunderli H, Mickey GH, Paulson DF: Isolation of a human prostate carcinoma cell line (DU145). Int J Cancer 21:274-281, 1978.
37. Dekken van H, Alers J: Loss of chromosome Y in prostate cancer cells, but not in stromal tissue Cancer Genet Cytogenet 66:131-132, 1993.
38. Mandahl N, Baldetorp B, Ferno M, Akerman M, Rydholm A, Heim S, Willen H, Killander D, Mitelman F: Comparative cytogenetic and DNA flow cytometric analysis of 150 bone and soft-tissue tumors. Int J Cancer 53:358-364, 1993.

CHAPTER 6

GAIN AND LOSS OF CHROMOSOMES 1, 7, 8, 10, 18 AND Y IN 46 PROSTATE CANCERS

Josée J.König, Wilma Teubel, Johannes C.Romijn, Fritz H.Schröder and Anne Hagemeijer

Published in: Human Pathology 27: 720-727, 1996

ABSTRACT

Fluorescence in situ hybridization (FISH) with centromere probes was used to investigate numerical aberrations of chromosomes 1, 7, 8, 10, 18 and Y in 46 prostate carcinoma (PC) and 11 benign prostatic hyperplasia (BPH) samples. None of the benign specimens showed any chromosomal aberration. 41/46 PC specimens showed numerical aberrations of one or more chromosomes. All investigated chromosomes showed numerical aberrations in at least 30% of the specimens, gain being more frequent than loss. Comparison of DNA-flow cytometry and FISH results showed that not only aneuploid tumors, but also most diploid tumors harbored numerical chromosome aberrations. Chromosome 10 was the most frequently gained (65%), and Y the most frequently lost chromosome (14%). Nonmetastatic and metastatic tumors differed significantly ($p<0.05$) in the number of copies for #7, 8 and 10, but not for #1, 18 and Y. These results suggest strongly that gains of chromosomes 7, 8 and 10 are involved in PC progression.

INTRODUCTION

By cytogenetic analysis, structural and numerical aberrations of chromosomes 1, 7, 8, 10, 16 and/or Y (1) were identified in about 25% of the PC's studied. However, this figure is probably an underestimation of the true extent of the aberrations. This is due to selective isolation and preferential in vitro growth of nonmalignant prostate epithelium (2, 3). The use of interphase cytogenetic techniques for characterization of uncultured PC material has been stimulated by these findings. Application of in situ hybridization with centromere specific DNA probes to fixed sections of PC has shown numerical aberrations for chromosomes 1, 7, 8, 10, 12, 17, 18, X and Y (4-7). The finding of numerical aberrations in different chromosomes is not surprising, since about 50% of the PC's have an aneuploid DNA content (8). In the present study we investigated numerical changes of chromosomes 1, 7, 8, 10, 18 and Y using FISH with centromere specific DNA probes on nuclear suspensions of fresh tissue samples from 11 BPH and 43 PC patients. Selection of this chromosome panel was based on evidence from the literature and previous studies (9-12) that these chromosomes were possibly implicated in PC development or progression. Study of recurring patterns of specific chromosomal aberrations might provide new information about the genetic events involved in these processes.

The BPH specimens showed no deviation from normal diploidy, so consequently the BPH results were used as a control. Based on this, significant chromosome gains and losses in the PC samples could be analysed. Possible correlations with clinical parameters and ploidy as determined by DNA flow cytometry (FCM) were investigated.

MATERIALS AND METHODS

Tumor tissues

Eleven BPH specimens were studied, obtained at transurethral resection (TUR) or prostatectomy for BPH. The mean age of the BPH patients was 72.4±5.6 (range 64-80 years). Forty-six PC specimens, from 43 different patients were studied, obtained at radical prostatectomy (15 specimens), TUR (26 specimens), or pelvic lymph node dissection (5 specimens). From one lymph node specimen (PC295) a cell line emerged after xenografting on nude mice. Tissue from this cell line at mouse passage 4 was used instead of the original tissue. The mean age of the PC patients was 66.7±10.5 (range 49-93 years). The percentage of tumor cells present in the tissue specimens was assessed from paraffin sections of adjacent tissue. Only specimens with more than 50% tumor cells were included in the study.

From 23 patients detailed clinical data could be obtained (Table 1). Twelve patients were lymph node negative (nonmetastatic), and 11 were node positive (metastatic) at the time of

first surgery. Three patients had organ confined disease (T2N0), whereas 4 tumors showed periprostatic spread (T4). The remaining 16 patients showed extracapsular extension of the tumor (T3N0) and/or had positive lymph nodes (N+, TNM system for PC 1992 (13)). Three of these tumors were well differentiated (G1, all from primary tumors obtained at radical prostatectomy), 10 were moderately differentiated (G2) and in 10, poorly differentiated (G3) areas were found. From three patients (cases 4, 5 and 10) two consecutive tumor samples were obtained. The second sample of case 4 was obtained from a local recurrence, diagnosed 37 months after radical prostatectomy.

Table 1. Detailed clinical data, FCM and FISH results from 21 patients

CASE	PC NR	TISSUE	G	TNM AT SURGERY	FU *	STATUS	FCM	ANEUSOMIES †
1	202	TUR	2	T4N3M0	52(44)	D	A	P1,P7,P8,P10,P18 M1,M7,M8,M10
2	236	LM	3	T3N2M0	50(0)	DN	D	P10
3	244	LM	2	T2N2M0	56(0)	D	A	P7,P8,P10,P18
4	256	P	2	T3N1M0	53(37)	P	T	P8,P10,P18 M8,M10,M18
	384	TUR					T	P1,P7,P8,P10,P18 M7
5	269	LM	3	T3NxM0	15(0)	D	AH	P1,P7,P8,P10,P18
	291	TUR					A	P1,P8,P10,P18 M10
6	270	LM	3	T0NxM2	17(0)	D	T	P1,P7,P8,P10,P18,P Y
7	288	P	2	T3N0M0	40	NP	D	P1,P8,P10 M8,MY
8	289	P	3	T3N0M0	15(5)	D	A	P1,P7,P18
9	290	P	2	T3N0M0	38(31)	P	A	P1,P7,P8,P10,P18 M8,M10,MY
10	295	LM	3	T1NxM0	9(0)	D	AH	P8,P10 M1,M18,MY
	320	TUR					A	P10 M7,M8,M10,MY
11	296	TUR	3	T4N2M0	13(0)	P	A	P8,P18 M18
12	341	P	1	T2N0M0	30	NP	D	-

Table 1, continued. Detailed clinical data, FCM and FISH results from 21 patients

13	342	P	1	T2N0M0	29	NP	A	P1,P7,P8,P10,P18,P Y
14	343	P	2	T3N0M0	29	NP	A	-
15	352	P	2	T3N0M0	17	NP	D	M8
16	354	P	3	T3N0M0	26	NP	A	P7,P8,P10,P18
17	362	P	3	T4N0M0	23(19)	P	A	P1,P8,P18 M18
18	371	TUR	2	T1NxM0	40(0)	D	A	-
19	382	P	1	T2N0M0	19	NP	D	-
20	389	P	2	T3N0M0	20	NP	D	P7,P10,P18 M7,M10
21	395	P	2	T3N0M0	18	NP	D	M18,MY
22	400	TUR	3	T4NxM2	25(0)	D	NE	-
23	420	TUR	3	T2NxM0	13(0)	P	NE	P10 M1,M10

Notes
P= primary tumor, TUR= trans urethral resection, LM= lymph node metastasis, G= tumor grade, Nx= one or more positive lymph nodes, FU= total follow up time in months, NP= not progressed, P= progressed, D= deceased from PC, DN= deceased, not from PC, D= diploid, T= tetraploid, A= aneuploid, AH= hypodiploid aneuploid, NE= not evaluable. * Time to progression, when appropriate, in brackets. † P= polysomy, M= monosomy or nullisomy; P and M percentages at or above cut-off percentage for each chromosome as specified in Table 2.

Tissue processing and sample preparation
Suspected benign hyperplasia or carcinoma tissues were excised and cut into several smaller fragments, snap frozen in liquid nitrogen and stored at -80°C. For isolation of cells from tissue, approximately 0.5 ml of phosphate buffered saline (PBS) was added to a thawed specimen. Subsequently, the tissue pieces were minced with a scalpel into a suspension of small cell clumps and single cells. The clumps were discarded after sedimentation for 3-5 min in 5 ml of PBS. The supernatant was centrifuged and the resulting pellet washed and resuspended in PBS.
The cell suspension was incubated with hypotonic solution (0.075M KCl) for 10 min at 37°C and fixed in methanol/acetic acid (3:1). Fixed cells were stored in methanol at -20°C until used for FISH. The above described procedure was adequate for tissue processing and fixation of nuclei from both primary tumor tissue and lymph node metastases. As nuclei from TUR tissue tended to coagulate in suspension, an additional sedimentation step at unit gravity was necessary for these preparations.

FCM procedure
Samples were processed for DNA-FCM as described (3). The ploidy of the different peaks in histograms from tumor samples was calculated from their position, relative to the G0/G1 peak (C = 2) in a histogram of cultured normal diploid prostate fibroblasts. Diploid: C= 1.9-2.2; hypodiploid C≤ 1.8; hyperdiploid C= 2.3-2.7; triploid: C= 2.8-3.4; tetraploid: C= 3.5-4.2. Samples had a significant tetraploid cell population when the tetraploid peak, representing diploid G2/M as well as tetraploid tumor G0/G1 nuclei, contained more than 10% of the nuclei.

FISH procedure
The chromosome specific probes used were: PUC 1.77 for chromosome 1 (14); p7t.1 for chromosome 7 (15); D8Z2 for chromosome 8 (16); D10Z1 for chromosome 10 (17); L1.84 for chromosome 18 (18) and DYZ5 (Amprobe, Amersham) for the Y chromosome.
Hybridization and detection were performed as described before (2). Hybridization of the

biotinylated probe (15 ng per slide) to the nuclei occurred during overnight incubation at 37°C in a moist chamber in 65% formamide for #1, 18 and Y and in 60 % formamide for #7, 8 and 10.

Evaluation and statistics

For the evaluation of FISH signals we used the criteria defined by Hopman et al. (19): a) nuclei should be intact and should not overlap; b) FISH signals within one nucleus should be completely separated (split or paired spots should be counted as one) and of the same intensity. When these criteria could not be met, such nuclei were excluded from counting. When more than 10% of the nuclei on a slide had to be excluded, the hybridization was repeated. When there were more than 5% nuclei with one spot on a slide, the hybridization was also repeated. At least 300 nuclei were scored per sample and per probe.

In mixed tumor-normal samples small aberrations will not be detectable when the percentage of non-tumor cells is too large. To compensate for this heterogeneity, only tumor samples in our study which contained more than 50% tumor cells were electable for this study. So, depending on the cut-off percentage (ranging from 5-9%, (see Table 2)), aberrations occurring in as few as 10% of the tumor cells could still be detected.

For each chromosome in each specimen the average number of copies (ANC) was calculated (total number of spots counted/ total number of nuclei counted). Consequently, an ANC of 0.90 for Y means that 10% of the nuclei in a tissue sample show no signal; an ANC of 2.15 means that a maximum of 15% of the nuclei show gain of one or more signals. The Kruskal-Wallis test was used to test the relation between the ANC and clinical stage (11).

RESULTS

BPH specimens

For the 11 BPH specimens investigated the ANC's for each investigated chromosome were very narrowly distributed around the diploid values (Table 2). The ranges found were 1.93-2.05 (#1), 1.94-2.03 (#7), 1.93-2.04 (#8), 1.96-2.05 (#10), 1.98-2.05 (#18) and 0.97-1.07 (Y).

Loss of signals as well as extra spots were generally scored with low frequency. Only the Y chromosome showed a relatively high mean (4.4%) for extra spots scored. Since no evidence for numerical abnormalities of the investigated chromosomes was shown, BPH could be considered a diploid control. Thus the BPH results were used to assess cut-off ANC's (Table 2). Significant gain and loss (further referred to as gain and loss) of the investigated chromosomes in PC are defined as respectively at or above cut-off ANC for gain and at or below cut-off ANC for loss.

Table 2. Combined results of FISH analysis of 6 centromere probes in 11 BPH specimens

CHR	LOSS OF SIGNALS *		EXTRA SIGNALS †		ANC		
	mean % ± SD	CUT OFF % ‡	mean % ± SD	CUT OFF % ‡	mean ± SD	CUT OFF §	
						GAIN	LOSS
1	3.5±2.4	8.3	2.1±1.4	5.0	1.99±0.03	2.08	1.90
7	2.4±2.1	6.6	2.1±1.0	5.0	2.00±0.03	2.09	1.91
8	3.2±2.5	8.2	1.8±1.0	5.0	1.99±0.03	2.08	1.90
10	2.6±2.2	7.0	1.8±1.3	5.0	2.00±0.02	2.06	1.94
18	1.2±1.1	5.0	2.2±1.6	5.4	2.01±0.02	2.07	1.95
Y	1.0±1.3	5.0	4.4±2.3	9.0	1.03±0.03	1.12	0.94

Notes

CHR= chromosome. ANC = average number of copies. * Loss of signals: percentage of <2 spots for #1, 7, 8, 10 and 18, <1 spot for Y. † Extra signals: percentage of >2 spots for #1, 7, 8, 10 and #18, >1 spot for Y. ‡ cut off percentage = mean + 2x SD, at least 5%. § cut off ANC for gain = mean ANC + 3x SD; cut off ANC for loss = mean ANC - 3x SD.

PC specimens

Detailed data per specimen and per chromosome are listed in Appendix I. Significant numerical aberrations were detected for all six investigated chromosomes. Gain (trisomy and tetrasomy combined, occasionally penta- and hexasomy) was more common than loss (mono- and nullisomy). Gain was highest for #10 (64.7%) and lowest for Y (16.3%). Loss was highest for Y (14%) and lowest for #7 (2.6%). In metastatic tumors more aberrations were detected than in nonmetastatic tumors. Five tumors did not show any abnormalities, while in 21 tumors one or more chromosomes showed simultaneous gain and loss. This resulted mostly in normal values for the ANC.

Chromosome 1

The results for chromosome 1 showed gain in 37.8%, but loss in less than 10% of the tumors (Figure 1). Two cases (PC202 and PC302) showed simultaneous loss and gain. In metastatic tumors gain was more frequent than in nonmetastatic tumors. Loss was found only in two metastatic tumors (PC295 and PC420). Polysomy was seen in all stage groups irrespective of long or short survival time of the patient (Table 1). Statistics did not reveal any correlation of numerical aberrations with increasing stage, metastatic disease or tumor site (Table 3).

Chromosome 7

Numerical aberrations of chromosome 7 were observed in 41% of the tumors. While only one tumor (PC320) showed loss, gain was seen in 38.8% of the tumors. 7 tumors showed simultaneous loss and gain. Gain of #7 was more frequent in metastatic tumors and a significant difference between the ANC's of nonmetastatic and metastatic tumors was found (Figure 1). However, high percentages of polysomy were observed in two patients that were >2 years disease free (PC290 and PC342, Table 1). The ANC's of radical prostatectomy specimens and lymph node metastasis (LM) specimens were significantly different (Table 3).

Chromosome 8

The copy number of chromosome 8 was shown to be aberrant in 51% of the tumors. Gain of #8 was more frequently found in metastatic tumors, while loss occurred more often in nonmetastatic tumors (Figure 1). 7/10 tumors with loss also showed gain. 4/5 LM showed high percentages of polysomy (PC244, PC269, PC270 and PC295, Table 1). All these patients died within 2 years. However, also in one specimen from a >2 years disease free patient (PC342) a high percentage of polysomy was found. High percentages of monosomy were observed in two lymph node positive T3 tumors (PC256 and PC352). Significant differences in ANC values were found between nonmetastatic and metastatic tumors (Figure 1), between radical prostatectomy and TUR specimens and between radical prostatectomy and LM specimens (Table 3).

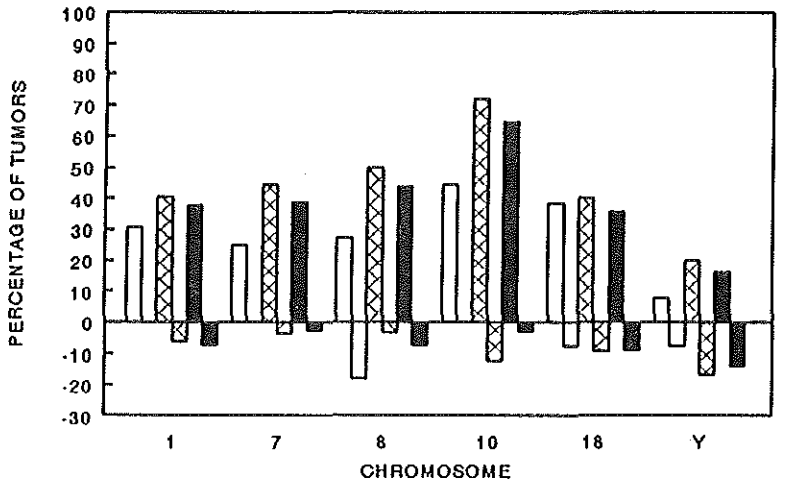


Figure 1. Percentages of gain and loss of chromosomes 1, 7, 8, 10, 18 and Y in respectively nonmetastatic, metastatic and all PC specimens. For chromosomes 1 and 18 all investigated tumor specimens could be evaluated, i.e. 15 nonmetastatic and 31 metastatic specimens. For chromosomes 7, 8, 10 and Y respectively 14, 14, 11 and 15 nonmetastatic and respectively 25, 29, 24 and 29 metastatic specimens were evaluable. Positive percentages (standing bars) represent gain, negative percentages (hanging bars) represent loss.

Chromosome 10

Gain of #10, the most frequently found numerical aberration (64.7% of the tumors) correlated with metastatic disease (Figure 1). Significant loss of #10 occurred in only one metastatic tumor (PC479). However, concurrent loss and gain of #10 was found in another ten tumors. ANC values tended to increase with stage ($p=0.05$), but showed no relation to the survival time. The ANC's of LM were significantly higher than those of radical prostatectomy specimens, but were not different from TUR values (Table 3).

Chromosome 18

Gain and loss of #18 were about equally frequent in nonmetastatic and in metastatic tumors (Figure 1). High polysomy percentages were present in all stage groups, the highest values being found in patients with short survival (e.g. PC269, PC270 and PC291, Table 1). Statistics did not reveal any correlation of numerical aberrations with tumor site (Table 3).

Y chromosome

Aberrations of Y, equally divided between loss and gain, did not correlate with any clinical parameter (Table 3), although the frequency of loss was higher in metastatic tumors (Figure 1). Only two cases showed simultaneous loss and gain (PC290 and PC332). The mean age of PC patients with a tumor that showed loss of Y was 65.4±13.2 (range 54-86 years, N=7). The mean age of patients without Y loss was 67.0±10.2 (range 49-93, N=36).

Table 3. Correlations of ANC values per chromosome with tumor site

CHROMOSOME	P-VALUES		
	RP/TUR *	RP/LM †	TUR/LM ‡
1	0.81	0.41	0.24
7	0.09	***0.04***	0.29
8	***0.03***	***0.04***	0.12
10	0.08	***0.03***	0.26
18	0.91	0.22	0.34
Y	0.48	0.54	0.39

Notes
Kruskal-Wallis test. P values in bold type indicate statistical significance. * radical prostatectomy versus TUR. † radical prostatectomy versus LM. ‡ TUR versus LM.

DNA-FCM compared with FISH results of PC patients with follow-up and staging data

Of the 24 specimens, investigated both by FISH analysis and DNA-FCM, 7 tumors were DNA diploid, 3 were tetraploid and 14 were aneuploid (Table 1). 6/7 DNA diploid tumors occurred in node negative patients, while the three tetraploid tumors were all from node positive patients. Aneuploid tumors were found in patients that were at least node positive or staged T3, except case 13 (T2N0).

Investigated with FISH, five tumors did not show any numerical aberration. Two of these tumors were diploid (PC341 and PC382) and two were aneuploid (PC343 and PC371). The fifth, PC400, was not evaluable by FCM. All other tumors showed numerical aberrations of one or more chromosomes. The average number of aberrant chromosomes in diploid tumors was 2.0±2.2 and in aneuploid and tetraploid tumors together was 4.7±2.3. Chromosome gain was most prominent in highly DNA aneuploid tumors (PC244, PC269, PC270, PC290, PC291, PC295, PC342 and PC354). Tumors with hypodiploid cell populations could show chromosome loss (PC295), or not (PC269). However, also tumors which did not show hypodiploid cells with FCM (e.g.PC202, PC256 and PC290), showed loss for more than one chromosome.

Cases with multiple specimens

PC256 and PC384 (case 4) showed a DNA histogram with about the same percentage of tetraploid cells (Figure 2). FISH showed numerical aberrations for all investigated chromosomes in both specimens, but loss of chromosome 7 and gain of chromosome 10 were more pronounced in the second sample PC384.

