INTERPHASE CYTOGENETICS OF PROSTATIC ADENOCARCINOMA

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INTERPHASE CYTOGENETICS OF PROSTATIC ADENOCARCINOMA

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PROEFSCHRIFT

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Voor mijn ouders

CONTENTS

LIST OF ABBREVIATIONS

AAS	aminoacetylsilane
ABC	avidin-biotinylated-horseradish-peroxidase complex
bp	base pair
BPH	benign prostatic hyperplasia
BT	biotinylated tyramine
CARD	catalyzed reporter deposition
CCD	charge coupled device
CGH	comparative genomic hybridization
CT	computer-tomography
DAB	diaminobenzidine
dATP	deoxyadenosinetriphosphate
DHT	5α-dihydrotestosterone
DNA	deoxyribonucleic acid
DRE	digital rectal examination
FCM	flow cytometry
FISH	fluorescent *in situ* hybridization
HE	hematoxylin eosin
ISH	*in situ* hybridization
Kb	kilobase pairs
LH	luteinizing hormone
LHRH	luteinizing hormone-releasing hormone
LOH	loss of heterozygosity
Mb	megabase pairs
MRI	magnetic resonance imaging
PAP	prostate acid phosphatase
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PIN	prostatic intraepithelial neoplasia
PPV	positive predictive value
PSA	prostate-specific antigen
PTHrP	parathyroid hormone-related peptide
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
SEER	surveillance, epidemiology, and end results
SSC	standard saline citrate
TNM	tumor, nodes and metastasis
TRUS	transrectal ultrasonography
TUR	transurethral resection
YAC	yeast artificial chromosome

CHAPTER 1

GENERAL INTRODUCTION

This chapter was published in part in "Interphase Cytogenetic Analysis of Solid Tumors by Non-Isotopic DNA *in situ* Hybridization" by J.C. Alers and H. van Dekken. Progr. Histochem. Cytochem. Series Vol. 31 (3), pp. 1-137, G. Fisher Verlag, Stuttgart, Germany (1996)

In the first part of this chapter an overview will be presented on the structural, histological and functional aspects of the normal human prostate. The second part describes the epidemiological and clinicopathological features of prostatic adenocarcinoma. Further, a state of the art of (cyto)genetic aberrations occurring in prostatic cancer is given. The third part of this introduction will discuss methodological aspects of this thesis, i.e., the development and methodology of non-isotopic in situ hybridization. Finally, the scope of this thesis will be presented.

1 THE HUMAN PROSTATE

1.1 Anatomy and Histology

The morphogenesis of the human prostate starts around the 10th week of gestation. Solid epithelial outgrowths (prostatic buds) that emerge from the endodermal urogenital sinus below the developing bladder start to evaginate into the surrounding mesenchymal tissue. The ducts grow rapidly in length, branch and canalize. By 13 weeks over 70 primary ducts are formed of which some show secretory cytodifferentiation. The prostatic buds arise from different parts of the prostatic urethra in five separate groups. Prostate and seminal vesicle development is elicited via mesenchymal-epithelial interactions. Urogenital sinus mesenchyme induces prostatic ductal morphogenesis, regulates epithelial proliferation, and specifies expression of prostate secretory proteins. While mesenchymal-epithelial interactions play a fundamental role in male urogenital development, the overall developmental process is elicited by androgens. Testosterone is the primary androgen secreted by the testes, but its conversion into the biologically more active metabolite 5α-dihydrotestosterone (DHT) by the enzyme 5α-reductase is critical for differentiation and development of the prostate and the male external genitalia. Both testosterone and DHT bind to the intracellular androgen receptor to regulate androgen-dependent cellular differentiation and function. (reviewed in Cunha *et al.*, 1987, 1996; Chung *et al.*, 1991). Between birth and puberty there is little prostatic development. At puberty, under the influence of testosterone, the prostate grows to a retroperitoneal chestnut-shaped organ of 3-4 cm in diameter with an average adult weight of 20 grams.

Classically, the prostate has been divided into anterior, middle, posterior and two lateral lobes by drawing divergent lines from the centrally located urethra. These subdivisions, however, can be recognized only in the embryo (reviewed in Cotran *et al.*, 1994). A division that correlates better with the physiologic and pathologic features of the organ is into an inner (periurethral) and an outer (cortical) zone. The inner zone is the primary site for benign prostatic hyperplasia (BPH), whereas the outer zone is the site of predilection for adenocarcinoma arising from peripheral ducts and acini. A modification of this scheme divides the prostate into peripheral, central, transitional, and periurethral gland regions. The peripheral zone is roughly

equivalent to the lateral and posterior lobes, and comprises about 70% of the mass of the glandular prostate. The central zone makes up about 25 % of the glandular prostate mass and its location approximates that of the originally described middle lobe. The transition zone contains glands that terminate in the proximal urethra and grow laterally around the distal end of the internal urethral sphincter. It represents only about 5-10% of the prostatic glandular tissue. The periurethral gland region is confined to a sleeve of the proximal urethra and is only a fraction of the size of the transition zone. According to this system, the transitional and periurethral regions are the predominant sites of origin of BPH, whereas the peripheral zone is most susceptible to inflammation and carcinoma (Fig. 1). The prostate is enveloped by the prostatic capsule, but this is not a well-defined anatomic structure with constant features. It is more evident along the base of the prostate and less so along the anterior and apical surfaces (McNeal, 1988) .

Histologically, the prostate is a compound tubuloalveolar gland, consisting of glandular structures supported by a stroma of fibromuscular connective tissue, blood vessels, lymphatics and nerves. The glandular component of the organ is composed of acini, large (primary, major, excretory), and peripheral (secondary, minor) ducts. Characteristically, within each prostate zone, the entire duct-acinar system, except for the large ducts near the urethra, is lined by two layers of cells: a basal layer of low cuboidal cells covered by a layer of columnar mucus-secreting cells lining the lumen of the gland (Fig. 2, p.17). The highly differentiated secretory cells contribute a wide variety of products to the seminal fluid. They produce prostatic acid phosphatase (PAP) and prostate-specific antigen (PSA), both of which can be readily identified immunohistochemically and have been proved of great diagnostic utility because of their organ specificity (Allsbrook and Simms, 1992). Secretory cells also coexpress various keratins and vimentin (Leong *et al.*, 1988). The secretory epithelium requires continuous support by androgens for its maintenance and widely express the nuclear androgen receptor (Masai *et al.*, 1990; Ruizeveld de Winter *et al.*, 1990, 1991; van der Kwast *et al.*, 1991). Because most prostatic adenocarcinomas express cytokeratin profiles typical of the secretory cells, usually secrete PSA and often contain androgen receptors, it is postulated that prostatic adenocarcinomas have a secretory luminal origin (reviewed in Ware, 1994). However, this issue remains rather controversial (e.g., reviewed in Bonkhoff and Remberger, 1996).

The undifferentiated basal cells form a thin continuous layer that separates the secretory cells from the basement membrane. They characteristically express high molecular weight cytokeratins, but do not express PSA or PAP (Okada *et al.*, 1992; Bonkhoff and Remberger, 1996). The basal cell layer is composed of androgen-independent cell populations and generally lacks immunoreactivity for the nuclear androgen receptor. The function of the basal cells in the non-malignant prostate is still uncertain. Some suggest that they represent a multipotential

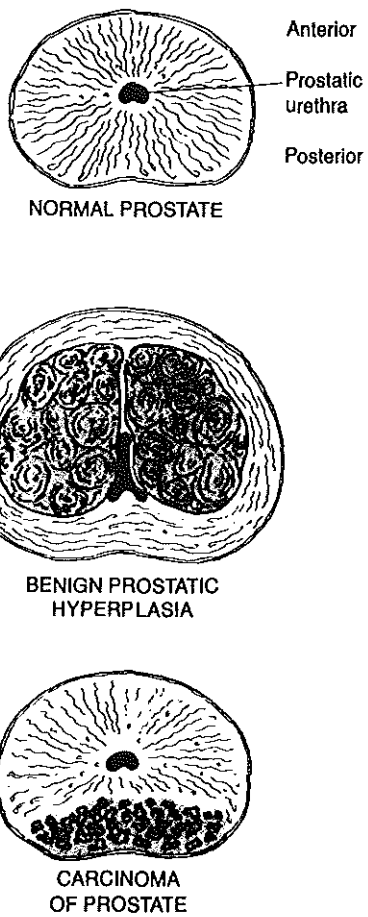


FIGURE 1.
Normal prostate, nodular hyperplasia, and adenocarcinoma. In benign prostatic hyperplasia the nodules distort and compress the urethra and exert pressure on the surrounding normal prostatic tissue. Prostatic carcinoma usually arises from peripheral glands, in which case it does not compress the urethra (After Rubin and Farber, 1984).

population that gives rise to all epithelial lineages present in the normal, hyperplastic, and cancerous prostate (Ware, 1994; Bonkhoff and Remberger, 1996). Neuroendocrine or endocrine-paracrine cells also reside among the secretory luminal cells in a scattered fashion. Neuroendocrine cells are terminal differentiated, post-mitotic cell types which lack androgen receptors (Krijnen *et al.*, 1993; Bonkhoff *et al.*, 1995; Noordzij *et al.*, 1996). These cells secrete chromogranin A and B, secretogranin II, neuron-specific enolase and serotonin. In addition, some neuroendocrine cells express various peptide hormones such as calcitonin and related peptides, as well as somatostatin and bombesin (reviewed in Noordzij *et al.*, 1995). They can coexpress PSA in a focal fashion, suggesting a common origin with the secretory cells (Aprikian *et al.*, 1993).
In contrast to the peripheral ducts, which are lined by cuboidal epithelium, the large

prostatic ducts are lined by transitional epithelium that is continuous and indistinguishable from that lining the prostatic urethra. In contrast to bladder epithelium, its surface does not display umbrella cells but rather a single layer of columnar secretory cells that are immunoreactive for PSA and PAP (McNeal, 1988).
The prostatic stroma is notable because of its large content of smooth muscle fibers, which function is to squeeze out the prostatic secretion when properly stimulated. Prostatic stromal cells have been found to express androgen receptors in a focal fashion (Ruizeveld de Winter *et al.*, 1991).

1.2 Function of the Prostate

The prostate is the largest of the male accessory sex glands (prostate, seminal vesicles and bulbourethral glands). The specific products of these glands probably have the common purpose to increase the efficiency of conception.
During spermatogenesis and epididymal maturation of the spermatozoa, the sperm cells are prepared for flagellar movement. However, the spermatozoa do not exhibit any progressive movements upon storage in the epididymal cauda. At ejaculation, the sperm-rich epididymal fluid mixes with the secretions produced by the prostate and the seminal vesicles, the seminal vesicles secretions constituting some 60% and the prostatic secretion some 30% of the ejaculated volume. The bulk of the ejaculate is immediately turned into a gelatinous meshwork in which the spermatozoa are entrapped. Analysis of the secretion produced by the seminal vesicles has shown high molecular mass complexes of semenogelin to be the predominant protein component in this fluid, as well as the major protein constituent of the seminal gel. Within 15 minutes from ejaculation, the seminal coagulum is liquefied, and the spermatozoa display vigorous progressive motility. Liquefaction of the seminal gel parallels proteolytic fragmentation of semenogelin (Lilja *et al.*, 1989). This is mainly due to the proteolytic activity of PSA, which is a major constituent of the prostatic secretion (Lilja, 1985; Lundwall and Lilja, 1987). PSA is a serine protease (Ban *et al.*, 1984; Watt *et al.*, 1986) and a member of the kallikrein gene family (Riegman *et al.*, 1989a, b). Other proteases, which are secreted by the prostate gland include kallikreins, plasminogen activator, pepsinogen II, metalloproteases, and caseinolytic and gelatinolytic activities (reviewed in Wilson, 1995). These proteases secreted into the semen have been postulated to produce liquefaction of the seminal coagulum and to possibly interact with sperm so as to modify their cell surfaces and affect their fertilizing ability.
Two other proteins, which are secreted in large amounts by the prostate are PAP and Prostatic Secretory Protein (PSP_{94}) or β-inhibin/β-microseminoprotein (Lilja and Abrahamson, 1988). Another prostate product that is secreted in relatively large amounts is Zn-α_2-Glycoprotein, a truncated secretory major histocompatibility complex related protein (Araki *et al.*, 1988). The specific physiological functions of

these proteins are still unknown.

2 PROSTATE CANCER

2.1 Epidemiology and Etiology

The incidence of prostate cancer has dramatically increased during the last two decades. It is now the most commonly diagnosed cancer (excluding skin cancer) in males in Western countries with a high socio-economic standard and its mortality is only surpassed by that of lung cancer (Potosky *et al.*, 1995; Jacobsen *et al.*, 1995; Severson *et al.*, 1995). Data from the Surveillance, Epidemiology, and End Results (SEER) program demonstrate that the age-adjusted incidence of prostate carcinoma per 100,000 American individuals has increased from 84,4 to 163 between 1984 and 1991 (Stephenson *et al.* 1996). The incidence of prostate cancer was found to increase along a linear trend in the period between 1973 to 1987, which became exponential from 1987 to 1991 (Carter and Coffey, 1990; Potosky *et al.*, 1995; Stephenson *et al.*, 1996). The linear increase in the incidence and mortality rate on an age-adjusted basis is most likely caused by a shift in the age distribution of Western males towards an increased life expectancy, as well as a possible change in risk factors for the development of prostate cancer (reviewed in Carter and Coffey, 1990). The exponential increase in incidence has been largely attributed to the increased use of PSA and transrectal ultrasonography (TRUS) in prostate carcinoma detection and population-based screening programs, rather than a true increase in incidence (Jacobsen *et al.*, 1995; Potosky *et al.*, 1995; Schröder et al., 1996; Stephenson *et al.*, 1996). Interestingly, a recent study of prostate cancer incidence trends in Utah, USA indicates that as from its top in 1992 the age-adjusted incidence rate has started to decline rapidly (Stephenson *et al.*, 1996). This phenomenon also occurred following the introduction of mammography as a population screening test for breast cancer (Harris *et al.*, 1992) and is most likely explained by the fact that these new diagnostic modalities enabled the diagnosis of cases that would otherwise have been either diagnosed later or even missed completely. These cases are progressively depleting the population of potential new cases ("cull effect"). For 1996 about 317,000 new prostate cancer cases and 41,400 prostate cancer deaths have been predicted for the USA (Parker *et al.*, 1996). The age-adjusted SEER average mortality rate in the USA in the period from 1973 to 1990 slightly increased to a number of 23.4 per 100,000 men per year (Stephenson *et al.*, 1996).

Likewise, in Western Europe the incidence of prostatic cancer has increased the past few years, albeit less dramatical than in the USA: In The Netherlands the age-adjusted incidence increased from 36 to 64.4 per 100,000 men (European Standardized Rate) between 1971 and 1992 (Eindhoven Cancer Registry). Data from the Comprehensive Cancer Centre Amsterdam reported an increase of 38% more cases in 1994 compared to 1989 (Visser and Horenblas, 1996). On the basis

of a prevalence of 253 per 100,000 men, there were about 18,000 men with a diagnosis of prostate cancer alive in 1995. The absolute number of deaths from prostate cancer in The Netherlands in 1994 was 2354 (Centraal Bureau voor de Statistiek, 1996). The mortality/incidence ratio was 0.45 in 1992. The age-adjusted mortality rate amounted to approximately 25 per 100,000 men in 1992 and this number did not change very much in the period between 1971 and 1992 (Eindhoven Cancer Registry). The cumulative risk for the development of prostatic cancer in Dutch males before the age of 75 was 4.3% in 1992 (Netherlands Cancer Registry, 1992). Of all cancers in man, the incidence of clinical prostate cancer increases most rapidly with age (Carter and Coffey, 1990; Potosky *et al.*, 1995). In The Netherlands in the period between 1989 to 1992 a 60-fold increase was observed in the incidence of clinical cancer with age from around 14 cases at the age of 50 to 54 to 889 cases per 100,000 individuals in males over the age of 85 (Netherlands Cancer Registry, 1992).

From autopsy studies and examination of prostatectomy specimens removed for BPH or cystoprostatectomy specimens excised for bladder cancer it is known that approximately 25 to 30% of men over the age of 50 years, who have no clinical evidence of prostate cancer, harbor microscopic foci of well or moderately differentiated cancer within the prostate (latent prostate cancer; reviewed in Scardino et al., 1992 and Matzkin *et al.*, 1994; Ohori *et al.*, 1994). The prevalence of latent prostate cancer increases dramatically with age: Every decade of aging doubles the incidence of such tumors from 10 % of men in their 50s to 70% in men in their 80s (reviewed in Gittes, 1991). When these numbers are extrapolated to the American male population over 50 years of age, approximately 11,000,000 American men have latent prostate cancer. However, only a small percentage (1%) become clinically manifest since in 1990 approximately 100,000 men were diagnosed with the disease (Carter and Coffey, 1990; reviewed in Coffey, 1993). Although the annual clinical incidence of prostate cancer is relatively low, the lifetime risk for a 50-year-old white male of developing *"autopsy cancer"* in 1985 was 42%, the risk of developing the disease clinically 9.5%, and the risk of dying from the disease is 2.9% (Scardino *et al.*, 1992). For a 50-year-old male it is therefore estimated that about 1 out of 4 latent prostate cancers become clinically apparent and that about 1 out of 3 patients with clinical prostate cancer eventually die of it (Scardino *et al.*, 1992). This discrepancy between the prevalence of latent and clinically manifest prostate cancer is poorly understood with respect to the factors responsible for the progression of these microscopic cancers to a more aggressive form that is clinically evident.

There are some remarkable and puzzling geographic and racial differences in the incidence of clinically diagnosed prostatic cancer. Prostatic cancer is extremely rare in Asians. The age-adjusted incidence rate (per 100,000 population) in the early 1980s (pre-PSA era) was only 0.8 case in Shanghai, China, and 4 per 100,000 in

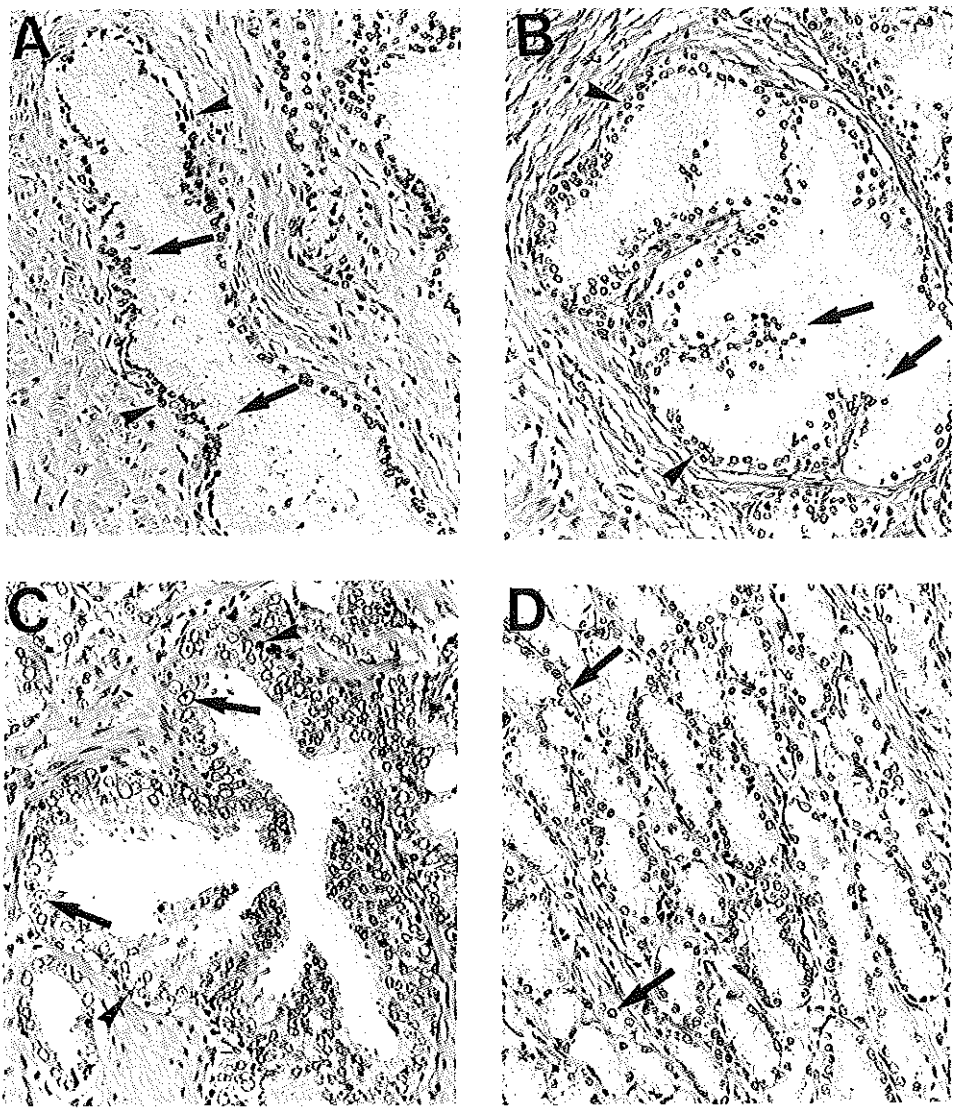


FIGURE 2.
Hematoxylin-eosin stained tissue sections of **A)** Normal prostate. Note the two cell layers, i.e. a basal cell layer (*arrowheads*) and a luminal, secretory cell layer (*arrows*). **B)** Nodular hyperplasia of the prostate. Note the papillary buds (*arrows*) and infoldings of the luminal cell layer and the presence of a basal cell layer (*arrowheads*). **C)** High-grade prostatic intraepithelial neoplasia (PIN). Note the crowding and the stratification of the nuclei, which show prominent nucleoli (*arrows*). The basal cell layer is still intact (*arrowheads*). **D)** Well-differentiated adenocarcinoma of the prostate. Note the presence of numerous small acini lying "back to back". The basal cell layer is absent. The nuclei are vacuolized and show prominent nucleoli (*arrows*). Magnification: 176x.

Japan. Recent reports, however, show an increase in the incidence of prostatic cancer in Japan, possibly due to Western dietary influences (Uchida *et al.*, 1995). In Europe, the highest incidences are seen in Scandinavian countries and Switzerland (30 to 40 per 100,000) and the lowest in Eastern Europe (e.g., Slovakia 8.1 per 100,000; Silverberg, 1987). The highest rates in the world are seen in blacks in the USA, with incidences up to 127 per 100,000 black males compared to 80 per 100,000 in age matched white controls (SEER data; reviewed in Morton, 1994). However, the recent increase in incidence rate is not equal for black and white elderly men and the black to white incidence ratio reached a low of 1 in 1992 (Severson *et al.*, 1995). This might be due to a greater availability of screening of the white population (Gilliland *et al.*, 1994). The mortality rate in African American males is almost twice as high as in white males, which has been ascribed in part to the more advanced stage at presentation (Brawn *et al.*, 1993; Morton, 1994). The racial disparity in incidence rate appeared to be independent of socioeconomic status (Baquet *et al.*, 1991). Interestingly, when Japanese males migrate to California or Hawaii, the age-adjusted yearly incidence rate for prostatic cancer increases in the first and second generations and becomes similar to the high rates of US males (Silverberg, 1987; Carter and Coffey, 1990). This clearly demonstrates that prostatic carcinogenesis can be directly affected by environmental and not merely by genetic factors.

Little is known about the etiology of prostatic cancer. Apart from age and race, several risk factors, such as family history of the disease, hormone levels, and environmental influences are suspected of playing roles. Men with a father or brother affected with prostate cancer have about twice the risk for developing the disease than do men without affected relatives. The risk is higher still for men with more than one affected relative and for men with a relative who developed the disease at a young age (Carter *et al.*, 1993; reviewed in Giovannucci, 1995). Segregation analysis revealed that the familial clustering of prostate cancer could be explained by the autosomal dominant inheritance of a rare high risk allele giving a cumulative lifetime risk of prostate cancer of about 90%, being responsible for about 10% of all prostate cancers by age 85 (Carter *et al.*, 1992; Coffey, 1993; reviewed in Key, 1995; Monroe *et al.*, 1995).

The role of hormones in the induction of prostate cancer is poorly understood. It is speculated that the endocrine changes of old age are related to its origin. Support for this general thesis lies in the regression of the disease after therapeutic castration or estrogen therapy. Additional support for this is suggested by the absence of prostatic cancer in eunuchs castrated before puberty, its relationship to fertility and an above-average sex drive, and its relative infrequency in patients with hyperestrogenism resulting from liver cirrhosis (Silverberg, 1987). Further, the fact that (young) black men have serum testosterone levels 15% higher than white men may be suggestive for a partial endocrine role in the high prevalence of prostatic

cancer in blacks (Ross *et al.*, 1986; Gittes, 1991; Morton, 1994; reviewed in Brown, 1996).
It is interesting to note that despite the striking racial and geographic differences in the incidence of clinically evident prostatic cancer, the incidence of latent carcinoma at autopsy and the frequency of PIN is almost similar in different ethnic groups (Yatani *et al.*, 1982; Carter and Coffey, 1990; Sakr *et al.* 1993). This implies that the rate at which progression takes place from latent to clinically manifest cancer is markedly different and is probably influenced by environmental factors. It has been proposed that high animal dietary fat or red meat intake as seen in Western societies, increases risk for this cancer (Morton, 1994; Giovannucci, 1995; Key, 1995). Dietary fat intake may influence the levels of hormones such as testosterone, which in turn affect the growth of the prostate (Hill *et al.*, 1979). However, this potential link between diet and hormonal levels needs to be further unraveled (Giovannucci, 1995). Dietary intake of vitamin A and β-carotene have frequently been suggested to render a significant protective effect against prostatic cancer (Giovannucci, 1995; Key, 1995). More data are needed on other, possible (prostatic) cancer preventing constituents in fruit and vegetables such as fiber and phyto-estrogens, and also on other factors related to diet, such as vitamin D_3 (Morton, 1994; Key, 1995). Many other risk factors for prostatic cancer, including occupational exposure to cadmium, vasectomy, and a past history of veneral disease, have been suggested. However, none of these factors has been established fully, and all must be confirmed by larger studies (Silverberg, 1987; Coffey, 1993; Giovannucci, 1995; Key, 1995).

2.2 Early Detection of Prostate Cancer

Prostate cancer is a notoriously silent disease with few early symptoms. Symptoms associated with bladder outlet obstruction are not common, since prostatic carcinoma most predominantly develops in the peripheral zone, in which case it does not compress the urethra (Fig. 1, p. 13). Traditionally, a yearly digital rectal examination (DRE) in men above the age of 40 has been considered the most reasonable screening technique for prostatic cancer (Gittes, 1991). The peripheral posterior location of most tumors renders them relatively easy palpable. The mean size of a palpable cancer was reported to be 5.4 cc (range 0.01 to 25.5 cc; Stamey *et al.*, 1989a). The positive predictive value (PPV) of a palpable abnormality to be prostatic cancer ranged from 22% to 36% (Scardino *et al.*, 1992).
TRUS has a proven ability to detect hypoechoic lesions (Gittes, 1991). The mean size of tumor volume detected by TRUS is 4.2 cc (range 0.04 to 19 cc). In general, the size of a hypoechoic lesion corresponds reasonably well with the actual size of the tumor measured in the surgical specimen (Scardino *et al.*, 1992). However, ultrasound scanning can fail to detect up to 30% of the prostatic lesions that are easily palpable on DRE, because they are isoechoic instead of hypoechoic. The

PPV of TRUS ranged from 15 to 41% (Scardino *et al.*, 1992). Perhaps the most important use of TRUS in the early detection of prostate cancer is as a guide for directed needle biopsies of the prostate (Gittes, 1991; Scardino *et al.*, 1992). A relatively new diagnostic imaging modality for the clinical staging of prostatic cancer is the endo-rectal magnetic resonance imaging (MRI) technique. Endo-rectal MRI was reported to reliably identify extraprostatic spread preoperatively. However, the reported accuracy of this imaging modality, as well as its (costly) application for prostatic cancer screening, varies markedly in literature (D'Amico *et al.*, 1995; Perrotti *et al.*, 1996).

In the past decade, the search for organ-specific substances in the prostate has led to the discovery of PSA. As stated above the use of PSA for the detection of prostatic cancer has increased exponentially from 1988 onwards (Potosky *et al.*, 1995; Jacobsen *et al.*, 1995). Moreover, serum PSA level was reported to be an important prognostic marker for tumor volume, stage and relapse, as well as a clinical utility for the monitoring of response to treatment, e.g., for imminent local and/or distant recurrent disease ("biochemical relapse") after radical prostatectomy (Stamey *et al.*, 1989a; Oesterling, 1991; Kupelian *et al.*, 1996; Olsson *et al.*, 1996). PSA is secreted by all but the most undifferentiated prostatic tumors. The PSA test has a high sensitivity and specificity, is rapid and inexpensive, and is minimally invasive (Bostwick, 1994). Mild serum elevations of PSA can be seen with nodular hyperplasia, but levels above 10 ng/ml are most unlikely to be due to BPH alone: Catalona *et al.* (1991) found that patients with serum PSA values above 10 ng/ml had an incidence of prostatic cancer of about 60%. The PPV of PSA levels > 4 ng/ml and >10 ng/ml amounts to 22-35% and 65-67%, respectively (Scardino *et al.*, 1992). Elevations of serum PSA also occur in prostatitis, prostatic infarct, and major trauma to the prostate such as needle biopsy or TURP, but these elevations should be transitory and resolve with proper treatment (Gittes, 1991). Further, several recent studies have shown that PSA is not entirely organ-specific, e.g. it can be expressed by male breast tumors (Monne *et al.*, 1994). Apart from these false-positive results, the other disadvantage is that an elevated PSA level provides no information about the location and the extent of a possible tumor (Scardino *et al.*, 1992).

Two surgical procedures that are primary responsible for the histological detection of prostatic carcinoma include TURP and needle biopsy. Different regions of the prostate are sampled by these methods: TURP specimens usually consist of tissue from the transition zone, urethra, periurethral area, bladder neck and anterior fibromuscular stroma, whereas most needle biopsy specimens consist of tissue from the peripheral zone (reviewed in Bostwick, 1995a).

DRE, TRUS and PSA are each limited in the ability to detect early prostate cancer. The integration of these methods represents a powerful diagnostic triad for the detection of early prostatic carcinoma (Cooner *et al.*, 1990; Scardino *et al.*, 1992;

Cupp and Oesterling, 1993). Nowadays, in several screening studies combination of DRE, PSA and TRUS is performed. In the Rotterdam pilot screening study, prostatic biopsies are taken if a suspicious DRE or TRUS or PSA level (≥ 4.0 ng/ml) was found. The PPV for the presence of biopsy-proven cancer were 19%, 38%, and 57% for the combination of TRUS+DRE, TRUS+PSA, and DRE+PSA, respectively. Combination of all three methods rendered a PPV of 68% (Schröder *et al.*, 1996). However, the value of screening for prostatic cancer remains controversial (reviewed in Schröder and Boyle, 1993). Nevertheless, the wide-spread use of PSA test and TRUS followed by systematic biopsy to screen for prostatic cancer has resulted in the early detection of many tumors. An increasing number of tumors are being diagnosed and treated when they are non-palpable on DRE. Concerns have been expressed that the increased detection rate occurring through the use of especially PSA tests will lead to the "over-diagnosis" of latent cancers and subsequent "over-treatment". However, recent studies show that the percentage of "clinically irrelevant" tumors detected by present screening methods amounts to 6 to 17% only, and that the majority of tumors detected this way are in a curable state (Mettlin *et al.*, 1993; Stormont *et al.*, 1993; Epstein *et al.*, 1994; Ohori *et al.*, 1994; Scaletscky *et al.*, 1994).

2.3 Patterns of Spread of Prostatic Cancer

Prostate cancer is considered to develop multifocally in about half of the cases (Miller and Cygan, 1994). This is probably an expression of true multicentricity rather than intraglandular tumor spread. Most clinically organ-confined prostate cancers grow very slowly with doubling times exceeding 4 years, whereas advanced tumors grow faster (Schmid *et al.*, 1993; Davidson *et al.*, 1995). The extension within the prostate first of all takes place into the outer area and intermediate part. The inner peri-urethral zone and the prostatic urethra itself are infiltrated in later stages of intraprostatic spread. Prostatic cancer frequently grows peripherally through the prostatic capsule favoring the passage along the conduits provided by the perineural spaces that perforate the capsule only at the upper outer corner and at the apex (Villers *et al.*, 1989; reviewed in McNeal, 1992). Most studies have suggested that tumor volume and grade are directly related with the ability to penetrate the prostatic capsule (McNeal, 1992). However, some reports suggest that also relatively small and low grade tumors can exhibit locally invasive behavior (Miller and Cygan, 1994).

Advanced tumor may extend into the seminal vesicles, the apex, and the neck of the bladder. Seminal vesicle invasion almost always results from direct extension of the tumor in the ejaculatory duct wall inside the prostate. Penetration of the apex is seen in approximately 20% of cases. Apical capsule penetration has a great clinical importance. Positive surgical resection margins are particularly common at the apex because of the short extraprostatic course of the nerve branches and the

close proximity of many deep pelvic structures to the prostatic capsule (McNeal, 1992). Rectal invasion *per continuitatem* is much less common, supposedly because of the resistance offered by the tough fibromuscular structure covering the posterior aspect of the prostate known as Denonvilliers' fascia (Villers *et al.*, 1993).
It is generally believed that prostatic tumors begin as small lesions, which must reach a certain volume (> 1cc) before metastasis can occur (Brawn, 1992; McNeal, 1992). The metastatic spread of prostate cancer is both lymphatic and hematogenous. Lymphatic spread is usually orderly and affects the regional nodes first. Regional lymphatic dissemination involves the iliac and para-aortic lymph nodes. Involvement of distant nodes such as the left supraclavicular and pulmonary hilar nodes, is usually a late event in prostatic spread via the lymphatic system, but this may also result from spread via hematogeneous routes. Regional lymph node metastases in patients with clinically localized palpable prostatic carcinoma are most likely to be found on the same side as the tumor (Harrison *et al.*, 1992). The involvement of lymph nodes may be detected by computer-tomography (CT) scans or MRI. Because microscopic metastases may be missed by either of these two procedures, many centers use pathological evaluation of pelvic lymphadenectomy specimens as a staging procedure prior to radical prostatectomy. However, staging lymphadenectomies often become lymph node samplings rather than total, meticulous lymph node dissections and are not intended to identify all pelvic lymph node metastases. Non-regional lymph nodes may be identified as of a prostatic origin by immunohistochemical staining for PSA or PAP.
The most common form of hematogenous spread of prostate cancer is to the bone marrow. Autopsy series on patients with prostate carcinoma reveal that 80 to 85% have bone metastases (Shoskes and Perrin, 1989). Bone metastases are usually multiple but can be solitary. The lesions are characteristically osteoblastic. Sometimes the appearance of a bone metastasis precedes the urologic manifestations. The vertebral column, especially the lumbar spine and the sacrum, is the most common location of bony metastases (Harada *et al.*, 1992). Spinal involvement frequently extends into the epidural space and is a cause of extrinsic compression of the spinal cord and weakness in the legs, progressing to paraplegia (Shoskes and Perrin, 1989). Other frequent sites of osseous metastasis include the ribs, the pelvis, the sternum, and the upper part of the femur, but any other bone can be involved through the systemic circulation (Saitoh *et al.*, 1984).
Several studies have begun to reveal possible underlying mechanisms for the predilection of prostatic cancer to metastasize to the bone. An acceleration of prostate cancer growth by factors derived from bone fibroblasts has been reported in an *in vivo* tumor model (Gleave *et al.*, 1991). Study of adherence of Dunning prostate carcinoma cells to tissue cultures of bone marrow cells suggested that the adhesion of prostate carcinoma cells to, specifically, the endothelium of the bone marrow, may play a role (Haq *et al.*, 1992). More recently, several studies have

implicated parathyroid hormone related peptide (PTHrP) as a potential factor in the pathogenesis of bone metastases. Asadi *et al.* (1996) demonstrated enhanced expression of PTHrP in prostate cancer compared with BPH. Prostate cancer cells derived from a bone metastatic lesion (PC3) secrete significantly greater amounts of PTHrP than cell lines derived from other (hematogenous) metastases, suggesting that the secretion of PTHrP by these tumor cells offers them an ability to infiltrate and grow in bone (Iwamura *et al.*, 1994). Further, PSA is known to cleave PTHrP thereby disrupting its osteolytic ability. This may explain why prostatic bone metastases, in contrast to bone metastases of other tumors, have usually an osteoblastic phenotype and are rather accompanied by hypocalcemia than by hypercalcemia (Iwamura *et al.*, 1996).
The most common sites of visceral metastases are the lungs, liver, adrenals and kidneys, pleura, pancreas, spleen, and brain, in decreasing order of frequency. Hence, almost every organ or structure may display involvement (Saitoh *et al.*, 1984). Visceral metastases may be detected by CT scan or MRI. The diagnosis of osseous metastases is usually performed by skeletal surveys or the much more sensitive radionucleotide bone scanning. Further, in metastases of unknown origin, immunohistochemistry with PSA and PAP markers, may reveal a prostatic origin.

2.4 Staging of Prostatic Cancer

The stage of a malignant neoplasm reflects the extent of spread of a cancer within the patients and is an important parameter for the clinical gravity of the disease. The stage of prostatic cancer is assessed clinically by a combination of DRE, TRUS, CT scans, MRI, and X-ray photos.
Two commonly used staging systems for prostate cancer are the (American) Whitmore-Jewett classification (Jewett, 1975; Gittes, 1991; Table 1) and the tumor, nodes and metastasis (TNM) 1992 system of staging (Schröder *et al.*, 1992), as has been advocated by the UICC (International Union Against Cancer) and the AJCC (American Joint Committee on Cancer). Nowadays, the TNM classification is the most widely used staging system for prostatic cancer (Table 2). In recent years the percentage of clinically organ confined disease has sharply increased, whereas a slight decrease was found in the percentage of patients who presented with distant prostate cancer. As stated above this was mainly due to the application of PSA,TRUS, and screening studies (Gilliland *et al.*, 1994; Jacobsen *et al.*, 1995; Potosky *et al.*, 1995; Severson *et al.*, 1995; Stephenson *et al.*, 1996).
Several studies showed a reasonable overall correlation between pathological tumor stage and the likelihood of the development of distant metastatic or local recurrent disease. Approximately half of the patients with clinically manifest prostate cancer will have extraprostatic disease (N+M+) at the time of diagnosis (Scardino *et al.*, 1992). These patients have a dismal prognosis with 10-year cancer-specific survival rates of 10% and 40% for patients with distant metastases

Table 1. Clinical staging according to the Whitmore-Jewett classification

Stage	*Description*
A	No palpable lesion
	A1: Focal
	A2: Diffuse
B	Confined to prostate
	B0: Cancer detected by needle biopsy (e.g. because of elevated PSA)
	B1: Small, discrete nodule, ≤ 1.5 cm
	B2: Large (>1.5 cm) or multiple nodules or areas
C	Localized to periprostatic area
	C1: No involvement of seminal vesicles, prostate ≤ 70 g
	C2: Involvement of seminal vesicles, prostate >70 g, tumor not fixed
D	Metastatic disease
	D1: Pelvic lymph node metastases or urethral obstruction causing hydronephrosis
	D2: Bone or distant lymph node or organ or soft tissue metastases

After Gittes (1991) and Rosai (1996).

and regional lymph node metastases, respectively (Gervasi *et al.*, 1989; Lerner *et al.*, 1991; Scardino *et al.*, 1992). Tumors with locally extensive disease (stages T3/T4) showed a 10-year cancer-specific survival of 60% (Scardino *et al.*, 1992). For patients treated by radical prostatectomy with pathological stage C/T3 an actuarial 10-year cancer-specific survival rate of 58-76% was reported and progression or disease-free survival rates were 59-77% at 5 years, 52-54% at 10 years, and 11-42%% at 15 years (Schellhammer, 1988; Stein *et al.*, 1992; van den Ouden *et al.*, 1994; Lieber *et al.*, 1995). For pathological stage B/T2 tumors the actuarial 10-year cancer-specific survival was 95-96% and disease-free survival rates amounted to 87-91%, 76-80%, and 47-63%, at 5, 10 and 15 years, respectively (Middleton *et al.*, 1986; Schellhammer, 1988; Stein *et al.*, 1992; Lieber *et al.*, 1995). For patients treated by radiotherapy the actuarial 10-year cancer-specific survival for clinical stage C (node-negative) was 48% and stage C disease-free survival rates were 59%, 44% and 30% at 5, 10 and 15 years, respectively. For clinical stage A2/B (node-negative) the actuarial 10-year cancer-specific survival amounted to 87% and stage B disease-free survival was 82%, 59%, and 59%, at 5, 10, and 15 years, respectively (Lerner *et al.*, 1991; Zagars *et al.*, 1993). Further, several studies based on radical prostatectomy specimens found that capsular involvement, invasion into the seminal vesicles, as well as the presence of positive surgical resection margins, are important predictors of recurrent disease, implicating a poor prognosis (Middleton *et al.*, 1986; Schellhammer, 1988; Stein *et al.*, 1992; Humphrey *et al.*, 1993; Kupelian *et al.*, 1996).

Incidental cancers (T1), detected by TURP (T1a and T1b) for the treatment of BPH in 10% to 15% of cases, appear to be somewhat different entities than carcinomas arising in the peripheral zone, detected by biopsy (T1c). The transition zone accounts for about 24% of all prostate cancers, but 78% of stage T1 cancers

Table 2. The TNM classification of prostate cancer

Stage	*Description*
Tx	Primary tumor cannot be assessed
T0	No evidence of primary tumor
T1	Clinically unapparent tumor, not palpable nor visible by imaging
	T1a Tumor an incidental histologic finding in ≤ 5% of tissue resected by TURP
	T1b Tumor an incidental histologic finding in > 5% of tissue resected by TURP
	T1c Tumor identified by needle biopsy (e.g. because of elevated PSA)
T2	Tumor confined within the prostate
	T2a Tumor involves ≤ half of lobe
	T2b Tumor involves > half of lobe
	T2c Tumor involves both lobes
T3	Tumor extends through the prostatic capsule
	T3a Unilateral extracapsular extension
	T3b Bilateral extracapsular extension
	T3c Tumor invades seminal vesicle(s)
T4	Tumor is fixed or invades adjacent structures other than seminal vesicles
	T4a Invasion of bladder neck and/or external sphincter and/or rectum
	T4b Invasion of levator muscles and/or fixation to pelvic wall
Nx	Regional lymph nodes cannot be assessed
N0	No regional lymph node metastasis
N1	Metastasis in a single regional lymph node, ≤ 2cm
N2	Metastasis in a single regional lymph node, 2-5 cm or multiple regional lymph nodes, all ≤ 5cm
N3	Metastasis in regional lymph node(s), > 5 cm
Mx	Presence of distant metastasis cannot be assessed
M0	No distant metastasis
M1	Distant metastasis
	M1a Non-regional lymph node(s)
	M1b Bone(s)
	M1c Other site(s)

After Schröder *et al.* (1992).

originate in this zone, including 75% and 79% of stage T1a and T1b cancers, respectively. This high percentage of incidental cancer in the transition zone is probably due to selective sampling by TURP. Transition zone cancer appears to be better differentiated than peripheral zone cancer and has a smaller tumor volume. The risk of extracapsular extension and spread to regional lymph nodes is significantly lower than in peripheral zone cancer, since the transition zone

boundary is suggested to function as an anatomic barrier to the spread of cancer (McNeal, 1992; Bostwick, 1995a). The clinical significance of, especially T1a, incidental carcinoma remains controversial. Age of the patient seems to be the most important predictor of crude survival (Lowe, 1991). The rate of local and/or metastatic progression of untreated T1a cancer varies from 2-50%, with a median time to progression varying from >4 to 13.5 years (Matzkin *et al.*, 1994). In contrast, clinical progression is more likely in stage T1b cancer, which frequently has a greater tumor burden and is clinically more aggressive than stage T2a cancer. Progression, either locally or systemically, is seen in 25 to 50% of cases (Schröder and Boyle, 1993). Lowe and Listrom (1988) have calculated the median time for progression, i.e. symptomatic local recurrence or distant metastases, of T1b cancers, to be 4.75 years. Stage T1b cancer resembles T2b/T3 cancer, when treated only palliatively, with 10-year cancer-specific survival rates of only 40% (Aus *et al.*, 1995).

The concordance between clinical and pathological (p)T stage, i.e. tumor stage after histological evaluation, has been investigated in several studies. Of the patients with stage T1c (B0) prostate cancer treated by radical prostatectomy, 23 to 40% showed extracapsular extension and 5 to 9% showed seminal vesicle invasion. Further, up to one third of cases showed resection margin positivity after surgery (Oesterling *et al.*, 1993; Stormont *et al.*, 1993; Epstein *et al.* 1994; Scaletscky *et al.*, 1994; Bostwick, 1995a; Kupelian *et al.*, 1996; Visser and Horenblas, 1996). These results indicate that the T1c stage identifies a heterogenous population of patients and therefore fails to assist in stratification of patients for therapy. In clinical stage T2a-b (B1) tumors extracapsular spread occurred in 25 to 81%, seminal vesicle invasion in 5 to 26%, margin positivity in 20 to 64%, and regional lymph node involvement in 2 to 14% of cases (Oesterling *et al.*, 1993; Stormont *et al.*, 1993; Bostwick, 1995a; Kupelian *et al.*, 1996). For stage T2c cancers these numbers were 81%, 56%, 58%, and 25%, respectively (Kupelian *et al.*, 1996). Thus, a patient with clinically localized prostate cancer has a substantial risk of extraprostatic extension by pathological examination, which can be removed less easily, and consequently, carries a higher chance of progressive disease. The correlation between clinical stage T3/C cancer and pathological stage pT3/C is fairly good, with overstaging occurring in 15 to 19% of cases (Schellhammer, 1988; Visser and Horenblas, 1996). However, the number of patients with clinical stage T3 and especially T4, treated by radical prostatectomy, is relatively small, since these patients often have regional and/or distant metastatic disease, and therefore receive other forms of treatment.

2.5 Histology of Prostatic Cancer

The primary malignant tumors occurring in the prostate may originate in both the epithelial and the stromal components. Over 95% of prostatic cancers are

adenocarcinomas arising from the epithelium of the prostatic acini. The other malignant epithelial and stromal neoplasms are beyond the scope of this thesis.The WHO classification for the histologic typing of prostatic cancer is shown in Table 3.

Table 3. Histological classification of prostate tumors

Description	
I. Epithelial tumors	
A. Benign	
B. Malignant	**1. Adenocarcinoma**
	2. Transitional cell carcinoma
	3. Squamous cell carcinoma
	4. Undifferentiated carcinoma
II. Non-epithelial tumors	
A. Benign	
B. Malignant	**1. Rhabdomyosarcoma**
	2. Leiomyosarcoma
	3. Others
III. Miscellaneous tumors	**1. Neuroendocrine tumors**
	2. Carcinosarcomas
	3. Others
IV. Secondary tumors	
V. Unclassified tumors	
VI. Tumor-like lesions and epithelial abnormalities	

After Mostofi *et al.* (1980).

Histologically, most adenocarcinomas produce well-defined, readily demonstrable gland patterns. The glands are either small or medium-sized with a single uniform layer of cuboidal or low columnar epithelium instead of a double layer of cells seen in normal prostatic tissue and hyperplasia (Fig. 2, p.17). Occasionally, the glands are somewhat larger, with a papillary or cribriform pattern. Typically, the neoplastic acini are closely spaced, "back-to-back" with little intervening stroma. They have an irregular shape and are dispersed haphazardly in the stroma, representing stromal invasion. The nuclei are usually larger than those of benign cells. They show variation in size, shape, and staining. The chromatin is condensed at the periphery, and there is vacuolization of the nuclei. One or two prominent nucleoli clumped near the nuclear membrane of the secretory cells is the most frequent nuclear feature. The cytoplasm of the carcinoma cells has a non-descript finely granular appearance. Mitotic figures and giant cells are rare. Not all prostatic carcinomas, however, are well differentiated. In poorly differentiated tumors, the glandular pattern is apparent only after careful examination. In these cases the tumor cells tend to grow in cords, nests, or sheets (Mostofi *et al.*, 1992a).

2.6 Precancerous Lesions of the Prostate

The first description of a prostate premalignant change was provided by Oerteil in 1926. Subsequently, a number of reports have appeared in the literature describing these conditions (McNeal, 1965; McNeal and Bostwick, 1986; reviewed in Brawer, 1992). One source of great confusion in the literature as well as in surgical pathology reporting has been the wide range of synonyms used to describe prostatic premalignant conditions. Terms such as "atypical epithelial hyperplasia", "cytologic atypia", "duct-acinar dysplasia", "glandular atypia", "intraductal dysplasia", "intraglandular dysplasia", and "large acinar atypical hyperplasia" have been used. Partly because of this confusion, a consensus conference was held in 1989 and the term prostatic intraepithelial neoplasia (PIN), introduced in 1987 by Bostwick and Brawer, was considered to be the most appropriate nomenclature for the most common premalignant prostatic change (Drago *et al.*, 1989).

The peripheral zone of the prostate, the area in which most prostatic carcinomas occur, is also the most common location for PIN. For example, Troncoso *et al.* (1989) noted that 81% of the foci of PIN occurred in this region. PIN was originally divided into three grades, depending on the severity of the following alterations: cell crowding and stratification; nuclear enlargement, pleomorphism and chromatin pattern; and nucleolar appearance (McNeal and Bostwick, 1986; Brawer, 1992; Fig. 2, p. 17). More recently, it has been proposed that PIN1 (mild dysplasia) be considered low-grade PIN and PIN2-3 (moderate to severe dysplasia) be considered high-grade PIN (Drago *et al.*, 1989; reviewed in Bostwick, 1995b). Low-grade PIN is difficult to distinguish from benign prostatic tissue in needle biopsies and TUR specimens (Epstein *et al.*, 1995). The atypical features of the cells making up high-grade PIN are indistinguishable from those of invasive cancer. The major distinction between carcinoma and PIN is that the latter is confined to the presence of a basal cell layer and, thus, preservation of at least the resemblance of the normal acinar system with two distinct epithelial cell types, basal and luminal. However, the basal cell layer is frequently disrupted in high grade PIN (Bostwick, 1995b). The proliferation of the luminal cells in PIN might be quite severe. In the most extreme case there might be a cribriform pattern within the PIN acini or ductule, which can make this variant difficult to distinguish from cribriform patterns in adenocarcinoma (Brawer, 1992; Epstein *et al.*, 1995).

PIN is associated with progressive abnormalities of phenotype and genotype, which are intermediate between normal prostatic epithelium and cancer. There is progressive loss of secretory differentiation, including PSA, cytoskeletal proteins, glycoproteins, and neuroendocrine cells. Other markers show progressive increase, including *c-erbB-2* and *bcl-2* proto-oncoproteins, proliferating cell nuclear antigen (PCNA) expression, aneuploidy and genetic abnormalities (Bostwick, 1995b).

The incidence and extent of PIN appear to increase with patient age according to most studies (Bostwick, 1995b). In a review of 429 step-sectioned whole prostates

derived from autopsies, Kovi *et al.* (1988) found that the prevalence of PIN in prostates with cancer increased with age, predating the onset of carcinoma by more than 5 years. A similar study by Sakr *et al.* (1993) revealed the onset of PIN in men in their 20s and 30s (9% and 22%, respectively). Most foci of PIN in young men were low grade, with increasing frequency of high grade PIN with advancing age. The prevalence of PIN was almost similar in blacks and whites.

The clinical importance of recognizing PIN is based on its strong association with prostatic carcinoma. Because PIN has a high predictive value as a marker for adenocarcinoma, its identification in prostatic biopsy specimens warrants further search for concurrent invasive cancer (Bostwick, 1995b). High grade intraepithelial neoplasia was reported to be a frequent finding in needle biopsies and was present in over 15% of cases (Bostwick *et al.*, 1995). A retrospective case-control study of 100 patients with high grade PIN and 112 patients without PIN on needle biopsies, matched for clinical stage, age, and PSA level and taken because of elevated PSA level and/or abnormal DRE or TRUS, revealed adenocarcinoma in 35% of follow-up biopsies from cases with PIN, compared with 13% in the control group (Davidson *et al.* 1995). The frequency of PIN in prostates with cancer is significantly higher than in prostates without cancer (Brawer, 1992; Bostwick, 1995b). McNeal and Bostwick (1986) observed PIN in 82% of step-sectioned autopsy prostates with cancer, but in only 43% of benign prostates from patients of similar age. For high grade PIN these numbers were 72% and 26%, respectively. Likewise, Qian and Bostwick (1995) found that 86% of a large series of whole-mount radical prostatectomies with cancer contained high-grade PIN, usually within 2 mm of cancer. Further, McNeal (1993) demonstrated a direct continuity between dysplasia and invasive carcinoma in near half of cases in his study of microcarcinomas.