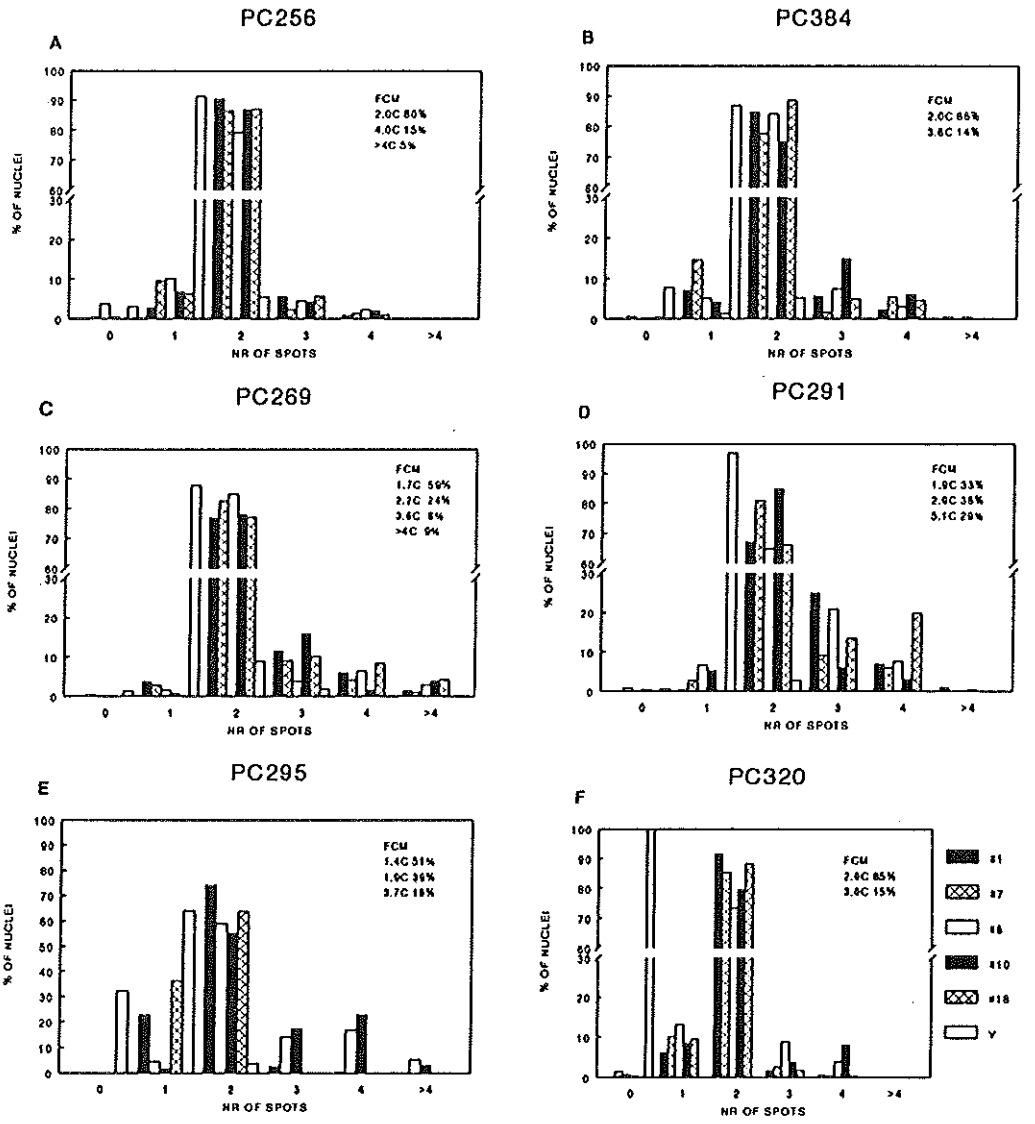


Figure 2. FISH and DNA-FCM results of cases with multiple specimens.
Case 4: a and b; case 5: c and d; case 10: e and f.

The two samples obtained from case 5 (PC269 and PC291) displayed a profound dissimilarity in FCM results, with different proportions of different DNA content (Figure 2). With FISH all investigated chromosomes showed numerical aberrations in both samples. While polysomy of chromosomes 1, 8 and especially 18 was far more extended in PC291, in PC269 chromosome 10 was more polysomic than in the second sample.

The two samples obtained from case 10 were also heterogeneous with respect to FCM results. In the TUR (PC320, sample contained 80% tumor cells), a triploid stemline (3.0C) was found, while in a sample from the xenografted cell line (PC295),

which consequently consisted exclusively of tumor cells, a hypotetraploid (3.7C) and a hypodiploid stemline (1.4C) were observed (Figure 2). The histograms of the FISH results showed however that these tumors were clearly related: both samples showed polysomy for chromosomes 8 and 10 and loss for more than one chromosome. Losses were more extensive in PC295 than in PC320, except for Y which was completely lost in PC320 and only partly lost in PC295.

DISCUSSION

In the BPH samples tested essentially no numerical aberrations were detected for the six chromosomes investigated. These results are in contrast with the report of Aly et al (20), who found loss of Y and gain of 7 in several cultured BPH specimens. Based on our findings we assumed that occasional BPH cells would not contribute to any chromosomal abnormalities found in PC specimens.

DNA-FCM studies on about half of the tumors showed that the frequency of DNA aneuploidy increased with increasing tumor stage. Combined FISH and DNA-FCM showed that, as expected, the majority of aneuploid tumors had numerical chromosomal aberrations. It was however also shown that most diploid tumors also had one or more numerically aberrant chromosomes. This can be explained by the fact that only chromosomal aberrations which comprise more than 4% of the total amount of DNA can be detected with DNA-FCM. No chromosome seemed to be specifically aberrant in DNA diploid tumors.

41/46 PC's showed numerical aberrations of one or more chromosomes of the six chromosome panel. All investigated chromosomes individually showed numerical aberrations in at least 30% of the specimens, and gain was more frequent than loss. Gain of #10 was the most frequent numerical aberration found (65%), chromosome 8 was the second most frequently gained chromosome (44%) and chromosomes 1, 7 and 18 showed gain with a frequency between 35 and 40%. The Y chromosome showed the lowest frequency of gain (16%), but the highest frequency of loss (14%).

Gain of chromosomes 7, 8 and 10 correlated with metastatic disease. Moreover, the ANC of #10 was significantly higher in advanced tumors. Gain of #10 in PC has been reported before (10), and deletions of part of 10q have been reported in cytogenetic (1, 21) and LOH studies (10, 22-24). However, the association of chromosome 10 aberrations with metastatic disease is a new finding.

In a previous study we were the first to suggest that 8p deletions are possibly important aberrations in PC (25). Since then, 8p deletions have been reported in several cytogenetic studies of PC's (1, 10, 26-28). Recently the importance of loss of #8p sequences in PC and the putative presence of at least two tumor suppressor genes on 8p has been established by several LOH studies (26-31). The deletions that were found by LOH mapping quite often spanned most of the 8p arm and sometimes involved the centromeric region. In the present study loss of #8 was seen primarily in nonmetastatic tumors, while in metastatic tumors gain was more prominent.

Deletions of 7q are amongst the first aberrations reported in PC (1). Gain of #7 has been found in cytogenetic (5, 21, 22, 32) and FISH studies (10, 11, 33, 34). In agreement with our findings, Bandyk et al.(11) observed that gain of #7 was significantly increased in PC metastases compared to primary tumors.

Loss of chromosomes 7, 8 and 10 was observed mostly simultaneously with gain in the same tumor samples, which is suggestive for heterogeneity in such tumors. In fact, chromosome 8 centromere gains and losses were shown to exist in different areas of the same tumors (5). Loss of (part of) a homolog followed by

multiplication of the other homolog is one possible mechanism through which these numerical aberrations could be generated. Another mechanism could be the formation of an isochromosome, followed by a nondisjunction event. This has been suggested as an explanation for the simultaneous loss of 8p and gain of 8q (27). In a recent comparative genome hybridization study of PC it was reported that loss of 8p and gain of 8q occur frequently, but that gain of (the whole) 7 and loss of 10q are infrequent observations (35).

Chromosome 18 likewise showed simultaneous loss and gain in most tumors, suggesting similar possible mechanisms as stated above. The percentage of numerical aberrations of #18 however, did not correlate with metastatic disease. Moreover, we have reported before that DNA diploid PC's, which mostly are early tumors, often already show gain of #18 (12). So numerical aberrations of #18 are likely to be an early event in PC. In fact, recently allelic losses of 18q were reported in 6/20 early stage PC's (36). It will be of interest to investigate if #18 aberrations are already present in the preneoplastic prostatic intraepithelial neoplasia (PIN) lesions.

Aberrations of both #1 and Y also did not correlate with metastatic disease. Furthermore, simultaneous loss and gain were only rarely observed. Previously we described (12) that gain of #1 and Y was largely restricted to DNA aneuploid tumors and this finding was confirmed in the present study. Y loss in the tumor did not correlate with the patients age. So alternative mechanisms, like multiplication through tetraploidization of the whole genome (37), or loss of Y as a reflection of a general state of hyperproliferation (38) are more likely explanations for the aberrations found for these chromosomes.

Comparison of two consecutive samples from the same patients revealed similar results with FCM in only 1/3 cases. However, FISH analysis of these specimens revealed clear evidence of karyotypic evolution towards a more aberrant karyotype.

The present report has substantiated the value of centromere FISH as a means to look at the ploidy of individual chromosomes in prostate cancer tissue. Numerical aberrations were found for all six investigated chromosomes and no doubt most other chromosomes, when eventually investigated, will also show numerical aberrations in at least part of the PC's. The most important finding was however, that combined with clinical data, gains of chromosomes 7, 8 and 10 were shown to be definitely involved in the progression of PC. This not only substantiates the postulated presence of tumor suppressor genes on 8p, but also justifies renewed interest in the commonly deleted regions on 7q and 10q (1).

Acknowledgements

This work was supported by grant IKR 90-12 from the Dutch Cancer Society.

LITERATURE

1. Sandberg, AA. Chromosomal abnormalities and related events in prostate cancer. Hum Pathol 1992;23:368-80.

2. König, JJ, Teubel, W, Dongen, JJW van, Hagemeijer, A, Romijn, JC, Schröder, FH. Tissue culture loss of aneuploid cells from carcinomas of the prostate. Genes Chrom Cancer 1993;8:22-7.

3. König, JJ, Dongen, JJW van, Schröder, FH. Preferential loss of abnormal prostate carcinoma cells by collagenase treatment. Cytometry 1993;14:805-10.

4. Breitkreuz, T, Romanakis, K, Lutz, S, et al. Genotypic characterization of prostatic carcinomas: a combined cytogenetic, flow cytometry, and in situ DNA hybridization study. Cancer Res 1993;53:4035-40.

5. Micale, MA, Sanford, JS, Powell, IJ, Sakr, WA, Wolman, SR. Defining the extent and

nature of cytogenetic events in prostatic adenocarcinoma: paraffin FISH vs. metaphase analysis. Cancer Genet Cytogenet 1993;69:7-12.
6. Brothman, AR, Watson, MJ, Zhu, XL, Williams, BJ, Rohr, LR. Evaluation of 20 archival prostate tumor specimens by fluorescence in situ hybridization (FISH). Cancer Genet Cytogenet 1994;75:40-4.
7. Baretton, GB, Valina, C, Vogt, T, Schnelderbanger, K, Diebold, J, Lohrs, U. Interphase cytogenetics of prostatic carcinomas by use of nonisotopic in situ hybridization. Cancer Res 1994;54:4472-80. 8. Zincke, H, Bergstralh, EJ, Larson-Keller, JJ et al. Stage D1 prostate cancer treated by radical prostatectomy and adjuvant hormonal treatment. Evidence for favorable survival in patients with DNA diploid tumors. Cancer 1992;70:312-23.
9. Lundgren, R, Heim, S, Mandahl, N, Anderson, H, Mitelman, F. Chromosome abnormalities are associated with unfavorable outcome in prostate cancer patients. J Urol 1992;147:784-8.
10. Macoska, JA, Micale, MA, Sakr, WA, Benson, PD, Wolman, SR. Extensive genetic alterations in prostate cancer revealed by dual PCR and FISH analysis. Genes Chromos Cancer 1993;8:88-97.
11. Bandyk, MG, Zhao, L, Troncoso, P et al. Trisomy 7: A potential cytogenetic marker of human prostate cancer progression. Genes Chromos Cancer 1994;9:19-27.
12. König, JJ, Teubel, W, Dongen JJW van, Romijn, JC, Hagemeijer, A, Schröder, FH. Loss and gain of chromosomes 1, 18 and Y in prostate cancer. Prostate 1994;25:281-91.
13. Schröder, FH, Hermanek, P, Denis, L, Fair, WR, Gospodarowicz, MK, Pavone-Macaluso, M. The TNM classification of prostate cancer. Prostate Suppl 1992;4:129-138.
14. Cooke, HJ, Hindley, J. Cloning of human satellite III DNA: different components are on different chromosomes. Nucl Acids Res 1979;6:3177-97.
15. Waye, S, England, SB, Willard, HF. Genomic organization of " satellite DNA on human chromosome 7 : evidence for two distinct alphoid domains on a single chromosome. Mol Cell Biol 1987;7:349-56.
16. Donlon, T, Wyman, AR, Mulholland, J, Barker, D, Bruns, G, Latt, S, Botstein, D. " Satellite-like sequences at the centromeres of chromosome #8. Am J Hum Genet 1986;39: A196.
17. Devilee, P, Kievits, T, Waye, JS, Pearson, PL, Willard, HF. Chromosome-specific " satellite DNA : isolation and mapping of a polymorphic alphoid repeat from human chromosome 10. Genomics 1988;3:1-7.
18. Devilee, P, Cremer, T, Slagboom, P et al. Two subsets of human alphoid repetitive DNA show distinct preferential localization in the pericentric regions of chromosomes 13, 18 and 21. Cytogenet Cell Genet 1986;41:193-201.
19.Hopman, AHN, Ramaekers, FCS, Raap, AK, Beck, JLM, Devilee, P, Ploeg, M van der, Vooijs, GP. In situ hybridization as a tool to study numerical chromosome aberrations in solid tumors. Histochemistry 1988;89:307-16.
20. Aly, M, Dal Cin, P, Voorde, W van de, Poppel, H van, Ameye, F, Baert, L, Berghe, H van den. Chromosome abnormalities in benign prostatic hyperplasia. Genes Chromos Cancer 1994;9:227-33.
21. Arps, S, Rodewald, A, Schmalenberger, B, Carl, P, Bressel, M, Kastendieck, H. Cytogenetic survey of 32 cancers of the prostate. Cancer Genet Cytogenet 1993;66:93-9.
22. Bergerheim, USR, Kunimi, K, Collins, VP, Ekman, P. Deletion mapping of chromosomes 8, 10, and 16 in human prostatic carcinoma. Genes Chrom Cancer 1991;3:215-20.
23. Carter, BS, Ewing, CM, Ward, WS et al. Allelic loss of chromosomes 16q and 10q in human prostate cancer. Proc Natl Acad Sci 1990;87:8751-8755.
24. Kunimi, K, Bergerheim, USR, Larsson, IL, Ekman, P, Collins, VP. Allelotyping of human prostatic adenocarcinoma. Genomics 1991;11:530-6.
25. König, JJ, Kamst, E, Hagemeijer, A, Romijn, JC, Horoszewicz, J, Schröder, FH. Cytogenetic characterization of several androgen responsive and unresponsive sublines of the human prostatic carcinoma cell line LNCaP. Urol Res 1989;17:79-86.
26. Macoska, JA, Trybus, TM, Sakr, WA, Wolf, MC, Benson, PD, Powell, IJ, Pontes, JE. Fluorescence in situ hybridization analysis of 8p allelic loss and chromosome 8 instability in human prostate cancer. Cancer Res 1994;54:3824-30.
27. Cher, ML, MacGrogan, D, Bookstein, R, Brown, JA, Jenkins, RB, Jensen, RH. Comparative genomic hybridization, allelic imbalance, and fluorescence in situ hybridization on chromosome 8 in prostate cancer. Genes Chromos Cancer 1994;11:153-62.
28. Matsuyama, H, Pan, Y, Skoog, L et al. Deletion mapping of chromosome 8p in prostate cancer by fluorescence in situ hybridization. Oncogene 1994;9:3071-6.

29. Bova, GS, Carter, BS, Bussemakers, MJG et al. Homozygous deletion and frequent allelic loss of chromosome 8p22 loci in human prostate cancer. Cancer Res 1993;53:3869-73.
30. MacGrogan, D, Levy, A, Bostwick, D, Wagner, M, Wells, D, Bookstein, R. Loss of chromosome arm 8p loci in prostate cancer : Mapping by quantitative allelic imbalance. Genes Chromos Cancer 1994;10:151-9.
31. Trapman, J, Sleddens, HFBM, Weiden, MM van der et al. Loss of heterozygosity of chromosome 8 microsatellite loci implicates a candidate tumor suppressor gene between the loci D8S87 and D8S133 in human prostate cancer. Cancer Res 1994;54:6061-4.
32. Micale, MA, Mohamed, A, Sakr, W, Powell, IJ, Wolman, SR. Cytogenetics of primary prostatic adenocarcinoma. Clonality and chromosome instability. Cancer Genet Cytogenet 1992;61:165-173.
33. Jones, E, Zhu, XL, Rohr, LR, Stephenson, RA, Brothman, AR. Aneusomy of chromosome 7 and 17 detected by FISH in prostate cancer and the effects of selection in vitro. Genes Chromos Cancer 1994;11:163-70.
34. Alcaraz, A, Takahashi, S, Brown, JA et al. Aneuploidy and aneusomy of chromosome 7 detected by fluorescence in situ hybridization are markers of poor prognosis in prostate cancer. Cancer Res 1994;54:3998-4002.
35. Visakorpi, T, Kallioniemi, A, Syvanen, A et al. Genetic changes in primary and recurrent prostate cancer by comparative genomic hybridization. Cancer Res 1995;55:342-7.
36. Latil, A, Baron, J, Cussenot, O et al. Genetic alterations in localized prostate cancer : Identification of a common region of deletion on chromosome arm 18q. Genes Chromos Cancer 1994;11:119-25.
37. Shackney, SE, Smith, CA, Miller, BW et al. Model for the genetic evolution of human solid tumors. Cancer Res 1989;49:3344-54.
38. Casalone, R, Portentoso, P, Granata, P et al. Chromosome changes in benign prostatic hyperplasia and their significance in the origin of prostatic carcinoma. Cancer Genet Cytogenet 1993;68:126-30.

Appendix I

Average number of copies of investigated chromosomes per tumor sample.

I.Non-metastatic tumors

PC NR	ANC #1	ANC #7	ANC #8	ANC #10	ANC #18	ANC Y
256†	2.05	1.95	1.92*	2.01*	2.02*	1.02
285	*2.08*	2.01	NE	NE	*2.11*	1.06
288	*2.11*	2.00	1.95*	*2.17*	1.99	*0.94*
290	*2.49*	*2.46*	*2.31**	*2.16**	*2.26*	1.02*
341‡	1.96	2.02	1.99	1.99	1.97	1.01
342	*2.32*	*2.38*	*2.51*	*2.12*	*2.17*	*1.22*
343‡	1.93	1.99	1.97	2.00	1.98	0.97
352	1.97	2.01	*1.90*	NE	1.96	1.01
354	2.03	*2.14*	*2.09*	*2.11*	*2.35*	1.02
362	*2.29*	2.02	*2.11*	2.05	2.03*	1.06
382	2.01	2.05	1.95	2.00	2.01	1.03
389	2.05	2.02*	2.00	*2.09**	*2.12*	1.08
395	1.95	1.93	2.03	1.97	*1.94*	*0.91*
432	1.96	1.98	*1.79*	NE	1.99	1.01
435‡	2.04	NE	2.02	NE	2.04	1.09

Appendix I, continued. II.Metastatic tumors

PC NR	ANC #1	ANC #7	ANC #8	ANC #10	ANC #18	ANC Y
202	2.03*	2.07*	1.98*	1.95*	***2.08***	0.97
236	2.04	2.06	2.02	***2.12***	1.99	1.01
244	2.03	***2.37***	***2.31***	***2.34***	***2.35***	0.98
262	2.06	2.08	NE	2.02*	1.97	NE
269†	***2.24***	***2.17***	***2.24***	***2.30***	***2.40***	1.11
270	***2.30***	***2.28***	***2.80***	***2.44***	***2.36***	***1.26***
289	***2.28***	***2.12***	1.91	2.03	***2.29***	1.06
291†	***2.42***	***2.17***	***2.30***	***2.08****	***2.53***	1.02
295†	***1.80***	NE	***2.60***	***2.71***	***1.64***	***0.72***
296	2.01	2.06	***2.11***	NE	2.02*	1.05
302	2.06*	2.03*	***2.11***	***2.21****	***2.17***	0.99
320†	1.97	***1.90***	2.04*	***2.11****	***1.93***	***0***
324	2.04	2.08	2.03	***2.11***	***1.94***	***0.21***
332	2.07	NE	1.99*	NE	1.98	1.05*
351	***2.20***	***2.52***	***2.34***	***2.51***	***2.12***	1.11
353	***2.09***	***2.28***	NE	***2.36***	1.99*	NE
355	***2.37***	***2.09****	***2.12****	***2.10***	***2.29***	1.03
366	***2.28***	***2.35***	***2.13***	***2.23***	***2.14****	***0.79***
371‡	2.00	2.04	1.99	2.03	2.00	0.98
384†	***2.16***	1.97*	***2.13***	***2.23***	***2.12***	0.97
392	2.00	2.07	2.00*	NE	2.01	1.01
400‡	1.94	2.06	1.95	NE	2.01	1.02
403	1.96	2.01*	2.07	2.03*	1.98	1.08
405	2.04	NE	***2.09***	NE	1.98	***1.15***
417	1.95	NE	***2.17***	NE	2.05	1.02
418	2.03	***2.48***	***2.27***	***2.21***	***2.49***	1.03
420	***1.90***	NE	2.06	***2.14****	1.98	1.00
449	***2.18***	***2.21***	***2.09***	NE	2.03*	***1.27***
461	2.01	NE	2.03	***2.06***	2.01	***0.92***
465	***2.26***	***2.18***	***2.33***	***2.14***	***2.18****	***1.23***
479	1.96	1.98*	***1.89***	***1.85***	***1.90***	1.00

Notes
NE= not evaluable. Bold figures: significant gain or loss. * : simultaneous significant gain and loss. † specimen from double sampled patient. ‡ specimen without chromosomal aberrations

CHAPTER 7.1

CYTOGENETIC CHARACTERIZATION OF SEVERAL ANDROGEN RESPONSIVE AND UNRESPONSIVE SUBLINES OF THE HUMAN PROSTATIC CARCINOMA CELL LINE LNCAP

Josée J. König, Eric Kamst, Anne Hagemeijer, Johannes C. Romijn, Julius Horoszewicz and Fritz H. Schröder

Published in Urological Research 17: 79-86, 1989

ABSTRACT

The cytogenetic evolution of the prostatic adenocarcinoma cell line LNCaP was investigated during long term in vitro culture. Study of five different sublines demonstrated that the original karyotype was well preserved in all sublines, with respect to the chromosome number as well as to the primary markers. All sublines showed additional, subline specific secondary marker chromosomes. Comparison of these markers in androgen responsive and nonresponsive sublines showed rearrangement of the short arm of chromosome 8 in both nonresponsive sublines. The breakpoints were in 8p21 and 8p23, respectively, resulting in deletion of the 8p23 pter region in both sublines. In contrast, the hormone responsive sublines did not show any aberrations in chromosome 8. Review of published karyotypes of patients and cell lines seems to support our finding of partial deletion of 8p in androgen nonresponsive prostate tumor cells.