2.7 Grading of Prostatic Adenocarcinoma

Numerous systems have been designed for the grading of prostatic adenocarcinoma over the past decades. The grading systems attempt to help in predicting clinical patient outcome based on tumor characteristics like tissue architecture and/or nuclear features. The grading systems most often used for adenocarcinoma of the prostate are the Gleason grading system (Gleason, 1966, 1992), the (modified) MD Anderson grading system (Brawn *et al.*, 1982), and the WHO/Mostofi-Schröder system (Mostofi, 1975; Mostofi *et al.*, 1980; Schröder *et al.*, 1985). Both the Gleason and the MD Anderson grading system were used for the studies described in this thesis and therefore we will focus on these two grading systems.

The grading system developed by Gleason in conjunction with the Veterans Administration Cooperative Urologic Research Group (VACURG) is based on the degree of glandular differentiation and the growth patterns of the tumor in relation to the stroma as evaluated on low-power examination (Gleason, 1992). At present it is

the most widely used grading system and most often reported in (American) literature. The Gleason system is unique in that it takes into account the histomorphologic heterogeneity of prostatic adenocarcinoma. Gleason observed that a majority of prostatic carcinomas had either a single growth pattern or at the most, two patterns of differentiation: A dominant pattern and a secondary pattern. The system recognizes five primary growth patterns representing the transition from well differentiated (pattern 1) to anaplastic (pattern 5; Table 4; Fig. 3). A tumor is assigned a certain Gleason score, ranging from 2 to 10, by adding the two most common growth patterns or doubling the grade in case of a single growth pattern.
Several studies have shown that relationships exist between Gleason score and a number of other known prognostic variables, including tumor volume, pathological stage, anatomical zone of origin (i.e., transition or pheripheral zone), and metastases in pelvic lymph nodes (McNeal *et al.*, 1988, 1990; Stamey *et al.*, 1988; Greene *et al.*, 1991; McNeal, 1992; Bostwick, 1995a). The prognostic value was found to be the largest in tumors with either the lowest (score of 2 through 4) or highest (score of 8 through 10) Gleason scores (Epstein *et al.*, 1996; Kupelian *et al.*, 1996). However, half of patients present with tumors with intermediate Gleason scores and in these patients the Gleason score cannot distinguish the non-aggressive and aggressive tumors on an individual basis (Stephenson *et al.*, 1996). In stage pT1 to pT3b (all N0) tumors with a Gleason score of 2 to 4 the risk of progression, i.e., (biochemical) evidence of local or distant recurrent disease, within 10 years was only 14%, whereas a Gleason score of 8 to 9 was associated with a chance of progression of 65%. In intermediate grades, i.e. scores 5 to 6, and 7, the chance of progression was 18% and 48%, respectively (Epstein *et al.*, 1996). Ten year cancer-specific survival rates in pathological stages B to D1 was reported 94%, 88% and 56% for Gleason scores 2 to 4, 5 to 7, and 8 to 10, respectively (Lieber *et al.*, 1995).
Since the Gleason system is based on tumor growth patterns instead of nuclear features, Gleason grading is difficult to apply to biopsies, in which very small amounts of adenocarcinoma are present. "Undergrading" of the original prostatic biopsy compared with the grade of the resected specimen has been found in as many as 45% to 50% of the original biopsies (Gleason, 1992). Recently, Thickman *et al.* (1996), found that the Gleason grade of resected specimens was within one histologic score of the Gleason grade as determined by biopsy in 75% of cases when 4 biopsies were investigated. In biopsy specimens the degree of nuclear atypia seems to be the most suitable basis for grading (Mostofi *et al.*, 1992b).The original MD Anderson classification system as proposed by Brawn *et al.*, (1982) was composed of four grades, i.e, well, moderately, poorly and undifferentiated tumors. This grading method is based on the assumption that differentiated (gland forming) prostate carcinomas have a better prognosis than undifferentiated (non-gland) forming prostate carcinomas (Table 5). At present, the modified MD

Table 4. Brief description of the Gleason grading system

Grade	*Description*
1	Simple round glands, closely packed in round masses with well-defined edges
2	Simple round glands, loosely packed in vague, rounded masses with loosely defined edges
3	Variable sized single glands of irregular shape and irregular spacing with ill-defined infiltrating edges and/or smoothly circumscribed masses of cribriform or papillary epithelium; no necrosis
4	Raggedly infiltrating masses of fused glandular epithelium, frequently with many large clear cells resembling "hypernephroma"
5	Papillary and cribriform epithelium in smooth, rounded masses, more solid than grade 3 and with central necrosis and/or anaplastic adenocarcinoma in ragged sheets, diffusely infiltrating prostatic stroma

After Gleason (1992).

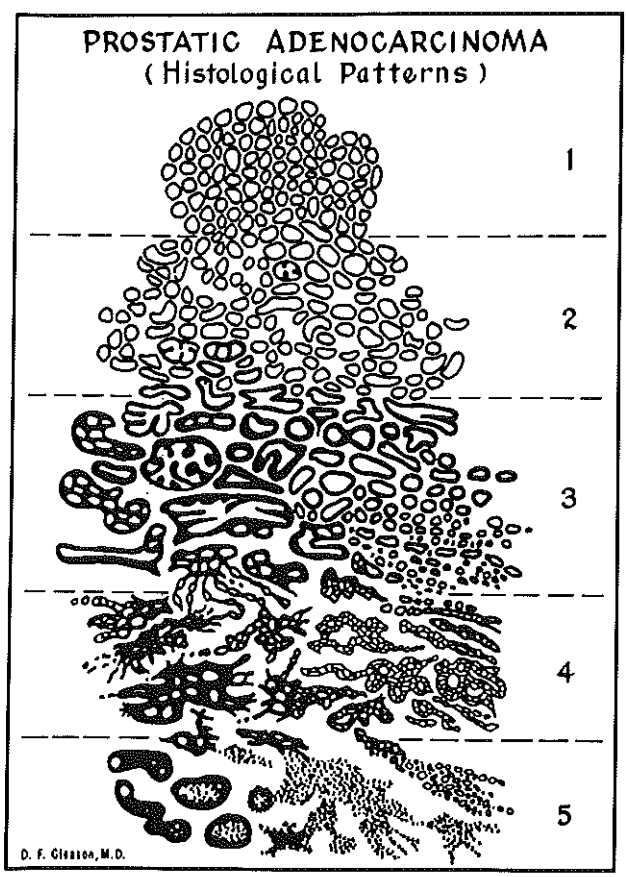


FIGURE 3.
Simplified drawing of the different histologic Gleason growth patterns in prostatic adenocarcinoma, emphasizing the degree of glandular differentiation and relation to stroma.
(After Tannenbaum, 1977).

Anderson system that recognizes three grades, i.e., well, moderately and poorly differentiated, is most commonly used. According to this modification, moderately differentiated tumors contain 25-75% glands, whereas poorly differentiated tumors replace the former criteria for undifferentiated tumors. Using the four grade MD Anderson grading system, a study of the Comprehensive Cancer Center in Amsterdam reported that at presentation 25%, 36%, 32%, 0% and 7% of prostatic carcinomas were well, moderately, poorly, undifferentiated and of unknown grade, respectively. Further, higher clinical tumor stages showed an increase in the percentage of less differentiated tumors (Visser and Horenblas, 1996). This is in agreement with the general observation that well differentiated lesions rarely metastasize and metastases are rarely well differentiated (Brawn, 1992).

Table 5. MD Anderson grading system

Grade	*Description*
Well differentiated	**75-100% of the tumor forms glands; 0-25% does not. Excluded are cribriform-papillary tumors**
Moderately differentiated	**50-75% of the tumor forms glands; 25-50% does not. Included are tumors consisting of 50% or more of a cribriform-papillary pattern**
Poorly differentiated	**25-50% of the tumor forms glands; 50-75% does not**
Undifferentiated	**0-25% of the tumor forms glands; 75-100% does not**

Brawn *et al.*, 1982.

Histological grading, no matter how well defined, is setting atypical steps on a biological grey-scale. It is substantially subjective, and intraobserver and interobserver variation occurs. On re-examining routine clinical samples, including 50% needle biopsy specimens, Gleason himself duplicated exactly his previous histological scores approximately 50% of the time and within ± 1 histological score approximately 85% of the time (Gleason, 1992). Harada *et al.* (1977) reported an intraobserver reproducibility of 64% for the primary Gleason pattern and 44% for the second pattern. The interobserver reproducibility of the Gleason, the MD Anderson and three other grading systems has been investigated by Ten Kate *et al.* (1986). The authors found that none of the grading systems investigated demonstrated a high degree of reproducibility. Reproducibility of the MD Anderson (4 grades) grading system was reasonably good compared to the Gleason system.

2.8 Treatment of Prostatic Cancer

The clinical course of prostatic cancer is highly variable and difficult to predict in individual cases. The treatment modality chosen depends largely on clinical staging. Since prostatic cancer is a disease of elderly men, also the age, general health and motivation of the patient has to be taken into account. The management

choices of localized prostatic carcinoma include radical prostatectomy, external radiation therapy, and "deferred" or "expectant" treatment. There is still uncertainty (and therefore great controversy) which of these modalities is to be preferred in patients with localized disease.
The treatment of incidental carcinoma remains especially controversial. Some centers suggest that T1a lesions progress sufficiently slowly to warrant observation rather than aggressive treatment in elderly men. The group at risk seems to be the young patient (below the age of 60), who is likely to survive more than 10 years after diagnosis. These patients should undergo a TRUS-guided biopsy, as well as monitoring of PSA levels. If subsequent tissue sampling identifies other than well differentiated tumor or indicates more extensive cancer than the T1a staging, curative treatment is suggested (Matzkin *et al.*, 1994). Treatment is warranted for stage T1b lesion which represents an intermediate threat to anyone who has a life expectancy of at least 5 years (Schröder and Boyle, 1993). Most urologists treat stage T1b cancer therefore aggressively by radical prostatectomy or radiation therapy, particularly when it is detected in younger patients (Matzkin *et al.*, 1994).
Expectant management (i.e. no treatment at first) of localized prostatic cancer strongly increases with age and low tumor stage and grade (Severson *et al.*, 1995; Visser and Horenblas, 1996). Warner and Whitmore (1994) reported the outcome of expectant management of 75 patients with well or moderately differentiated clinical stage B tumors. These patients were selected from a larger panel on the basis of no tumor progression after one year of follow-up. The disease-free survival rate of this group was 88-96% at 10 years, but decreased to 66-82% and 23-67% at 15 and 20 years, respectively. Local progression was relatively slow but all tumors were judged to have increased in size during observation intervals (mean time to progression 78 months). Distant progression also occurred, but at a much slower rate (mean 186 months). Johansson *et al.* (1992) emphasized the slow rate of progression and low rate of death from prostate cancer. These authors found that in selected patients with early stage prostate cancer, who did not receive treatment, only 8.5% died of prostate cancer after 10 years. The progression-free 10 year survival rate was 53% and local growth was the only sign of tumor progression in 66% of cases. Similar high patient survival data were reported by other groups (e.g. Adolfsson *et al.*, 1994; Chodak, 1994). These data are in contrast with those of Aus *et al.* (1995). These authors found in a long-term survival study of a large panel of patients treated with palliative intent only (stages T1 to T4, all M0) a cancer mortality rate of 50%. Among the patients who survived at least 10 years the mortality rate due to prostate cancer was 63%. Both in the US and in The Netherlands the proportion of patients treated conservatively declined in the period from 1986 to 1994, especially in younger patients (<75 years) with clinically organ-confined disease (Mettlin *et al.*, 1996; Visser and Horenblas, 1996).
Most patients with clinical stages T1c to T3 are offered a form of curative treatment,

i.e. radical prostatectomy or radiation therapy. Neither method has proved statistical superiority in its effectiveness (Gittes, 1991). Radical prostatectomy entails the removal of the entire prostate, including the capsule, a layer of surrounding connective tissue, and the attached seminal vesicles. New techniques of radical prostatectomy have markedly reduced the chance of post-surgery complications, most importantly impotence and incontinence (Gittes, 1991). Soon after radical prostatectomy PSA levels become undetectable. The finding of measurable PSA levels after radical prostatectomy leaves no doubt that there is local residual or distant metastatic disease (Gittes, 1991; Stein *et al.*, 1992; Oefelein *et al.*, 1995; Kupelian *et al.*, 1996). In the US radical prostatectomy rates in men between 50 and 79 years have been more than tripled between 1983 and 1989 (Lu-Yao and Greenberg, 1994; Mettlin *et al.*, 1996). The shift towards a more aggressive form of therapy was even more prominent in elderly men (>75 years), where the incidence of radical prostatectomy as first course of treatment increased from 0 in 1973 to 38.4 per 100,000 individuals in 1992 (Severson *et al.*, 1995). In The Netherlands, there was a six-fold increase in the number of radical prostatectomies, especially in men under the age of 60 in the period from 1989 to 1994 (Visser and Horenblas, 1996).

Interstitial radiation using iodine-125 or gold-198 was in vogue for several years. However, nowadays external-beam radiotherapy is an established and well-tested curative treatment for localized prostate cancer. Potence is preserved in over half of patients who undergo radiation. Further, the incidence of rectal-wall damage, formerly an important complication, has been greatly reduced. The course of serum levels of PSA after definitive radiation for presumably localized prostatic cancer involves a much delayed decline, as compared to the drop after surgical excision, but one that remains very useful prognostically (Stamey *et al.*, 1989b). Progressive reelevation of PSA levels indicates either failure of local treatment or the appearance of distant metastases. There has been a controversy for several years about the importance of residual cancer after radiation therapy. Systematic biopsies of fully treated patients have shown a 35 to 91% incidence of apparently viable tumor cells (Gittes, 1991). In the US the proportion of patients between 50 and 79 years receiving radiotherapy remained stable between 1983 and 1989 (Lu-Yao and Greenberg, 1994). In elderly men (>75 years) with local and regional stage disease an exponential increase was seen in the period between 1973 and 1992 (Severson *et al.*, 1995). In The Netherlands patients with stage T3 tumors and men between 60 and 75 years of age with stage T1 to T3 tumors were more often treated with radiotherapy than with radical prostatectomy (Visser and Horenblas, 1996).

Endocrine management was introduced into the treatment of prostate cancer by Huggins and his associates in 1941 (e.g., Huggins and Hodges, 1941). Since then hormonal therapy has been mainstay for palliative treatment of locally advanced and metastatic prostatic cancer, but is also the first choice of therapy for patients

with T2/T3 staged tumors over the age of 75 (Severson *et al.*, 1995; Visser and Horenblas, 1996). Because prostatic cancer cells depend on androgens for their sustenance, the aim of endocrine manipulations is to deprive the tumor cells of testosterone. This can be achieved by androgen deprivation or androgen blockade. Androgen deprivation can be achieved by bilateral orchiectomy or by administration of estrogens or synthetic agonists of luteinizing hormone-releasing hormone (LHRH). Although estrogens can inhibit testicular androgen synthesis directly, their principal effect appears to be suppression of the secretion of pituitary luteinizing hormone (LH) to such a point that the testicular output of testosterone is essentially at castrate levels. Synthetic analogues of LHRH (e.g., Buserelin) act similarly. Long-term administration of LHRH agonists (after an initial transient increase in LH secretion) suppresses LH release, achieving in effect a pharmacological orchiectomy. Anti-androgens can be defined as substances that are able to counteract the biological effects of androgens by competing with these hormones at the receptor level: Steroidal anti-androgens (e.g., Cyproterone acetate) interfere with the androgen receptor, block 5-α reductase activity and have an anti-gonadotropic activity, whereas the non-steroidal type (e.g., Flutamide) displaces testosterone and DHT from the androgen receptor. The concept of total androgen blockade is the combination of surgical or pharmacological castration and blockade of the adrenal androgen pathway, which constitutes 5% of the androgenic stimulation to the prostate or to prostatic cancer. This can be achieved by adrenalectomy or administration of (non)-steroidal anti-androgens. At present, the advantage of total androgen blockade over monotherapy remains controversial (Gittes, 1991; reviewed in Schröder, 1991, Stearns and McGarvey, 1992, Daneshgari and Crawford, 1993 and Newling, 1995).

Surgical or pharmacological androgen deprivation in patients with metastatic carcinoma leads to a rapid reduction of symptoms, as well as a dramatic decrease in PSA levels (Stamey *et al.*, 1989c; Schröder, 1991). Approximately 70% of prostate cancer patients respond favorably to hormonal therapy and achieve at least partial remission (Stearns and McGarvey, 1992). Originally, it was assumed that this palliation was also associated with an increased survival rate. Median time to further progression of metastatic prostatic cancer patients after hormonal treatment is 12 to 18 months and median survival is 24 to 30 months (Schröder, 1991; Newling, 1995). However, eventually all androgen-dependent tumors progress to a state of autonomous growth and then they are no longer sensitive to androgen withdrawal. Progression to endocrine therapy resistance may become manifest by a rise in PSA serum levels, an increase in primary tumor size, and/or new or growing bone or visceral metastases (Stamey *et al.*, 1989c; Newling, 1993). Median survival time of patients with hormone-refractory tumors is only 5 to 10 months (Stearns and McGarvey, 1992). At present, there is no effective chemotherapeutic agent to induce a meaningful response in hormone-refractory

disease (Newling, 1995). Symptoms of urethral obstruction in hormone-refractory carcinoma can be handled by palliative TURP. Symptomatic metastatic bone lesions can be treated by radiotherapy.
Since most hormone refractory carcinomas express a structural intact human androgen receptor, androgen ablation therapy apparently does not cause a selective outgrowth of androgen receptor-negative prostatic tumor clones (Ruizeveld de Winter *et al.*, 1994). Recently, it was suggested that failure of conventional androgen deprivation therapy may be caused by clonal expansion of tumor cells that are able to continue androgen-dependent growth due to amplification and increased expression of the wild-type androgen receptor gene (Koivisto *et al.*, 1997).

2.9 Ploidy Status in Prostatic Cancer

Overall, low grade, low stage tumors are generally DNA diploid, whereas high grade, high stage tumors are more frequently DNA aneuploid (Tribukait, 1991; reviewed in Shankey *et al.*, 1993). Several studies have suggested that nuclear DNA ploidy, as determined by DNA flow cytometry (FCM) or image cytometry (ICM), may offer additional prognostic information beyond clinical staging and histological grading for patients with prostatic adenocarcinoma (reviewed in Deitch and deVere White, 1992; Shankey *et al.*, 1993). In univariate analysis, DNA diploidy is strongly associated with favorable outcome, whereas DNA aneuploidy is strongly associated with poor outcome irrespective of stage or therapy. In multivariate studies, the status of DNA ploidy as an independent prognostic marker was controversial (Shankey *et al.*, 1993).
A good correlation of DNA ploidy with histologic grade has been reported. DNA ploidy may add clinically useful prognostic information for patients with intermediate grade tumors (Lieber *et al.*, 1995). Further, DNA content analysis of prostatic needle biopsies was reported to directly correlate with radical prostatectomy specimen ploidy status and was associated, independently, with the presence of metastasis, post-prostatectomy disease recurrence and extracapsular spread (Ross *et al.*, 1994). However, for the individual patient, the added information of DNA FCM may have limited value, since approximately 15% of those with diploid disease will experience disease progression within 5 years as compared with half of those with nondiploid disease. Further, ploidy does not predict length of survival once prostate cancer becomes disseminated (Deitch and deVere White, 1992). In the near future, randomized trials of patients might take DNA content into account to further elucidate the clinical utility of DNA FCM in the management of prostatic cancer.

2.10 Cytogenetic Aberrations and Allelic Loss in Prostatic Cancer

Knowledge concerning cytogenetic aberrations in prostate cancer is relatively

limited when compared with other common malignancies, and a consistent primary cytogenetic change has yet to be identified (reviewed in Sandberg, 1992). Conventional cytogenetic analyses have revealed that most prostatic tumors are diploid and have normal karyotypes. This is most likely due to overgrowth of normal stromal cells in culture (reviewed in Sandberg, 1990). Numerical aberrations encountered in prostate cancer include loss of the Y chromosome, trisomy of chromosome 7 and del(7)(q22), monosomy 8 and del(8)(p21), monosomy 10 and a del(10)(q24) (Brothman *et al.* 1990, 1991; Lundgren *et al.* 1992; Arps *et al.* 1993).
On the molecular level, restriction fragment length polymorphism (RFLP) and microsatellite studies showed frequent (> 30% of cases) loss of heterozygosity (LOH) on 7q31.1, 8p12-p22, 10p, 10q23-q25, 13q14, 16q, 17q21 and 18q (Carter *et al.* 1990a; Bergerheim *et al.*, 1991; Kunimi *et al.*, 1991; Latil *et al.*, 1994, 1997; Gao *et al.*, 1995a; Gray *et al.*, 1995; reviewed in Isaacs *et al.*, 1995; Takahashi *et al.*, 1995; Cooney *et al.*, 1996; Cunningham *et al.*, 1996). Further, a major susceptibility locus for familial forms of prostate cancer was mapped to chromosome 1q24-q25 by linkage analysis (Smith *et al.*, 1996). Recently, attention has been drawn to chromosome arm 8p, especially to the 8p21-p22 and the 8p12-p21region (Bova *et al.*, 1993, 1996; MacGrogan *et al.*, 1994; Trapman *et al.*, 1994; Kagan *et al.*, 1995; Vocke *et al.*, 1996). The results suggest the existence of two distinct chromosome 8p sites for candidate tumor suppressor genes important in prostate cancer development. Also in PIN loss of alleles on 8p was noted (Sakr *et al.*, 1994; Emmert-Buck *et al.*, 1995), indicating a possible initiating role of a tumor-suppressor gene at 8p in prostatic tumorigenesis. Further, widespread genomic instability of microsatellite repeats, indicative of aberrant DNA mismatch repair function, occurred in 20-64% of prostatic carcinoma of Japanese patients. Genomic instability was significantly associated with high tumor grade and extraglandular spread (Egawa *et al.*, 1995; Uchida *et al.*, 1995). Interestingly, in prostatic carcinomas in Western countries, an extremely low replication error frequency was found (Terrell *et al.*, 1995; Cunningham *et al.*, 1996).

2.11 Molecular Cytogenetics of Prostatic Cancer

The application of (F)ISH to nuclear suspensions and paraffin sections of prostatic tissue revealed numerical aberrations of chromosomes 7, 8, 10, 16, 17, 18, X and Y (van Dekken et al., 1990a; Persons *et al.*, 1993a; van Dekken and Alers, 1993; Baretton *et al.*, 1994; Brown *et al.*, 1994; Henke *et al.*, 1994; Jones *et al.*, 1994; Visakorpi *et al.*, 1994). Furthermore, FISH studies of nuclear suspensions or touch imprints of prostatic tumors revealed that alterations of chromosomes 7 and 8 are potential markers of poor prognosis in prostate cancer (Alcaraz *et al.*, 1994; Bandyk *et al.*, 1994; Takahashi *et al.*, 1994). Double-target FISH with cosmid probes for two chromosome 8p loci was applied to touch preparations in 42 cases of prostatic cancer by Matsuyama *et al.* (1994). In 71% of cases deletion of especially 8p22

sequences was seen. Likewise, Macoska *et al.*, (1994) showed the loss of 8p22 sequences in frozen tissue sections of prostatic tumors by means of FISH. FISH analysis of 16 radical prostatectomy samples with YAC-probes specific for 8p12 and 8p22 sequences demonstrated loss of one or both 8p loci in 62.5% of cases (Huang *et al.*, 1996).
Recently, a CGH study by Cher *et al.* (1994) demonstrated loss of 8p sequences in a panel of 17 prostatic tumors. Changes that occurred commonly in tumors with 8p allelic loss included 8q gain and loss of 13q, 16p, 16q, 17p, 17q, 20q, and Y sequences. Further, 8p loss and 8q gain as detected by CGH correlated with allelic imbalance mapping by PCR/RFLP study in 90% of cases. Furthermore, a CGH study of 31 primary and 9 recurrent prostatic carcinomas by Visakorpi *et al.* (1995a) revealed DNA sequence copy number changes in 74% of cases. Losses were five times more common than gains and most often involved 8p (32%), 13q (32%), 6q (22%), 16q (19%), 18q (19%), and 9p (16%). Allelic loss studies with five polymorphic markers for four different chromosomes were done on 13 samples and showed a 76% concordance with CGH results. Local recurrences that developed during endocrine therapy, showed significantly more gains and losses of DNA sequences than did primary tumors. Particularly involved were gains of 8q (found in 89% of recurrences versus 6% in primary tumors), X (56% versus 0%), and 7 (56% versus 10%), as well as loss of 8p (78% versus 32%; Visakorpi *et al.*, 1995a). The same authors demonstrated by CGH that amplification of the X-linked androgen receptor gene occurs in about 30% of recurrent tumors during androgen deprivation therapy (Visakorpi *et al.*, 1995b; Koivisto *et al.*, 1997). A recent study of a panel of 20 regional lymph node and 2 bone metastases, as well as prostatic tissue from 11 patients who had developed hormone-refractory metastatic disease, showed gain of 8q (85%), 11p (52%), 1q (52%), 3q (52%), and 2p (42%) sequences. Loss was seen on chromosome arms 8p (80%), 13q (75%), 16q (55%), 17p (50%), 10q (50%), 2q (42%), 5q (39%), 6q (39%), and 15q (39%; Cher *et al.*, 1996). No significant differences in the occurrence of chromosomal aberrations were found between the untreated and hormone-refractory tumors. In Table 6 a summary is given of karyotyping, LOH and ISH data. The results show that several chromosomal loci are implicated in prostatic tumor development.