INTRODUCTION

The study of chromosome abnormalities in cancer, first in leukemia and in the last decade in an increasing number of solid tumors, has revealed that chromosome aberrations can be important to diagnosis as well as to the prognosis of a number of human tumors. In many tumors specific chromosomal aberrations have been identified which seem to be unique to that tumor [15]. The cytogenetics of urogenital solid tumors has advanced greatly through the improvement in cytogenetic techniques. This has resulted in the definition of specific chromosomal rearrangements in bladder [9], kidney [14] and testis tumors [11]. In prostatic cancer however, cytogenetic reports about patient material are still anecdotal [1, 2, 10] and so far there is only preliminary evidence concerning the involvement of the long arm of chromosome 10 in PC [1, 2, 8, 10, 21]. In the present study we investigated the evolution of chromosomal abnormalities in the PC cell line LNCaP [8, 16]. During long term in vitro culture several sublines were derived from the parental cell line. These sublines arose at different times and sometimes under specific selective conditions [13, 16]. They differed in their response to the addition or depletion of androgens in the culture medium [33]. This study investigated any cytogenetic characteristics which might distinguish between the androgen responsive and nonresponsive cell lines.

MATERIALS AND METHODS

Cell lines

The LNCaP (Lymph Node Carcinoma of the Prostate) – FGC (Fast Growing Colony) cell line as well as LNO (Lymph Node Original) and LNO-DHT (grown in the presence of dihydrotestosterone) were established at the Roswell Park Memorial Institute [16]. LNO was established at an early passage number of LNCaP by growing cells continuously in medium depleted of androgens. The scheme in Fig. 1 shows the relationship between the various sublines used in this study. FGC in this scheme is the tetraploid line as described by Gibas et al. [8]. The suffix "GJ" was chosen to discriminate in this diagram between the original FGC and the cell line presently in culture at our Institute. In the rest of this paper it will be referred to only as FGC. LNCaP-EM is identical to FGC at a very early passage number. LNCaP-R (Resistant) [13] spontaneously arose from LNCaP-FGC and was kindly provided by Dr. Hasenson (Karolinska Institute, Sweden). LNCaP-JB arose spontaneously also from LNCaP-FGC at the Biochemical Department of the University of Rotterdam.

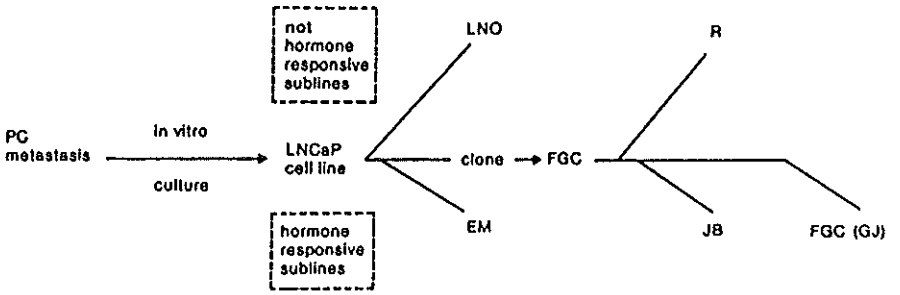


Fig.1. Schematic presentation of the derivation of sublines of the LNCaP cell line.

Cell culture and cytogenetic methods

All cell lines were cultured in their respective optimal medium according to a culturing schedule, adapted to each individual cell line. FGC, JB, EM and R were routinely grown in RPMI with 10% fetal calf serum (FCS) plus 2 mM glutamine and antibiotics. LNO was grown in the same medium with 5% "stripped" FCS. Serum was stripped from steroids by treating it with dextran coated charcoal.

After about three passages the cells were grown to half confluency. Then fresh medium at room temperature was added. After ±28 h the cells were incubated with colcemid (0.04 Fg/ml) for 1 h, subsequently incubated with KC1/EGTA/HEPES solution [36] for 20 min and fixed in a standard way. Chromosomes were stained for R-, Q- and G-bands. Chromosome nomenclature was according to the ISCN of 1985 [18].

Table 1. Growth characteristics of LNCaP sublines

SUBLINE	IN VITRO PROLIFERATION	
	WITHOUT ANDROGENS[a]	WITH ANDROGENS[b]
FGC	-	++
JB	-	++
EM	-	ND
LNO	+	+
R	+	+

Notes

[a] culture medium with "stripped" serum.

[b] culture medium with "stripped" serum, supplemented with the optimal concentration of androgen [33].

-= no growth; += normal growth; ++= fast growth.

RESULTS

In Table 1 the in vitro growth characteristics of all karyotyped sublines on media with and without androgens are summarized. The original cell line FGC as well as the sublines JB and EM remained responsive to androgens during long term tissue culture. This has been tested regularly. In contrast, LNO and the R subline both grew without androgens and did not respond to the addition of androgens. Detailed

characteristics and results of hormonal manipulation of LNCaP sublines are described in the paper by van Steenbrugge et al. [33]. In Fig. 2 the chromosome distribution of all sublines is presented. All sublines were nearly tetraploid, with most cells having a chromosome number of around 90, with the exception of LNO, which had a median of 83 chromosomes. EM had the most narrow chromosome distribution, followed by JB and FGC. The R line was shown to have a broad distribution from 82 to 94 chromosomes and in LNO some near-octoploid cells were present. The triploid cells in FGC as well as in LNO were all shown to be incomplete and thus represented no real subpo- pulations.

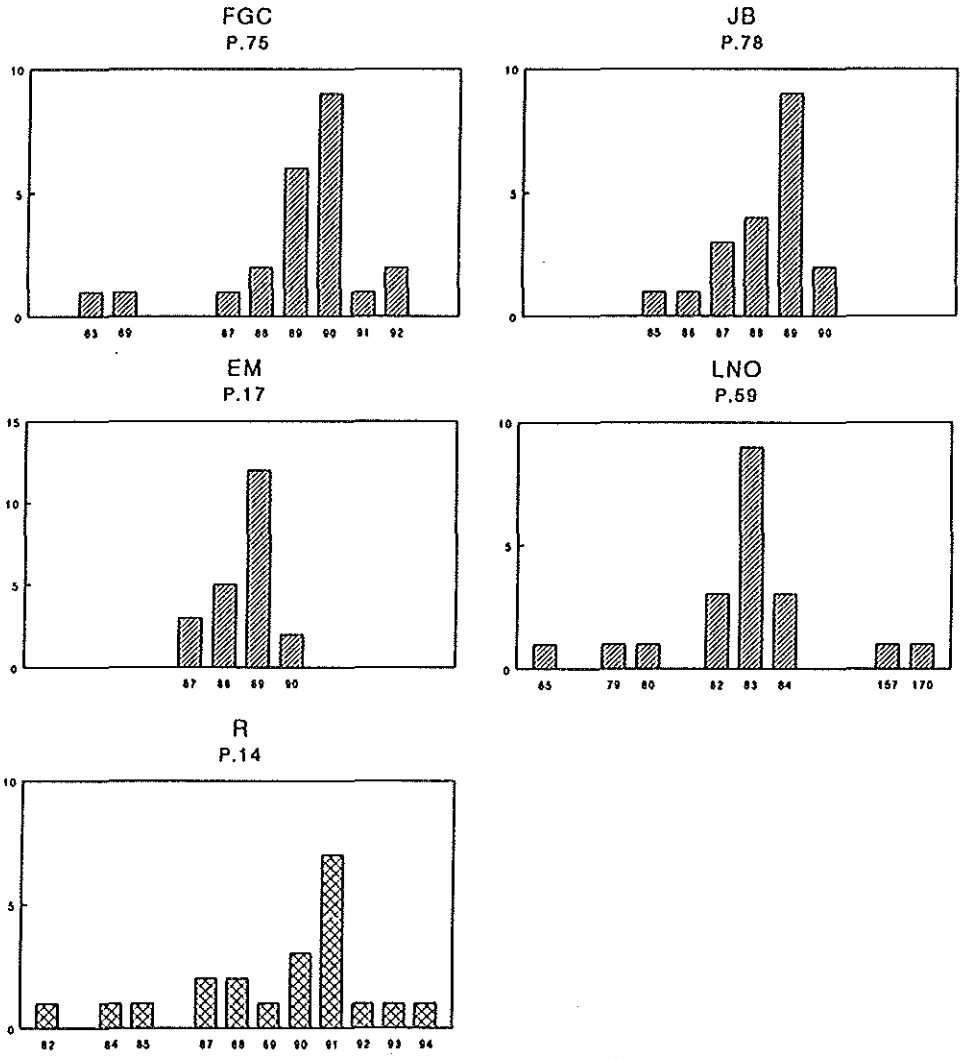


Fig.2. Chromosome distribution in LNCaP sublines at the passage number indicated. Horizontal axis= number of chromosomes; vertical axis= number of cells.

All sublines had the same basic karyotype in common. This was essentially the karyotype as reported by Gibas et al. [8] with the addition of two markers that had arisen from a probable reciprocal translocation between chromosomes 4 and 6 (see Table 3). Since these markers could only be clearly distinguished by R- banding (see Fig. 5) it was possible that they were already present in the karyotype of the LNCaP cell line. Figure 3 shows a representative G-banded karyotype of JB. This subline has the basic karyotype of near-tetraploidy and nine consistent markers (M 1 to M9). In most common markers we identified similar breakpoints as Gibas et al. have described [8], with the exception of M3, M6 and M7 (see Table 3). High resolution banding analysis of JB showed the (6: 16) translocation to be reciprocal, with breakpoints at 6p21 and 16q22 in M3 and M6, respectively (see Fig. 5). We reconsidered the interstitial deletion in M7 and assigned the breakpoints to bands 13q32.3 and 13q22.2, which resulted in a larger homozygous region in chromosome 13 (see Table 3 and Fig. 5). Markers MA2, MA3, MB1, MC1, and MD1 were specific for the JB subline. Table 2 presents a summary of the loss and gain of normal chromosomes and common markers in all sublines. The absence of M7 (13q-) in a third (FCG, JB), nearly half (LNO, R) or all cells karyotyped (as in EM) as well as the fact that this marker is usually present in only one copy, strongly suggests that the 13q- is a secondary marker and arose only after tetraploidization in vitro had taken place. M 1 (1p-) on the contrary was present in three copies in most cells of FGC, EM and R. In LNO a new marker chromosome (MA4) was formed from one copy of M I. No other markers were present in more than two copies. Partial or complete loss of one copy of a marker or a normal chromosome was mostly cell line specific. Examples of this are: loss of one homologue of #9 in FGC, loss of one normal chromosome 4 as well as one #17 in EM, loss of one copy of M9 (6q+) in LNO and also loss of one #15 and one #17 in this subline. In the R line one copy of #19 was lost in nearly all cells as well as one copy of each of the G group chromosomes 21 and 22. Other chromosome losses did not occur in more than 40% of all cells.

In several cases loss of a normal chromosome or a marker was associated with the appearance of a new unique marker chromosome. These markers can be found in Table 3, which lists the most probable construction of the marker chromosomes that were specific for each subline. These new marker chromosomes resulted from nine non-reciprocal translocations, six deletions and two duplications. Two isochromosomes were formed. All new markers were present in one copy, except for MA7 (2q-) from the R line. One copy of #2 and two copies of M2 (2p-) were present, so MA7 originated probably from a normal chromosome 2 and was duplicated independently from tetraploidization of the total genome. Figure 4 shows a G-banded pre- sentation of all specific markers present in over 50% of all cells from each subline. Marker ME1 (16q+) is represented with R- and Q bands as well, to show the attachment of the Y chromosome to the distal part of 16q.

Table 2. Gains and losses of normal chromosomes and common markers in LNCaP sublines

LNCAP KARYOTYPE		SUBLINE KARYOTYPES				
CHROMO-SOME	COPY NR	FGC	JB	EM	LNO	R
1	1	1 (45; MA1)	-	-	-	1 (75; MA6)
M1 (1p-)	2	3 (90)	-	3 (95)	1 (95; MA4)	3 (70)
2	2	-	1 (100;MA2)	-	1 (25)	1 (95; MA7)
M2 (2p-)	2	1 (20)	-	-	-	1 (60)
3	4	-	3 (35; MA3)	-	3 (95; MA5)	-
4	2	-	-	1 (100)	-	-
M8 (4q+)	2	-	-	-	-	-
5	4	-	-	-	-	-
6	0	-	-	-	-	-
M3 (6p-)	2	-	-	-	1 (20)	-
M9 (6q+)	2	1 (25)	-	-	1 (95)	1 (25)
7	4	-	3 (50)	-	3 (40)	-
8	4	-	-	-	3 (40; MC3)	3 (75; MC4)
9	4	3 (95)	-	-	-	3 (40)
10	2	-	-	1 (100;MC2)	-	-
M4 (10q-)	2	-	-	-	-	-
11	4	-	-	-	-	-
12	4	-	3 (100; MC1)	-	-	3 (95; MC5)
13	2	-	-	-	1 (35; M7)	3 (20; M7) 4 (20; M7)
M7 (13q-)	1	0 (25)	0 (25; #13)	0 (100)	0 (55; #13)	0 (50; #13) 2 (45)
14	4	-	3 (20)	-	-	-
15	2	-	1 (90; MD1)	-	1 (80)	1 (70; MA8)
M5 (15q+)	2	-	-	-	-	1 (75)
16	2	-	1 (35; MB1)	-	0 (100;ME1)	-
M6 (16q+)	2	-	-	-	1 (20)	1 (70; MB2)
17	4	-	-	3 (100)	3 (75)	3 (25)
18	4	-	- (25)	-	3 (30)	3 (25)
19	4	-	-	-	-	3 (80)
20	4	-	-	3 (100;MF1)	3 (20)	-
21	4	3 (20)	3 (45)	-	3 (70) 2 (20)	3 (85)
22	4	-	-	-	3 (35)	3 (75)
X	2	-	-	-	-	-
Y	2	1(35)	1 (30)	-	1 (80; ME1) 0 (20)	1 (20)

Notes

The aberrant copy number is given with, in brackets, the percentage of cells in which it was observed (>20%), together with the denomination of the new marker eventually formed.

[a]marker numbering and usual copy number is according to Gibas et al. [8]; -= no deviation.

When we compared the nonresponsive sublines LNO and R with the hormone responsive lines FGC, JB and EM we found that with respect to numerical variations loss of chromosome 19 only occurred in LNO and R and not in the other three sublines. Loss of other normal and marker chromosomes was either cell line specific as mentioned above or evently distributed over all sublines. A comparison of the new subline specific markers resulted in the identification of 8p- (MC4) in LNO and 8p+ (MC5) in R that were not present in the other three sublines. As a result of these 8p rearrangements in LNO and R the 8p236pter region was in both cell lines lost in one copy of #8.

Table 3. Possible identification of subline-specific marker chromosomes

SUBLINE	MARKER	SHORT DESIGNATION	PERCENTAGE	DETAILED STRUCTURAL DESIGNATION
FGC	MA1	1p+	45	dir dup(1)(p33→pter)
JB	MA2	2q-	100	del(2)(q31→qter)
	MA3	3p+	35	dup(3)(p23→p25)
	MC1	16q+	45	16pter→16q22::?
	MC2	12q-	90	del(12)(q22→q24.1)
	MD1	15q+	80	15pter→15q24::?
EM	MC3	10q+	100	10pter→10q24?::12q22→12qter
	MF1	20p+	100	20qter→20p12::17p12→17pter
LNO	MA4[a]	1p+	90	1qter→1q21::15q26→15q24::1p22→1qter
	MA5	i(3q)	95	i(3q)
	MC4	8p-	40	del(8)(p21→pter)
	ME1	16q+	85	16pter→16q24::Yp11→Yqter
R	MA6	1q-	45	del(1)(q32→q42)
	MA7	2q-	95	del(2)(q32→q35)
	MA8	15p+q+	70	1pter→1p22::15q24→15p12::15q22→15q24::1p22→1pter
	MB1	16q+	70	16pter→16q13::?::2q12→2qter
	MC5	8p+	70	8qter→8p23::8q13→8qter
	MC6	12q-	95	del(12)(q22→qter)
	MC7	16q+	70	16pter→16q13::?::16q21→16qter
	MF2	i(21q)	70	i(21q)
COMMON MARKERS	M3	6p-	100	6qter→6p21.1::16q22→16qter
	M6	16q+	100	16pter→16q22::6p21.1→6pter
	M7	13q-	100	del(13)(q21.3→q22.2)
	M8	4q+	100	4pter→4q25::6q15→6qter
	M9	6q+	100	6pter→6q15::4q25→4qter

Notes
All markers were present in one copy, with the exception of M8, M9 and MA7. These were present in two copies.
[a] MA4 was derived from M1: der(1)t(1;15)(p22.3;q24).

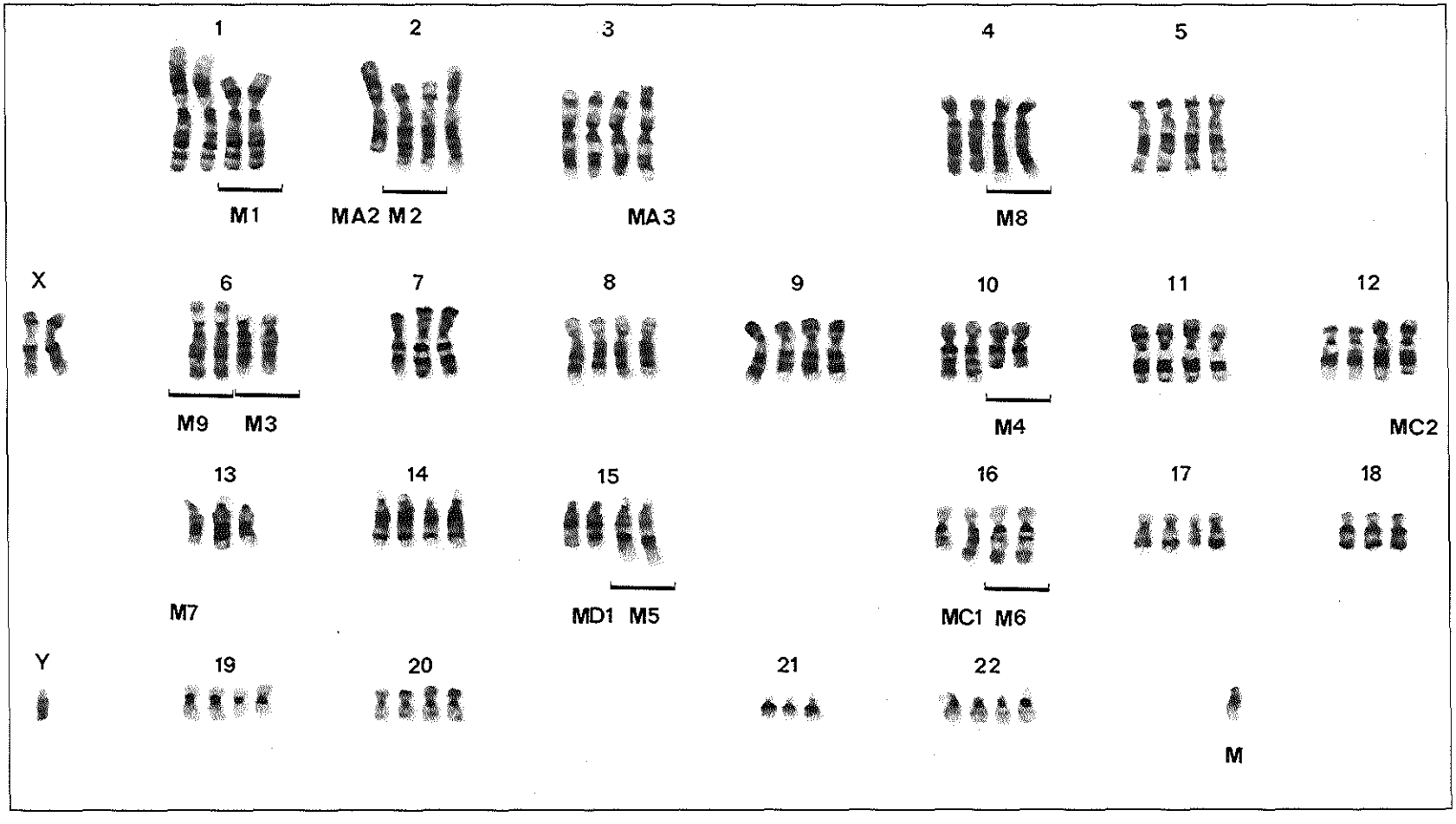


Fig.3. Representative G-banded karyotype of LNCaP FGC-JB.

Fig.5. G- and R-banded presentation of M3, M6 and M7. Arrows point to the breakpoints in the normal chromosomes.