2.12 Oncogenes, Tumor Suppressor Genes and Miscellaneous genes in Prostatic Cancer

Many studies indicate that the genomic sites involved in tumor-associated chromosomal rearrangements may include (one of the) two major classes of cancer associated genes, i.e. the dominantly-acting oncogenes and the recessive tumor suppressor genes (reviewed in Bishop, 1991). The oncogenes code for factors that control cellular proliferation and differentiation processes. Tumor suppressor genes

Table 6. Summary of karyotyping, molecular genetic and *in situ* hybridization (including comparative genomic hybridization) data of prostatic cancer

Method of investigation	*(Cyto)genetic aberration*
KARYOTYPING	
Numerical aberrations	+7, -Y
Structural rearrangements	del(7)(q22), del(8)(p21), del(10)(q24)
LOH	7q, 8p, 10pq, 13q, 16q, 17q, 18q
ISH	+7, +8, -8, -10, -17, -18, +X, -Y, del(8)(p22)
CGH	loss of 5q, 6q, 8p, 9p, 10q, 13q, 16pq, 17pq, 18q, 20q, Y sequences; gain of 1q, 7pq, 8q, Xq sequences

For details and references see text.

generally suppress cell proliferation. In Knudson's two mutation hypothesis (Knudson, 1971) mutational inactivation of one copy of the gene combined with a deletion of the other copy deregulates cell growth. In this paragraph the involvement of some of the most well known (proto)oncogenes, i.e. *ras*, *myc*, and *bcl-2*, and tumor suppressor genes, i.e. *p53*, *Rb1*, *MTS1*, and *p21* in prostate cancer will be briefly discussed, together with some other genes potentially involved in prostatic tumor progression.

Combination of three independent studies on mutations in the *ras* oncogene in prostatic cancer samples showed a total of three *ras* mutations in 94 samples (overall frequency of 4%). All mutations occurred in the Ha-*ras* gene, mapped to chromosome 11p15.5 (Carter *et al.*, 1990b; Gumerlock *et al.*, 1991; Moul *et al.*, 1992; Isaacs *et al.*, 1995). In contrast to these studies however, several reports have appeared which suggest that *ras* gene mutations do occur at significant frequencies (up to 33%) in prostatic carcinoma, both in latent carcinoma (Konishi *et al.*, 1992), and in clinical disease (Anwar *et al.*, 1992; Konishi *et al.*, 1995). In both studies, prostate tissue from Japanese men was examined, raising the possibility that significant differences may exist in the genetic events associated with prostate cancer in American men vs Japanese men. In latent prostate cancer mutations were found in the Ki-*ras* gene (located on chromosome 12p12.1), whereas in the clinical cancers mutations were found in both the Ha-*ras* gene and the Ki-*ras* gene.

There is only limited evidence that the *c-myc* gene (mapping to chromosome 8q24) itself is amplified in prostate cancer specimens. However, the c-*myc* gene is amplified, rearranged, and overexpressed in the cell line LNCaP, which is derived from a prostatic lymph node metastasis. Further, amplification of the c-*myc* gene is observed in the metastatic cell line PC-3 and in some metastatic prostatic tumors (reviewed in Peehl, 1993; Van Den Berg *et al.*, 1995; Jenkins *et al.*, 1997).

The *bcl-2* (proto)oncoprotein (mapped to chromosome 18q21.3) has been shown to

prolong cell survival by inhibiting apoptosis. Gene transfection experiments using the androgen-responsive LNCaP cell line have demonstrated that *bcl-2* overexpression permits continued growth *in vitro* and tumor formation *in vivo* despite androgen deprivation (Raffo *et al.*, 1995). Several immunohistochemical studies of *bcl-2* protein levels in prostate tumors have revealed a correlation between the accumulation of *bcl-2* protein and high tumor grade and stage, as well as resistance to anti-androgen therapy (McDonnell *et al.*, 1992; Colombel *et al.*, 1993; Westin *et al.*, 1995; Bubendorf *et al.*, 1996; Krajewska *et al.*, 1996).

p53, located on 17p13.1, is the most commonly mutated gene in human cancers. Varying frequencies of mutations of *p53* have been identified in prostatic cancer, although most investigators agree that the overall frequency of p53 in primary prostate cancer is low, ranging from 6 to 14% (Visakorpi *et al.*, 1992; Bookstein *et al.*, 1993; Navone *et al.*, 1993; Dinjens *et al.*, 1994; Ittman *et al.*, 1994; Mirchandani *et al.*, 1995). Further, a wide range of (mutated) *p53* overexpression (6%-79%) has been reported from immunohistochemistry analyses of prostatic specimens. These marked differences are likely caused by intratumoral heterogeneity for mutated *p53* within the prostatic tumor specimens, as well as variations in experimental protocols (Mirchandani *et al.*, 1995; Wertz *et al.*, 1996). Mutations in the *p53* gene and/or accumulation of *p53* protein were infrequent in clinically localized cancers, but more common in aggressive and/or advanced cancers, indicating that *p53* gene mutation is a late event in prostatic tumorigenesis. A statistically significant correlation was found between *p53* mutation and/or nuclear expression, and high histologic grade, advanced (metastatic) tumor stage, and the transition from androgen-dependent to androgen-independent growth (Visakorpi *et al.*, 1992; Bookstein *et al.*, 1993; Navone *et al.*, 1993; Ittman *et al.*, 1994; Mirchandani *et al.*, 1995). In contrast, Dinjens *et al.* (1994) showed that *p53* mutations were infrequent (10 to 15%) in both primary and lymph node metastatic prostate cancer, suggesting that there is no strict correlation between *p53* mutation and tumor metastasis, at least to regional lymph nodes.

The importance of *Rb1* gene (located on 13q14) inactivation in prostate cancer has been suggested by Bookstein *et al.* (1990a, b). The authors found reduced or absent expression of the *Rb* protein in two out of seven predominantly metastatic prostate cancers, of which one showed a mutation in the *Rb* gene and simultaneous loss of the wildtype allele. Further, they showed that introduction of a normal *Rb1* homologue in the Dunning 145 prostatic cell line resulted in loss of the ability to form tumors in nude mice. A recent study showed that despite frequent LOH on 13q14, no significant correlation between LOH in the *Rb1* region and the absence of *Rb1* protein product could be established, suggesting that additional genes in this region, e.g., *BRCA2* on 13q12, may be important in prostatic tumorigenesis (Cooney *et al.*, 1995; Cunningham *et al.*, 1996).

Investigation of the involvement of the *MTS1*, located on chromosome 9p21, in 4

cell lines and 20 primary prostate tumors showed mutations in two cases only, one in the Dunning 145 cell line, and one in a primary tumor (Tamimi *et al.*, 1996). The *WAF1/CIP1* (*p21*) gene, localized to chromosome 6p21.2, is a *p53* mediator gene and an inhibitor for G1 cyclin-dependent kinases. Preliminary data demonstrate that mutations of the *p21* gene occurred in 17% of prostatic cancer patients (Gao *et al.*, 1995b).

Other genes possibly involved in the pathogenesis of prostatic cancer include *MXI1*, a protein that negatively regulates *myc* activity, which gene is located on chromosome 10q24-25. It shows mutations in primary tumors with concomitant 10q24-25 deletions (Eagle *et al.*, 1995). Another candidate might be *PAC1*, mapping to chromosome arm 10p, which is known to suppress tumorigenicity and induce apoptosis of prostatic cancer cells in nude mice (Sanchez *et al.*, 1996). Dong *et al.* (1995) reported that a gene from chromosome 11p11.2, designated *KAI1* was able to suppress metastasis when introduced into rat prostate cancer cells. Further, expression of this gene was reduced in human cell lines derived from metastatic prostate tumors, suggesting that decreased expression of this gene may be involved in prostatic tumor progression. Another locus recently implicated in prostatic cancer is *BRCA1* or other genes located on chromosome 17q. This gene, located at chromosomal region 17q21, is involved in hereditary breast/ovarian cancer and possibly also in prostate cancer (Gao *et al.*, 1995a). Introduction of fragments of normal chromosomal region 17q, including *BRCA1*, suppressed the malignant phenotype of human prostate cancer cell line PPC-1 (Murakami *et al.*, 1995).

Other factors likely involved in the acquisition of invasiveness and subsequent metastatic spread of prostatic cancer are the genes of the E-cadherin/catenin complex. E-cadherin is an epithelial cell adhesion molecule, which maps to chromosome 16q22.1. E-cadherin is closely associated with α-catenin, which is a necessary component of the E-cadherin-mediated cell adhesion complex, and maps to chromosome 5q31. Loss of E-cadherin expression correlated well with the invasive phenotype of prostatic cancer cell lines (Bussemakers *et al.*, 1992). Recently, Graff *et al.* (1995) showed that loss of E-cadherin expression in both breast and prostatic carcinomas results from hypermethylation of CpG islands in the E-cadherin promotor region, thereby preventing transcription of this gene. Further, Morton *et al.*, (1993) demonstrated that in cell lines most human prostatic cancer cells have reduced or absent levels of α-catenin or E-cadherin as compared with normal prostatic epithelial cells. Transfer of a normal chromosome 5 into the PC3 prostate cancer cell line, which has a homozygous deletion of this gene, resulted in re-expression of α-catenin and subsequent restoration of E-cadherin function (Ewing *et al.*, 1995). A statistically significant inverse correlation was found between E-cadherin expression and tumor grade, stage and overall survival (Umbas *et al.*, 1994; Isaacs *et al.*, 1995).

Recently, attention was drawn to isoforms of CD44. This transmembranous glycoprotein, located on chromosome 11p13, participates in specific cell-cell and cell-extracellular matrix interactions and has been proposed to play a major role in tumorigenicity or metastasis of different types of tumor cells. In prostate cancer it was shown that down regulation of the standard form of CD44 correlates with metastatic potential in Dunning rat cell lines. Further, enhanced expression of standard CD44 in a highly metastatic cell line suppressed its metastatic capability (Gao *et al.*, 1997). Kallakury *et al.* (1996) found that down regulation of the standard form of CD44 correlated with high tumor grade and aneuploidy. Moreover, loss of CD44 (standard) expression in prostate adenocarcinoma predicted a poor prognosis, independent of stage and grade (Noordzij *et al.*, 1997).

3 NON-RADIOACTIVE *IN SITU* HYBRIDIZATION

3.1 The development of Non-Isotopic *In Situ* Hybridization

The technique of molecular hybridization was introduced by Gall and Pardue, John *et al.*, and Buongiorno-Nardelli and Amaldi in 1969, using radioactively labelled probes applied to tissue sections. At that time it was called *in situ* hybridization (ISH) to emphasize the difference with the biochemical hybridization method, introduced in 1961 by Hall and Spiegelman. Using radioactive isotopes, the insulin gene was mapped to chromosome 11 by Harper *et al.* (1981). The first reports on non-isotopic ISH came from Rudkin and Stollar in 1977. After hybridization of the 5S-rRNA genes on *Drosophila* polytene chromosomes with a RNA probe, they used a rabbit-derived antibody against DNA-RNA hybrids for detection purposes. The antibody was visualized by a second fluorochrome-labeled antibody against rabbit immunoglobulins. A direct approach was used by Bauman *et al.* (1980), who applied fluorochrome-labeled RNA as a probe for the detection of kinetoplast DNA in the insect *Chrithidae Luciliae* and of 5S rRNA genes in *Drosophila*.

The most important labeling techniques for ISH came with the introduction of enzymatically or chemically modified nucleic acid probes. In 1981 Langer *et al.* reported the first enzymatic synthesis of biotin-labeled polynucleotides. In 1982 the first applications were reported in the mapping of genes on *Drosophila* chromosomes (Langer-Safer *et al.*, 1982) and of centromeric DNA on mammalian chromosomes (Manuelidis *et al.*, 1982). The visualization of biotin-labeled hybrids was accomplished by anti-biotin antibodies or avidin, coupled to fluorochromes or enzymes. The first chemical introduction of a molecule, capable of serving as a hapten for immunological detection, into the DNA was described by Tchen *et al.* and Landegent *et al.* (both in 1984). These authors used 2-acetylaminofluorene (AAF) to modify the guanosine residues in the nucleic acids. This technique was further refined by Landegent *et al.* (1985), who was the first to report detection of a single copy sequence by non-isotopic ISH. Using AAF-modified DNA probes and anti-AAF antibodies, these authors were able to localize part of the human

thyreoglobulin gene. Hopman *et al.* (1986) modified the cytosine bases within a human DNA probe with mercury acetate to achieve the simultaneous detection of mercurated human DNA and biotinylated mouse satellite DNA in a hybrid cell line, using different fluorochromes for visualization of the probes. The first triple color ISH was reported by Nederlof *et al.* (1989), using a combination of biotinylated, mercurated and AAF-modified probes. The latest modification procedures for ISH were the use of sulfonated probes (Morimoto *et al.*, 1987), and incorporation of fluorescein (Bauman, 1985) and digoxigenin (Herrington *et al.*, 1989) into DNA and RNA probes. Biotin and digoxigenin labeling systems are now widely used and labeling materials and specific antibodies are commercially available. Recently, directly labeled probes have been applied by incorporation of fluorochromated dUTPs into the DNA by a simple nick translation reaction (Wiegant *et al.*, 1993).

3.2 The Sensitivity and Application of ISH

When non-isotopic ISH was initially introduced, only large targets could be detected. This was in contrast with the extremely sensitive radioactively labeled probes, with which very small DNA targets could be visualized. However, ISH with radioactively-labeled probes is very time-consuming; resolution and morphology are poor and also carries the burden of radioactive waste. The more rapid results and the more precise localization made non-radioactive ISH very attractive for cytogeneticists. At first only repetitive sequences (e.g., Cooke and Hindley, 1979; Burk *et al.*, 1985; Willard, 1985; Buroker *et al.*, 1987) were used as targets, ranging from 100 Kb up to a few Mb's. Among those targets were the rRNA genes in the satellites of the acrocentric chromosomes, the α-satellite DNA's on the centromeres and the heterochromatin block on the Y chromosome (Bauman *et al.*, 1981; Manuelidis, 1982; Pinkel *et al.*, 1986). Refinement of the procedure allowed visualization of single copy sequences, a few Kb in size (Garson et al, 1987; Ambros and Karlic, 1987; Lawrence *et al.*, 1988). Regional chromosome-specific DNA probes, harboring large pieces of genomic DNA, are nowadays provided by cosmid clones (Van Dilla and Deaven, 1990), yeast artificial chromosomes (Riethman *et al.*, 1989; Lengauer *et al.*, 1992a, b) and lately, P1 phages (Kroisel *et al.*, 1994; reviewed in Monaco and Larin, 1994). Recently, an important refinement of the signal detection procedure (Kerstens *et al.*, 1995; Raap *et al.*, 1995) has been introduced for (fluorescent) ISH. Also visualization by charge-coupled device (CCD) cameras and the use of sophisticated image analysis systems (e.g., Viegas-Pequignot, 1989; Ried *et al.*, 1992), have made non-radioactive ISH suitable for gene-mapping purposes and as sensitive as radioactive ISH.

Non-radioactive ISH with chromosome-specific repetitive DNA probes, mostly α-satellite DNA, appeared very useful in detecting numerical chromosome aberrations in interphase nuclei (Pinkel *et al.*, 1986; Moyzis *et al.*, 1987; Devilee *et al.*, 1988; Hopman *et al.*, 1988; van Dekken and Bauman, 1988; see Fig. 4). Cremer *et al.*

FIGURE 4.
Fluorescent *in situ* hybridization (FISH) with a biotin-labeled chromosome 15-specific probe to metaphase and interphase cells. Hybridized probe was visualized with fluoresceinated avidin (white), total nuclear DNA was counterstained with propidium iodide (grey). The chromosome 15 hybridization signals are recognized on both metaphase chromosomes (*arrows*) and within the interphase nucleus (*arrowheads*). Magnification: A 100x objective was used.

(1988a) coined the term *"Interphase Cytogenetics"* for this procedure, in a study using bicolor double-target ISH to detect numerical chromosomal aberrations in neurogenic tumors. At present, satellite DNA probes for the majority of human chromosomes are commercially available.

An important development with respect to the detection of numerical and structural chromosomal aberrations in metaphase and interphase chromosomes came with the application of chromosome-specific libraries as probes for ISH the so-called *"chromosome paints"* (e.g. Cremer *et al.*, 1988b; Lichter *et al.*, 1988; Pinkel *et al.*, 1988; Kuo *et al.*, 1991). These probes proved very suitable for detection of chromosomal translocations (Pinkel *et al.*, 1988). A combination of cosmid- and phage-cloned DNA sequences was used by Arnoldus *et al.*, and Tkachuk *et al.* (both 1990), to visualize the *bcr/abl* fusion in the Philadelphia translocation within blood and marrow interphase cells of leukemia patients.

Recently, new methods were introduced for a simultaneous and unequivocal discernment of all human chromosomes in different shades of colors (Schröck *et*

al., 1996; Speicher *et al.*, 1996). It is based on labeling painting probes for each chromosome with different combinations of five spectrally overlapping fluorochromes ("Multiplex" or "Spectral FISH"). However, the fluorochrome colors are not distinct enough for the unaided human eye to distinguish which combination a chromosome carries. The two teams solved this problem in different ways: Speicher *et al.* examined the different colors of the stained chromosomes by series of filters. Sophisticated computer-aided analysis then combined the data and displayed each chromosome in pseudo-colors. In contrast, Schröck *et al.* used a so-called interferometer to determine the full spectrum of light emitted by each stained chromosome. The resulting interferogram was analyzed by Fourier transformation and the measured spectra were then converted to classification (pseudo) colors. The sensitivity of spectral FISH with currently available painting probes is between 0.5 and 1.5 Mb. This method is especially suitable for the detection of translocations and for the identification of marker chromosomes (Le Beau, 1996).

Another fairly recent development is the so-called *in situ* PCR, originally introduced by Haase *et al.* (1990). This technique combines the properties of the PCR technique with ISH, permitting the localization of specific amplified DNA or RNA sequences within isolated cells and tissue sections (reviewed in Nuovo, 1992; Komminoth and Long, 1993). At present, this technique has proven its suitability predominantly in the detection of (latent) viral infections in tissues. A minor modification of this *in situ* PCR method is primed in situ labeling (PRINS). It is based on the rapid annealing of unlabeled specific oligonucleotide primers to its complementary target sequence *in situ* followed by extension by a PCR in the presence of labeled nucleotides, the latter being detected with conventional fluorescent detection methods (Koch *et al.*, 1989). The detection of multiple DNA targets using different fluorochromes in sequential PRINS reactions has been recently reported (Hindkjaer *et al.*, 1994; Speel *et al.*, 1995).

A potential powerful application is the combination of immunocytochemistry (ICC), or immunohistochemistry (IHC), and ISH. It allows simultaneous examination of gene structure and gene expression in the same cellular material. For successful combination of ICC/IHC and ISH several requirements must be met, such as preservation of cell morphology and protein epitopes, accessibility of target DNA, absence of cross reaction between the different detection procedures, and good color contrast and stability of enzyme precipitates or fluorochromes. A variety of such combined ICC/IHC and ISH procedures have now been reported using either enzyme precipitation reactions (Mullink *et al.*, 1989; Speel *et al.*, 1994a), fluorochromes (Weber-Matthiessen *et al.*, 1993; Bridge *et al.*, 1994; Leger *et al.*, 1994), or a combination of both (Herbergs *et al.*, 1994; Speel *et al.*, 1994b).

Most applications mentioned above were based on dissociated tissue, which eliminates the possibility of assessing the architecture of the tissue. In this situation,

it is difficult to correlate specific chromosomal changes with histological characteristics of the cells of interest. Recently, ISH techniques have been adapted for application to tissue sections, permitting a combination of cytogenetic and histopathological analysis (e.g. Hopman *et al.*, 1991a; van Dekken *et al.*, 1992).

3.3 Comparative Genomic Hybridization

Comparative genomic hybridization (CGH) was introduced by Kallioniemi *et al.* in 1992. Since then, this elegant technique was applied for the analysis of human malignancies (e.g. DuManoir *et al.*, 1993; Kallioniemi *et al.*, 1994). This procedure provides a global analysis of gains and losses of genetic material of the whole chromosomal complement of solid tumors. Importantly, DNA derived from paraffin embedded tumor tissue is also applicable in CGH analysis (Isola *et al.*, 1994; Ghazvini *et al.*, 1996). In the CGH assay normal human metaphase chromosomes are competitively hybridized with two differentially labeled (red and green) genomic complements (normal reference vs. isolated tumor DNA) which, upon fluorescence microscopy, reveal the chromosomal locations of copy number changes in DNA sequences between the two genomes (reviewed in Houldsworth and Chaganti, 1994; Waldman *et al.*, 1996). Differential fluorescent hybridization signals represent gains and losses of the tumor DNA relative to the reference DNA. Ratios of the green:red signals can be quantitated along the length of each homologue and provide a so-called *"copy number karyotype"* of the tumor DNA. These ratio measurements require sophisticated multicolor image analysis (Kallioniemi *et al.*, 1994; DuManoir *et al.*, 1995). Although the CGH method represents an important step forward in cancer genetics there are some limitations and technical difficulties. Firstly, CGH only detects loss or gain of DNA sequences and therefore balanced translocations or inversions are not detectable. Secondly CGH can not detect ploidy changes in tumors (e.g., discrimination of diploid from true triploid tumors). Finally, CGH can not detect intratumoral heterogeneity (Kallioniemi *et al.*, 1994). In spite of these limitations CGH technology in its current state of development is a promising research tool and with further optimization can prove useful for routine applications in both tumor genetics and clinical genetics (Bryndorf *et al.*, 1995; Waldman et al, 1996).

3.4 Methodological Aspects of Non-Isotopic *In Situ* Hybridization

3.4.1. Isolated Nuclei vs Tissue Sections

During the development of interphase *in situ* hybridization (ISH), most investigators applied fluorescent ISH (FISH) to cell suspensions made from disaggregated fresh or frozen tumor material (e.g. Hopman *et al.*, 1988, 1991b; van Dekken and Bauman, 1988; van Dekken *et al.*, 1990a, b; Anastasi *et al.*, 1990). Application of FISH to isolated nuclei results in high accuracy with respect to the copy number assessment of the target chromosome. Other advantages of working with nuclear

suspensions are (Table 7): 1) A fast, standardized protocol can be used with uniform pretreatment of a variety of specimens. 2) Double- or even triple-color experiments involving two or more target chromosomes are possible with FISH (Wiegant *et al.*, 1993). 3) Signal amplification is optional and relatively easy with FISH. The disadvantages of this method are: 1) The tissue architecture is lost which eliminates the detection of relationships between chromosome changes and histopathological characteristics. 2) Admixture of stromal (non-tumor) elements that cannot be distinguished from the tumor cells may be included in the cell sample. Thus, a chromosomal abnormality in tumor cells can be obscured by the presence of stromal cells. The number of non-tumor cells might vary between different types of solid tumors. In tumors with a poorly developed stroma, e.g. small cell carcinomas, this admixture-effect might be much less than in, e.g. adenocarcinomas with abundant stroma. 3) Detection of potentially important, intratumoral cytogenetic heterogeneity is not possible in suspensions.

Table 7. A methodological comparison between in situ hybridization with chromosome-specific DNA probes to isolated tumor cell nuclei and routinely processed (paraffin-embedded) tissue sections

	Cell suspensions	*Tissue sections*
Cell preparation	disaggregation	cutting slices
Pretreatment	uniform	variable[a]
Hybridization	standard	standard
Detection	fluorescence	absorption[b]
Signal amplification	optional	pre-planned
Evaluation	whole nuclei	nuclear slices

[a] Differences in fixation type and time often require optimization of pretreatment.
[b] Due to autofluorescence phenomena in (fixed) tissues absorption microscopy is favorable.

In recent years, several investigators have developed protocols for ISH on tissue sections (e.g. Hopman *et al.* 1991a; van Dekken *et al.* 1992; Kim *et al.* 1993; Krishnadath *et al.* 1995). Visualization of the signal is usually performed by standard immunoperoxidase methods. Such are preferred above fluorescent methods, since tissue morphology is better appreciated after hematoxylin counterstaining. Moreover, FISH on tissue sections may be hampered by autofluorescence phenomena and fixation pigments. The main advantages are therefore (Table 7): 1) Preservation of tissue architecture enabling a precise analysis of tumor cells only. 2) Cytogenetic heterogeneity is easily recognized. 3) Archival material is well suited for evaluation. The main side effect is that sectioning of the tissue blocks results in truncated nuclei. This phenomenon makes it more difficult to establish the exact chromosome copy number per nucleus. Fig. 5 shows

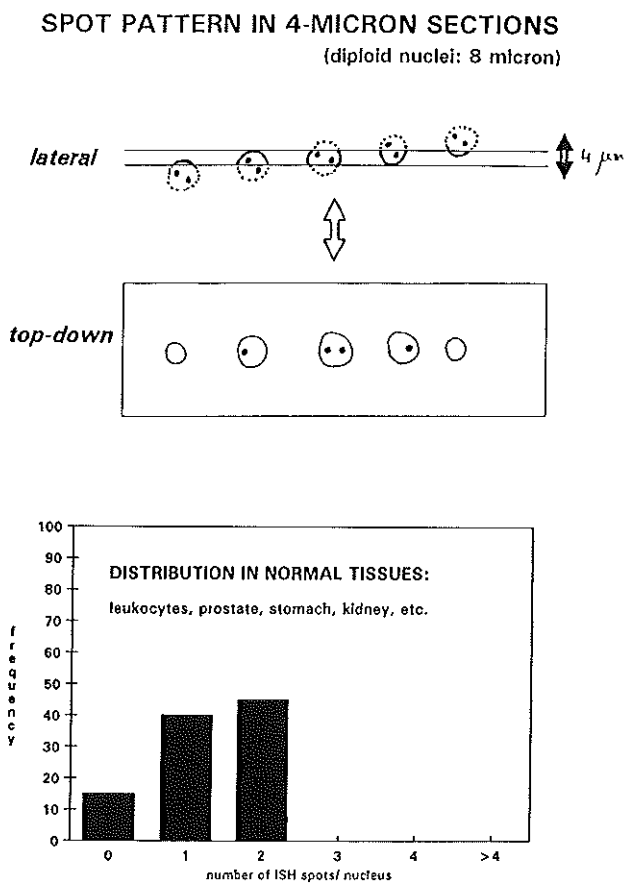


FIGURE 5.
Ideogram representing hybridization spot patterns of cell nuclei in 4 µm tissue sections. Due to the truncation effect (top), cells are seen that contain 0, 1, or 2 spots per nucleus (middle). This results in various cell types in a distribution pattern with about half of the cells containing 2 spots per nucleus (bottom).

a diploid probe spot pattern for 4 µm tissue sections. Due to truncation, the normal control cells generally display 0 or 1 spot for the autosomes in about 10% and 40% of nuclei respectively. The main disadvantages of ISH to tissue sections are: 1) Since the specimens show more variation, the protocol is more time consuming. The optimal protease digestion time needs to be determined for each tissue block. 2) The enzymatic chromogenic reaction product cannot be as easily amplified as in FISH (Table 7).