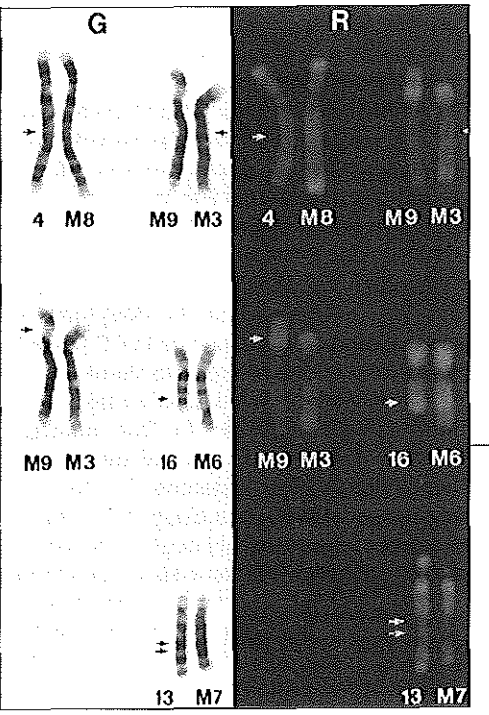
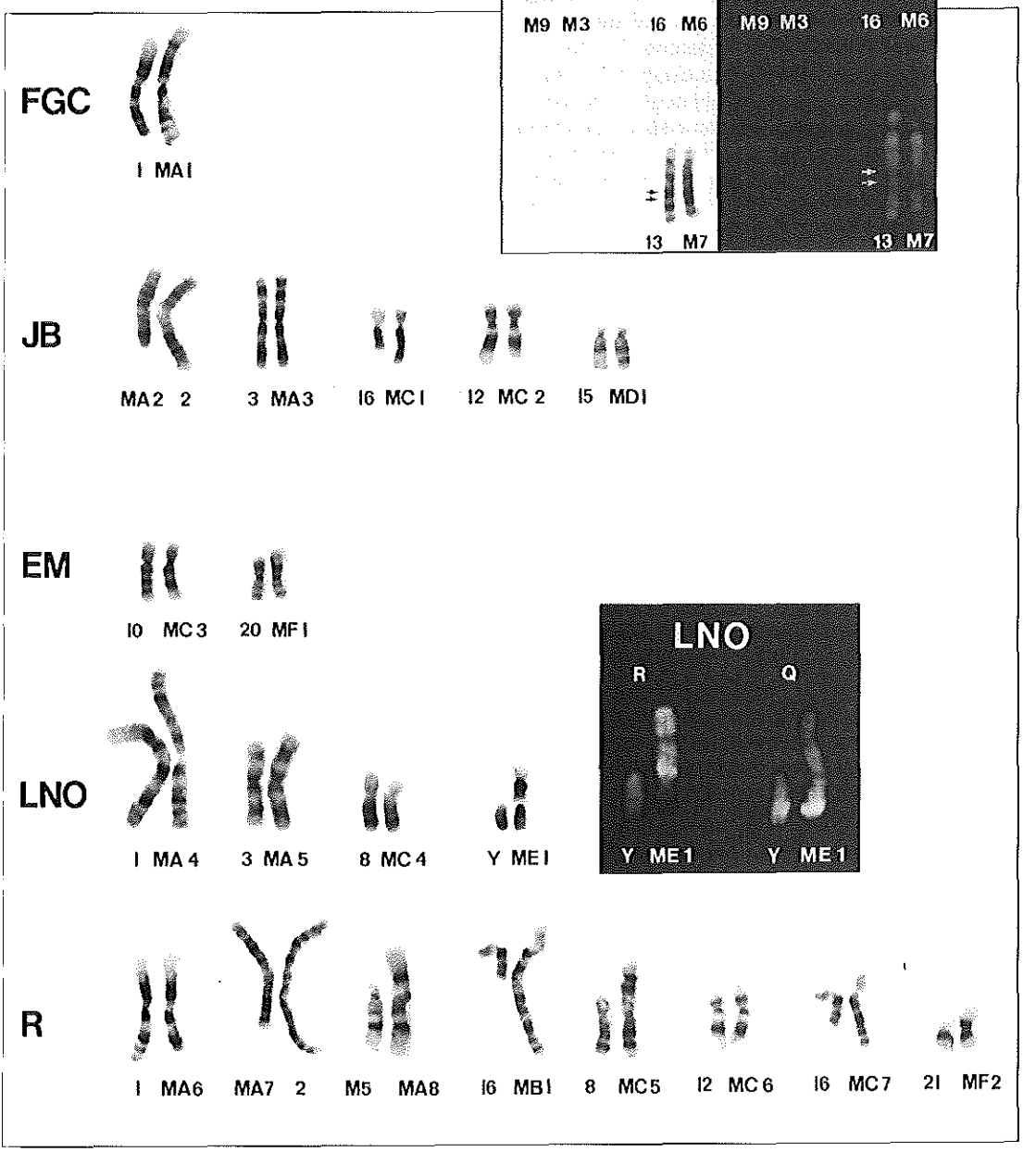


Fig.4. G-, R- and Q-banded new marker chromosomes.



DISCUSSION

In this cytogenetic investigation of several sublines derived from the PC cell line LNCaP we found that the basic karyotype of the parental cell line was well preserved. In other words, all sublines showed the same karyotypic evolutionary pattern, e.g. a doubling of the stem line to a near-tetraploid cell population, preservation of the original markers (most of them present in two copies) and generation of some new, subline specific marker chromosomes (present in one copy). This same pattern has been observed in malignant gliomas [3] as well as in an ovarian carcinoma cell line [22]. Involvement of 10q (M4) has previously been indicated to be a possible specific marker chromosome in PC [2]. In the 10q246qter region genes are located that code for proteins linked to steroid metabolism (the multigene family Cytochrome P-450 PB-1) [6, 24] as well as to malignant growth in general (plasminogen activator urokinase (PLAU)) [29, 30]. One member of the Cytochrome P-450 family has a function in the oxidation and degradation of steroid hormones, e.g. the 16a-hydroxylation of testosterone [6]. Moreover, it has been indicated that Cyt P-450 activities are linked to carcinogen activation. The exact role of plasminogen activators in cancer is unclear, although it has been established that these enzymes play a role in pericellular proteolysis and invasive neoplastic growth [30]. Deletion of the 10q246qter region is not restricted to prostatic malignancies. Recently the same region was indicated to be important in the early stages of melanocytic neoplasia [28] and it was postulated that an oncogene, or more likely a TSG was possibly located in this region.

The aim of the present study was to compare the karyotypes of the androgen responsive with the nonresponsive sublines. An acquired deletion of the distal part of the short arm of chromosome 8 was the only specific aberration which the nonresponsive sublines LNO and R had in common and which at the same time was not present in the responsive sublines FGC, EM and JB. In the near-tetraploid cells of LNO and R, three homologues of #8 remained that seemed unaffected. However, a mutation that was undetectable by cytogenetic methods remains a distinct possibility in this case. In general the establishment of monosomy is sufficient for a cell to behave differently from the parental cell when a recessive feature is unmasked by deletion of the dominant feature [5, 23]. We reviewed the karyotype of five hormone nonresponsive PC cell lines, e.g. the xenografted cell lines PC133 and PC135 [20, 32] and the tissue culture cell lines PC93 [7], DU145 [34] and PC-3 [19]. We found that in all these cell lines the 8p216pter region was rearranged or deleted in at least one copy of chromosome 8 (unpublished results). In the literature examples of similar #8p aberrations were found in nonresponsive cell lines [17, 27] as well as in patient material [10]. In contrast, there was no cytogenetic evidence that this same region was ever affected in hormone responsive PC material [2 (case 1), 8, 21]. At present no aberrations in the #8p region are known that are considered to be specific for a certain type of tumor and generally rearrangements in this region are rare [4, 25, 26]. In one report, formation of isochromosomes of the long arm of #8 or deletion of #8p is implicated as a secondary event in transitional cell carcinoma of the urinary bladder [9]. It is very speculative however at this point to assume a similar karyotypic evolution for different urogenital tumors.

Few genes have been mapped in the 8p region. It is striking however, that the gene for tissue type plasminogen activator (PLAT) maps relatively close to the deleted region at #8p12 [37], while PLAU maps in the 10q region that has become homozygous in these PC cell lines. Relevant genes that are located in the deleted region of chromosome 8 are the luteinizing hormone releasing hormone (LHRH) precursor gene [38] and the gene for the enzyme glutathione reductase (GSR) [12]. LHRH has an important function in androgen metabolism, but is normally not

synthetized in the prostate. No data are available at present that this might be the case in PC. GSR reduces glutathionedisulfide to glutathione, which is a substrate for glutathione S-transferase (GST), an important enzyme in detoxication processes. GST is a catalyst of steroid isomerisation, it binds steroid hormones [35] and it is present in high quantities in many tumors [31].

In conclusion, this comparative cytogenetic study of five LNCaP sublines demonstrated a good conservation of most original markers, in particular of the 10q marker that has been proposed as a specific prostate associated aberration. In addition to this, a deletion of the 8p216pter region tends to be present in all the hormone unresponsive PC cell lines investigated by us, and reported by others. This finding is new, the implications of which should be confirmed and investigated further.

Acknowledgements

This study was supported by grant no. IKR 86-14 of the Netherlands Cancer Foundation.

LITERATURE

1. Ahmann FR, Woo L, Hendrix M, Trent JM. Growth in semisolid agar of prostate cancer cells obtained from bone marrow aspirates. Cancer Res 46:3560-3564, 1986.
2. Atkin NB, Baker MC. Chromosome study of five cancers of the prostate. Hum Genet 70:359-364, 1985.
3. Bigner SH, Mark J, Bigner DD. Chromosomal progression of malignant human gliomas from biopsy to establishment as permanent lines in vitro. Cancer Genet Cytogenet 24:163-176, 1987.
4. Camuto PM, Greco MA, Persky M, Wolman SR. Abnormality of 8p in a benign salivary gland tunor. Cancer Genet Cytogenet 31:301-303, 1988.
5. Cavenee WK, Dryja TP, Phillips RA, Benedict WF, Godbout R, Gallie BL, Murphee AL, Strong LC, White RL. Expression of recessive alleles by chromosomal mechanisms in retinoblastoma. Nature 305:779-784,1983
6. Cheng KC, Schenkman JB. Testosterone metabolism by cytochrome P-450 isozymes RLM3 and RLM5 and by microsomes. J Biol Chem 258:11738-11744, 1983.
7. Claas FHJ, Steenbrugge GJ van. Expression of HLA-like structures on a permanent human tumor line PC-93. Tissue Antigens 21:227-232, 1983.
8. Gibas Z, Becher R, Kawinski E, Horoszewicz J, Sandberg AA. A high-resolution study of chromosome changes in a human prostatic carcinoma cell line (LNCaP). Cancer Genet Cytogenet 11:399-404, 1984.
9. Gibas Z, Prout GR, Connolly JG, Pontes JE, Sandberg AA) Nonrandom chromosomal changes in transitional cell carcinoma of the bladder. Cancer Res 44:1257-1264, 1984.
10. Gibas Z, Pontes JE, Sandberg AA. Chromosome rearrangements in a metastatic adenocarcinoma of the prostate. Cancer Genet Cytogenet 16:301-304, 1985.
11. Gibas Z, Prout GR, Edson Pontes J, Sandberg AA. Chromosome changes in germ cell tumors of the testis. Cancer Genet Cytogenet 19:245 – 252, 1986.
12. Gutenson W, Rodewald A, Haas B, Schulz P, Cleve H. Refined mapping of the gene for glutathione reductase on human chromosome 8. Hum Genet 43:221-224, 1978.
13. Hasenson M, Hartley-Asp B, Kihlfors C, Lunding A, Gustafsson J, Pousette A. Effect of hormones on growth and ATP content of a human prostatic carcinoma cell line, LNCaP-r. Prostate 7:183-194, 1985.
14. Hecht F, Berger CS, Sandberg AA. Nonreciprocal chromosome translocation t(5; 14) in cancers of the kidney: adenocarcinoma of the renal parenchyma and transitional cell carcinoma of the kidney pelvis. Cancer Genet Cytogenet 14:197-203, 1985.
15. Hecht F. Solid tumor breakpoint update. Cancer Genet Cytogenet 31:129 – 131, 1988.
16. Horoszewicz JS, Leong SS, Kawinski E, Karr JP, Rosenthal H, Ming Chu T, Mirand EA, Murphy GP. LNCaP model of human prostatic carcinoma. Cancer Res 43:1809-1818, 1983.
17. Iizumi T, Yakasi T, Kanoh S, Kondo I, Koiso K. Establishment of a new prostatic carcinoma cell line (TSU-PRI). J Urol 137:1304-1306, 1987.

18. ISCN (1985) An International system for human cytogenetic nomenclature. In: Harnden DG, Klinger HP (eds) In collaboration with Cytogenet Cell Genet. Karger, Basel.
19. Kaighn ME, Shankar Narayan K, Ohnuki Y, Lechner JF, Jones LW. Establishment and characterization of a human prostatic carcinoma cell line (PC-3). Invest Urol 17:16-23, 1979.
20. König JJ, Hagemeijer A, Smit BE, Romijn HC, Schröder FH. Cytogenetics of prostatic carcinoma: studies on two human tumor cell lines (abstract). Cancer Genet Cytogenet 28:43, 1987.
21. König JJ, Hagemeijer A, Smit B, Kamst E, Romijn JC, Schröder FH. Cytogenetic characterization of an established xenografted prostatic carcinoma cell line (PC-82). Cancer Genet Cytogenet 34:91-99, 1988.
22. Kunzmann R, Holzel F. Karyotype alterations in human ovarian carcinoma cells during long-term cultivation and nude mouse passage. Cancer Genet Cytogenet 28:201-212, 1987.
23. Lundberg C, Skoog L, Cavenee WK, Nordenskjold M. Loss of heterozygosity in human ductal breast tumors indicates a recessive mutation on chromosome 13. Proc Natl Acad Sci USA 84:2372-2376, 1987.
24. Meehan RR, Gosden JR, Rout D, Hastie ND, Friedberg T, Adesnik M, Buckland R, Van Heiningen V, Fletcher J, Spurr NK, Sweeney J, Wolf CR. Human Cytochrome P-450 PB1: a multigene family involved in mephenytoin and steroid oxidations that maps to chromosome 10. Am J Hum Genet 42:26 – 37, 1988.
25. Mitelman F. Restricted number of chromosomal regions implicated in aetiology of human cancer and leukemia. Nature 310:325-327, 1984.
26. Mitelman F. Catalog of chromosome aberrations in cancer. In: Sandberg AA (ed) Progress and topics in cytogenetics, vol 5. Liss, New York, pp 203-248, 1985.
27. Nelson-Rees WA, Flandermeyer RR. DU145 cell line karyotype. Int J Cancer 21:796 – 797, 1978.
28. Parmiter AH, Balaban G, Clark WH, Nowell PC. Possible involvement of the chromosome region 10q24-q26 in early stages of melanocytic neoplasia. Cancer Genet Cytogenet 30:313-317, 1988.
29. Rajput B, Degen SF, Reich E, Waller EK, Axelrod J, Eddy RL, Shows TB. Chromosomal locations of human tissue plasminogen activator and urokinase genes. Science 230:672 – 674, 1985.
30. Saksela 0.Plasminogen activation and regulation of pericellular proteolysis. Biochim Biophys Acta 823:35-65, 1985.
31. Shea TC, Kelley SL, Henner WD. Identification of an anionic form of glutathione transferase present in many human tumors and human tumor cell lines. Cancer Res 48:527-533, 1988.
32. Steenbrugge GJ van, Groen M, Gallee MPW, Schröder FH (in press) Two androgen-independent human prostatic carcinomas established in athymic nude mice: the PC-133 and PC-135 cell lines. Urol Res .
33. Steenbrugge GJ van, Groen M, Dongen JW van, Korput H van der, Trapman J, Hasenson M, Horoszewicz J. The human prostatic carcinoma cell line LNCaP and its derivatives. An overview. Urol Res 17:71-77, 1989.
34. Stone KR, Mickey DD, Wuderli H, Mickey GH, Paulson DF. Isolation of a human prostate carcinoma cell line (DU 145). Int J Cancer 21:?74 – 281, 1978.
35. Tew KD, Clapper ML, Greenberg RE, Weese JL, Hoffman SJ, Smith TM. Glutathione S-transferases in human prostate. Biochim Biophys Acta 926:8 – 15, 1987.
36. Trent J, Crickard K, Gibas Z, Goodacre A, Pathak S, Sandberg AA, Thompson F, Whang-Peng J, Wolman S. Methodologic advances in the cytogenetic analysis of human solid tumors. Cancer Genet Cytogenet 19:57 – 66, 1986.
37. Yang-Feng TL, Opdenakker G, Vockaert G, Francke U.Mapping of the human tissue-type plasminogen activator (PLAT) gene to chromosome 8 (8p126q11.2) Cytogenet Cell Genet 40:784, 1985.
38. Yang-Feng TL, Seeburg PH, Francke U. Human luteinizing hormone-releasing hormone gene (LHRH) is located on short arm of chromosome 8 (region 8p11.26p21). Som Cell Mol Genet 12:95 – 100, 1986.

CHAPTER 7.2

CHARACTERIZATION OF CHROMOSOME 8 ABERRATIONS IN THE PROSTATE CANCER CELL LINE LNCAP-FGC AND SUBLINES

Josée J.König, Wilma Teubel, Gert-Jan van Steenbrugge, Johannes C.Romijn and Anne Hagemeijer

In press Urological Research, 1998

ABSTRACT

In two androgen dependent (FGC and P70) and two androgen independent (LNO and R) sublines of the prostate cancer model LNCaP numerical and structural aberrations of chromosome 8 were investigated in detail. The techniques used were whole chromosome paint (WCP) and fluorescence in situ hybridization (FISH) with three cosmid probes mapping to different parts of the p-arm (D8S7 (8p23.3), LPL (8p22) and PLAT (8p11.1)). By WCP all four cell lines showed four copies of chromosome 8 in most cells. However, FISH demonstrated that in all sublines deletions in the 8p region were present. The majority of both FGC and P70 had two copies of cosmids D8S7 and LPL. The cosmid PLAT showed a broader distribution (1-4 copies), especially in P70. Compared to FGC and P70, both LNO and R showed a larger number of copies (3-4) of all three cosmid loci. It is discussed that this difference is probably the result of nondisjunction as a reaction to loss of other sequences on 8p, possibly the tumor suppressor gene (TSG) mapping to 8p21. The fact that both sublines LNO and R are androgen independent raises the possibility of a link between TSG loss on 8p and androgen independence.

INTRODUCTION

Recently we demonstrated that in patients with PC, abnormalities of the number of copies of chromosome 8 in the tumor are correlated with an advanced tumor stage [9]. In a previous cytogenetic study of sublines of the prostatic carcinoma cell line LNCaP [8], we already observed that the two androgen independent sublines R and LNO both had breakpoints in chromosome 8p, which resulted in various marker chromosomes. In that study such breakpoints and markers were not observed in the other, androgen dependent, sublines. Since it is well known that advanced tumors are often non-responsive to hormonal therapy, it was concluded that these rearrangements would possibly have a relation with progression to androgen independence in these cell lines.

In the last years the interest for the 8p region has greatly increased, thanks to the postulated location in that region of one or more tumor suppressor genes (TSG's), that may be also important in prostate carcinoma (PC) progression [1, 2, 12, 16].

The present study adds new information to the cytogenetic data of the LNCaP PC model. A high passage of the parental FGC line [11] has been characterized separately and additional information about the chromosome 8 rearrangements in the other four sublines is reported. These results were obtained by molecular cytogenetic techniques, like whole chromosome paints (WCP) and fluorescence in situ hybridization (FISH) with cosmid probes.

The different rearrangements found in the sublines are discussed in relation to their biological behavior.

MATERIALS AND METHODS

PC cell lines, culture and androgen responsiveness

All PC cell lines used in this study: FGC at passage 20 (FGC), FGC at passage 70 (P70), LNO and LNCaP-r (R) were sublines established from the original LNCaP cell line (Figure 1)[6, 11, 15]. FGC and LNO were kindly provided by Dr.J.Horoszewicz (Roswell Park Memorial Institute, Buffalo, USA). It is important to note here that the cell line we call FGC is identical to the cell line LNCaP-FGC that is available through the ATCC. The R line [5] was a gift from Dr.M.Hasenson (Karolinska Institute, Sweden). The P70 subline is a higher passage culture which originated from FGC after prolonged (1 year) in vitro culture, replating once weekly [11].

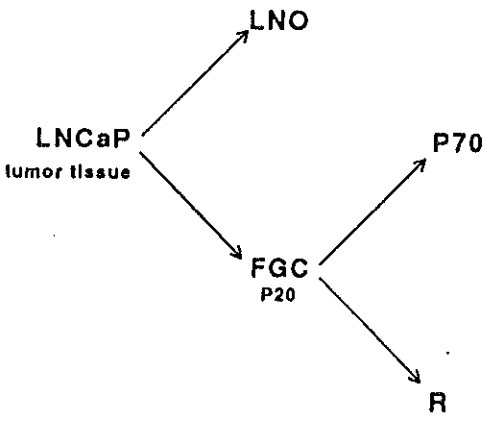


Figure 1. Schematic presentation of the lineage of sublines from LNCaP.

FGC, P70 and R were routinely cultured in RPMI medium, supplemented with 10% fetal calf serum (FCS), glutamin and antibiotics. For the culture of LNO, regular FCS was replaced by 5% steroid depleted FCS.
FGC is androgen dependent, which means that the cells will not grow in androgen depleted medium [15]. FGC cells respond with dose-dependent stimulation of proliferation to the addition of androgens. P70 has lost the complete dependence of androgens and thus also grows in their absence, but it still responds to the addition of androgens with increased proliferation [11], i.e. these cells are androgen independent, but sensitive. LNO and R are both androgen independent and insensitive; both grow well on steroid depleted serum and will not grow faster when androgens are added [5, 15]. However, these two sublines are different with respect to their lineage from the parent cell line (see Fig.1) and with respect to their karyotype [8].

Karyotypes
The karyotypes of FGC, LNO and R have been described in detail before [8]. For the present study the karyotype of these cell lines was checked by R-banding of metaphases as described before. P70 arose later and was characterized in the same manner. All four cell lines were near tetraploid with median chromosome numbers of respectively 86 (FGC), 80 (LNO), 87 (R) and 91 (P70). All showed the original LNCaP marker chromosomes, involving chromosomes 1, 2, 3, 4, 6, 10, 13, 15 and 16, as well as each of their subline specific additional marker chromosomes. P70 showed only one specific marker, namely the same 1p+ marker as its parent cell line FGC (in all karyotyped cells).