An intermediate form between ISH performed on nuclear suspensions and tissue section ISH is a touch-imprint of fresh or frozen tumor material. In this technique, the tumor releases whole nuclei in the imprint on the slide, exhibitting a fair cell

morphology (e.g. Waldman *et al.*, 1991; Persons *et al.*, 1993a, b). Architectural details, however, cannot be analyzed.

3.4.2 Methodological Aspects of ISH Applied to Tissue Sections

Denaturation, hybridization and post-hybridization washing procedures are essentially the same for both ISH on metaphase chromosomes, nuclear suspensions and tissue sections. However, some additional steps have to be implemented for the successful application of ISH to tissue sections. Proper pretreatment of the specimen is crucial. The chromosomal DNA in a nuclear suspension or in a tissue sections requires proper fixation before ISH is applied. Without fixation, rigorous treatments like heat denaturation will lead to inevitable loss of target DNA and destruction of tissue morphology. ISH to paraffin-embedded archival tissue is influenced by the type and duration of fixation. Some fixatives are better suited for ISH than others. Certain fixatives may decrease the hybridization signal (Nuovo and Silverstein, 1988). Buffered formalin (pH 7.0) is an excellent fixative for ISH. With fixation times ranging from several hours to several days, reproducible strong hybridization signals may be obtained (Nuovo, 1991). In our experience, relatively long fixation yields better results than shorter fixation. Fortunately, in most pathology departments buffered formalin is used nowadays and standardization of fixation procedures has been introduced. However, adequate fixation of the tissue is only one prerequisite for high quality ISH, also the condition of the tissue before fixation is important. In this respect, tissues derived from autopsies often show poor quality ISH due to autolysis. Also, decalcification of bony tissues by aggressive methods, such as formic acid containing substances, often results in a specimen unsuitable for ISH. Fixatives that contain heavy metals, such as mercury (Zenker's solution) or picric acid (bouin's solution) are not very suitable for ISH. The duration of fixation is crucial for these fixatives and therefore they are not very suitable for routine applications. Furthermore, it has been documented that fixatives such as Bouin's lead to a marked degradation of DNA (Nuovo and Silverstein, 1988; Nuovo, 1992).

Preparation of the slides is an important aspect of ISH experiments, especially concerning tissue sections. Without proper adherence of tissue sections to the glass slides, the specimens might disintegrate or float from the slide during the ISH procedure. Organosilane or aminoalkylsilane (AAS), a chemical used in the glass industry, is a very good coating that provides optimal adherence much more than other coatings (e.g. poly-L-lysin; Nuovo, 1992). A disadvantage is its toxicity. Silane-coated slides are now available from commercial sources.

Most fixatives exert primary effects on cellular proteins, either by cross-linking them to other macromolecules such as DNA (buffered formalin), or by denaturation (ethanol). In this way endogenous tissue degrading enzymes are inactivated (Nuovo and Silverstein, 1988; Nuovo, 1992). However, cross-linking also hinders

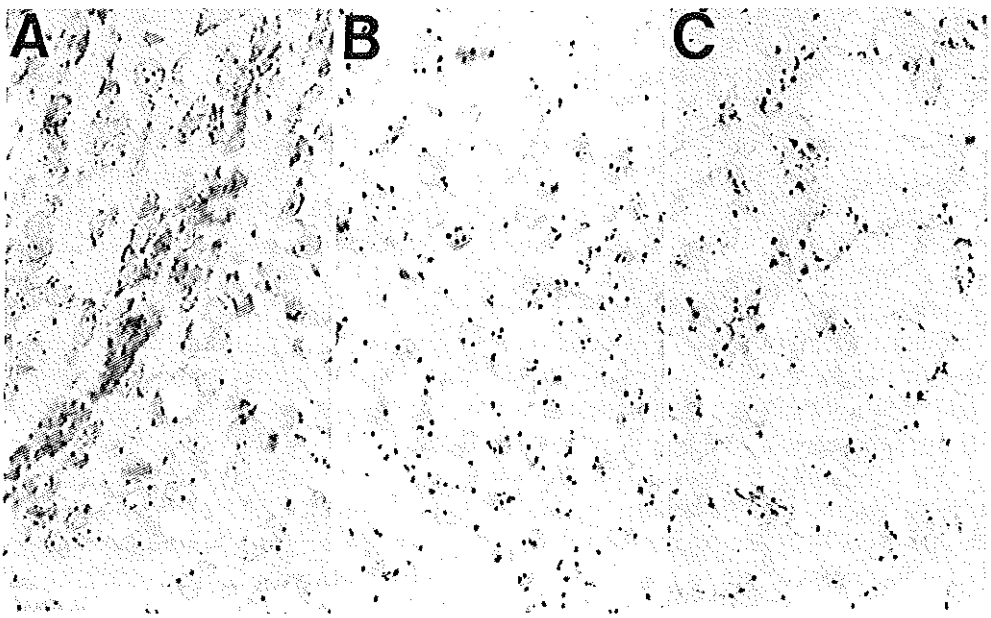


FIGURE 6.
In situ hybridization (ISH) with the chromosome 1-specific alpha-satellite DNA probe to a 4 µm section of an aneuploid poorly differentiated prostatic adenocarcinoma. The ISH spots were visualized with immunoperoxidase/DAB (black), hematoxylin was used as a counterstain (grey). **A)** ISH signals are not optimal due to a too short pepsin digestion time (5 min). Also a strong background is seen. **B)** The same area of this adenocarcinoma. A good signal intensity with fair tissue morphology is obtained with a 10 min pepsin digestion. **C)** Same area, 20 min pepsin digestion. The ISH signals are still intense, but the morphology is destroyed. Magnification: 361x.

penetration of the probe to target DNA. Different methods have been used to facilitate probe penetration including treatment with hot standard saline citrate (SSC), heating in a microwave oven and proteases. For tissue section ISH, pretreatment by pepsin digestion renders best results. This may vary from specimen to specimen (Hopman *et al.*, 1991a; van Dekken *et al.*, 1992).
Pretreatment of the sections before pepsin digestion, e.g. in hot 2xSSC buffer, allows shorter digestion times and results in better tissue morphology (Hopman *et al.*, 1991a). The effects of pepsin digestion on tissue sections are depicted in Fig. 6. In general, too short digestion times result in poor ISH signals (and strong background) but preserved morphology; in contrast, overdigestion results in a strong signal but poor tissue morphology. An optimal combination of signal strength and tissue morphology can be obtained by varying the digestion time.
Immunoperoxidase methods are often applied for the detection of ISH signals in tissue sections. A three-step reaction is most commonly performed using anti-biotin

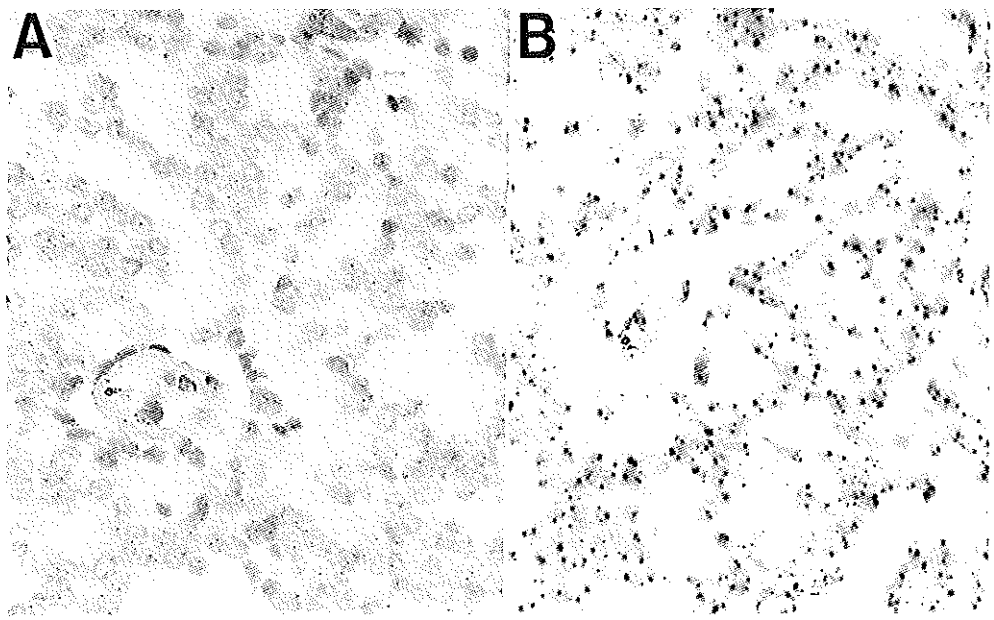
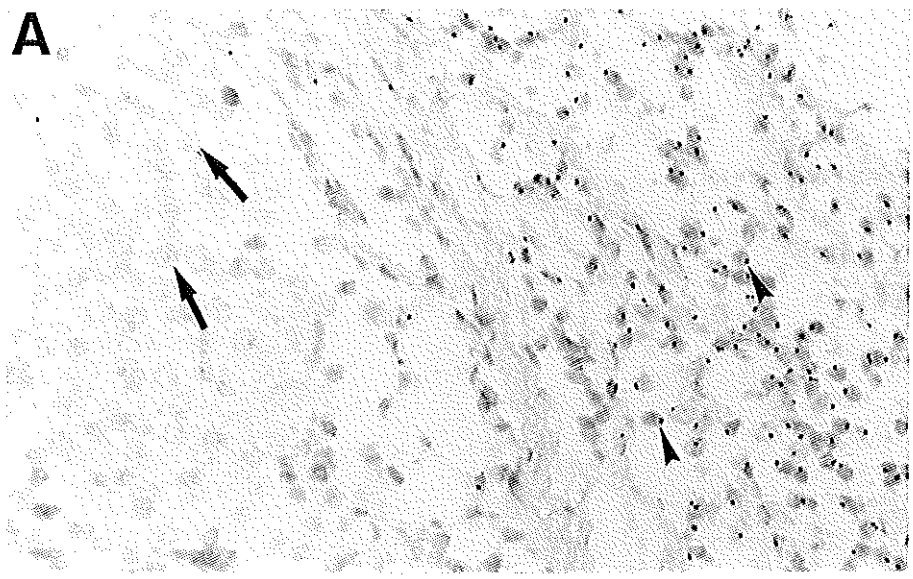
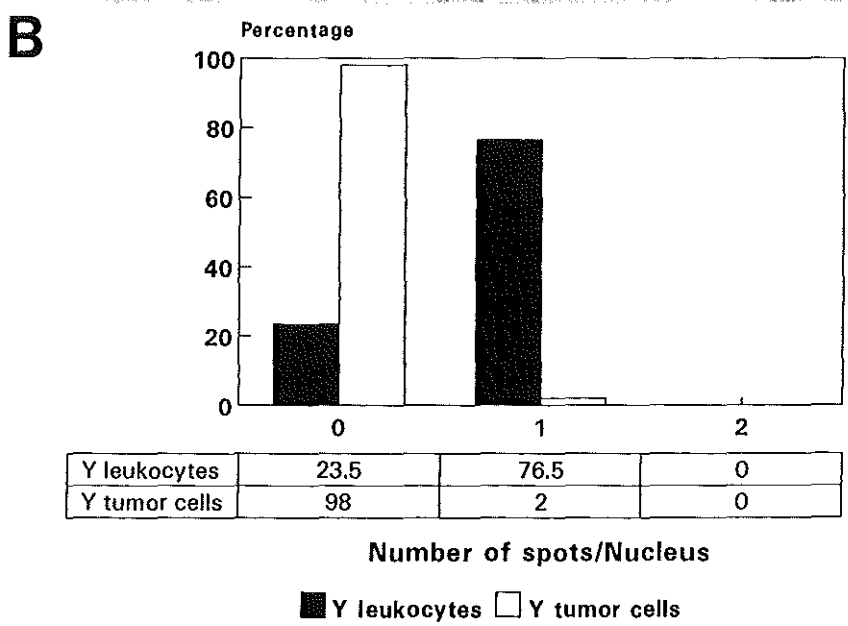


FIGURE 7.
ISH with a biotin-labeled DNA probe, specific for chromosome 1p36.3, to a 4 µm tissue section of a bone metastasis of prostatic adenocarcinoma. **A)** Standard immunoperoxidase reaction. ISH signals are barely seen in the nuclei. **B)** CARD amplification procedure resulting in strong ISH signals in the tumor nuclei. Magnification 361x.

or anti-digoxigenin as primary antibody, followed by a biotinylated secondary antibody, with subsequent incubation in ABC complex (avidin conjugated to biotinylated horseradish peroxidase). Visualization is mostly performed by diaminobenzidine (DAB) in imidazole containing buffer. Imidazole enhances chromogen deposition and thus the intensity of the ISH signals (Hopman *et al.*, 1991a; Speel *et al.*, 1992; van Dekken *et al.*, 1992).
Recently, an efficient signal-amplification method was introduced for ISH by Kerstens *et al.* (1995) and Raap *et al.* (1995). It was originally developed for immunoassays and immunohistochemistry (Bobrow *et al.*, 1989; Adams, 1992). This Catalyzed Reporter Deposition (CARD) method is based on the deposition of biotinylated tyramine (BT) at the site of the hybridized DNA probe. The BT precipitate is subsequently visualized with fluorochrome- or enzyme labeled (strept)avidin, resulting in strong hybridization signals. Nowadays, a commercial BT amplification kit has become available. The CARD method is especially suitable for visualization of (very) weak ISH signals, such as single copy probes. The principle is illustrated for ISH applied to tissue sections with a 1p telomeric DNA probe (Fig. 7). However, at present the efficiency of the CARD method for the visualization of

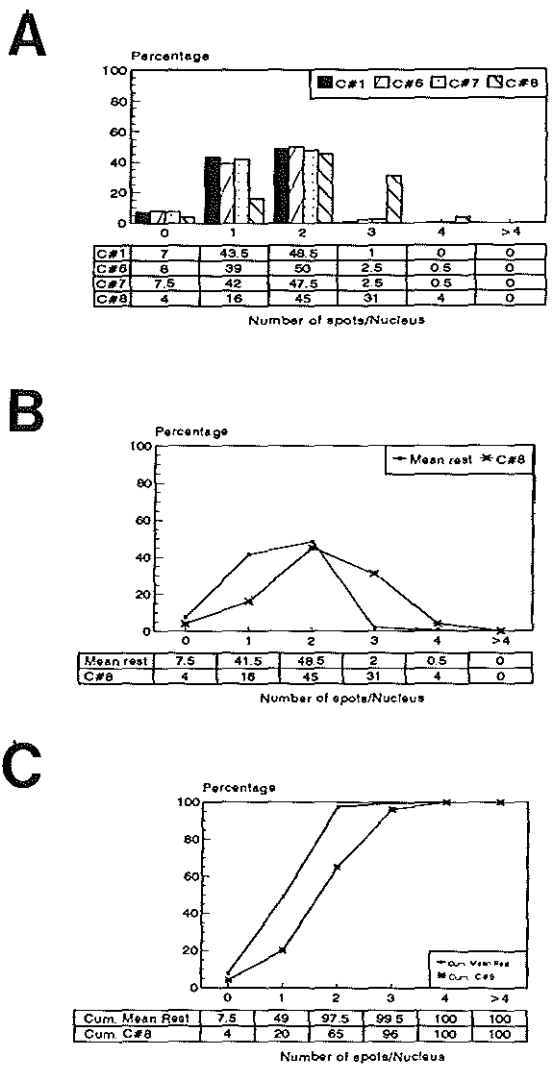




Y leukocytes	23.5	76.5	0
Y tumor cells	98	2	0

FIGURE 8.
ISH with the chromosome Y-specific probe to a 4 µm tissue section of a poorly differentiated prostatic adenocarcinoma. **A)** Loss of Y is seen in the tumor nuclei (*arrows*), but not in the surrounding leukocytes (*arrowheads*; Magnification 361x). **B)** Bar histogram displaying the spot profile for the chromosome Y in the leukocytes and tumor nuclei. Note loss of Y in the tumor cells.

single copy probes on archival tissue sections is insufficient.
Evaluation of ISH signals on tissue sections involves counting of ISH-related spots in interphase nuclei in morphologically identifiable tissue. We have chosen a section thickness of 4 µm after evaluating how section thickness affects overlap and spot countability. The effect of section thickness on ISH spot distribution was extensively analyzed by Dhingra *et al.* (1994). For each of the DNA probes, we count 100 "intact" (=spherical) and non-overlapping 4 µm truncated nuclei (van Dekken *et al.*, 1992, 1993). For the detection of aberrations in tissue sections, it is important to compare ISH spot distributions for different DNA probes with each other in the same cell type. Furthermore, on each tissue section normal diploid cells such as leucocytes, normal epithelium, nerve cells, etc., can serve as an internal control (Fig. 8). A model for evaluation of ISH spot distributions in tissue sections was recently proposed by Pahlplatz *et al.* (1995).
When evaluating (F)ISH signals, it is also important to distinguish between aneuploidy and aneusomy for a certain chromosome. To this end a probe set should include at least one probe for which no cytogenetic aberrations are expected. The probe set should therefore consist of at least three probes: Two target chromosome probes plus one or more "reference" probe(s) for correction of ploidy. The reference probe dot counts will always follow changes in ploidy, and this can be used to determine the proportion of aneuploid cells. Counting of probes identifying non-aberrant chromosomes will follow the spot distribution of the reference probe, whereas aberrant chromosomes will display a different spot distribution pattern. For statistical analysis the mean can be taken of the non-aberrant probes and compared with the aberrant probe (van Dekken *et al.*, 1992, 1993).
Whereas Chi-square and derivative statistical analyses are sufficient for suspension ISH, due to the truncation phenomenon, a more specialized test is needed for section ISH. Here the probe spot distributions can statistically be evaluated by means of e.g. the Kolmogorov-Smirnov test (Young, 1977; Kibbelaar *et al.*, 1993). This cumulative statistical test is very suitable for two-sided comparisons of histograms or other distributions. Underrepresentation of a specific chromosome shows a shift to the left of the DNA probe distribution, as compared with non-aberrant probe distributions. Conversely, overrepresentation is seen as a shift to the right. In Fig. 9 this is illustrated with chromosome 1, 6, 7, and 8 specific DNA probes showing gain of chromosome 8 in a prostatic tumor recurrence. This test is very suitable for evaluation of gains and losses of chromosomes (van Dekken *et al.*, 1992, 1993). However, the Kolmogorov-Smirnov test is not very apprpriate for recognition of small aberrant subsets. Good alternatives are then provided by, e.g., the multiple-proportion test and the z'-max test (Kibbelaar *et al.*, 1993).



C#1	7	43.5	48.5	1	0	0
C#6	8	39	50	2.5	0.5	0
C#7	7.5	42	47.5	2.5	0.5	0
C#8	4	16	45	31	4	0

Mean rest	7.5	41.5	48.5	2	0.5	0
C#8	4	16	45	31	4	0

Cum. Mean Rest	7.5	49	97.5	99.5	100	100
Cum. C#8	4	20	65	96	100	100

FIGURE 9.
Barhistograms (plus tables) illustrating statistical analysis of section ISH by the Kolmogorov-Smirnov test. **A)** Barhistogram displaying spot distributions for chromosomes 1, 6, 7, and 8 in a local prostatic tumor recurrence. Chromosomes 1, 6, 7 display a normal diploid profile and hence they are designated as non-aberrant probes. Chromosome 8 shows an overrepresentation. **B)** Same, line histogram for the mean of the non-aberrant probes (rest) compared to the chromosome 8 probe, which shows a shift to the right. **C)** Line histogram, cumulative: The maximum difference between the mean of the non-aberrant chromosomes and chromosome 8 is seen at 2 spots per nucleus, representing a P value of <0.01, if 100 cells are counted and a maximum difference of ≥ 16 is reached.

4. Scope of This Thesis

As discussed in this chapter, present methods of assessing the prognosis for prostate cancer include staging and grading parameters. Unfortunately, these methods fail to provide consistent predictive information regarding the clinical outcome of an individual tumor. Thus, there is a need to identify characteristics of prostate tumor cells that would help in defining the biological aggressiveness of individual tumors and guide the choice of therapy. The goal of the present study was the identification of chromosomal abnormalities specifically involved in prostatic tumor development and prostatic cancer progression towards metastatic disease.
The main questions addressed in this thesis were:
1) Which chromosomal aberrations occur in different stages of the prostatic cancer spectrum, i.e. precancerous lesions, organ-confined tumors, regionally advanced tumors, metastatic tumors and tumor recurrences?
2) Are specific chromosomes involved in prostatic tumor progression towards metastatic disease?
3) Are the same chromosomes also implicated in progression towards local recurrence and distant metastatic disease in individual patients?
4) What is the ploidy status of the tumors, representing different stages of prostatic tumor development?
5) Does the histological heterogeneity of prostatic cancer reflect a cytogenetical heterogeneity?
To this end interphase ISH with centromere-specific probes to archival, routinely processed tissue sections was performed. This allowed simultaneous detection of both chromosomal alterations and ploidy status of the tumor together with histopathological characteristics. By performing interphase ISH on tissue sections a precise analysis of only tumor cells can be made. This approach seemed especially suited for the detection of chromosomal abnormalities in prostate cancer, since this tumor type shows a remarkable multifocality and histological heterogeneity, as well as a profound admixture of non-tumor cells. The selection of the probe set used in this study was partially based on chromosomes implicated in prostatic cancer as indicated by other methods, i.e. karyotyping and LOH studies. Also probes for other, not previously implicated chromosomes, were added to the probe panel. Further, the probe set included probes for chromosomes, for which no chromosomal aberrations were expected, to determine the ploidy status of the tumors.
The chosen methodological approach was validated in Chapter 2: This part describes a comparative study of ISH to archival tissue sections of prostatic tumors derived from radical prostatectomy specimens and nuclear suspensions isolated from the same tissue block. The accuracy of defining chromosomal alterations within truncated nuclei on tissue sections compared to whole nuclei is discussed. Chapter 3 reports the cytogenetic heterogeneity for both chromosomal alterations

and ploidy status in organ-confined prostate cancer. This chapter evaluates the capacity of ISH on tissue sections for the detection of chromosomal alterations occurring in a focal fashion. In Chapter 4, an inventory is made of the chromosomal abnormalities occurring in early stages of prostatic cancer by ISH applied to PIN lesions and organ-confined prostatic carcinomas obtained from radical prostatectomies. Chapter 5 discusses the numerical aberrations occurring in tumor cells of patients at different stages of prostatic tumor progression. Therefore, ISH was applied to archival material from localized prostatic tumors, regional lymph node metastases and distant metastases. This study identified aberrations of chromosomes 7 and/or 8 as potential markers for a dismal prognosis. Additionally, the nature of chromosome 8 aberrations in bone metastasis was further studied using comparative genomic hybridization (CGH). To define whether these chromosomes are also implicated in prostatic tumor progression in individual cases, the cytogenetic status of all archival material available from patients with local recurrences and distant metastases was evaluated (Chapter 6). The genetic status of primary tumor tissue from cancers that recur in time and tumors that (have) displayed metastatic potential is compared with a disease-free reference group. Finally, Chapter 7 gives a brief discussion of the results of the studies described in this thesis.

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CHAPTER 2

INTERPHASE IN SITU HYBRIDIZATION TO DISAGGREGATED AND INTACT TISSUE SPECIMENS OF PROSTATIC ADENOCARCINOMAS

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ABSTRACT

A comparative study was performed of interphase in situ hybridization (ISH) to deparaffinized 4-µm tissue sections and nuclear suspensions from eight prostatic adenocarcinomas, as well as one normal prostatic control. Whole nuclear suspensions were derived from the same tumor areas to evaluate differences of ISH to truncated versus whole nuclei. DNA probes specific for the centromeres of chromosomes 1, 7, 8, 10, and Y were used for detection of numerical chromosomal changes and aneuploidy. In six adenocarcinomas chromosome aberrations (+7, +8, -8, -10, -Y) were seen. However, ISH to sections revealed focal aberrations (-10, -Y) in four cases that could not be distinguished in the suspensions. Chromosomal alterations occurring in larger tumor areas were also detected in the nuclear suspensions. Chromosome copy number changes, especially gains, were better discriminated in the nuclear suspensions. The rate of ISH aneuploidy seen in nuclear suspensions corresponded with that observed in the tissue sections ($P<0.01$). Ploidy patterns as assessed by ISH to sections and nuclear suspensions were in concordance with DNA flow cytometry (both $P<0.001$). We conclude that both section and suspension ISH were able to accurately detect aneuploidy and numerical chromosomal aberrations, occurring in larger histological areas. However, section ISH was also capable of revealing (small) focal cytogenetic abnormalities, due to a precise analysis of only target cells. Focal abnormalities were not detected by suspension ISH, probably due to admixture of non-aberrant tumor cells and stromal elements.

INTRODUCTION

Interphase cytogenetic analysis by in situ hybridization (ISH) is increasingly being used to detect specific karyotypic aberrations in human malignancies (Cremer *et al.*, 1988; Hopman *et al.*, 1989; Anastasi *et al.*, 1990; van Dekken *et al.*, 1990). Analysis of chromosome copy number is possible using probes that recognize chromosome-specific repeat sequences, such as (peri)-centromeric alpha satellite DNA. Results from interphase ISH studies on chromosome number are comparable to those obtained by classical karyotyping, and yield extra information in most cases (Poddighe *et al.*, 1991; Micale *et al.*, 1993). Also, ploidy of tumors can be established by interphase ISH, rendering data highly comparable with those measured by DNA flow cytometry (FCM; van Dekken *et al.*, 1993; Persons *et al.*, 1994). Most investigators have used tumor cells or nuclei disaggregated from fresh tumors or tissue blocks. However, the inevitable loss of tissue architecture prevents the analysis of relationships between chromosome changes and histopathological characteristics. Further, no discrimination between tumor and non-tumor cells can be made. To circumvent these problems, investigators have adapted ISH to routine paraffin sections (Hopman *et al.* 1991; van Dekken *et al.* 1992; Kim *et al.* 1993; Persons *et al.* 1993; Krishnadath *et al.* 1994; Zitzelsberger *et al.*, 1994). A disadvantage of this technique is, however, that sectioning of the tissue blocks

leaves the nuclei truncated. The latter phenomenon makes it more difficult to establish the precise chromosome copy number per nucleus.
The goal of the present study was to define the correlation of ISH spot numbers of truncated nuclei (4-µm sections) versus whole nuclei (suspensions), isolated from the same tissue area. Nine prostate specimens (eight adenocarcinomas, one normal) were chosen from an ongoing ISH study, in which the section method was used (Alers *et al.*, 1995a). A protocol for isolation of nuclei from deparaffinized tissue (Wang *et al.*, 1993) was further optimized for ISH. We have used non-fluorescent (peroxidase/diaminobenzidene) visualization of the DNA probes, thus providing an optimal histological examination of target cells. Further, this approach is not biased by autofluorescence and fixation artefacts. The accuracy of detecting numerical chromosome changes was then compared between section ISH and suspension ISH. In addition, we evaluated the ploidy status of the specimens as assessed by ISH to both sections and suspensions with DNA ploidy as measured by FCM.