FISH procedure
The cosmid probes for three unique chromosome 8p loci (D8S7, 11E1/8p23.3, LPL, 114C11/8p22 and PLAT, 105H8/8p11.1) were kindly provided by Dr.S.Wood (Vancouver, Canada)[18]. The order of the cosmids from telomere to centromere is: D8S7-LPL-PLAT. The chromosome 8 centromere probe was D8Z2 [3]. The chromosome 8 paint was from Cambio (UK). Hybridization and detection of cosmids and paint were performed essentially as described before for chromosome specific DNA probes [9], with some modifications. For cosmid hybridization 100ng biotinylated cosmid DNA and 10µg Cot-1 DNA (Gibco BRL) per slide were added to the hybridization mixture in 50% formamide. This mixture was denatured at 72°C for 4 min, followed by preannealing at 37°C for 2 hours. Hybridization to metaphases on slides occurred during overnight incubation at 37°C in a moist chamber. For the chromosome 8 paint 200ng biotynilated chromosome 8 DNA and 15µg Cot-1 DNA were used per slide. The same protocol as for cosmid DNA was followed. In both cases detection was by FITC-avidin. For double color FISH analysis with both the centromere probe and either D8S7 or LPL, the cosmid was digoxygenated (Boehringer). Detection was by Texas Red-anti-dig. Combination of the centromere probe with PLAT was not possible, due to the near-centromeric localization

of PLAT.
DNA was counterstained with propidium iodide. For identification of chromosomes DAPI (4',6-diamidino-2-phenylindol) stain (0.8 µg/ml) was also applied to the same slides.

FISH efficiency, use of WCP and definition of deletion
Between 50-100 metaphases of each cell line were scored per hybridization experiment. Hybridization efficiency for the three cosmid probes was evaluated using normal lymphocytes and was about 80% for all three, like described by Matsuyama et al [13].
We used WCP in stead of the centromere probe to determine the ploidy of chromosome 8 for the following reasons: 1) both techniques gave comparable results. As an example: in LNO the ploidy distribution of 8 by WCP versus by centromere probe was 4 vs.6% for 2copies, 17 vs.18% for 3 copies, 74 vs.72% for 4 copies and 5 vs.4% for >4 copies), 2) by employing WCP, the ploidy as well as structural rearrangements could be evaluated in the same experiment, 3) because we evaluated metaphases, and with WCP a bigger target is coloured, detection was easier.
The partially tetraploid (63% of the cells) human B lymphoblastoid cell line JY [14], that has no aberrations in chromosome 8 (checked by karyotype analysis) was used as a control for hybridization of the cosmids to tetraploid cells. In all tetraploid metaphases of JY three or four signals were detected for D8S7. The percentage of cells with three signals was 22±3.6%. The cut-off level of deletion was set to 30% (mean + 2xSD). This cut-off was also applied to LPL and PLAT, because these behaved similarly. So in cosmid experiments with four signals in less than 70% of the tumor metaphases, at least one copy of the probe was considered deleted. Because no metaphases with less than three signals were seen in the control cell line, in tumor metaphases with 0, 1 or 2 signals respectively all, three or two copies were considered deleted.

RESULTS

Aberrations of chromosome 8 by paint analysis

FGC

Paint analysis of metaphases of FGC demonstrated that the main population of cells showed four copies of chromosome 8 (82%). Ten percent of the cells had more than four, 8% three and a minor population two copies of chromosome 8 (Table 1). Marker chromosomes with microscopically visible 8p and 8q aberrations were mainly detected in cells with in total four copies of chromosome 8 (Table 2). These were mostly resulting from translocations of other material to 8p or 8q (Figure 2A), concurrent with partial deletion of 8p or 8q DNA. Also several independent chromosome fragments entirely consisting of chromosome 8 DNA were detected (3.2%).

P70

Paint analysis of P70 showed the close relationship to FGC in the nearly equal distribution of chromosome 8 copies (Table 1). However, 8p- and q-arm aberrations occurred less frequently in P70 than in FGC (Table 2). The most prominent aberration was an A-sized marker, consisting only of chromosome 8 DNA (in 4.4% of the cells). With DAPI stain, this marker showed a banding pattern that could fit an i(8q) (Figure 2B). In P70 metaphases the highest percentage of unidentified chromosome 8 material was found (4.4%). Both independent chromosome fragments, like in FGC, and translocated material to other chromosomes were seen.

LNO

Paint analysis of LNO showed 74% of the cells with four copies of chromosome 8, 17% cells with three copies and some cells with two or more than four copies (Table 1). Occasionally q-arm deletions were seen restricted to cells with three copies of 8). However, the majority of aberrations found were p-arm deletions of different sizes in cells with four copies of 8 (Table 2 and Figure 2C). The 8p- marker that was described earlier (del(8)(p216pter)) was still present, but its frequency had declined to 6%. Unidentified fragments were not detected

Table 1. Results of WCP and cosmid hybridization studies; percentages of metaphases with a positive hybridization signal.

Nr.of Co-pies	FGC				P70				LNO				R			
	WCP	D8S7	LPL	PLAT	WCP	D8S7	LPL	PLAT	WCP	D8S7	LPL	PLAT	WCP	D8S7	LPL	PLAT
1	0	23	12	28	0	7	19	4	0	3	7	7	0	0	8	0
2	2	68	69	39	3	47	52	26	4	42	36	21	2	39	20	22
3	8	8	20	33	7	37	27	52	17	52	46	36	24	32	28	27
4	82	3	0	0	84	9	2	17	74	3	11	25	59	19	44	40
>4	10	0	0	0	6	0	0	0	5	0	0	12	15	0	0	11

Notes
WCP= whole chromosome paint.

R

In R substantial proportions of three (24%) and more than four (15%) copies of 8 were detected by WCP (Table 1). P-or q-arm abnormalities occurred in cells with three, four, as well as more than four copies of chromosome 8 (Table 2). However, the 8p+ marker that was described previously (8qter68p23::8q1368qter)) occurred far less frequently (3.3%). Probably this marker was further modified, resulting in other chromosome 8 markers as demonstrated in Figure 2D. Independent fragments consisting of chromosome 8 material were seen only occasionally (0.8%).

FGC and R showed the highest overall percentage of metaphases with chromosome 8 aberrations (17.6% and 17.8% respectively).

Aberrations of 8p detected with cosmids in relation to the number of copies of chromosome 8

In all four cell lines investigated and for all three cosmids, the percentages of cells with four copies of cosmids were invariably below the cut-off value of 70% that marks loss of one copy. Consequently the frequency distributions presented in Table 1 can be regarded also as distribution patterns for loss of cosmid sequences.

PLAT was present on 100% of the 8p- marker chromosomes, found in any subline.

Paint analysis of FGC showed predominantly four copies of 8, but by cosmid analysis mainly two copies of D8S7 and LPL were detected. However, also populations with one or three copies of these two cosmids were seen with moderate frequency. PLAT showed less cells with two copies, but more cells with one and three copies. D8S7 showed four copies in some cells, but LPL and PLAT did not.

In P70, like in FGC, with WCP most cells had shown four copies of chromosome 8. In concordance with FGC, P70 mainly showed two copies for both D8S7 and LPL. However, the populations of cells with three copies of both these cosmids had increased in P70. PLAT even showed mainly three copies, and a significant population with four copies, although still more than a quarter of the cells maintained two copies. Also in contrast to the results for FGC was the observation that in P70 LPL showed more cells with one copy than D8S7 and PLAT.

Double color FISH analysis with D8Z2 for the centromere and the cosmids D8S7 or LPL (PLAT could not be evaluated in this way due to its position near the centromere) demonstrated that in cells with three copies of 8, D8S7 and LPL were present in only two copies. This indicated that in these cells one of the aberrant chromosomes was lost (not shown).

Although paint analysis of LNO showed largely the same distribution as FGC, the cosmid analysis showed different results. Two as well as three copies of D8S7 and LPL were most frequent in this subline. Moreover, PLAT demonstrated a completely different distribution, showing a clear shift to more copies. Double color FISH analysis showed that in cells with three copies of 8, like in P70, one of the aberrant chromosomes was lost.

In R, of which 15% of the cells had more than four copies of chromosome 8, only PLAT was present in more than four copies. However, in relation to the other sublines, all three cosmids demonstrated a clear shift towards more copies, whereby LPL and PLAT both showed a majority of cells with four copies. Double color FISH analysis showed the same result as for P70 and LNO.

Table 2. Percentages of chromosome 8 aberrations as detected by WCP.

	FGC	P70	LNO	R
P-ARM	8.0	4.4	4.0	9.9
Q-ARM	6.4	1.5	1.0	7.1
UNIDENTIFIED	3.2	4.4	0.0	0.8
ALL	17.6	10.3	5.0	17.8

DISCUSSION

Painting analysis showed that in all four LNCaP sublines the majority of the cells had four, apparently normal, copies of chromosome 8. FGC and P70 showed only minor populations with aberrations from tetraploidy, never exceeding 10% of the cells. However, LNO, and especially R, both androgen independent and both descended from the parental cell line at an early stage of in vitro culture, displayed larger cell populations with three -(LNO,R) or even more than four copies (R). The findings described here demonstrate that tumor cell lines growing in vitro are dynamic entities, just like tumors growing in vivo. Inherent chromosomal instability allows the cells to constantly generate new chromosomal aberrations. These will either quickly be lost again or be retained, dependent on the benefit the cell will have from them, while reacting on genetic or environmental pressure.

The presence of the 8p aberrations found before in LNO and R [8] were confirmed by WCP in the present study. Also new, not previously described aberrations of chromosome 8, involving both 8p and 8q sequences, were discovered in these cell lines. Such aberrations were also for the first time demonstrated by WCP in the sublines FGC and P70.

The most surprising finding of the present study was that, despite the apparent presence of four normal copies of chromosome 8 in most cells of the investigated cell lines, only few cells seemed to have retained all 8p sequences on all chromosomes. The deletion patterns found reflect the lineage of the different sublines as depicted in Figure 1.

Molecular genetic analysis in the 8p22-8cen region showed the close relationship between FGC and P70 through their identical microsatellite repeat patterns (pers. comm. W.Dinjens, Dept.Pathology, EUR). It is conceivable that in the original diploid cell line (LNCaP in Figure 1, refs.4 and 6) only one chromosome 8 had one or more deletions and that these mapped only distal to 8p22. Combined with the results of the FISH analysis of FGC and P70 for PLAT that maps proximal, and D8S7 and LPL that both map distal to 8p22, it can be assumed that after tetraploidization several new structural aberrations were generated in the normal- as well as in the preexisting aberrant chromosomes. These events then finally resulted in the deletion patterns observed for both the low passage cells (FGC) and the high passage cells

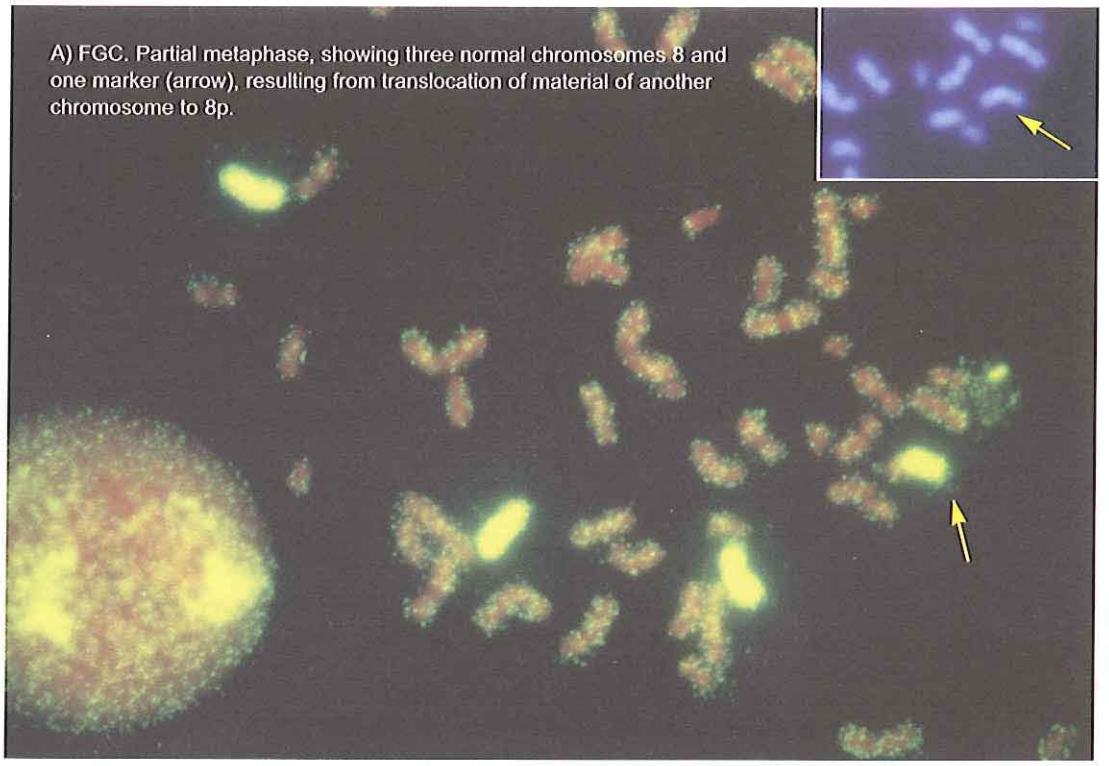
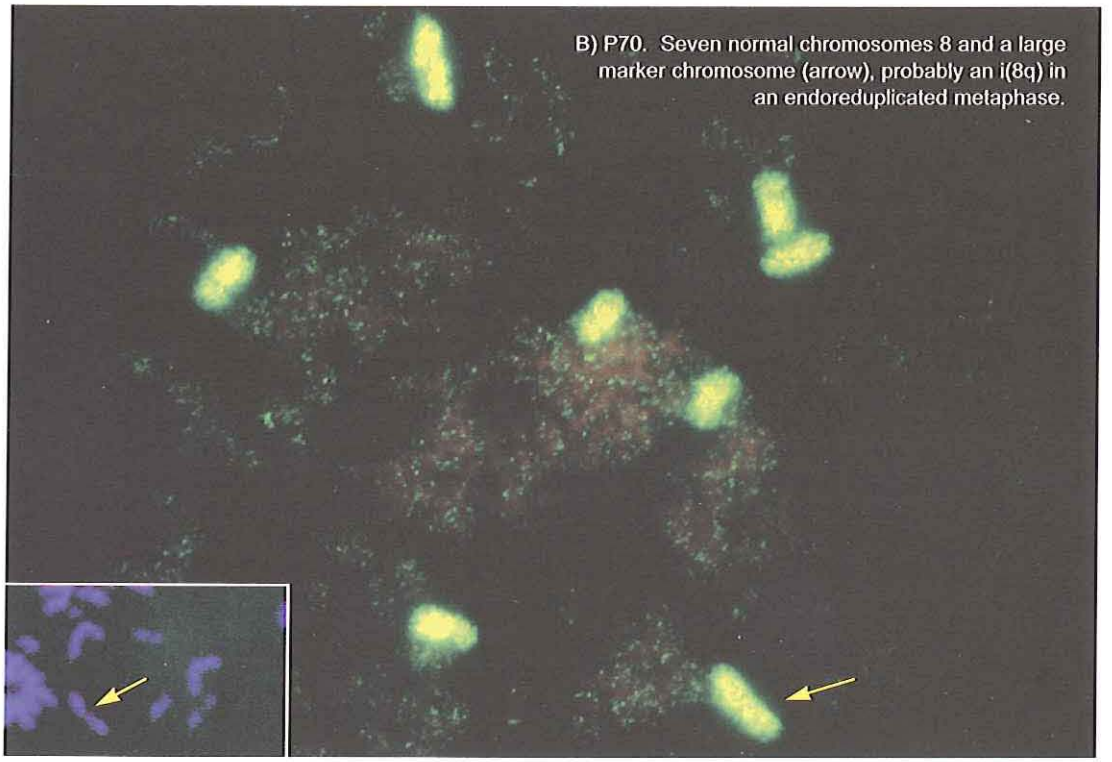
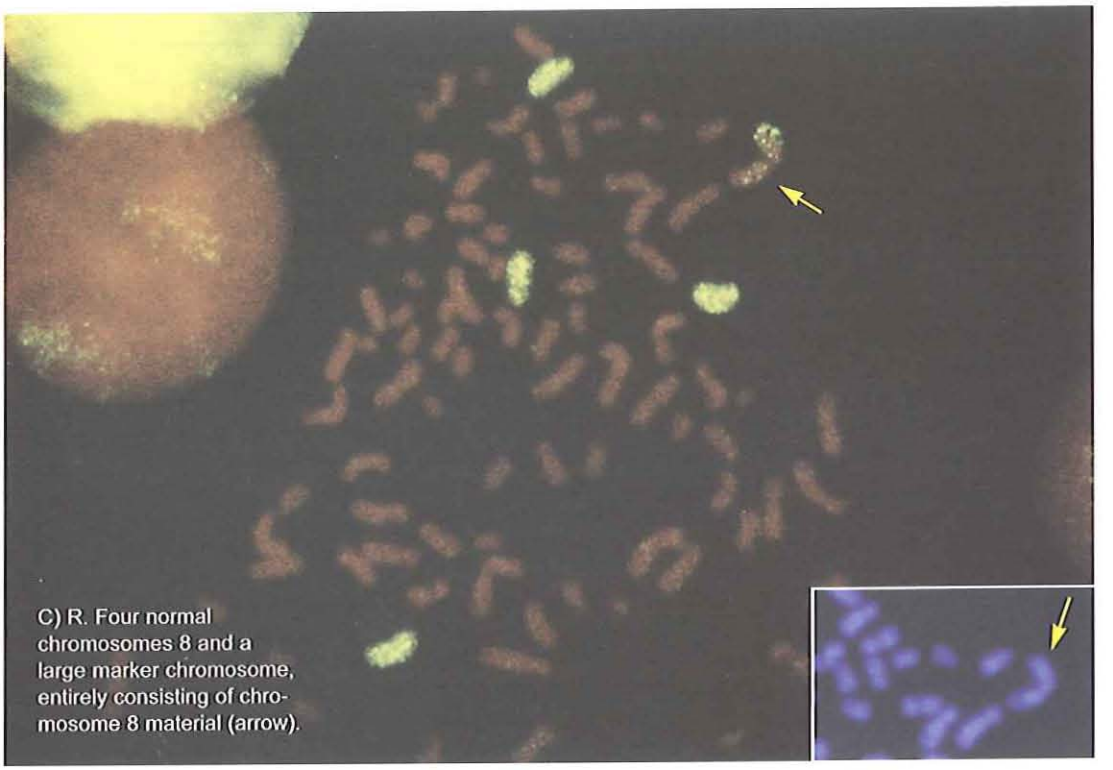
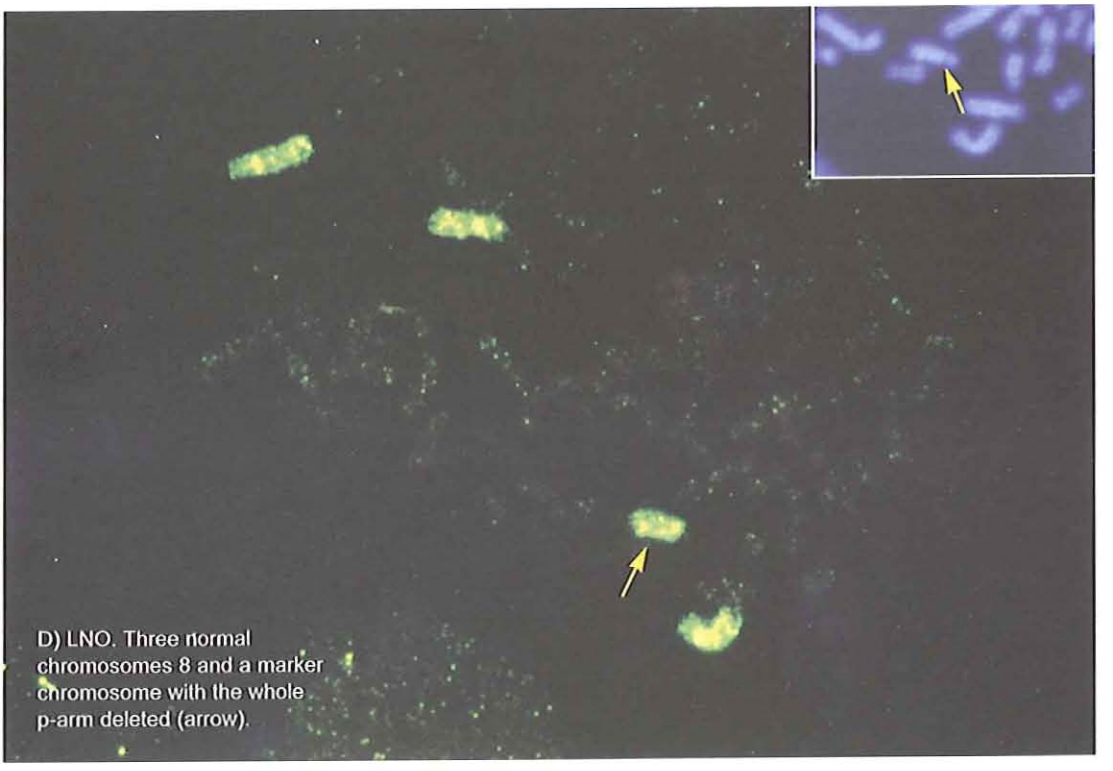


Figure 2. Detection of chromosome 8 paint DNA on all metaphases shown was by FITC avidin, propidium iodide counterstain. Verification of chromosomes by DAPI stain. Magnification: 312.5x.





C) R. Four normal chromosomes 8 and a large marker chromosome, entirely consisting of chromosome 8 material (arrow).



D) LNO. Three normal chromosomes 8 and a marker chromosome with the whole p-arm deleted (arrow).

(P70). The deletion patterns observed for the sublines LNO and R can only be explained by the occurrence of several nondisjunction events in addition to the tetraploidization and chromosome breakage, as described above for FGC and P70. Interestingly, analysis of CA repeats on 8p proximal to band p22 in both LNO and R showed differences in several markers when compared to FGC (pers. comm. W.Dinjens, Dept.Pathology, EUR). However, these data are preliminary and they need to be reconfirmed before definite conclusions can be drawn.

If the deletions found by cosmid analysis were continuous between the regions investigated, two chromosomes with microscopically visible 8p deletions were expected in most cells of FGC, P70 and LNO, and one in R. However, by paint analysis maximally 10% of the metaphases with such aberrations could be identified. A more likely explanation for these findings would be that the deletions were mostly discontinuous and relatively small, i.e. spanning less than one band. Using comparative genome hybridization, large deletions on 8p, sometimes spanning most of the p-arm, have been detected in a number of PC specimens [2, 7, 17]. However, with LOH mapping also interspersion of deleted and retained regions on chromosome 8 has been observed [12, 16], so it is conceivable that this is also the case in the LNCaP sublines investigated.

Matsyama et al.[13] found that in poorly differentiated clinical PC both D8S7 and LPL were lost. This is in concordance with our results, since the LNCaP cell line was derived from a poorly differentiated lymph node metastasis [6]. However, in more differentiated clinical tumors, it was found that D8S7 was mostly retained [13]. This result is suggestive of two TSG's that might be inactivated at different stages in the progression of PC. In other studies allelic loss on 8p generally was associated with higher tumor grade, and the deletion patterns found were suggestive of one or two TSG's [2, 12, 16]. Discordant results in two separate LOH studies, one narrowing down the locus for a putative TSG to 17cM in the 8p21 region [16], and the other reporting on a patient with a homozygous deletion in the 8p22 region [1], can be brought together by the concept of two TSG's on 8p, as was also suggested for colorectal cancer [19]. Following this concept, it is conceivable that in FGC and P70 only the 8p22 TSG was inactivated, and in LNO and R both TSG's.

We postulate that the shift towards more copies of certain 8p sequences in LNO and also in R is probably the result of nondisjunction as a reaction to partial loss of other sequences on 8p [2], possibly the TSG mapping to 8p21. This mechanism may even be typical for more advanced tumors [10].

As LNO is a very early descendant from the parental cell line, its differences from FGC and P70 could have been already present before in vitro culture. However, in the case of R, which originated from FGC long after its establishment as an in vitro cell line [5], the significant chromosomal rearrangements must have taken place during in vitro culture.

The present results once more emphasize the many ways in which tumor models can be employed. Moreover, if other variant cell lines of FGC can be generated by culture without androgens and if they show similar rearrangements, the link between the androgen independent and insensitive status and the phenomenon of loss of sequences on 8p in the LNCaP human prostate tumor cell line can be made more firmly. Then, to confirm the significance of this finding, patient tissues at various clinical stages should be investigated for these same rearrangements.

Acknowledgements
This work was supported by grant IKR 90-12 from the Dutch Cancer Society.

LITERATURE

1. Bova GS, Carter BS, Bussemakers MJG, Emi M, Fujiwara Y, Kyprianou N, Jacobs SC, Robinson JC, Epstein JI, Walsh PC, Isaacs WB Homozygous deletion and frequent allelic loss of chromosome 8p22 loci in human prostate cancer. Cancer Res 53: 3869-3873, 1993.
2. Cher ML, MacGrogan D, Bookstein R, Brown JA, Jenkins RB, Jensen RH. Comparative genomic hybridization, allelic imbalance, and fluorescence in situ hybridization on chromosome 8 in prostate cancer. Genes Chromos Cancer 11 :153-162, 1994.
3. Donlon T, Wyman AR, Mulholland J, Barker D, Bruns G, Latt S Botstein D. " satellite-like sequences at the centromere of chromosome 8. Am J Hum Genet 39: A196, 1986.
4. Gibas Z, Becher R, Kawinski E, Horoszewicz J, Sandberg AA. A high-resolution study of chromosome changes in a human prostatic carcinoma cell line (LNCaP). Cancer Genet Cytogenet 11: 399-404, 1984.
5. Hasenson M, Hartley-Asp B, Kihlfors C, Lundin A, Gustafsson J, Pousette A. Effect of hormones on growth and ATP content of a human prostatic carcinoma cell line, LNCaP-r. Prostate 7 :183-194., 1985.
6. Horoszewicz JS, Leong SS, Kawinski E, Karr JP, Rosenthal H, Ming Chu T, Mirand EA, Murphy GP. LNCaP model of human prostatic carcinoma. Cancer Res 43: 1809-1818, 1983.
7. Joos S, Bergerheim USR, Pan Y, Matsuyama H, Bentz M, Manoir S du, Lichter P. Mapping of chromosomal gains and losses in prostate cancer by comparative genomic hybridization. Genes chromos Cancer 14 :267-276, 1995.
8. König JJ, Kamst E, Hagemeijer A, Romijn JC, Horoszewicz J, Schröder FH. Cytogenetic characterization of several androgen responsive and unresponsive sublines of the human prostatic carcinoma cell line LNCaP. Urol Res 17: 79-86, 1989.
9. König JJ, Teubel W, Dongen JW van, Romijn JC, Hagemeijer A, Schröder FH. Loss and gain of chromosomes 1, 18 and Y in prostate cancer. Prostate 25: 281-291, 1994.
10. König JJ, Teubel W, Romijn JC, Schröder FH, Hagemeijer A. Gain and loss of chromosomes 1, 7, 8, 10, 18 and Y in 46 prostate cancers. Hum Pathol 27: 720-727, 1996.
11. Langeler EG, Uffelen van CJC, Blankenstein MA, Steenbrugge van GJ, Mulder E. Effect of culture conditions on androgen sensitivity of the human prostatic cancer cell line LNCaP. Prostate 23 :213-223, 1993.
12. MacGrogan D, Levy A, Bostwick D, Wagner M, Wells D, Bookstein R. Loss of chromosome arm 8p loci in prostate cancer : Mapping by quantitative allelic imbalance. Genes Chromos Cancer 10 :151-159, 1994.
13. Matsuyama H, Pan Y, Skoog L, Tribukait B, Naito K, Ekman P, Lichter P, Bergerheim USR. Deletion mapping of chromosome 8p in prostate cancer by fluorescence in situ hybridization. Oncogene 9 :3071-3076, 1994.
14. Reboul A, Arvieux J, Wright JF, Colomb MG. Proteolytic fragmentation of tetanus toxin by subcellular fractions of JY, a B lymphoblastoid cell line. Biochem J 277: 47-51, 1991.
15. Steenbrugge GJ van, Uffelen CJC van, Bolt J, Schröder FH. The human prostatic carcinoma cell line LNCaP and its derived sublines: An in vitro model for the study of androgen sensitivity. J Ster Biochem 40: 207-214, 1991.
16. Trapman J, Sleddens HFBM, Weiden MM van der, Dinjens WNM, Konig JJ, Schroder FH, Faber PW, Bosman FT. Loss of heterozygosity of chromosome 8 microsatellite loci implicates a candidate tumor suppressor gene between the loci D8S87 and D8S133 in human prostate cancer. Cancer Research 54: 6061-6064, 1994.
17. Visakorpi T, Kallioniemi AH, Syvanen AC, Hyytinen ER, Karhu R, Tammela T, Isola JJ, Kallioniemi OP. Genetic changes in primary and recurrent prostate cancer by comparative genomic hybridization. Cancer Res 55 :342-347, 1995.
18. Wood S, Schertzer M, Drabkin H, Patterson D, Longmire JL, Deaven LL. Characterization of a human chromosome 8 cosmid library constructed from flow-sorted chromosomes. Cytogenet Cell Genet 59 :243-247, 1992.
19. Yaremko ML, Wasylyshyn ML, Paulus KL, Michelassi F, Westbrook CA. Deletion mapping reveals two regions of chromosome 8 allele loss in colorectal carcinoma. Genes Chromos Cancer 10 :1-6, 1994.

CHAPTER 8

GENERAL DISCUSSION

GENERAL DISCUSSION

In vitro culture of PC cells

Before tissue culture of PC cells was applied for cytogenetic analysis, it already had an interesting history. PC cells had been cultured in several ways, from cell suspensions (after disaggregation of the tumor tissue by collagenase) or tissue cubes (organ culture); in monolayers or in an anchorage independent way as single cells and cell clumps in agarose. It was known that growth factors (e.g.EGF, HC), as well as hormones (e.g.DHT) could stimulate in vitro proliferation. This knowledge led to the development of several specialized tissue culture media, that contained varying mixtures of defined supplements like growth factors, hormones and trace elements. The necessity to add FCS in order to sustain proper proliferation of PC cells, was an indication that not all substances that were needed for in vitro growth could be defined. Moreover, the application of these media did not lead to the expected development of a large number of permanent in vitro cell lines, although short term proliferation of PC cells was thought to be optimal.

Cytogenetic analysis of PC cells, cultured according to the techniques described above, soon revealed another problem. Namely, 73% of the investigated PC's showed a normal diploid karyotype. With DNA-FCM however, over 50% of PC's were found to be aneuploid, so it was clear that many abnormal karyotypes were missed. Part of our investigations were carried out to elucidate the reasons for this discrepancy. In chapter 4.1 we specifically studied all aspects of the first step of the tissue disaggregation-tissue culture-metaphase preparation sequence. We found a 50% lower yield of aneuploid cells after collagenase digestion, although collagenase itself was non-toxic during digestion and also the overnight incubation at 37°C did not decrease total cell yield. Presently we think that the tumor cell membranes are made fragile by the digestion. Consequently the mechanical abrasion of the pipetting step, needed after digestion to disintegrate tissue fragments into the small cell clumps that are suitable for in vitro culture, causes many cells to be disrupted. However, collagenase digestion yields a higher number of viable cells that are able to attach and grow in tissue culture, compared to other methods like mechanical disaggregation or the application of other enzymes. So by lack of an acceptable alternative it has remained the method of choice for the preparation of cell suspensions.

In chapter 4.2 it was demonstrated that even starting with a high percentage of (aneuploid) PC tumor cells does not guarantee that such cells will ultimately be detected at the end of the whole sequence of tissue disaggregation-tissue culture-metaphase preparation. The reduction of aneuploid cells ranged from 44 to 85%, which often caused the initially present abnormal tumor cells to be missed in the final cytogenetic analysis. We estimated that in at least 25% of the reported tumor karyotypes aneuploid cell populations were missed, because they did not proliferate in culture. This meant that the techniques used for culturing PC cells were not adequate.

In chapter 3 we described the cytogenetic analysis of 39 PC specimens. We adapted the standard procedures for cytogenetic analysis of hematological malignancies and developed our own medium for short term tissue culture of PC. In the course of our studies we tested every new promising culture method for solid tumor cytogenetic analysis. These modifications resulted in a greatly increased culture success rate, and in a modestly increased karyotyping success rate. Clonal aberrations were found in 8 out of the 39 cases that yielded metaphases (21%). The most effective method came from Lund, Sweden. It employed collagenase digestion to obtain cell suspensions followed by size fractionation and short term culture in a completely synthetic medium. This culture medium was very complex.

It included virtually every trace element, vitamin and hormone that could in any way have a stimulating effect on PC cells, but contained no chemically undefined products, like fetal calf serum. In our hands 11/15 PC cultures handled by this method yielded metaphases, which was a higher percentage than achieved by any other method in our series. In three of these cultures clonal chromosomal aberrations were defined (27%). This indicated that, even under these optimized conditions, (normal) diploid epithelial cells apparently still had a growth advantage. Lundgren, in who's department the Lund method was developed and first used, postulated that the tumors that were cytogenetically normal could reflect a less aggressive tumor subtype. This was based on the observation that tumors with clonal abnormalities were associated with shorter survival times than tumors without aberrations [Lundgren et al '92]. However, in our series such a relationhip could not be demonstrated. Recently, Webb et al achieved good results by supplementing the medium with conditioned medium from various PC cell lines. They found chromosome aberrations in 33% of their cultured specimens. Although it is probable that also in this series not all abnormal karyotypes were detected, a comparison of their cytogenetic results with clinical parameters would be of interest.

Cytogenetics and molecular cytogenetics of patient tumors

General remarks

Comparison between FCM and FISH results indicated that, as expected, with an increasingly aberrant histogram by FCM, more chromosomes became numerically aberrant. In FISH analyses aneuploid tumors always showed cell populations with triploid as well as tetraploid copy numbers of the chromosomes investigated. Also, minor populations with polysomic (>4 copies) cells were present in some tumors. We conclude that these observations are manifestations of the genetic evolution of a tumor, that is characterized by repeated rounds of tetraploidization and subsequent chromosome loss.

We established that a great advantage of FISH over DNA-FCM is, that the number of FISH signals does not change during the cell cycle, while with FCM the DNA distribution of proliferating (S and G2M) cells will mask the DNA aneuploid nuclei (chapter 4.2).

Because, as was stated above, not all tumor cells were able to proliferate equally well in tissue culture, it is now recognized that certain tumor cell populations that were clonal in the tumor could well appear nonclonal after culture [Zitzelsberger et al '96]. So not only the clonal, but also the relevant nonclonal aberrations found in our karyotype analyses (in 30/39 cases) are discussed.

In contrast to PC, in BPH tissue we did not find any chromosomal aberrations (chapters 5 and 6). We concluded that occasional BPH cells in a sample did not contribute to any chromosomal abnormalities found in our PC specimens. Others found loss of Y and gain of 7 by FISH analysis, but only in cultured BPH specimens [Aly et al '94]. This is an indication that these aberrations are probably culture artifacts.

Chromosome 1

By classical cytogenetic analysis (chapter 3), we did not detect any structural or numerical abnormalities of chromosome 1. When we monitored whole chromosome loss and gain of chromosome 1 by FISH analysis with a centromere specific DNA probe (chapters 5 and 6). In more than one third of the tumors, most of which were aneuploid by FCM, gain of chromosome 1 was recognized. Loss was an infrequent observation. The occurrence of numerical aberrations of chromosome 1 was independent of tumor grade and stage.

In recent reports on PC cytogenetics it was established that structural abnormalities of 1p and 1q are among the most frequent aberrations found [Webb et al '96]. LOH analysis also has indicated 1q as an area that is frequently rearranged [Bova et al '96]. CGH analysis showed that 1q gain is often accompanied by 1p loss, suggestive for isochromosome formation [Muleris et al '94, Cher et al '96]. Through linkage analysis of high risk families it was recently shown that the 1q24-q25 region is likely to contain a PC susceptibility gene, named *HPC1* [Smith et al '96, Cooney et al '97]. The clinical features in families that showed linkage were not different from those in families that did not show linkage.

Chromosome 7

Only numerical aberrations of chromosome 7 were found in our karyotypes, and those were all nonclonal (chapter 3). Gain of 7 was relatively common in the series of tumors we investigated by FISH analysis (chapter 6). In agreement to what was reported by others, we found that gain of 7 was correlated with metastatic disease. In some tumors simultaneous loss and gain in the same tumor, suggestive for heterogeneity, was observed.

In a recent karyotype analysis report [Zitzelsberger et al '96], gain of 7 was observed as a clonal aberration, which was confirmed by FISH analysis. In that study gain of 7 was associated with advanced stage and poor differentiation. In a CGH study [Visakorpi et al '95A], the minimal region of overlap in gain of (part of) 7 was band 7p13. The candidate oncogene in that region is the EGF receptor. Also by LOH [Bova et al '96] 7p was indicated, albeit as a minor region, for the presence of a TSG.

The regions 7q22 and 7q31 are among the most frequently involved in rearrangements in PC, also by LOH, which is an indication for the location of a possible TSG [Kallioniemi et al '96, Webb et al '96, Zenklusen et al '94, Bova et al '96]. In a combined CGH/LOH study [Joos et al '95], the most frequent abnormality of 7q detected by CGH was gain of the whole q arm. LOH of 7q has been indicated as an early event in the development of PC [Bova et al '96, Latil et al '96].

Chromosome 8

Different deletions of the p-arm of chromosome 8 were observed as nonclonal abnormalities in our karyotype analysis study (chapter 3). It was the most frequently observed nonclonal change. Gain of 8 by FISH analysis was observed in almost half of the tumors and was correlated with tumor progression. In contrast, loss of 8 occurred in less than 10% of the tumors, mostly in nonmetastatic tumors, and in most of these, loss was combined with gain (chapter 6).

We were the first to link 8p aberrations with tumor progression, when we reported that the two androgen insensitive sublines of LNCaP both had lost one copy of the 8p23-pter region, while the androgen sensitive sublines had retained that region (chapter 7.1).

At the moment, chromosome 8 and more specifically the 8p region is the most intensively studied chromosomal region in prostate cancer. When all abnormalities found by karyotype analysis are taken together, 8p21 is the fifth most frequently found breakpoint in PC [Webb et al '96].

Detailed LOH studies have indicated that, as mentioned above, at least two distinct regions on 8p (8p11-12 and 8p22)harbor TSG's [Netto et al '94, Kallioniemi et al '96, MacGrogan et al '96, Vocke et al '96]. Recently a candidate TSG was cloned from the 8p22-p21.3 region. This gene, named *PRLTS*, shows frequent LOH, but is rarely mutated in PC [Komiya et al '97].

More detailed investigations applying FISH with several cosmids mapping to

different regions on 8p revealed that few cells in any subline, androgen sensitive or -insensitive, had retained all 8p sequences on all copies of 8 (chapter 7.2). However, we found different patterns of loss in the different sublines. We concluded that in both androgen sensitive sublines (FGC and P70), and probably also in the original tumor tissue, the postulated TSG on 8p22 was knocked out. In both androgen insensitive sublines (LNO and R) then, both TSG's on 8p22 and 8p21 would be inactivated. In R, which was generated from FGC after its establishment as an in vitro cell line, loss of the second TSG must have occurred during tissue culture, possibly under environmental pressure through culture without androgens. For LNO, which originated from part of the original tumor cell suspension, it is also possible that due to heterogeneity in the original tumor, the LNO cell line arose from an insensitive cell population that already had both TSG's inactivated. It will be interesting to check these contentions by generating new sublines and testing them for the same deletions.

FISH and CGH studies have, in agreement with our results, shown that numerical aberrations of 8 occur frequently and that gain of 8 is a marker of clinically aggressive PC [Lieber '94, Alers et al '95A, Alers et al '95B, Joos et al '95, Visakorpi et al '95A, Qian et al '95, Bova et al '96, Huang et al '96, Geburek et al '97, Cher et al '96, Alers et al '98]. Gain of 8q has a possible utility as a marker of progression. The candidate gene here is *C-MYC* on 8q24, that is overexpressed in some poorly differentiated PC [Jenkins et al '97].

Recent chromosome transfer studies bringing parts of human 8p into highly metastatic rat PC cells to search for metastasis suppressor genes (MSG's) of rat PC, indicated 8p21-p12 as a candidate region. It is probably no coincidence that this overlaps with the candidate regions for the human TSG's [Nihei et al '96, Dong et al '96].

Chromosome 10

Rearrangements of chromosome 10 were observed by us only by FISH analysis (chapter 6). In our PC series chromosome 10 was the most frequently gained as well as the most frequently lost chromosome. Loss of 10 was always accompanied by gain in the same tumor specimen, which was also observed for chromosome 7, suggesting scattered areas of gain and loss within one tumor. This concept was later confirmed by studies on PC tissue sections that showed focal heterogeneity [Alers et al '95B].

We were the first to report that numerical aberrations of 10 were correlated with metastatic disease and also found that the average number of copies (ANC) of 10 was significantly higher in advanced tumors. This led us to conclude that one or more genes may be located on chromosome 10 that confer an aggressive cancer phenotype and are potentially important in the process of metastasis. This conclusion was confirmed in other studies also investigating aberrations of 10 by FISH [Alers et al '95A, Qian et al '95], karyotype [Webb et al '96] or CGH analysis [Cher et al '96].

Chromosomal aberrations of 10q were among the first aberrations identified in PC. Recently, intriuging new results are reported. Different LOH studies and a microcell transfer experiment have shown that the 10p area (more specifically 10p11.2-pter) harbors a TSG, important in the early stages of the disease [Komiya et al '96, Bova et al '96, Trybus et al '96, Murakami et al '96]. Also, it is now established that one or more TSG's related to tumor progression are located in the 10q area [Netto et al '94, Komiya et al '96, Kallioniemi et al '96, Bova et al '96, Latil et al '96, Trybus et al '96]. This was confirmed by the observation that microcell-mediated transfer of part of chromosome 10 (10cen-q23) into rat PC cells, suppressed the

metastatic ability of the host cell line [Dong et al '96]. A candidate MSG is the *PTEN* gene on 10q23 [Li et al '97]. It codes for a tyrosine phosphatase, that may regulate cell invasion and metastasis through interactions at focal adhesions. It is mutated in 4/4 PC cell lines. The 10q24-qter region is thought to contain another TSG. A candidate gene here is *MXI1*, a negative regulatory protein for *C-MYC*.

Chromosome 18

Structural or numerical abnormalities of chromosome 18 were not found by classical cytogenetic analysis (chapter 3). Investigation of whole chromosome loss and gain of 18 by FISH analysis (chapters 5 and 6) showed it to be gained in more than half of the tumors, irrespective of their total ploidy. Loss was an infrequent observation. The occurrence of numerical aberrations of chromosome 18 was independent of tumor grade and stage.

Chromosomal abnormalities of 18 have only become evident with molecular cytogenetic techniques. This means that most aberrations are probably subtle changes at gene level. By LOH and CGH it was established that most deletions occur in the 18q region and that it is not a major change in PC [Joos et al '95, Visakorpi et al '95A, Kallioniemi et al '96]. In agreement with our results, in these reports no relation with grade or stage was found. However, when LOH at the *DCC* gene locus (18q21.3) was investigated, an indication for a correlation with progression was found. In contrast to this, a recent report indicated 18q loss as an early event [Bova et al '96].

Y chromosome

Karyotype analysis revealed loss of Y as a recurring clonal and nonclonal change in PC cultures. By FISH analysis, Y loss was detected in less than one fifth of the tumors (chapters 5 and 6). Gain of Y was detected in an equal number of tumors and was in a few cases combined with loss. Numerical abnormalities of Y were unrelated to either age of the patient, grade or stage of the tumor. Loss of Y is a common change in PC, that is also recently described in cultured specimens [Zitzelsberger et al '96], as well as in sections [Alers et al '95A, Qian et al '95]. Previously it has also been indicated as a clonal change in cultured BPH [Aly et al '94]. However, we were unable to detect Y loss in uncultured cell suspensions of our BPH patients (chapters 5 and 6). Moreover, since Y loss has also been found in some other tumors and even in normal cells, Y loss is probably not a prostate specific change. It may be that cells without a Y chromosome have a tissue culture advantage, which explains their frequent occurrence in karyotype analysis of cultured prostate cells. This notion is supported by the fact that the majority of the known PC cell lines lack Y.

Other chromosomes and mechanisms

There are other chromosomes and chromosomal regions, like 3, 5q, 6q, 9p, 11p, 12, 13q, 15, 16q, 17 and X, that are relevant in PC that have not been investigated by us. Table 1 provides a state-of-the-art overview of all chromosomal regions that have been implicated in the development and progression of PC and the genes of interest in these regions. Inactivation of TSG's is thought to be the major consequence of the alterations found, since most alterations on gene level are deletions.