MATERIALS AND METHODS

Preparation of Tissue Sections

Routinely processed, formalin-fixed, paraffin-embedded materials from radical prostatectomies of eight patients with primary prostatic adenocarcinoma were used in this study. Patients had not received endocrine treatment prior to surgery. One autopsy specimen from a patient, who died from an unrelated disease, was used as a normal control. Tumors were graded according to the Gleason system, which recognizes nine growth patterns, which are arranged in five grades, with increasing loss of histological differentiation (Gleason, 1992). "Low grade" tumors have a total Gleason score of ≤ 6, "high grade" tumors a score of ≥ 7. In situ hybridization (ISH) was performed on consecutive 4-µm tissue sections. This section size was chosen after evaluating the degree of nuclear overlap (countability) and section thickness. Sections were mounted with distilled water on microscope glass slides coated with aminoalkylsilane (Sigma, St. Louis, MO, USA) and baked overnight at 60°C for better adherence.

Preparation of Nuclear Suspensions

The Gleason areas, analyzed on the tissue section, were selectively cut out from the paraffin blocks with a fine scalpel blade. The lower boundaries were then examined for the presence of tumor and the histological grade on hematoxylin and eosin-stained sections. Correspondence between upper and lower boundary was seen in all tumor samples. Nuclear suspensions were obtained according to an improved Hedley protocol (Hedley *et al.*, 1983; Heiden *et al.*, 1991; Wang *et al.*, 1993). Briefly, after deparaffination and rehydration, samples were digested in 1.5 ml of Carlsberg solution (0.1% Sigma protease XXIV [subtilisin], 0.1 M Tris, 0.07 M NaCl, pH 7.2) for 50 min at 37°C in a shaking water bath, with vigorous vortexing every 5 min. Subtilisin resulted in higher yields and less aggregation of the nuclei than digestion in 0.5% pepsin. Cell suspensions were centrifuged briefly and rinsed in PBS before filtering through a nylon mesh (pore size 40 µm) to remove aggregates of cells and debris. Suspensions were resuspended in PBS and sheared several times by vigorously passing the cells through a small syringe. Samples were then stored in ethanol:acetic acid (3:1) at -20°C.

Probe Set and Probe Labelling
A probe set specific for chromosome 1, 7, 8, 10, and Y was selected. Selection criteria were based on literature data considering numerical (and structural) aberrations in prostate and other solid tumors (Arps *et al.* 1993; Brothman *et al.*, 1990; Lundgren *et al.*, 1992). The sources of the (peri) centromeric probes were as follows: Chromosome 1 (clone pUC1.77; Cooke and Hindley, 1979); chromosome 7 (D7Z2, clone p7t1; Waye *et al.*, 1987); chromosome 8 (D8Z2, clone pJM128; Donlon *et al.*, 1986); chromosome 10 (D10Z1, clone pα10RP8; Devilee *et al.*, 1988); chromosome Y (DYZ3, clone pSP65; Cooke *et al.*,1982). The (peri)-centromeric repetitive satellite DNA probes were labelled with biotin-14-dATP by nick translation of complete plasmid DNA according to the manufacturer's directions (BRL, Gaithersburg, MD, USA). DNA probes were stored at -20°C.

In Situ Hybridization to Tissue Sections
ISH was performed as described by van Dekken *et al.* (1992, 1993). Briefly, tissue sections were deparaffinized with xylene followed by 100% ethanol and then treated with 0.3% H_2O_2 in methanol for 20 min to block endogenous peroxidase activity. To facilitate DNA probe accessibility to the cellular DNA, sections were digested with 0.4% pepsin (Sigma) in 0.2M HCl at 37°C for 5-30 min (mean: 14 min), after an incubation in 2x standard saline citrate (SSC; pH 7.0) at 70°C for 30 min to shorten the digestion time. Before applying the probe set, the optimal digestion time for each tumor was determined by a pepsin time series (5, 10, 15, 20 min).
Cellular DNA was heat denatured for 2 min in 70% formamide in 2x SSC (pH 7.0), followed by dehydration in graded ethanol series. Chromosome-specific repetitive DNA probes were denatured for 5 min at 70°C in a hybridization mixture containing 1-2 µg/ml probe DNA, 500 µg/ml sonicated herring sperm DNA (Sigma), 0.1% Tween-20, 10 % dextran sulphate, and 60% formamide in 2x SSC at pH 7.0. Then, 30 µl of probe mixture was applied to each slide. The slides were incubated overnight at 37°C in a moist chamber. Tissue sections were washed in 60% formamide in 2x SSC (pH 7.0) at 42°C for 10-15 min, then in 2xSSC at 42°C for 10-15 min.
Histochemical detection was performed by immunoperoxidase staining. Slides were subsequently incubated for 30 min at 37°C with mouse anti-biotin (Dakopatts, Glostrup, Denmark), biotin-labelled horse anti-mouse (Vector, Burlingame, CA, USA) and with avidin-biotin-complex (Vectastain Elite ABC Kit). The probe-related signal was developed with diaminobenzidine (DAB; 0.5g/l in 0.1M PBS+ imidazole with 0.05% H_2O_2). The signal was amplified with $CuSO_4$ (0.5% in 0.9% NaCl). Finally, the sections were counterstained with hematoxylin for 20 s, rinsed in tap water, dehydrated in graded ethanol and xylene solutions and mounted in Malinol (Chroma-Geselschaft, Köngen, Germany).

In Situ Hybridization to Nuclear Suspensions
Nuclear suspensions (concentration ranging from 0.5-5 10^6 nuclei/ml) in aliquots of 15 µl were spotted on aminoalkylsilane coated glass slides and air dried for at least 30 min. The pretreatment had to be optimized for accurate ISH. The following procedure yielded the best results: Slides were heated in 2xSSC (pH 7.0) in a microwave oven at 85°C for 10 min at 600W, and rinsed in 2xSSC at 37°C. Then the nuclei were digested in 0.1% pepsin (Sigma) in 0.2 HCl at 37°C for 5-7 min and rinsed again in PBS and 2x SSC, respectively. Denaturation, hybridization and detection was performed as described above.

Evaluation of ISH Results
The DNA probe set was analyzed for the normal prostate and for each prostatic adenocarcinoma on consecutive 4-µm sections in a previously defined tissue area. For

each of the probes, 100 "intact" (=spherical) and non-overlapping 4-µm nuclear slices were counted by two independent investigators (100 each) and the number of solid DAB spots per nuclear fragment was scored (0, 1, 2, 3, 4, >4 spots per nuclear slice). For the nuclear suspensions 200 intact and non-overlapping nuclei were scored by each investigator. Aggregates of nuclei were excluded from counting. The individual DNA probe spot distributions were then compared, and totalled, when no significant counting differences between the investigators were found. Chromosome 1 was used as a measure for aneuploidy, since no numerical aberrations were found for this probe (Alers *et al.*, 1995a). Further, in each case non-aberrant probes revealed identical ploidy patterns.
The efficiency of hybridization was checked by careful inspection of ISH signals in stromal cells adjacent to tumor glands present in one section. Further, the quality of ISH was controlled in adjacent sections, hybridized with other DNA probes. Performing these controls, we never observed areas within one section with loss of ISH signal in both tumor and neighbouring stromal cells for all probes. These artefacts might be seen, when the tissue has different accessibility for the probe in different areas (e.g., due to variable fixation).

DNA Flow Cytometry
Nuclear DNA content in the deparaffinized tissues was measured as described by Hedley *et al.* (1983). Gleason-graded tumor areas were selectively cut out of the paraffin blocks, and subsequently used for FCM and suspension ISH. Correspondence between upper and lower boundary was seen in all tumor samples. FCM and analysis of the nuclei stained with ethidium bromide (Sigma) from these areas was performed using a Facscan (Becton Dickinson, Mountain View, CA, USA). Tissue from a normal prostate served as a diploid control. A DNA index between 0.8 and 1.2 was considered diploid. The percentage of non-2C peak cells was derived from the flow histograms and used to the determine the percentage of aneuploid/tetraploid cells of the graded area(s) within one tumor.

Statistical Analysis
The probe spot distributions of both tissue sections and nuclear suspensions were evaluated statistically by means of the Kolmogorov-Smirnov (K-S) test (Young, 1977). This statistical test is very suitable for two-sided comparisons of histograms or other distributions. The Pearson correlation coefficient was used to measure the degree of association of aneuploidy in the tissue sections and in the nuclear suspensions, as well as aneuploidy detected by ISH with the percentage of non-2C peak cells as measured by FCM. This correlation coefficient r measures the degree of 'straight-line" association between the values of the two variables (Altman, 1991).

RESULTS

In Situ Hybridization to Tissue Sections and Nuclear Suspensions

The effect of nuclear slicing on cells in 4-µm thick sections of normal prostatic tissue, when compared with whole nuclei, is illustrated in Fig 1. In tissue sections approximately half of the truncated nuclei show no or one spot, which is as expected in view of the section thickness (Fig. 1A, 1C). More than 90% of the whole nuclei revealed the expected disomic signal for chromosome 1 (Fig. 1B, 1D). In the nuclear suspension 7% of the cells showed more than two spots, against 0% on the tissue sections (Fig. 1C, 1D). This is probably due to the focal presence of aneuploid cells or artifacts. It was also seen when fluorescent visualization of ISH

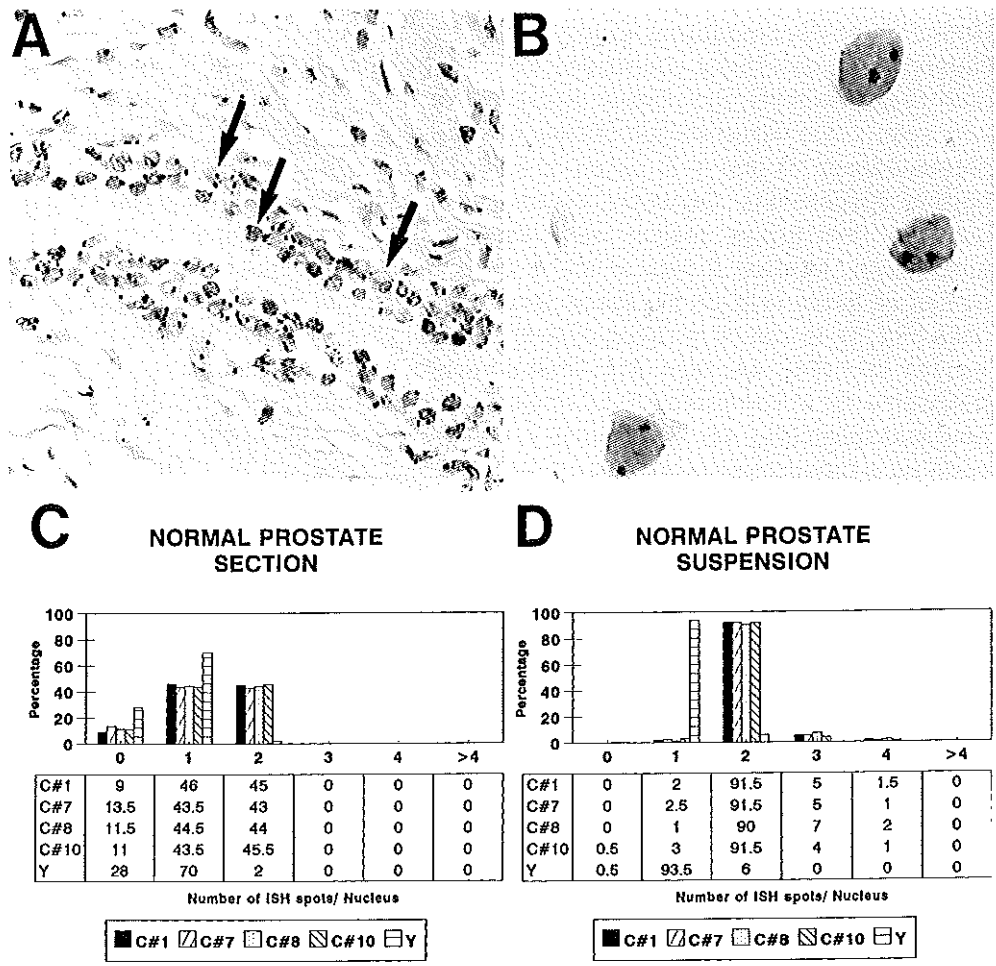

FIGURE 1.
A) In situ hybridization (ISH) with the chromosome-specific DNA probe set to a tissue section of a normal prostatic gland. Nuclei containing no, one, or two spots are seen for chromosome 1 (*arrows* from right to left). The ISH-related spots were visualized with immunoperoxidase/diaminobenzidine (black); hematoxylin was used as a counterstain (gray). **B)** ISH with the chromosome 1-specific probe to the corresponding nuclear suspension. Nuclei displaying two spots are seen. Magnification A 361x, B 880x. **C)** Bar histogram showing the ISH spot distributions for the whole probe set in the normal prostatic tissue section. In 4-µm sections about half of the nuclei show no or one spot for the autosomes. **D)** Bar histogram demonstrating the ISH-spot distributions in nuclear suspensions. In suspension over 90% of the cells shows the expected two spots per nucleus for the autosomes. Note the haploid distribution of chromosome Y in C and D.

signals was used (data not shown).

Detection of Chromosomal Aberrations

Numerical chromosomal aberrations for chromosomes 7, 8, and Y were seen in both tissue sections and nuclear suspensions (Table 1). Loss of chromosome 10 was seen in the tissue section of case 6 only. Histologically focal losses (Fig. 2) of chromosomes 10 and Y in cases 3, 4, and 7 could not be distinguished in the nuclear suspensions. Generally, chromosomal aberrations that occurred in large areas of the tissue section were seen in both section and corresponding suspension. In case 2 loss of the Y chromosome (nullisomy) was detected in almost all tumor cells on the tissue section and in about half of the disaggregated cells (Fig. 3). In case 6, a strong overrepresentation of chromosome 7 was seen by section ISH (Fig. 4A, 4C). This DNA tetraploid tumor (Table 1) showed a clear hexasomy for chromosome 7 in whole cell nuclei, suggesting trisomy 7 before polyploidization (Fig. 4B, 4D). In case 7, underrepresentation of chromosome 8 was seen as a monosomy in both tissue section and suspension ISH (Fig. 5). In case 4,a trisomy 7 was detected together with a high percentage of aneuploid cells in the nuclear suspension, but not on the tissue section (Table 1). ISH to sections at this lower level (± 150 µm) in the tissue block also revealed overrepresentation of chromosome 7 and a higher rate of aneuploidy than in the original (upper) section,

Table 1. In situ hybridization (ISH) to tissues sections, to nuclear suspensions, and DNA flow cytometry (FCM) of eight prostatic adenocarcinomas

Case	*Grade/Area*[a]	*FCM*[b]	*Aneuploidy*[c]		*Aberrations*[d]	
			Section	*Suspension*	*Section*	*Suspension*
1	Low/G2+G3	T (18)	20[e]	16	-	-
2	Low/G2+G3	D (8)	12.5[e]	13	-Y	-Y
3	Low/G3	D (10)	4.5	5	-10	-
4	High/G3+G5	D (4)	2	17.5	-Y	+7
5	High/G3+G5	A (23)	18.5[e]	39	-Y	-Y
6	High/G5[f]	T (44)	41	67	+7, +8, -10	+7, +8
7	High/G5	T (22)	6.5	28.5	-8, -Y	-8
8	High/G5	T (40)	42.5	52	-	-

[a]Gleason grade/area(s) investigated
[b]DNA flow cytometry: D(iploid): ≤10% of cells in non-2C peak; T(etraploid):>10% of cells in non-2C peak; A(neuploid): extra non-2C peak. The percentage of non-2C peak cells is given in parenthesis
[c]Percentage of cells with more than two ISH spots for chromosome 1 per nucleus. In normal control cells fewer than 2.5% cells contain more than two spots
[d]Chromosomal ISH aberration: P<0.01 in Kolmogorov-Smirnov test
[e]Mean percentage of aneuploid cells of two adjacent Gleason areas
[f]In this G4+G5 grade tumor only the grade 5 area was evaluated for both section and suspension. The grade 4 area was on a separate tissue block.

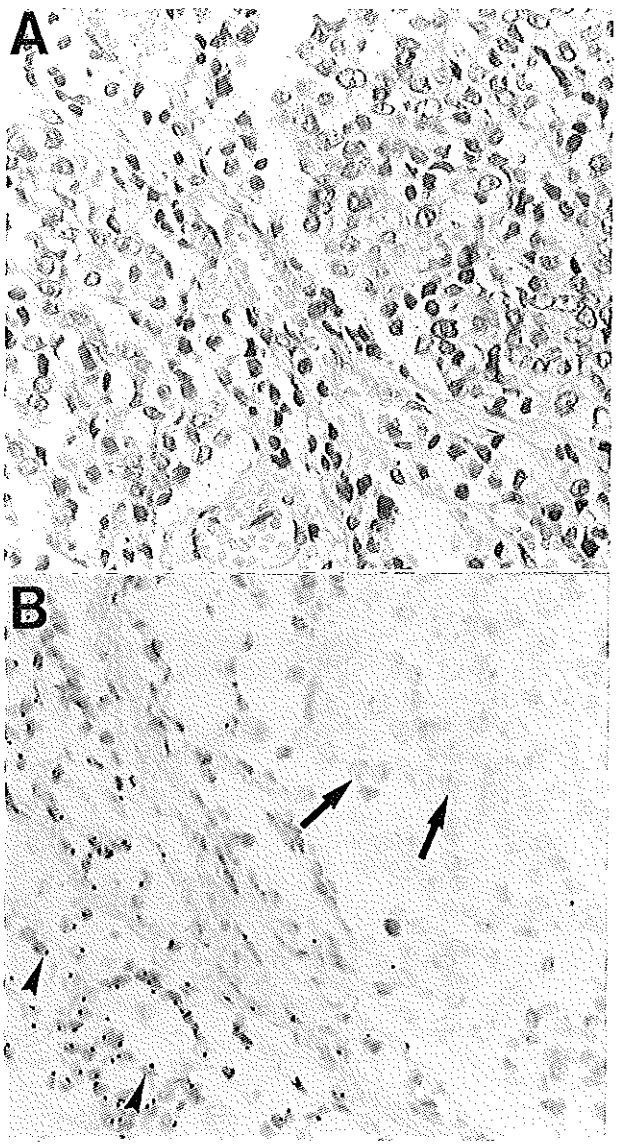

FIGURE 2.
A) Hematoxylin and eosin-stained tissue section of a Gleason 5 area of case 7, showing poorly differentiated tumor cells. **B)** Same case: ISH with the Y probe to this tissue section. The Y chromosome is lost in one part of the tumor (*arrows*), whereas neighboring cancer cell nuclei still retain this chromosome (*arrowheads*). This illustrates the focal cytogenetic heterogeneity in prostatic tumors. This chromosome Y aberration could not be distinguished by suspension ISH. Magnification: A, B 361x.

illustrating cytogenetic heterogeneity in this tumor. The same phenomenon accounts for the discrepancy in rate of aneuploidy in case 7 (Table 1).

Detection of Aneuploidy

The percentage of aneuploid nuclei observed in tissue sections and suspensions corresponded with each other (Pearson correlation r= 0.8755, $P<0.01$). Aneuploidy

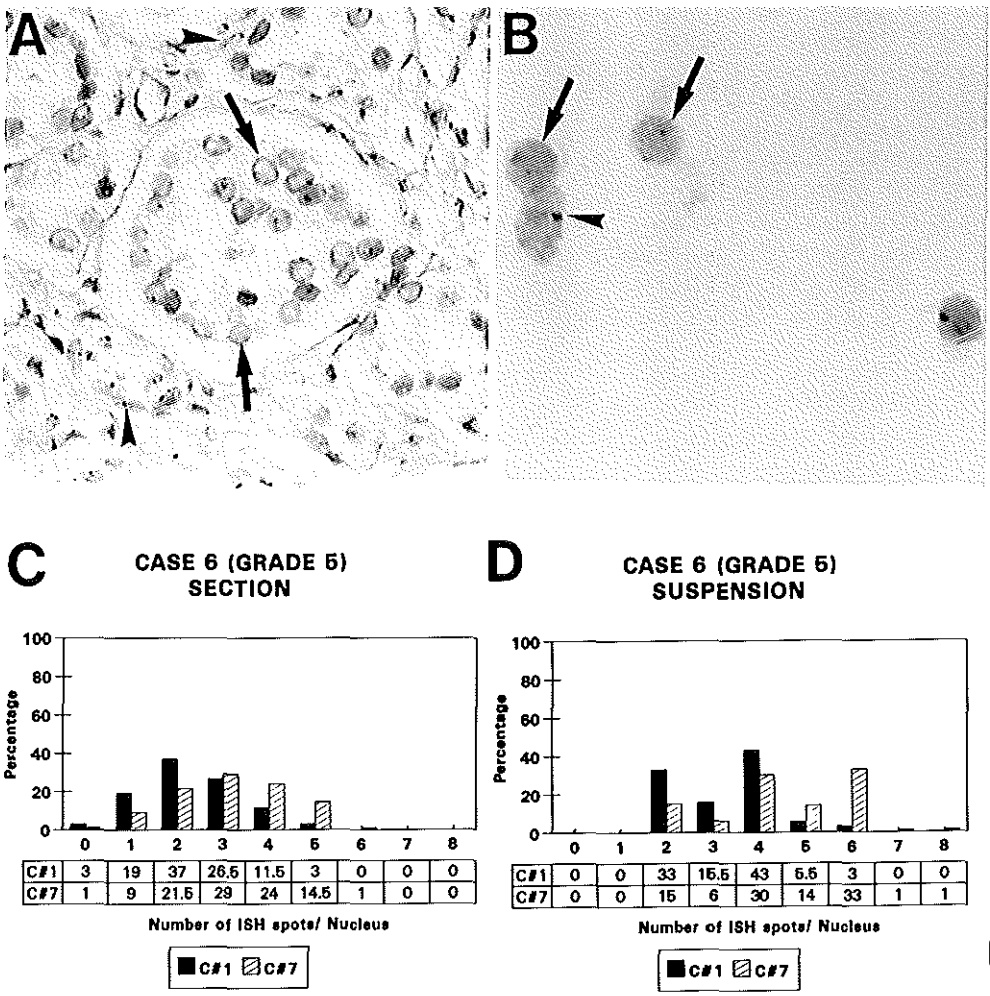

FIGURE 3.
A) ISH to a well-differentiated area of case 2 (Gleason grade 2). Loss of the Y chromosome is seen in the tumor glands (*arrows*), but not in stromal tissue cells (*arrowheads*). **B)** The corresponding nuclear suspension showing loss of Y in approximately half of the nuclei (*arrows*). The presence of Y-containing cells is seen, possibly of fibroblastic origin (*arrowhead*). Magnification A 361x, B 880x. **C)** Bar histogram showing a near-total Y loss in the tissue section. Chromosome 1 displays a few aneuploid nuclei. **D)** Disaggregated cells showing about half of the nuclei with loss of the Y chromosome. Admixtures of stromal cells or tumor cells that still contain the Y chromosome might cause this discrepancy. In comparison with C the number of (chromosome 1) tetraploid nuclei is slightly higher.

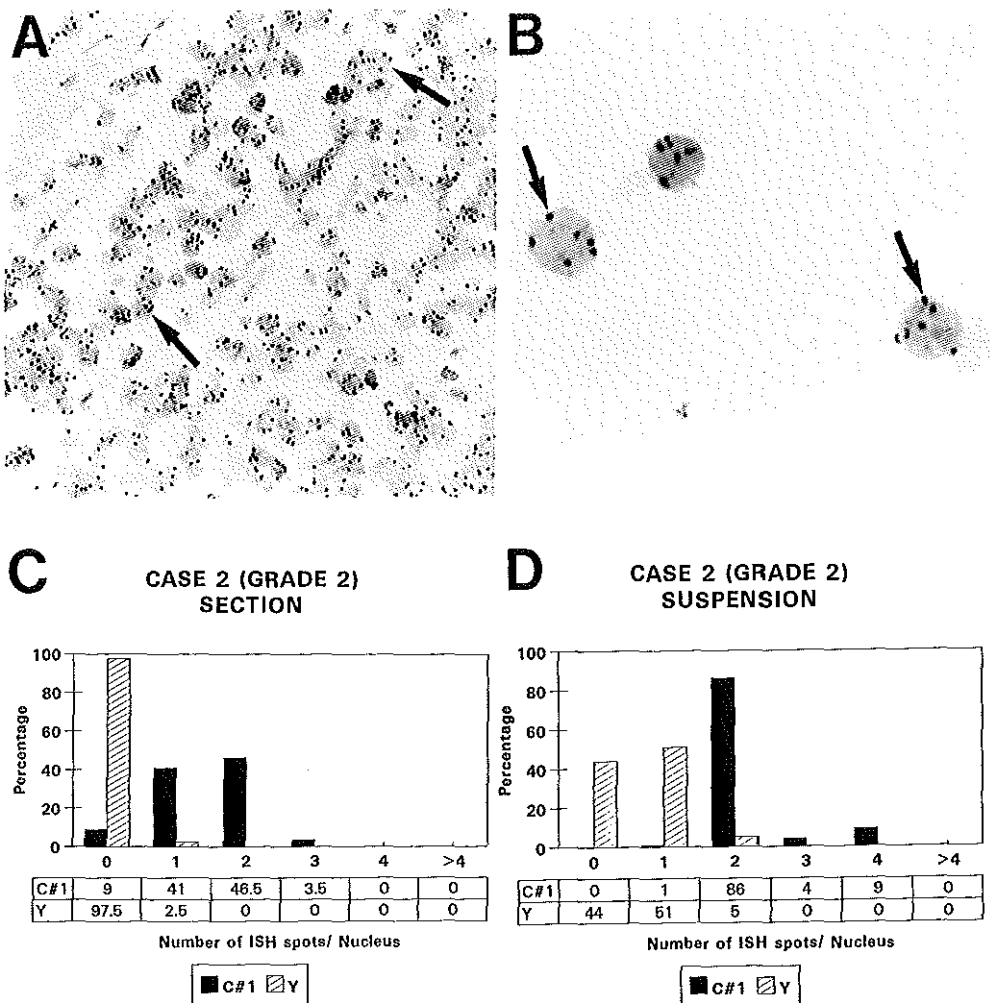

FIGURE 4.
A) ISH to a tissue section of the poorly differentiated case 6. In this tumor region (Gleason grade 5) many nuclei display more than two spots for chromosome 7, a few are *arrowed*. **B)** The corresponding nuclear suspension: Two nuclei hexasomic for chromosome 7 are seen (*arrows*). Magnification: A 361x, B 880x. **C)** Bar histogram showing overrepresentation of chromosome 7 in the tissue section of case 6, seen as a shift to the right of the spot distribution in comparison with chromosome 1. In this section about 40% of the nuclei are aneuploid for chromosome 1. **D)** Nuclear suspension showing distinct peaks at two, four and six spots per nucleus for chromosome 7. Distinct peaks at only two and four spots per nucleus are seen for chromosome 1.

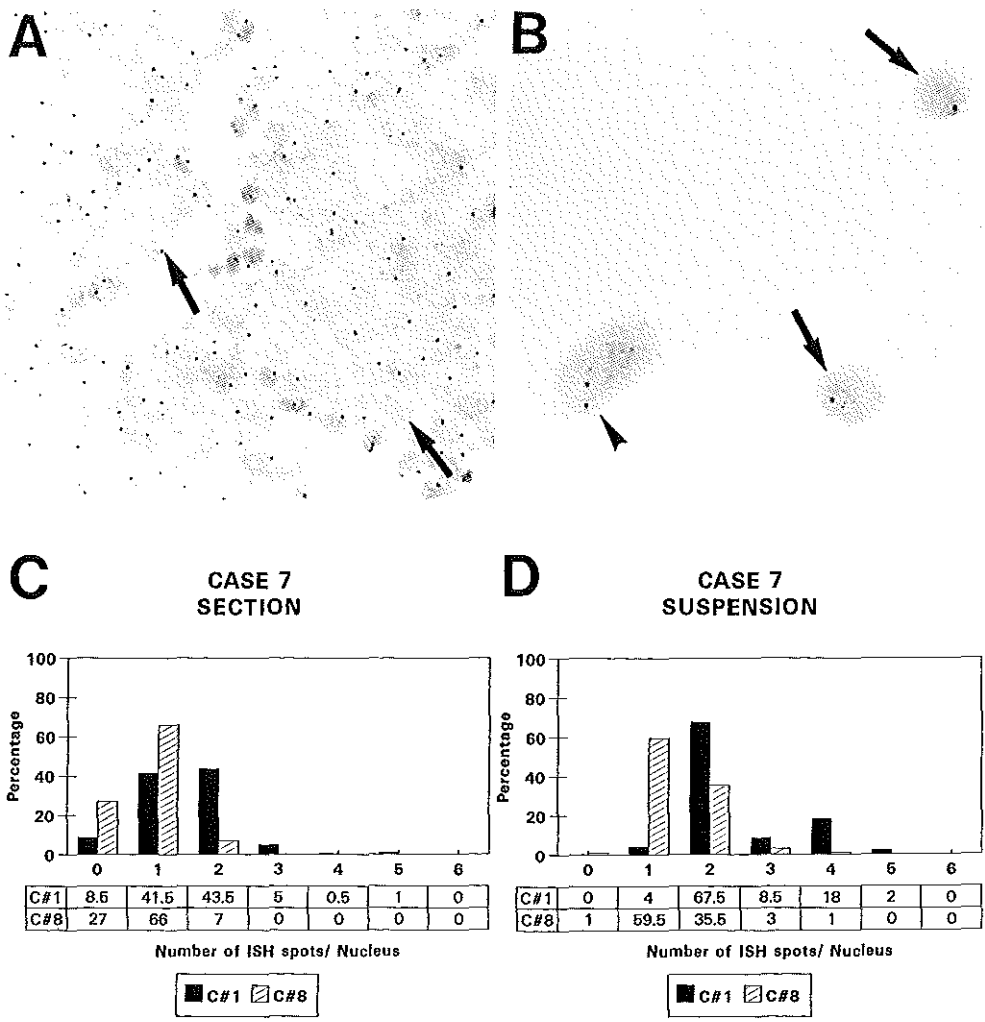

FIGURE 5.
A) ISH with the chromosome 8-specific probe to the tissue section of case 7 (Gleason grade 5). Many cells with no or only one spot per nucleus are seen, some are *arrowed*. Corresponding nuclear suspension showing monosomy 8 in two tumor cells (*arrows*), whereas another cell (*arrowhead*) reveals disomy for this chromosome. Magnification: A 361x, B 880x. **C)** Bar histogram showing underrepresentation (monosomy) of chromosome 8 in the tissue section, illustrated by a peak at one spot per nucleus. **D)** Nuclear suspension also revealing monosomy for chromosome 8. A distinct peak at four spots per nucleus for chromosome 1 can be observed, revealing the tetraploid fraction.

as assessed by ISH to sections and suspensions was in concordance with the percentage of non-2C peak cells, as measured by DNA FCM (r= 0.9301, P<0.001 and r= 0.9393, P<0.001, respectively). In general, the percentage of aneuploid nuclei was higher in the nuclear suspensions, especially in high-grade tumors (Table 1). In tissue sections, aneuploidy was seen by the presence of hyperdiploid cells. However, we could not distinguish between aneuploid (e.g., triploid) and tetraploid cells (Fig. 4C). Suspension ISH revealed distinct peaks for chromosome 1 at two and four spots per nucleus, representing diploid and tetraploid cells (Figs. 3D, 4D, 5D). Also, in all cases in the FCM tetraploid tumors, a peak at two spots per nucleus was distinguished, probably, due to the admixture of diploid stromal and/or diploid tumor cells.

DISCUSSION

In this series of prostatic tumors chromosomal aberrations (+7, +8, -8, -10, -Y) were seen in both suspensions and tissue sections. Moreover, ISH to sections revealed focal aberrations (-10, -Y) in four cases that could not be distinguished in the suspensions, probably due to dilution with non-aberrant cells. Therefore, only numerical aberrations that occurred in larger parts of the tumors were detected by the suspension method. In particular, gains of chromosomes were identified more clearly by suspension ISH (case 6, Fig. 4). In three tumors, ISH to disaggregated specimens confirmed nullisomy and monosomy for several chromosomes, already noted as chromosomal loss by section ISH (Table 1). The latter findings contradict the statement that due to truncation effects monosomy in a significant proportion of cells will be easily missed (Dhingra *et al.*, 1994).

In general, the number of aneuploid cells detected by section and suspension ISH were in agreement with each other (P<0.01), and with DNA flow cytometry data (both P<0.001). Therefore, in tissue sections, a good estimation of ploidy can be made, despite the truncation phenomenon. In our series the percentage of aneuploid cells was higher in the nuclear suspensions, especially in advanced tumors. In advanced prostate cancers the amount of stromal cells is often less than in well-differentiated cancers. This might lead to a relative increase in the percentage of aneuploid cells (Table 1). Further, heterogeneity in cellular DNA content in some specimens may account for the observed differences (cases 4 and 7).

Prostate adenocarcinomas are cytogenetically heterogeneous (Henke *et al.*, 1994; Alers *et al.*, 1995b). This is illustrated by case 4. Upper and lower boundaries of the tissue showed the same morphology, whereas the cytogenetic status appeared different. As shown above, loss of information concerning focal cytogenetic alterations is one of the major drawbacks of working with disaggregated specimens of prostatic tumors. Another issue is to distinguish tumor cells from non-tumor cells. Immunolabelling with a tumor-specific marker could solve this problem. Also sorting

of (labelled) tumor cells by flow cytometric methods with subsequent ISH can be applied (e.g. Beck *et al.* 1992). The major advantage of ISH to nuclear suspensions is that it allows direct visual assessment of the numerical chromosomal status, unbiased by truncation effects. Furthermore, it is a quick and easy to perform procedure. At present it is best suited for computer aided analysis of ISH (Mesker *et al.*, in preparation).

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CHAPTER 3

CYTOGENETIC HETEROGENEITY AND HISTOLOGIC TUMOR GROWTH PATTERNS IN PROSTATIC CANCER

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ABSTRACT

Twenty-five prostatic adenocarcinomas were studied for the presence of intratumoral cytogenetic heterogeneity by interphase in situ hybridization (ISH) to routinely processed tissue sections. ISH with a chromosome Y-specific repetitive DNA probe provided a model to investigate patterns of chromosomal heterogeneity within and between different pathological grades. The Gleason grading system was used, since it is based on a detailed classification of growth patterns. Heterogeneity with respect to ploidy of the tumor was examined by ISH with a repetitive DNA probe specific for chromosome 1. The ploidy status of these cancers was confirmed by DNA flow cytometry ($P<0.001$). Cytogenetic heterogeneity at the (Y) chromosomal level was observed between Gleason areas, within one area, and even within single tumor glands. The different patterns of chromosomal heterogeneity were seen in all tumor grades and stages. Differences in ploidy status were also found following the aforementioned histological patterns, again, in all grades and stages. Intraglandular heterogeneity was most frequently seen. No correlation was found between cytogenetic heterogeneity and proliferative activity (Ki-67 immunostaining). In contrast to current views on clonality, suggesting regional separation of subclones with different DNA content, this study demonstrates that these subclones can be interspersed.

INTRODUCTION

Prostate cancer, which is now exceeding lung cancer as the most commonly diagnosed cancer in American men [9], is known for its highly heterogeneous histological appearance [13]. Foci within a prostate show varying degrees of differentiation and may contain cells that differ genetically. Additionally, the complex tissue architecture [often comprising normal epithelium, stromal cells, benign prostatic hyperplasia (BPH), and prostatic intraepithelial neoplasia (PIN) within the tumor mass], has complicated analysis of prostatic tumors by conventional cytogenetic analysis. Karyotyping studies of prostate cancers are further hampered by the low-mitotic index of prostatic tumor cells and subsequent overgrowth in tissue culture of (normal) stromal or epithelial cells. Karyotyping of prostatic tumors has shown recurrent chromosomal aberrations of chromosome arms 7q and 10q, loss of Y, and trisomy of chromosome 7 [2, 5, 27, 29], and restriction fragment length polymorphism (RFLP) studies have revealed loss of alleles on 8p, 10p, 10q, 16q and 18q arms [3, 6, 25]. However, a clinically important chromosomal abnormality in prostatic cancer has yet to be identified.

In general, cytogenetic heterogeneity is a common feature of solid (epithelial) tumors [17, 21, 28, 32, 43]. Only limited data are available concerning cytogenetic heterogeneity in prostatic tumors [4, 26, 29]. Lundgren *et al.* [26] have demonstrated by karyotyping studies that patients with clonal chromosomal abnormalities had a poor outcome, compared with those who had non-clonal aberrations. Intratumoral heterogeneity in ploidy status of prostatic tumors has also been revealed by DNA flow cytometry (FCM) [4, 24, 31]. Both

aneuploidy/tetraploidy and diploidy have been detected when several biopsies per tumor were analyzed [4, 24, 31]. Likewise, FCM DNA studies of multiple samples from different sites in one tumor and/or metastases have shown heterogeneity in DNA in lung cancers [7, 38], gliomas [10], pancreatic tumors [39], gastrointestinal cancers [11, 38], and ovarian carcinomas [15]. In addition, in epithelial tumors such as bladder cancer [19, 20, 35], breast cancer [12] and lung cancer [22] cytogenetic heterogeneity has been revealed by interphase cytogenetics.
Interphase cytogenetic analysis by in situ hybridization (ISH) has been increasingly utilized to detect specific chromosomal abnormalities and their relation to progression in neoplasms. Most investigators have used cell suspensions from disaggregated tumor blocks [e.g., 19, 41]. However, the inevitable loss of tissue architecture prevents the detection of relationships between chromosome changes and histopathological characteristics. To circumvent these problems, we have applied ISH to archival paraffin-embedded tissue sections [23, 40, 42].
In the present study we addressed the following questions: 1) Does chromosomal heterogeneity exist within prostatic tumors, and if so, at what histological levels (glands, areas)? 2) Is the ploidy status of the tumors heterogeneous? 3) Are there any correlations between cytogenetic characteristics and proliferative activity of the tumor cells?

MATERIALS AND METHODS

Tissue Preparation

Routinely processed, formalin-fixed, paraffin-embedded tissues, obtained between 1990 and 1992, from radical prostatectomies of 25 patients with primary prostatic adenocarcinoma were used for this study. Tumors were staged according to the TNM classification [36] and graded according to the Gleason system [13]. The Gleason grading system recognizes five growth patterns with increasing loss of histological differentiation from grade 1 to 5. A tumor is assigned a certain Gleason score by adding the grades of the two dominant growth patterns [13]. Forty-one Gleason areas were discriminated in our panel of 25 prostatic tumors.
In situ hybridization (ISH) was performed on consecutive 4 µm tissue sections. Sections were mounted with distilled water on aminoalkylsilane (Sigma, St. Louis, MO) coated microscope glass slides and baked overnight at 60°C for better adherence.

Probe Selection

Probes specific for chromosome 1 and Y were selected. Selection criteria were based on literature data considering numerical (and structural) aberrations in prostate and other solid tumors. As described above loss of the Y chromosome is reported in prostatic cancer [2, 5, 27, 29, 40]. Further, the Y probe was best suited to visualize heterogeneity in the prostatic tumors, since loss of the Y chromosome is easily recognized. A probe specific for the centromeric region of chromosome 1 was chosen to quantify the rate of aneuploidy of the tumors. No recurrent numerical aberrations were found for this chromosome in karyotyping studies [2 ,5, 27, 29], as well as in an ongoing investigation by our own group [1].

In Situ Hybridization

ISH was performed as described before [23, 42]. The (peri)centromeric repetitive satellite

DNA probes were labeled with biotin-14-dATP by nick translation of complete plasmid DNA according to the manufacturer's directions (BRL, Gaithersburg, MD). Briefly, tissue sections were deparaffinized and then treated with 0.3% H_2O_2 in methanol for 20 min to block endogenous peroxidase activity. To facilitate DNA probe accessibility to the cellular DNA, sections were digested with 0.4% pepsin (Sigma) in 0.2M HCl at 37°C for 5-30 min (mean: 14 min), after an incubation in 2x standard saline citrate (SSC; pH 7.0) at 70°C for 30 min to shorten the digestion time.
Both cellular DNA and the chromosome specific repetitive DNA probes were heat denatured. The hybridization mixture contained 1-2 µg/ml probe DNA, 500 µg/ml sonicated herring sperm DNA (Sigma), 0.1% Tween-20, 10 % dextran sulphate, and 60% formamide in 2x SSC at pH 7.0. The slides were then incubated overnight at 37°C and subsequently washed.
Histochemical detection was performed by immunoperoxidase diaminobenzidine (DAB) staining as previously described. Finally, the sections were counterstained with hematoxylin, rinsed in tap water, dehydrated and mounted in Malinol (Chroma-Geselschaft, Köngen, Germany).

Evaluation of ISH Results
The centromere 1 and Y DNA probes, as well as the autosomal control DNA probes, were analyzed for each prostate adenocarcinoma on consecutive 4 µm sections in a tumor area to which a certain Gleason score had been assigned. A section size of 4 µm was chosen after evaluating the degree of nuclear overlap (i.e., countability) and section thickness. On each tissue section leukocytes, BPH, nerve cells, etc., served as internal controls to evaluate the quality of ISH and to detect probe polymorphisms. For each of the probes, 100 "intact" (i.e., spherical) and non-overlapping 4 µm nuclear slices were counted by two independent investigators and the number of solid DAB-spots per nuclear contour was scored (0, 1, 2, 3, 4, >4 spots per nuclear slice). The individual DNA probe spot distributions were then compared and totaled, when no significant counting differences between the investigators were found. In our series no discrepancies emerged using this approach. Tumor aneuploidy was determined by calculating the percentage of hyperdiploid cells in the dominant Gleason area(s). Heterogeneity for chromosome 1 and Y was scored by both careful inspection and counting of the aberrant areas.

Validation of ISH Results
Two types of artefacts could interfere with the analysis of heterogeneity, defined by ISH to routine tissue sections: 1) the effect of truncation of the nuclei, which causes disturbances, (most importantly) at the tumor glandular level; and 2) the efficiency of hybridization, leading to regional differences. Loss of the Y chromosome within one tumor gland was not caused by artefacts due to truncation of nuclei. Previous studies by our group [23, 42] revealed that an average of 65% of the cells displays an ISH Y-spot in truncated (normal) nuclei of various histologies. This distribution is in sharp contrast with tumor glands with loss of Y. Furthermore, to rule out a possible contamination of Y-less tumor cells within one gland with normal pre-existent cells that still carry the Y chromosome, cytopathology was checked in adjacent hematoxylin and eosin-stained slides. In case of intraglandular heterogeneity for ploidy, truncation of the nuclei cannot result in spot distributions as observed in this study. If a tumor gland were to be fully tetraploid or aneuploid, a distribution of ISH spots would be created with more than 50% of the cell nuclei carrying three or four hybridization spots for chromosome 1, as described previously by us [42]. By contrast, we most frequently observed a few tetraploid cells in a diploid background.
Insufficient ISH, leading to regional artefacts, can be ruled out for the following reasons : 1)

stromal cells adjacent to tumor glands always showed strong ISH signals, thus eliminating focal misinterpretations; and 2) ISH was controlled in adjacent sections hybridized with autosomal DNA probes; this prevented false interpretations at a more regional level. We never observed areas within one section with loss of ISH signal in both tumor and neighboring stromal cells. These artefacts could be seen when the tissue had different accessibility for the probe in different areas (e.g., due to variable fixation).

Immunohistochemistry
Primary labeling of the Ki-67 antigen was performed with a monoclonal antibody, MIB-1 (Immunotech, Marseille, France), diluted 1/100 in phosphate-buffered saline (PBS) [8]. Immunohistochemistry was performed using the routine ABC-immunoperoxidase method (Vector). For each specimen 200 tumor cells in the marked Gleason areas were counted randomly by two independent investigators.

DNA Flow Cytometry
DNA content of the paraffin material was measured as described by Hedley et al. [16]. Gleason-graded tumor areas were selectively cut out of the paraffin blocks. In eight cases (32%) the two dominant Gleason growth patterns were present within the same tissue block and could not be separated. Correspondence (presence of tumor, tumor grade) between upper and lower boundary was seen in 93% of the tumor samples. In only 3 of 41 areas (7%) was the tumor area not present at the lower boundary. Flow cytometry and analysis of the ethidium bromide (Sigma) stained nuclei from these areas was performed using a Facscan (Becton Dickinson, Mountain View, CA). Tissue from a normal prostate served as a diploid control. A DNA index between 0.9 and 1.1 was considered diploid. The percentage of non-2C peak cells was derived from the flow histograms and used to determine the percentage of aneuploid/tetraploid cells of the dominant Gleason area(s) within one tumor.

RESULTS

Chromosomal Heterogeneity

Loss of chromosome Y was used as a model system to study chromosomal heterogeneity. In our panel of 25 radical prostatectomies we found loss of the Y chromosome in five cases (seven Gleason graded areas; Fig. 1A). Loss of the Y chromosome was never observed in control cells, present on the same tissue section (Fig. 1B). Loss of Y was seen in both low and high grade tumors (Table 1). Heterogeneous loss of this chromosome was seen at three levels of aggregation: intraglandular, intraregional and interregional (Fig. 2). In detail, loss of the Y chromosome was seen in some cells within one tumor gland, whereas other cells in the same gland still contained the Y chromosome, thus displaying intraglandular heterogeneity (Figs. 2A, 3A). Foci of glands that lost chromosome Y and foci of glands that showed the normal spot distribution for Y alternated within one Gleason area (intraregional heterogeneity; Figs. 2B, 3B-E). The third distinct pattern of heterogeneity, termed interregional heterogeneity, defined loss of the Y chromosome in one Gleason area, whereas the other Gleason area retained the chromosome (Figs. 2C, 3F, G; Table 1). Furthermore, the three different patterns of chromosomal heterogeneity, illustrated by Y-loss, occurred in both low-grade (n=2; Gleason score ≤ 6) and high grade (n=3; Gleason score ≥ 7) tumors.

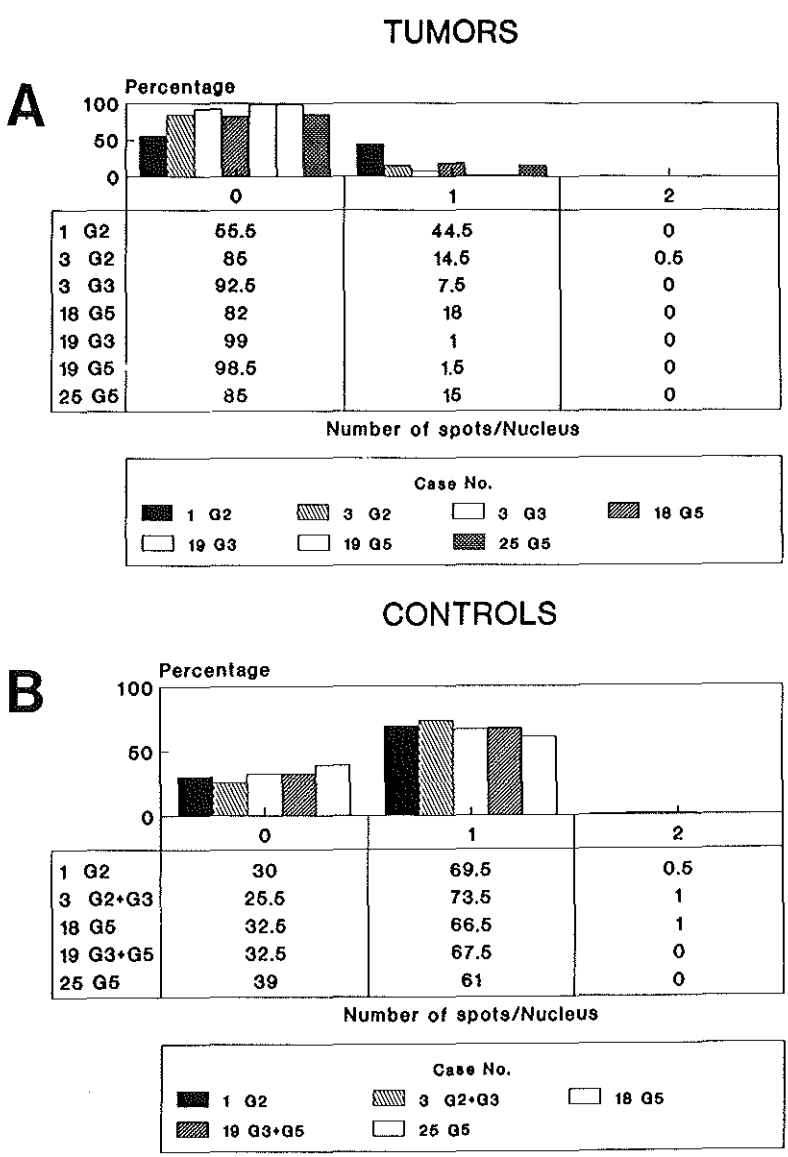


FIGURE 1.
Bar histograms showing ISH-spot distributions for the chromosome Y-specific DNA probe on 4 µm tissue sections. **A)** ISH patterns of Gleason areas with (partial) loss of Y in cases 1, 3, 18, 19, and 25. **B)** Control cells from the same sections of the aforementioned cases (e.g., leukocytes, normal prostatic glands). In control cells the percentage of cells with 1 spot for Y is between 60 and 70%. Note that Y-loss is seen as a shift to the left in the distributions.

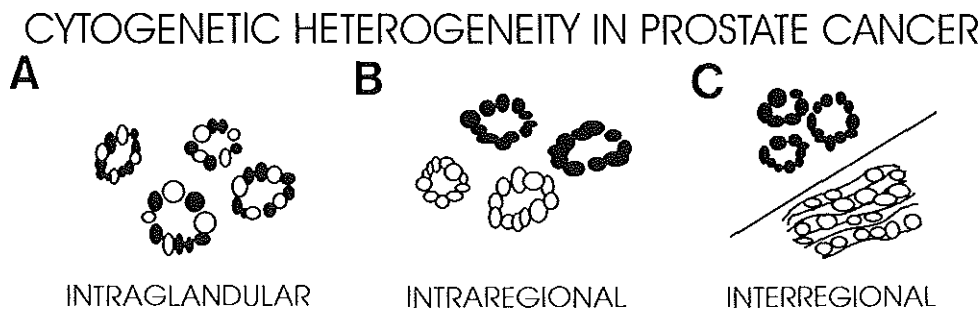


FIGURE 2.
Schematic drawing of histological patterns of cytogenetic heterogeneity in prostatic adenocarcinoma as detected by interphase cytogenetics. Nuclei that are different with respect to chromosomal and/or ploidy status are depicted in different shades. Three distinct histological patterns were discriminated: **A)** Intraglandular heterogeneity: neighboring cells are cytogenetically different. **B)** Intraregional heterogeneity: within one Gleason area, neighboring tumor glands show cytogenetical differences. **C)** Interregional heterogeneity. One Gleason area is cytogenetically different from another.