Other mechanisms for carcinogenesis, like gene amplification and microsatellite instability (MSI), seem less common in PC [Gao et al '94, Egawa et al '95, Watanabe et al '95, Ross et al '97]. However, this does not mean that these mechanisms cause minor changes. The meaning of MSI is not clear yet, it may occur during early stages of neoplastic transformation in a subset of PC. It is much more prevalent

in tumor material from US men than in Japanese. The amplification of e.g.the AR receptor seems a possible major mechanism whereby a subset of PC's relapse in spite of androgen deprivation [Visakorpi et al '95B, Koivisto et al '97]. Another example of a mechanism which may be important in PC is the amplification of the HER-2/neu oncogene that was found to correlate with tumor grade as well as with recurrence [Ross et al '97]. Telomerase activity, which is undetectable in normal cells, was found in in 84% of PC's [Kallioniemi '95]. This mechanism deserves wider attention in PC.

Concluding remarks and future

PC is characterized by both intratumor heterogeneity (the coexistence of multiple genetically related, but different cell clones) and multifocal growth (genetically unrelated clones), both suggestive of a multiclonal origin of the disease. We have shown that with molecular cytogenetic analysis of PC cell suspensions with a high percentage of tumor cells this heterogeneity can be identified, because populations with a different ploidy status may be distinguished within the same tumor.

The disease spectrum of PC is broad, ranging from latent lesions (histologic cancer, PIN) to clinical manifest adenocarcinoma, followed by aggressive metastatic and eventually hormone refractory cancer. A multistep hypothesis for gradual malignant transformation and cancer progression needs to be developed, which accounts for accumulation of genetic changes affecting the expression of critical genes. The major mechanism by which progression takes place is probably genetic instability, which renders tumors capable to adapt to changes in their environment therefore providing an advantage for survival of certain cells when environmental pressure (e.g.hormonal therapy) is applied. Possible molecular mechanisms for genomic instability include alterations of not only TSG's and oncogenes, but also genes encoding DNA repair enzymes and genes responsible of cell cycle control mechanisms. As we have seen, the damage is often extensive, and involves numerous genomic loci. A number of these loci can now be linked to defined stages in the development of the tumor. We have shown that aberrations of chromosomes 7, 8 and 10 are correlated to PC progression. Still, the picture is far from complete.

The major rate limiting step in PC tumorigenesis is progression from latent to clinical PC, since 9 out of 10 histologic lesions never become clinically manifest. Factors predicting which histologic PC will progress to aggressive behaviour are thus far lacking.

Further study of hereditary factors (what is the function of the HPC1 gene and is it the only susceptibility gene), but also of environmental factors (dietary factors, sexual activity, etc) hopefully will provide more insight.

Other important hallmarks are the development of metastasizing clones and progression to hormone independence. We have shown that a region on 8p is possibly linked to androgen insensitivity. Investigation of the genetic events that characterize the hallmarks in the biological history of PC must be the main future subjects of investigation. The basic outlines of these events are now emerging.

Future research needs to be multidisciplinary, cytogenetic as well as molecular (cyto)genetic, employing the specific point of view of each research field and using the different techniques with the aim to complement the results. In this perspective, karyotype analysis can be used to identify structural rearrangements at specific sites and provide targets for microdissection. Through FISH the DNA sequence copy number at specific loci can be investigated. FISH on tissue sections allows analysis of tumors within their histological context, e.g. to investigate the status of premalignant lesions, intratumor heterogeneity and multiclonality. CGH, combined with LOH analysis of specific loci, can give a comprehensive assessment of gene dosage imbalance throughout the genome.

Table 1. Chromosomal regions that are rearranged in PC

CHROMOSOME	CHROMOSOMAL REGION (CANDIDATE GENE)	TECHNIQUES, EXPRESSION STUDIES, MSI	COMMENTS
1	1p	cytogenetics, FISH	frequently rearranged region
	1q24-q25 (*HPC1*)	FISH, LOH, linkage analysis	first hereditary gene
2	2q	cytogenetics	-
3	3p24-p26 3p22-p12 3p14	cytogenetics, LOH, FISH	
4	-	-	-
5	-	cytogenetics	-
	5q21 (*APC*) 5q22 (α-catenin)	LOH, CGH, reduced expression	in recurrent tumors, possible TSG
6	6p	MSI	-
	6q23 6q14-q21	cytogenetics, CGH, LOH	possible TSG
7	-	cytogenetics, FISH	association with metastatic tumors and poor differentiation, gain also in PIN lesions
	7p13 (*EGFR*)	LOH, CGH	association with advanced stage and poor differentiation
	7q22 7q31.1-q31.2	cytogenetics, LOH	in early tumors, possible TSG
8	-	cytogenetics, FISH	gain marker for clinically aggresive tumors, gain also in PIN lesions
	8p21.3-p22 (*PRLTS*) 8p11-p12	cytogenetics, LOH, CGH, MSI	association with tumor progression, MSG activity, possible TSG
	8q24 (*C-MYC*)	LOH, CGH, overexpression	association with tumor progression
9	9p (*CDKN2*)	CGH, LOH	late event
	9q	CGH	-
10	-	cytogenetics, FISH, CGH	in clinically aggresive tumors, gain also in PIN lesions
	10p11.2-pter	CGH, LOH	association with progression and in less invasive tumors

Table 1, continued

CHROMOSOME	CHROMOSOMAL REGION (CANDIDATE GENE)	TECHNIQUES	COMMENTS
10	10q24-qter(*MXI1*)	cytogenetics, LOH	TSG activity; also in PIN lesions
11	11p11.2-p13 (*KAI1*)	chromosome and gene transfer, reduced expression	MSG activity
	11q	hypermethylation, reduced expression	-
12	12pter-q13	CGH, chromosome transfer	TSG activity
13	13q (*RB1*)	cytogenetics, LOH, CGH, MSI, reduced expression	-
14	-	-	-
15	-	cytogenetics, ISH	-
16	16p	CGH	-
	16q23-qter (*CDH1*) 16q22.1-q22.3	cytogenetics, FISH, LOH, CGH, reduced expression, MSI	reduced expression association with poor prognosis
	16q23.2-q24.1 (PBP of *C-MYC*) 16q24.3-qter	cytogenetics, FISH, LOH, CGH	possible TSG, MSG activity , invasiveness related
17	17pter-q23	chromosome transfer	MSG activity, possible TSG
	17p13.3 (*P53*)	LOH, CGH, MSI, hypermethylation, reduced expression	association with advanced stage and poor prognosis
	17q12-q22 (*BRCA1*)	cytogenetics, LOH	
18	-	FISH	-
	18q21.3 (*DCC*)	cytogenetics, LOH, CGH, reduced expression, MSI	-
	18q (*BCL-2*)	cytogenetics, overexpression	in recurrent tumors
19	19q (*C-CAM*)	reduced expression	also in BPH and PIN
20	-	CGH	-
21	-	-	-
22	-	CGH	-
X	Xq11-q13 (*AR*)	FISH, CGH, mutations, overexpression	in recurrent tumors
Y	-	cytogenetics, FISH	also in PIN lesions

Notes
[1]α-catenin is the e-cadherin binding protein. [2]PBP= promotor binding protein

LITERATURE

Alers JC, Krijtenburg PJ, Vissers KJ, Bosman FT, Kwast van der TH, Dekken van H. Interphase cytogenetics of prostatic adenocarcinoma and precursor lesions: Analysis of 25 radical prostatectomies and 17 adjacent prostatic intraepithelial neoplasias. Genes Chromos Cancer 12 :241-250, 1995.(A)

Alers JC, Krijtenburg PJ, Vissers CJ, Bosman FT, Kwast van der TH, Dekken van H. Cytogenetic heterogeneity and histologic tumor growth patterns in prostatic cancer. Cytometry 21: 84-94, 1995.(B)

Alers JC, Krijtenburg PJ, Hop WCJ, Bolle WABM, Schröder FH, Kwast van der TH, Bosman FT, Dekken van H. Longitudinal evaluation of cytogenetic aberrations in prostatic cancer: tumors that recur in time display an intermediate genetic status between non-persistent and metastatic tumors. Thesis Alers JC, submitted 1997.

Aly M, Dal Cin P, van de Voorde W, van Poppel H, Ameye F, Baert L, van den Berghe H. Chromosome abnormalities in benign prostatic hyperplasia. Genes Chromos Cancer 9: 227-233, 1994.

Bova GS, Isaacs WB. Review of allelic loss and gain in prostate cancer. World J Urol 14: 338-346, 1996.

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

Cooney KA, McCarthy JD, Lange E, Huang L, Miesfeldt S, Montie JE, Oesterling JE, Sandler HM, Lange K. Prostate cancer susceptibility locus on chromosome 1q: a confirmatory study. J Natl Cancer Inst 89: 955-959, 1997.

Dong JT, Rinker-Scheaffer CW, Ichikawa T, Barrett JC, Isaacs JT. Prostate cancer: biology of metastasis and its clinical implications. World J Urol 14: 182-189, 1996.

Egawa S, Uchida T, Suyama K, Wang C, Ohori M, Irie S, Iwamura M, Koshiba K. Genomic instability of microsatellite repeats in prostate cancer: relationship to clinicopathological variables. Cancer Res 55: 2418- 2421, 1995.

Gao X, Wu N, Grignon D, Zacharek A, Liu H, Salkowski A, Li G, Sakr W, Sarkar F, Porter AT, Chen Q, Honn KV. High frequency of mutator phenotype in human prostatic adenocarcinoma. Oncogene 9: 2999-3003, 1994.
Geburek BM, Kollmorgen TA, Qian J, D'Souza-Geburek SM, Lieber MM, Jenkins RB. Chromosomal anomalies in stage D1 prostate adenocarcinoma primary tumors and lymph node metastases detected by fluorescence in situ hybridization. J Urol 157: 223-227, 1997.

Huang SF, Xiao S, Renshaw AA, Loughlin KR, Hudson TJ, Fletcher JA. Fluorescence in situ hybridization evaluation of chromosome deletion patterns in prostate cancer. Am J Pathol 149: 1565-1573, 1996.

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

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

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

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

Komiya A, Suzuki H, Ueda T, Yatani R, Emi M, Ito H, Shimazaki J. Allelic losses at loci on chromosome 10 are associated with metastasis and progression of human prostate cancer. Genes Chrom Cancer 17 :245-253, 1996.

Komiya A, Suzuki H, Ueda T, Aida S, Ito N, Shiraishi T, Yatani R, Emi M, Yasuda K, Shimasaki J, Ito H. PRLTS gene alterations in human prostate cancer. Jpn J Cancer Res 88: 389-393, 1997.

Latil A, Fournier G, Cussenot O, Lidereau R. Differential chromosome allelic imbalance in the progression of human prostate cancer. J Urol 156: 2079-2083, 1996.

Li J, Yen C, Liaw D, Podsypanina K, Bose S, Wang SI, Puc J, Miliaresis C, Rodgers L, McCombie R, Bigner SH, Giovanella BC, Ittman M, Tycko B, Hibshoosh H, Wigler MH, Parsons R. PTEN, a putative protein tyrosine phosphatase gene mutated in human brain, breast, and prostate cancer. Science 275: 1943-1947, 1997.

Lieber MM. DNA ploidy in prostate cancer: potential measurement as a surrogate en point biomarker. J Cell Biochem Suppl 19: 246-248, 1994.

Lundgren R, Heim S, Mandahl N, Anderson H, Mitelman F. Chromosome abnormalities are associated with unfavorable outcome in prostate cancer patients. J Urol 147 :784-788, 1992.

MacGrogan D, Levy A, Bova GS, Isaacs WB, Bookstein R. Structure and methylation-associated silencing of a gene within a homozygously deleted region of human chromosome band 8p22. Genomics 35: 55-65, 1996.

Muleris M, Almeida A, Gerbault-Seureau M, Malfoy B, Dutrillaux B. Detection of DNA amplification in 17 primary breast carcinomas with homogeneously staining regions by a modified comparative genomic hybridization technique. Genes Chromos Cancer 10 :160-170, 1994.

Murakami YS, Albertsen H, Brothman AR, Leach RJ, White RL. Suppression of the malignant phenotype of human prostate cancer cell line PPC-1 by introduction of normal fragments of human chromosome 10. Cancer Res 56: 2157-2160, 1996.

Netto GJ, Humphrey PA. Molecular aspects of human prostatic carcinoma. Am J Clin Pathol 102(Suppl1): S57-S64, 1994.

Nihei N, Ichikawa T, Kawana Y, Kuramochi H, Kugoh H, Oshomura M, Hayata I, Shimazaki J, Ito H. Mapping of metastasis suppressor gene(s) for rat prostate cancer on the short arm of human chromosome 8 by irradiated microcell-mediated chromosome transfer. Genes Chrom Cancer 17 :260-268, 1996.

Qian J, Bostwick DG, Takahashi S, Borell TJ, Herath JF, Lieber MM, Jenkins RB. Chromosomal anomalies in prostatic intraepithelial neoplasia and carcinoma detected by fluorescence in situ hybridization. Cancer Res 55: 5408-5414, 1995.

Ross JS, Sheehan CE, Hayner-Buchan AM, Ambros RA, Kallakury BV, Kaufman RP, Fisher HA, Rifkin MD, Muraca PJ. Prognostic significance of HER-2/neu gene amplification status by fluorescence in situ hybridization of prostate carcinoma. Cancer 79: 2162-2170, 1997.

Smith JR, Freije D, Carpten JD, Gronberg H, Xu J, Isaacs SD, Brownstein MJ, Bova GS, Guo H, Bujnovszky P, Nusskern DR, Damber JE, Bergh A, Emanuelsson M, Kallioniemi OP, Walker-Daniels J, Bailey-Wilson JE, Beaty TH, Meyers DA, Walsh PC, Collins FS, Trent JM, Isaacs WB. Major susceptibility locus for prostate cancer on chromosome 1 suggested by a genome-wide search. Science 274: 1371-1374, 1996.

Trybus TM, Burgess AC, Wojno KJ, Glover TW, Macoska JA. Distinct areas of allelic loss on chromosomal regions 10p and 10q in human prostate cancer. Cancer Res 56: 2263-2267, 1996.

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

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

Vocke CD, Pozzatti RO, Bostwick DG, Florence CD, Jennings SB, Strup SE, Duray PH, Liotta LA. Emmert-Buck MR, Linehan WM. Analysis of 99 microdissected prostate carcinomas reveals a high frequency of allelic loss on chromosome 8p12-p21. Cancer Res 56: 2411-2416, 1996.

Watanabe M, Imai H, Shiraishi T, Shimazaki J, Kotake T, Yatani R. Microsatellite instability in human prostate cancer. Br J Cancer 72: 562-564, 1995.

Webb HD, Hawkins AL, Griffin CA. Cytogenetic abnormalities are frequent in uncultured prostate cancer cells. Cancer Genet Cytogenet 88 :126-132, 1996.

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

Zitzelsberger H, Szucs S, Robens E, Weier HU, Höfler H, Bauchinger M. Combined cytogenetic and molecular genetic analyses of fifty-nine untreated human prostate carcinomas. Cancer Genet Cytogenet 90 :37-44, 1996.

SUMMARY

SUMMARY

The incidence of clinical prostate cancer throughout the Western world has shown a gradual increase over the last decades. Since the prevalence of histologic PC is the same throughout the world, but clinical cancer is not, it is clear that not only the initial neoplastic transformation event followed by tumor growth can be responsible for the development of a clinically evident PC. This has triggered increasing interest in studies determining the factors that are implicated in the promotion of a histological tumor to a clinical cancer. These can be classified as either environmental, endogenous or familial.

Epidemiologic studies have indicated, among other factors, the involvement of diet, of sexual activity patterns and of occupational hazards. Family studies showed that about 9% of all PC's apparently are the result of a genetic predisposition through a single rare autosomally dominant susceptibility gene that has a very high penetrance rate.

Other research areas of continued interest and importance are the characterization of exactly those tumors that will metastasize and those that will escape hormonal control. In both areas efforts are focussed on finding suitable markers to discriminate between aggressive and non-aggressive tumors. The best prognostic markers at present still are pathological stage and grade. Promising new additional markers are IGF-I, nuclear features, neuroendocrine markers, adhesion molecules, growth fraction, DNA ploidy and genetic markers.

In general, (cyto)genetic study of PC has importance for all factors causing and promoting PC, since each permanent change of biological behavior in the tumor cells will be based on a change at the genetic level.

This thesis describes our contributions to the determination of some areas in the human genome that are important in the development and progression of PC. In this respect it can be regarded as a record of the developments in prostate cancer cytogenetics. It also contains several contributions to technical developments in the solid tumor cytogenetics research field.

In chapter 3 we report our investigations on the value of different short term in vitro culture- and karyotyping techniques and on a karyotyping study on short term cultured cells from PC. The most suitable technique for culture was collagenase digestion to obtain a cell suspension followed by size fractionation and short term culture in a completely synthetic medium that contained virtually every compound that could in any way have a stimulating effect on PC cells. The karyotyping techniques used for haematological malignancies were made suitable for PC, for instance through adaptations in the isolation of nuclei. We found clonal aberrations in 8 out of the 39 cases (21%) that yielded metaphases. The clonal numerical chromosomal changes found were loss of the Y chromosome and gain of chromosome 8. Breakpoints in clonal structural chromosomal aberrations were at 3q13, 3q21, 11p11, 17p11, 17p12, 21q11 and 21q22. Furthermore some karyotypes showed double minutes and numerous nonclonal aberrations were found.

Subsequently two aspects of the question why a much larger percentage of abnormalities is commonly detected in DNA flow cytometry (DNA-FCM) studies than by karyotype analysis were addressed. In chapter 4.1, comparison of DNA histograms of tumor samples obtained before and after collagenase treatment showed that through collagenase digestion about 50% of the PC cells were lost. It is conceivable that the collagenase renders the cell membranes of tumor cells so fragile that they may rupture easily when mechanically dissociated by pipetting.

After culture a reduction of DNA aneuploid nuclei ranging from 44 to 85% was found, suggestive of preferential growth of normal epithelial cells (chapter 4.2). So we calculated that after both collagenase digestion and short term culture the proportion of PC cells in the samples analysed was reduced by 75%.

In chapters 5 and 6 the cytogenetic characterization of PC specimens (and also some benign prostatic hyperplasia (BPH) samples) was focussed on numerical aberrations of a panel of chromosomes. These were selected based on the frequent aberrations of these chromosomes described in the literature. When the fluorescence in situ hybridization (FISH) technique with DNA probes was made applicable to solid tumor samples, prostate nuclei could be analysed without the need for tissue culture. In chapter 5 whole chromosome loss and gain of chromosomes 1, 18 and Y were investigated by FISH, in relation to each other, in relation to tumor stage and grade and in relation to overall ploidy. In chapter 6 numerical aberrations of chromosomes 1, 7, 8, 10, 18 and Y were investigated also by FISH analysis, in relation to overall ploidy, disease outcome, tumor stage and grade and patient survival.

Nearly all PC specimens showed numerical aberrations of one or more chromosomes, while none of the benign specimens showed any chromosomal aberration. Gain of a chromosome was more frequent than loss.

Comparison of DNA-FCM and FISH results showed that not only in DNA aneuploid tumors, but also in most DNA diploid tumors numerical chromosome aberrations occurred. This comparison also showed that, with an increasingly aberrant histogram by FCM, numerical changes became more frequent. In FISH analyses aneuploid tumors always showed cell populations with triploid as well as tetraploid copy numbers of the chromosomes investigated.

Simultaneous loss and gain of one or more chromosomes in the same tumor specimen was a recurrent observation. This finding could be explained by assuming that scattered areas of gain and loss can occur within one and the same tumor. The observation that in 19% of the tumors the numbers of copies for chromosomes 1, 18 and Y were not the same (chapter 5) supports this cytogenetic manifestation of tumor heterogeneity.

Gain of chromosomes 7, 8 and 10 correlated with metastatic disease. The average number of copies (ANC) of chromosomes 7 and 10 was significantly higher in lymph node metastasis (LM) than in radical prostatectomy (RP) specimens. For chromosome 8 the ANC was higher in LM and transurethral resection specimens than in RP specimens. While loss of chromosome 8 occurred mainly in samples from nonmetastatic patients, loss of chromosome 1 correlated with metastatic disease. For both chromosome 8 and 18 high polysomy percentages correlated with short survival.

In chapter 7.1 LNCaP, a human PC cell line with sublines with different androgen responsiveness, was cytogenetically characterized. In the sublines the original karyotype was well preserved, but all showed also additional, subline specific secondary marker chromosomes. Comparison showed that in both nonresponsive sublines a rearrangement of the short arm of chromosome 8 had occurred. The breakpoints were at 8p21and 8p23, respectively, resulting in the commonly deleted region 8p236pter. By describing this observation we were the first to link 8p aberrations with progression to androgen independence.

At the moment, the short arm of chromosome 8 is the most extensively studied chromosomal region in prostate cancer. The 8p116p12 area and the 8p22 area deserve special interest, because for both areas strong indications for the presence

of a tumor suppressor gene (TSG) were reported. In chapter 7.2 we investigated the chromosome 8 aberrations in the LNCaP sublines in more detail by performing FISH analysis with three cosmids mapping to the 8p region. Cosmid analysis demonstrated that in all sublines, androgen responsive or not, deletions in the 8p region were present, although by whole chromosome paint most cells seemed to have four normal copies of chromosome 8. The differences between androgen responsive and nonresponsive sublines were in the number of copies per cosmid. In both androgen responsive sublines most cells showed only two copies of cosmids D8S7 (8p23.3) and LPL (8p22), while the cosmid PLAT (8p11.1) was present in 1-4 copies. Both androgen unresponsive sublines however, showed a larger number of copies (3-4) for all three cosmid loci. We stated that this difference may be the result of nondisjunction as a reaction to loss of other sequences on 8p, possibly the TSG mapping to 8p12. Following the concept of two separate TSG's on 8p, it is conceivable that in both responsive sublines only the 8p22 TSG was inactivated, and in both nonresponsive both TSG's.

PC is characterized by both intratumor heterogeneity (the coexistence of multiple genetically related, but not identical clones) and multifocal growth (genetically unrelated clones), both suggestive of multiclonal development. We have shown that through molecular cytogenetic analysis of PC this heterogeneity can be identified, because populations with different ploidy could be detected in the same tumor.

We have made a contribution to the development of a multistep hypothesis for malignant transformation and cancer progression by showing that aberrations of chromosomes 7, 8 and 10 are correlated to PC progression.

An important hallmark in the development of PC is progression to hormone independence. We have shown that a region on 8p is possibly linked to this phenomenon.

Future research needs to be multidisciplinary, cytogenetic as well as molecular (cyto)genetic, optimally combining the specific points of view and the technical potentials of the contributing disciplines.

SAMENVATTING

SAMENVATTING

De laatste tientallen jaren is het aantal ziektegevallen van prostaatkanker in de gehele Westerse wereld geleidelijk gestegen. De ziektegevallen kunnen in twee categorieën verdeeld worden. Enerzijds zijn er de gevallen die bij toeval ontdekt worden, waarbij de patient zich niet ziek voelt en geen symptomen heeft: het latente prostaatcarcinoom. Anderzijds is er prostaatkanker waarbij de patient wel symptomen heeft, zich ziek voelt en indien niet succesvol behandeld, aan zal overlijden: het klinisch manifest prostaatcarcinoom. De stijging betreft de tweede categorie, terwijl het aantal latente gevallen gelijk is gebleven. Er moeten dus factoren zijn, die na het ontstaan van de latente kanker de tumor aanzetten tot de kwaadaardige groei die leidt tot een klinisch manifeste tumor. Er is dan ook een steeds groeiende interesse in studies waarbij gezocht wordt naar deze factoren. Zulke factoren kunnen bepaald worden door afkomst (familiair), de omgeving of ze kunnen vanuit de patient zelf ontstaan (endogeen).

In grootschalige studies zijn verhoogde risico's voor het krijgen van een klinisch manifest prostaatcarcinoom aangetoond met betrekking tot bepaalde eetgewoonten (bv. zuivelproducten en dierlijke vetten), het patroon van sexuele activiteit (bv. wisselende contacten, venerische ziekten) en bij bepaalde beroepen (bv. blootstelling aan zware metalen). Zeer opmerkelijk is, dat bij mensen die vanuit een land met een laag aantal ziektegevallen naar een land met een hoger aantal verhuisden, een verhoging van het aantal ziektegevallen gevonden werd. Dit is een sterke aanwijzing dat omgevingsfactoren belangrijk zijn.

In familiestudies, waarbij onderzocht werd in hoeverre prostaatkanker erfelijk is, kwam naar voren dat 9% van alle prostaattumoren waarschijnlijk erfelijk zijn.

Een belangrijke vraag voor de keuze van de soort behandeling is, of een tumor zal uitzaaien (metastaseren) of niet. Er worden studies gedaan die trachten een vroegtijdig onderscheid te maken tussen de tumoren die in de toekomst zullen metastaseren en degenen die dit niet zullen doen. Gezocht wordt naar eigenschappen die wel aanwezig zijn bij de ene groep tumoren en niet bij de andere (markers). Op het moment zijn de best onderzochte en betrouwbaarste prognostische (voorspellend omtrent het verdere verloop van de ziekte) markers de pathologische stagering en gradering. Veelbelovende markers zijn IGF-I (een groeihormoon), nucleaire (celkern) eigenschappen, neuroendocriene markers (verband houdend met hormonen), adhesie (hechtings-)factoren, de groeifractie (het percentage groeiende cellen), DNA ploidie (hoeveelheid DNA -erfelijk materiaal- per cel) en verschillende genetische markers.

In het algemeen is genetische studie (onderzoek aan het totale DNA -het genoom) van prostaatkanker belangrijk voor alle factoren die betrokken zijn bij de vorming en ontwikkeling van prostaatkanker. Dit proefschrift beschrijft onze bijdragen aan het aanwijzen van verschillende delen van het genoom die belangrijk zijn in de ontwikkeling en de progressie (voortschrijden) van prostaatkanker. Ook kan dit proefschrift beschouwd worden als een verslag van de ontwikkelingen in het prostaatkanker chromosoomonderzoek. Chromosomen zijn celkernlichaampjes waarop de erfelijke eigenschappen liggen, er zijn er 46 in een normale cel, 22 genummerde paren en twee zg.geslachtschromosomen, X en Y.

In hoofdstuk 4 hebben wij de waarde van verschillende in vitro (in een kweekbakje) kweek- en karyotyperings (chromosoomgroeperings)technieken onderzocht en tevens een karyotyperingsstudie op gekweekte cellen van tumoren van prostaatkankerpatiënten beschreven. De beste kweekmethode bleek degene te zijn waarbij gebruik gemaakt werd van collagenase (steunweefsel afbrekend

enzym) om een celsuspensie (losse cellen) te verkrijgen. Vervolgens werden de celklompjes op grootte gescheiden en tenslotte werd er gekweekt in een volledig synthetisch medium dat nagenoeg alle stoffen bevatte die op enigerlei wijze een stimulerend effect op de groei van prostaatkankercellen zouden kunnen hebben. De technieken gebruikt voor de karyotypering van bloedtumoren werden bruikbaar gemaakt voor prostaatkankercellen door onder andere de wijze van isolatie van de celkernen aan te passen. Karyotypering leverde clonale afwijkingen (=in meer dan twee cellen aanwezig) op in 8 van de 39 (21%) gevallen. De clonale veranderingen in aantal chromosomen waren verlies van het Y chromosoom en een extra chromosoom 8. Structurele (in één chromosoom) clonale veranderingen werden zichtbaar als breukpunten op 3q13, 3q21, 11p11, 17p11, 17p12, 21q11 en 21q22 (notatie: chromosoom nummer-korte (p) of lange arm (q)- plek op de arm). Verder werden in enkele karyotypes double minutes (losse, kleine stukjes chromosoom) gevonden. Niet clonale veranderingen, zowel numeriek als structureel, kwamen veel voor.

Vervolgens werden twee aspecten van een vraag onderzocht die velen die aan prostaatkanker chromosoomonderzoek werkten bezighield, nl: Waarom worden er in DNA flow cytometrie (methode om het DNA gehalte per cel te meten (DNA-FCM)) studies consequent zoveel meer afwijkende prostaattumoren gevonden dan door karyotypering? In hoofdstuk 5.1 werd door middel van DNA-FCM aangetoond dat door collagenase behandeling ongeveer 50% van de tumor cellen verloren gaat. Wij concludeerden dat het aannemelijk is dat de collagenase de celmembranen van de tumorcellen dermate kwetsbaar maakt dat ze gemakkelijk scheuren wanneer er enige mechanische druk (in dit geval door pipetteren- opzuigen in een holle buis) op uitgeoefend wordt. In hoofdstuk 5.2 werd na kweken een terugval in de hoeveelheid celkernen met een abnormaal DNA gehalte met 44 tot 85% gevonden, volgens ons veroorzaakt door de groei van bij voorkeur normale cellen. Over het geheel berekend, dus na collagenase behandeling en kortdurende weefselkweek, was de hoeveelheid prostaatkankercellen gedaald met 75%.

In de hoofdstukken 6 en 7 werd van een aantal kwaadaardige, en tevens van een aantal goedaardige (BPH's) prostaattumoren, eventuele numerieke afwijkingen van zes verschillende chromosomen bekeken. Deze chromosomen werden gekozen omdat ze in de vakliteratuur het meest genoemd waren in verband met afwijkingen bij prostaatkanker. Door de fluorescentie in situ hybridisatie (FISH) techniek met DNA probes (een methode om het aantal kopieën van een specifiek chromosoom direct op de kern zichtbaar te maken) hiervoor geschikt te maken, konden de prostaatkernen nu geanalyseerd worden zonder de noodzaak van weefselkweek. In hoofdstuk 6 werden door middel van FISH analyse de numerieke veranderingen in prostaatkanker en BPH bekeken van de chromosomen 1, 18 en Y. De uitkomsten werden vergeleken tussen de chromosomen onderling, en in relatie tot de totale DNA inhoud. In hoofdstuk 7 werden met dezelfde technieken de chromosomen 1, 7, 8, 10, 18 en Y in een uitgebreider aantal prostaattumoren en BPH's bekeken. Terwijl in geen van de onderzochte BPH's enige afwijking aangetoond kon worden, vertoonden bijna alle prostaattumoren numerieke afwijkingen voor één of meer chromosomen, waarbij extra kopieën meer voorkwamen dan chromosoom verlies.

Vergelijking van DNA-FCM en FISH resultaten toonde aan dat niet alleen in DNA aneuploide (abnormale hoeveelheid DNA) tumoren, maar ook in DNA diploide (normale hoeveelheid DNA) tumoren afwijkingen voorkomen. Ook werd bewezen dat, zoals verwacht, met het toenemen van de afwijkingen in de hoeveelheid DNA, meer chromosomen afwijkingen vertoonden. Verder werden er altijd zowel triploide-

(1,5x de normale hoeveelheid DNA, 3 kopiën van een chromosoom) als tetraploide (2x de normale hoeveelheid DNA, 4 kopiën van een chromosoom) cellen gezien. Herhaaldelijk werd aangetoond dat zowel cellen met verlies als met extra kopieën van hetzelfde chromosoom in één tumor sample (gedeelte van de tumor dat voor onderzoek gebruikt wordt) aanwezig waren. Dit werd verklaard door aan te nemen dat binnen één en dezelfde tumor verschillende gebieden bestaan waar cellen met verlies of extra chromosomen voorkomen. Dit wordt cytogenetische heterogeniteit genoemd. Ook het feit dat in 19% van de tumoren binnen dezelfde tumor een verschillend aantal kopieën voor de chromosomen 1, 18 en Y gevonden werden, ondersteunt dit. De aanwezigheid van extra kopieën van de chromosomen 7, 8 en 10 in de tumor was gecorreleerd met de aanwezigheid van metastasen bij de patiënt. Het gemiddeld aantal kopieën van de chromosomen 7, 8 en 10 was significant hoger in samples van lymfeknoop metastasen, dan in samples van operaties waarbij de gehele prostaat verwijderd werd. Verlies van chromosoom 8 kwam vooral voor in samples van patiënten zonder metastasen, terwijl verlies van chromosoom 1 juist correleerde met metastasering. Hoge percentages cellen met veel kopieën van zowel chromosoom 8 als 18 correleerden met een korte overleving.

Bij prostaatkanker is een belangrijk kenmerk van progressie, dat de tumor niet meer op androgenen reageert. In hoofdstuk 8.1 werd een menselijke prostaatkanker cellijn (LNCaP), waarvan de daarvan afgeleide cellijnen verschillend op androgenen (mannelijke hormonen) reageren, cytogenetisch gekarakteriseerd. Dit werd gedaan om cytogenetische verschillen tussen deze cellijnen op te sporen. In de afgeleide cellijnen was het oorspronkelijke karyotype goed bewaard gebleven, met per cellijn een aantal specifieke later gevormde marker chromosomen. Onderlinge vergelijking van deze cellijnen toonde aan dat de korte arm van chromosoom 8 was veranderd in de cellijnen die niet op androgenen reageren. De breekpunten lagen op 8p21 en 8p23, respectievelijk, zodat in beide 8p236pter verloren was. Met deze belangwekkende observatie waren wij de eersten die 8p afwijkingen verbonden aan de progressie van prostaatkanker.

Op dit moment is de korte arm van chromosoom 8 de meest bestudeerde chromosomale regio in prostaatkanker. Het 8p116p12 en het 8p22 gebied hebben speciaal de interesse, want er zijn sterke aanwijzingen dat in beide gebieden één of meer tumor suppressor genen (genen die in de normale cel het ontstaan van tumoren onderdrukken) gelokaliseerd zijn . In hoofdstuk 8.2 werden de afwijkingen van chromosoom 8 in LNCaP verder onderzocht door middel van FISH analyse met drie cosmiden (relatief grote stukjes DNA) die gelokaliseerd zijn op verschillende delen van de 8p regio. Deze analyse toonde aan dat in alle sublijnen, androgeen responsief (reagerend op androgenen) of niet, deleties (verlies van stukken DNA) in het 8p gebied voorkwamen, hoewel met een 'whole chromosome paint' (kleuring van het gehele chromosoom) in bijna alle cellen vier normale chromosomen 8 werden gezien. De verschillen tussen de sublijnen lagen vooral in het aantal kopieën per cosmide. In beide androgeen responsieve lijnen vertoonden de meeste cellen twee kopieën van D8S7 (8p23.3) en LPL (8p22), terwijl van PLAT (8p11.1) één tot vier kopieën aanwezig konden zijn. Beide niet responsieve lijnen vertoonden echter meer kopieën voor alle cosmiden (drie tot vier). Een verklaring kan zijn dat dit verschil het resultaat is van een reactie op het verlies van andere gedeelten van 8p, mogelijk het tumor suppressor gen op 8p12. Als je aanneemt dat op 8p twee afzonderlijke tumor suppressor genen liggen, dan is het aannemelijk dat in de responsieve lijnen alleen het tumor suppressor gen van 8p22 is geïnactiveerd, en in de niet-responsieve lijnen beide.

Prostaatkanker wordt gekarakteriseerd door zowel het naast elkaar groeien van meerdere verwante, maar niet identieke klonen (cellen oorspronkelijk afkomstig van één cel) als door het naast elkaar groeien van niet verwante klonen binnen dezelfde tumor. Dit wordt respectievelijk intratumor heterogeniteit en multifocale groei genoemd. Beide verschijnselen leiden tot de gedachte dat bij de groei van een prostaattumor meerdere klonen tot ontwikkeling komen. Door het aantonen van groepen cellen met een verschillende ploidie (DNA hoeveelheid, totaal per cel of per chromosoom) in één tumor hebben wij laten zien dat met moleculair cytogenetische analyse van prostaatkanker samples deze verschijnselen kunnen worden aangetoond.

Bovendien hebben wij aangetoond dat afwijkingen van de chromosomen 7, 8 en 10 zijn gecorreleerd met progressie, wat als een bijdrage beschouwd kan worden aan de ontwikkeling van een meerstaps hypothese voor gefaseerd ontstaan, ontwikkeling en voortschrijden van prostaatkanker. Een belangrijke stap in de ontwikkeling van prostaatkanker is progressie naar androgeen onafhankelijkheid. Ons onderzoek heeft de chromosomale regio 8p voor het eerst hiermee in verband gebracht.

Curriculum vitae

Josée Jeannette König
geboren op 13 december 1955 te s'Gravenhage

1962-1968	openbare lagere school te Sassenheim (ZH)
1968-1974	Gymnasium B aan het Thorbecke lyceum te s'Gravenhage
1974-1981	studie Biochemie aan de Rijksuniversiteit Leiden
1981-1982	Afd.PA Leiden, flow cytometrie onderzoek aan borsttumoren
1982-1994	Afd.Urologische Oncologie EUR, Projecten: Isolatie en karakterisering van prostate epithelium inhibiting factor; Prostaatkanker cytogenetica; Moleculaire cytogenetica van ontwikkeling en progressie van prostaatkanker.
5 oktober 1988	getrouwd met Jan Willem van Dongen
27 september 1990	geboorte Thomas en Julia
10 juni 1998	promotie

DANKWOORD

In dit stukje wil ik een poging wagen iedereen te bedanken die op één of andere manier heeft bijgedragen aan het tot stand komen van dit proefschrift en het onderzoek dat hieraan ten grondslag ligt. Hopelijk vindt iedereen die van belang is geweest zijn of haar naam hieronder terug. Zo niet, mea culpa, ik zal met liefde alsnog een persoonlijke dankzegging in zijn of haar boekje schrijven!

Ik bedank:

-Professor Schröder, die de aanzet tot het onderzoek heeft gegeven en mij in de gelegenheid heeft gesteld dit onderzoek op de afdeling Oncologische Urologie uit te voeren.

-Anne Hagemeijer voor haar uitstekende begeleiding, haar ideeën en het ter beschikking stellen van de expertise van haar afdeling. Anne, van jouw kritische kijk op alles heb ik enorm veel geleerd.

-De overige leden van de leescommissie: Wolter Oosterhuis, Ad Geurts van Kessel, Frans Ramaekers en Ton Hopman, voor de beoordeling van het manuscript.

-Alle dames van de Klinische genetica leukemiegroep voor het mij leren van hun métier, en voor de gezellige koffiepauzes, labuitjes en andere activiteiten.

-Eric Kamst voor het opstarten van het cytogenetische werk, hoewel het voor hem niet altijd gemakkelijk was, zo tussen de meisjes.

-Hans Romijn voor zijn begeleiding en zijn altijd relevante commentaar op mijn manuscripten.

-De medewerkers van het Chirurgisch lab, met name de varkens, voor het prettige werkklimaat tijdens de eerste jaren van het onderzoek.

-De medewerkers van de Pathologie voor hun bijdrage in de vorm van coupes, weefsel, uitslagen ed. Vooral de pathologen bedank ik, die altijd bereid waren steriel weefsel voor mij uit te snijden. JanTrapman en Winand Dinjens en consorten wil ik bedanken voor de prettige samenwerking mbt.de moleculair genetische experimenten.

-De fotografen Frank van der Panne van de Pathologie en Jan Tuin van het Thoraxcentrum , voor respectievelijk de goede foto's, en de hulp bij het maken van fotocompilaties op de computer.

-Ton Verkerk van de Celbiologie voor zijn hulp en grote geduld bij het helpen bedienen van de FACS II en het FCM computer programma.

-Annet Verkerk en Marjan Hettinga van het Trial bureau voor het leveren van gegevens uit de prostaatdatabank.

-Bert Eussen van de Klinische genetica voor zijn hulp bij het maken en verwerken van speciale in situ hybridizaties.

-De Nederlandse Kankerbestrijding voor het financieren van de twee projecten die tot dit proefschrift hebben geleid. De SUWO wil ik bedanken voor financiele ondersteuning bij buitenlandse reizen en de promotie. Ook alle andere sponsors bedank ik voor hun financiele ondersteuning van de promotie.

-Mijn directe collega's: GJ, Wytske, Wilma, Anko, Eric-Jan, Sigrun, Monique, Carl, en alle anderen voor hun steun en niet aflatende belangstelling. Ook wil ik dank zeggen aan de vele urologie assistenten en de buitenlandse gasten die het klimaat op het lab altijd verlevendigden. Met name wil ik Philip Weijerman bedanken die me het laatste zetje gaf mijn proefschrift af te maken. Robert Kraaij wil ik bedanken voor het herstellen van hoofdstuk 7.1 en tips mbt.het boekje.

-Mijn paranimfen Oscar König en Wilma Teubel voor al het werk en alle steun rondom de promotie.

-Wilma Teubel, want zonder haar was er geen proefschrift geweest. Wilma, jouw werklust, expertise, slimme invallen en vriendschap hebben het onderzoek mede gedragen. Ik kan je niet genoeg daarvoor bedanken.

-Hans Pijper voor zijn hulp met de lay-out van het boekje. Ook bedank ik Ad en Els den Houter, die mij in staat gesteld hebben bij hen op de drukkerij mijn foto's bij te werken. Ik bedank ook hun medewerkster Saskia van der Meer, die zo'n mooie omslag uit de computer heeft getoverd. Samen hebben ze van het boekje iets moois gemaakt.

-Pa en Ma van Dongen die altijd hebben klaargestaan als er opgehaald, opgepast enzovoort moest worden. Jullie bijdrage is van onschatbare waarde geweest, want het heeft me in staat gesteld op de momenten waarop het erop aan kwam door te kunnen werken.

-Jan Willem voor zijn liefde en steun. En onze geweldige kids, Thomas en Julia, die elke dag opvrolijken en tot een belevenis maken. Jullie onvoorwaardelijke liefde, ook voor een echtgenote en mama die weleens knorrig was en geen tijd had en moest schrijven, was en is inspirerend. Reden genoeg om het boekje aan jullie op te dragen.

BIBLIOGRAFIE

J.J.König, J.C.Romijn and F.H.Schröder. Prostatic Epithelium Inhibiting Factor (PEIF): Organ specificity and production by prostatic fibroblasts. Urol Res 15: 145-149, 1987.

J.J.König. Genetic changes associated with progression to endocrine independence in carcinoma of the breast and prostate. In: Mechanisms of progression to hormone-independent growth of breast and prostatic cancer, P.M.J.J.Berns, J.C.Romijn and F.H.Schröder (eds) Parthenon Publishing, New Yersey. pp123-134, 1991.

A.Kooistra, J.J.König, J.C.Romijn and F.H.Schröder. Negative control of epithelial cell proliferation by prostatic stroma. Anticancer Res 11: 1495-1500, 1991.

P.C.Weijerman, J.J.König, S.T.Wong, H.G.M.Niesters and D.M.Peehl. Lipofection-mediated immortalization of human prostatic epithelial cells of normal and malignant origin using human pappilomavirus type 18 DNA. Cancer Res 54: 5579-5583, 1994.

J.Trapman, H.F.B.M.Sleddens, M.M.van der Weiden, W.N.M.Dinjens, J.J.König, F.H.Schröder, P.W.Faber and F.T.Bosman. Loss of heterozygosity of chromosome 8 microsatellite loci implicates a candidate tumor suppressor gene between the loci D8S87 and D8S133 in human prostate cancer. Cancer Res 54: 6061-6064, 1994.

A.Kooistra, J.J.König, D.M.Keizer, J.C.Romijn and F.H.Schröder. Inhibition of prostatic epithelial cell proliferation by a factor secreted specifically by prostatic stromal cells. Prostate 26: 123-132, 1995.

A.Kooistra, N.M.J.Elissen, J.J.König, M.Vermey, T.H.van der Kwast, J.C.Romijn and F.H.Schröder. Immunocytochemical characterization of explant cultures of human prostatic stromal cells. Prostate 27: 42-49, 1995.

P.C.Weijerman, E.van Drunen, J.J.König, W.Teubel, J.C.Romijn, F.H.Schröder and A.Hagemeijer. Specific cytogenetic aberrations in two novel human prostatic cell lines immortalized by human papillomavirus type 18 DNA. Cancer Genet Cytogenet 99: 108-115, 1997.