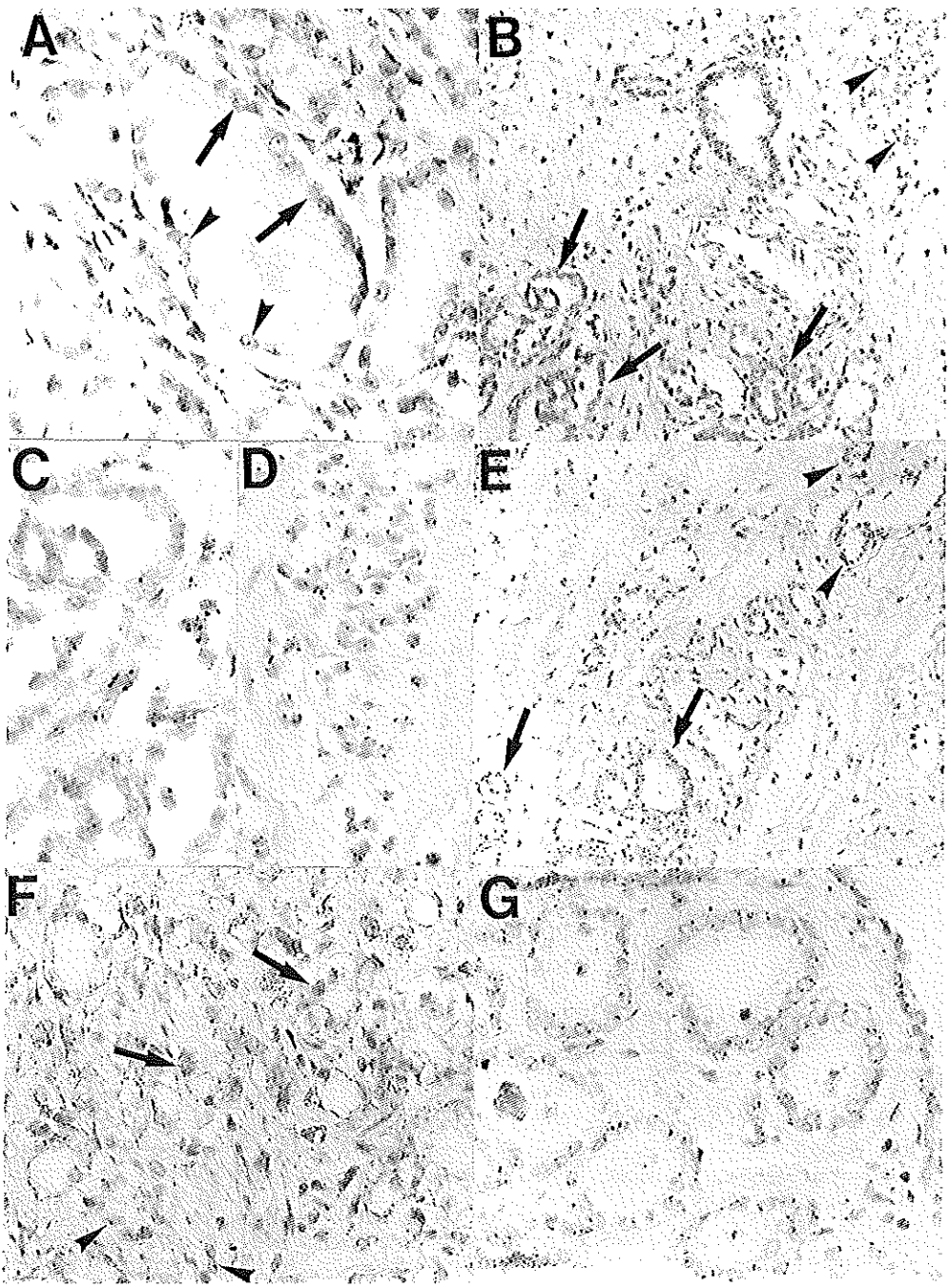
Heterogeneity in Ploidy

A probe specific for the centromere region of chromosome 1 was used to assess the ploidy status of the tumor cells within the dominant Gleason growth pattern(s) (Table 1). Aneuploidy defined by ISH correlated well with aneuploidy\tetraploidy measured by DNA flow cytometry (FCM; $P<0.001$, Pearson's correlation). Heterogeneity in ploidy, demonstrated by ISH with the DNA probe specific for chromosome 1, revealed the same three patterns that were distinguished for chromosomal heterogeneity (Fig. 2). Intraglandular heterogeneity resulted in differences in spot number for chromosome 1 between neighboring nuclei (Figs. 2A, 4A, B). Control probes for other chromosomes, e.g., chromosome 7, showed comparable hybridization patterns in the same tumor glands (Figs. 4C, 5A). Intraregional differences within one Gleason area were seen in, for example, the Gleason 2 area of case 5. One part of this area was highly aneuploid, whereas another part showed a more diploid distribution (Figs. 4D, E, 5B, C). Interregional heterogeneity for ploidy between different Gleason areas was distinguished in several cases (Table 1). This is illustrated by an aneuploid cribriform growth pattern (grade 3) and another poorly differentiated area (grade 4) of case 16, which displayed a rather diploid distribution (Figs. 4F, G, 5D, E). In all these cases control probes showed the same spot distribution as chromosome 1 (Fig. 5). No significant differences in the occurrence of the three aforementioned histological patterns were

Table 1. Results of pathological grading/staging, flow cytometry (FCM), and in situ hybridization (ISH)

Case	Grade	Type[a]	Stage[b]	FCM[c]	ISH aneuploidy[d] (%)	Y-ISH
1	4	G2	T3N0	D (7)	4	-Y
2	5	G2	T4N2	D (8)	6	
		G3			2.5	
3	5	G2	T2N0	D (8)	23	-Y
		G3			21.5	-Y
4	5	G2	T3N0	D (5)	8	
		G3		A*	6.5	
5	5	G2	T2N0	T (19)	37.5	
		G3			3	
6	5	G2	T2N0	D (7)	0.5	
		G3		T (12)	1	
7	6	G3c	T2N0	T (14)	8	
8	6	G3c	T3N0	D (9)	1.5	
9	6	G3c	T3N0	D (10)	4.5	
10	6	G3	T3N0	D (7)	1	
11	7	G3	T3N0	D (6)	2	
		G4		T (21)	5.5	
12	7	G3	T3N0	T (23)	2	
		G4			9.5	
13	7	G3c	T3N0	A (20)	12	
		G4		T (36)	30.5	
14	7	G3	T3N0	D (10)	1	
		G4		D (8)	2	
15	7	G3	T3N0	D (4)	2	
		G4			24.5	
16	7	G3c	T4N0	T (16)	46	
		G4			4.5	
17	8	G3	T3N0	A*	32	
		G5		T (36)	21.5	
18	8	G3	T4N0	D (1)	1.5	
		G5		D (4)	2	-Y
19	8	G3c	T3N0	A (23)	19	-Y
		G5			18	-Y
20	8	G4	T3N1	T (19)	23	
21	9	G4	T3N0	D (9)	4	
		G5			2.5	
22	9	G4	T4N0	D (7)	30	
		G5		T (44)	41	
23	10	G5	T2N0	T (40)	42	
24	10	G5	T3N0	D (5)	15.5	
25	10	G5	T3N0	T (22)	6.5	-Y

[a] Dominant Gleason growth pattern(s).
[b] TNM classification: All tumors M0.
[c] D(iploid): 0.9 <Di <1.1 and 4C peak ≤ 10%; T(etraploid): 0.9 <Di <1.1 and 4C peak > 10%; A(neuploid): Di <0.9 or Di >1.1. Values between parentheses are percentage non-2C peak FCM.
[d] Percentage of cells with > 2 spots/nuclei for chromosome 1. All control cells revealed < 2.5% hyperdiploid cells.
* Shoulder in DNA histogram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observed between low- and high-grade tumors (Fig. 6). Intraglandular heterogeneity was most frequently detected.

Proliferative Activity

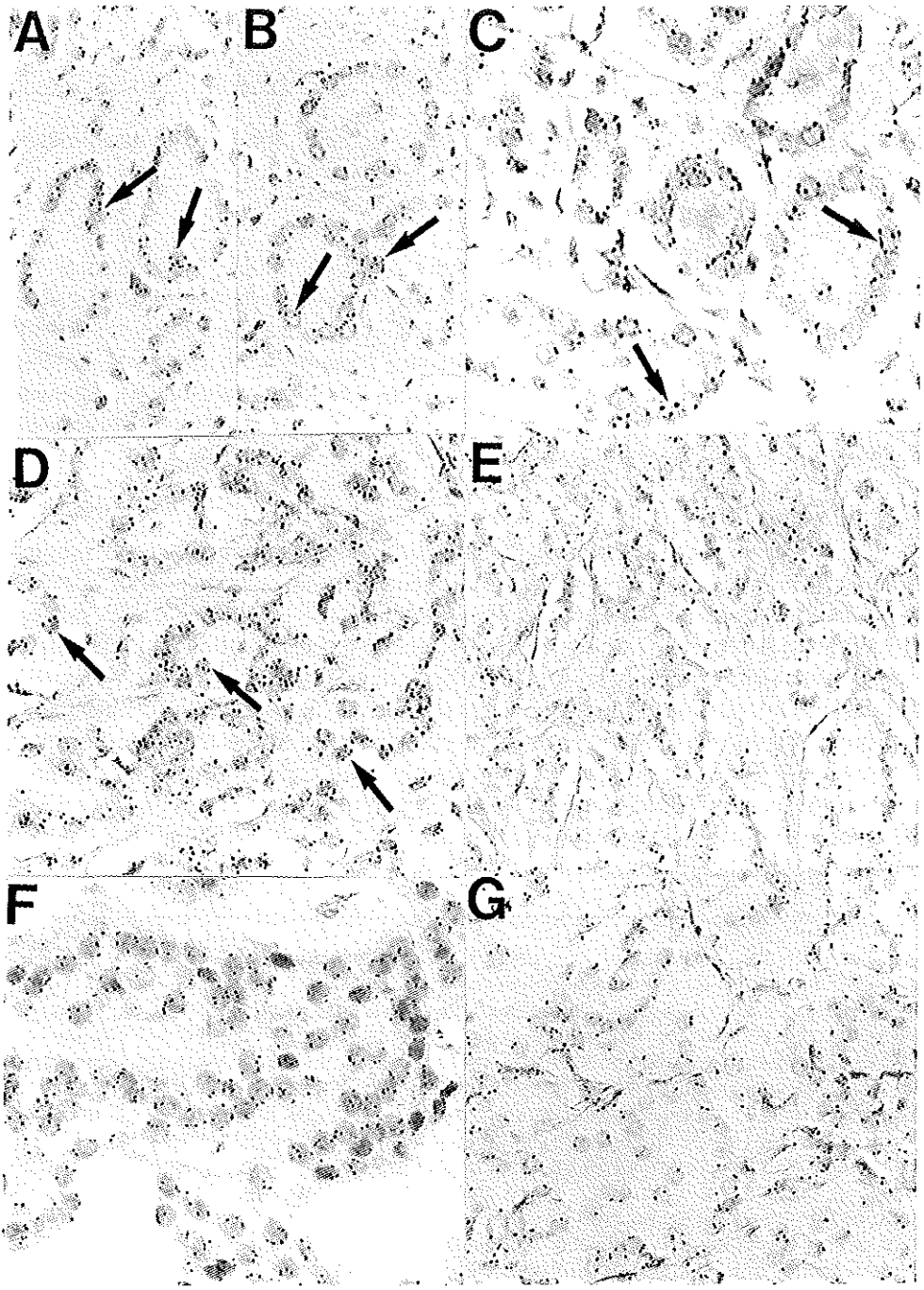
Immunohistochemistry (IHC) with a Ki-67 antibody (MIB-1) in normal and hyperplastic glands demonstrated less than 2.5% positive immunostaining of the nuclei. In tumor cells the percentages of stained nuclei varied from 1% to 28% (mean, 8%). No differences in MIB-1 staining patterns were observed between parts of a tumor with or without chromosome (Y) loss (not shown). Likewise, heterogeneity in ploidy of the tumor cell nuclei did not result in differences in proliferation rate as assessed by MIB-1 IHC.

DISCUSSION

In this study we were able to distinguish cytogenetic heterogeneity in prostatic adenocarcinomas by means of ISH to routine paraffin sections. This approach retained the tissue architecture, allowing detection of cell subsets with different karyotype. Control studies were performed for, e.g. the effect of truncation of the nuclei and variation in hybridization efficiency. Three patterns of cytogenetic heterogeneity could be distinguished: intraglandular, intraregional and interregional (Gleason areas). Heterogeneity for both chromosomal status and chromosome 1 ISH defined ploidy occurred in these three patterns. Cytogenetic heterogeneity at the chromosomal level was defined by loss of chromosome Y. Loss of Y is the most common chromosomal aberration in prostatic cancer [2, 5, 27, 29]. The importance of (loss of) the Y chromosome is not clear. In some cancers loss of Y is a possible

FIGURE 3.

A) ISH with the chromosome Y-specific DNA probe to the Gleason 2 area of case 3, showing intraglandular chromosomal heterogeneity: loss of chromosome Y can be seen in most tumor cell nuclei (*arrows*), whereas some cancer cells still contain the Y chromosome (*arrowheads*). At least ten neighboring cancer cell nuclei with Y-loss are seen to line up in this gland. The ISH-related spots were visualized with immunoperoxidase/DAB (black), and hematoxylin was used as a counterstain (gray). **B)** ISH with the Y-specific probe to the Gleason 2 area of case 1, showing intraregional heterogeneity. Foci of tumor glands without Y chromosome (*arrows*) and with chromosome Y (*arrowheads*), both situated in one Gleason area, are shown. **C)** Detail of B, showing Y-loss in tumor glands. **D)** Detail of B, showing retention of Y in other tumor glands. **E)** ISH with the chromosome 1-specific (control) probe to the same area of case 1. No differences in ISH-related spot pattern for this probe can be seen between foci without (*arrows*) and with (*arrowheads*) the Y chromosome, thus, eliminating the possibility of different hybridization efficiency between the two areas. **F)** ISH with the Y probe to the Gleason 5 area of case 18. Y-loss is seen in the cancer cells (*arrows*), but not in the stromal cells (*arrowheads*). **G)** Same case, showing the Gleason 3 area: in these tumor glands the Y chromosome is present. This case illustrates interregional chromosomal heterogeneity. A 40X objective was used in A, C, D, F and G and a 20x objective in B and E.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prognostic parameter [33]. In our panel no correlation was found between age of the patient and loss of the Y chromosome, as has been reported previously [34]. Further, control cells, e.g., leukocytes, always retained the Y-chromosome. We observed heterogeneity for Y-loss even within one tumor gland. In our series of tumors we did not observe significant differences in the occurrence of the described histological patterns for Y-loss heterogeneity between low- and high-grade tumors. Although the number of tumors with Y-loss is too small for statistical evaluation, our data suggest that with tumor progression the Y-loss pattern does not change dramatically.

Chromosome 1 was used as a measure for ploidy status. In a previous study no abnormalities of this chromosome occurred [1]. Further, tumor aneuploidy, defined by ISH for chromosome 1, was confirmed by DNA FCM. Similar results were obtained by us in other organ systems [23, 42]. FCM is not suitable for the detection of subtle focal differences. This study shows that neighboring nuclei within one tumor gland contained different copy numbers of chromosome 1 and therefore they differ in DNA content. Surprisingly, it appeared to be the most frequent ploidy pattern. These data suggest that "single cell heterogeneity" exists aside from focal differences. The latter is seen by us as intra- and interregional heterogeneity. In our study we did not observe significant variation in the incidence of the three types of heterogeneity between low- and high-grade tumors. Apparently, in high-grade tumors ploidy differences can still exist at various levels, due to continuous genetic instability, rather than expansion into one single pattern [30].

Clonal karyotypic changes in prostatic tumors were also found by others [4, 26, 29]. In most karyotyping studies a clone is defined as two or more cells with the same karyotype, or three or more cells with the same numerical aberration. Micale *et al.* [29] reported that clonal aberrations were confined to tumors in advanced stages.

FIGURE 4.
A) ISH with the chromosome 1-specific probe to the Gleason 2 area of case 3. Intraglandular differences in ploidy can be seen between neighboring cells. A few aneuploid cell nuclei with three or four spots are *arrowed*. **B)** Same case: intraglandular heterogeneity showing slightly more aneuploid nuclei (*arrows*). Note that, in general, these nuclei are larger in size than those with one or two spots. **C)** Same case: ISH with the chromosome 7-specific (control) probe to the same area. This probe displays the same ploidy pattern. A few aneuploid nuclei are *arrowed*. **D)** ISH with the centromere 1-specific probe to the Gleason 2 area of case 5. Many aneuploid nuclei can be distinguished, and a few are *arrowed*. **E)** Neighboring cancer glands within the same area contain cells mostly showing two spots per nucleus (intraregional heterogeneity). **F)** Gleason 3 area: ISH with the centromere 1 probe to a cribriform gland (case 16). Many aneuploid nuclei are visible. **G)** Same case, Gleason 4 area: most nuclei display two spots. Case 16 illustrates interregional heterogeneity in ploidy. A 40x objective was used in A-G.

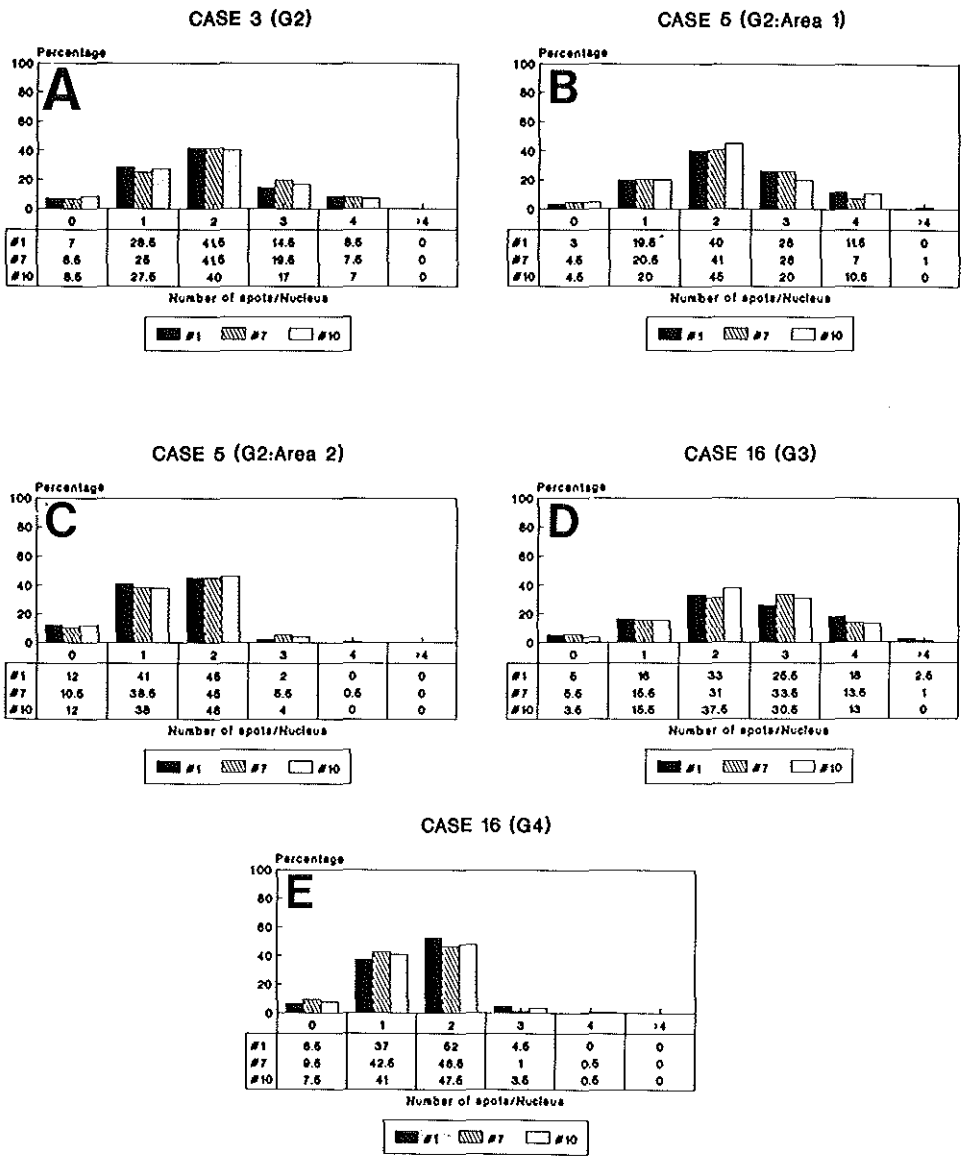

FIGURE 5.
ISH spot distributions for chromosomes 1, 7, and 10 of consecutive tissue sections of all cases shown in Figure 4. **A)** Gleason 2 area of case 3. **B)** Aneuploid part of Gleason 2 area of case 5. **C)** Less aneuploid part of Gleason 2 area of case 5. **D)** Gleason 3 area of case 16. **E)** Gleason 4 area of the same case. The spot distributions for these chromosomes are highly comparable. It illustrates equal hybridization conditions for all these probes.

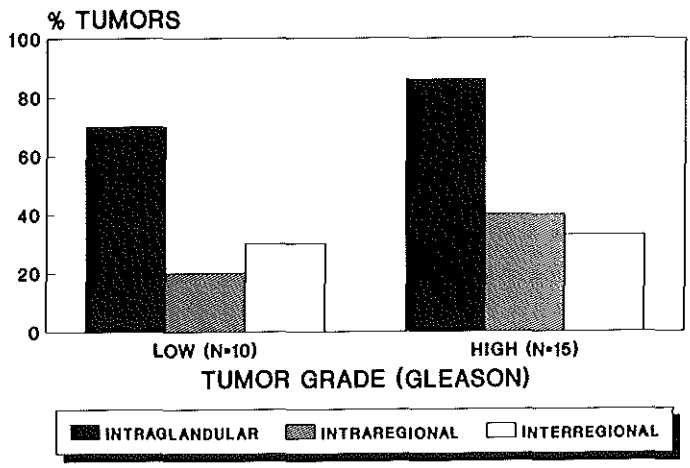

FIGURE 6.
Bar histogram of the distribution of the three different patterns of heterogeneity in ploidy with respect to pathological grade. Heterogeneity of ploidy status can be found in both low- and high-grade tumors.

Henke *et al.* [18] used interphase cytogenetics and found that focal abnormalities occurred only in higher tumor grades. These findings are in contrast with our data: We distinguished cytogenetic heterogeneity throughout the grading and staging spectrum. DNA FCM of multiple samples of prostatic adenocarcinomas showed heterogeneity in ploidy in more than half of cases [31]. This multiple site sampling demonstrated that single biopsy specimens, when used in karyotyping, DNA flow cytometry, and interphase cytogenetics on nuclear suspensions, are hardly representative for a given (prostatic) tumor [4, 7, 31, 37, 38]. At present, the clinical importance of DNA ploidy heterogeneity is not clear and varies among the tumors studied [10, 14, 15].

We conclude that cytogenetic heterogeneity is a very prominent feature of prostatic adenocarcinoma. ISH applied to routine sections provides us with a tool to discriminate this phenomenon even at the glandular level.

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CHAPTER 4

INTERPHASE CYTOGENETICS OF PROSTATIC ADENOCARCINOMA AND PRECURSOR LESIONS: ANALYSIS OF 25 RADICAL PROSTATECTOMIES AND 17 ADJACENT PROSTATIC INTRAEPITHELIAL NEOPLASIAS

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ABSTRACT

Twenty-five radical prostatectomy specimens were screened for the presence of numerical chromosome changes within the adenocarcinoma as well as in 17 adjacent prostatic intraepithelial neoplasias (PIN) by means of interphase in situ hybridization (ISH) to routinely processed tissue sections. To this end a defined alfoid repetitive DNA probe set was used, specific for the centromeres of chromosomes 1, 7, 8, 10, 15 and Y. The cytogenetic information was correlated with histopathological and clinical features as well as with DNA ploidy. Numerical aberrations of at least one chromosome were shown in 13 of 25 cases (52%). Alterations of chromosome 8 and loss of the Y chromosome were the most frequent findings (both 20%), followed by loss of chromosomes 15 (16%) and 10 (12%). Gain of chromosome 7 was seen in 8% of cases. No aberrations of chromosomes 7, 8, 10, and 15 were found in the adjacent PIN lesions, whereas loss of the Y chromosome in both PIN and tumor occurred in two cases. Also, (low level) aneuploidy was observed in 76% of these PIN lesions. Ploidy of the carcinomas as assessed by ISH correlated well with ploidy measured by DNA flow cytometry (FCM; $P<0.02$). Due to the more specific correspondence between ISH and tumor pathology, pathologic grade correlated with ISH aneuploidy ($P<0.05$), whereas FCM ploidy did not. Further, genetic heterogeneity within a tumor was seen, as judged by the focal appearance of chromosomal aberrations. Chromosomal alterations occurred in all grades and stages, although loss of chromosome 10, gain of chromosome 7, and aberrations of chromosome 8 tended to predominate in more advanced cancers.

INTRODUCTION

In Europe and the United States, prostate cancer is presently the second leading cause of male deaths from malignant neoplasms (Carter and Coffey, 1990). As the population ages it is predicted that the number of patients will increase steadily over the next decade (Carter and Coffey, 1990). The clinical course of prostate cancer is highly variable and unpredictable. Present methods of assessing the prognosis for prostate cancer include clinical staging and histopathological grading (Gleason, 1992; Schröder *et al.*, 1992). Unfortunately, these methods fail to provide consistent predictive information regarding the clinical outcome of an individual tumor, particularly in tumors confined to the prostate. Cellular DNA measurements provided useful information on the biological aggressiveness of the tumor (Deitch and deVere White, 1992). The therapeutic strategy in individual cases, however, is still difficult to design. Hence, there is a need to identify characteristics of prostate tumor cells that would help in defining the biological aggressiveness of individual tumors and guide the choice of therapy. An understanding of prostate cancer cytogenetics might provide such information.

Knowledge of cytogenetic alterations in prostate cancer is relatively sparse when compared with other common malignancies, and a consistent primary cytogenetic change has yet to be identified (Sandberg, 1992). In general, cytogenetic studies of

prostate cancer by karyotyping of metaphase spreads are hampered by preferential growth of normal (diploid) cells and by the low mitotic index of the tumor cells. Conventional cytogenetic analyses have revealed loss of the Y chromosome, trisomy of chromosome 7, and loss of 7q, 8p and 10q chromosome arms (Lundgren *et al.*, 1988, 1992a; Brothman *et al.*, 1990, 1991; Micale *et al.*, 1992; Sandberg, 1992; Arps *et al.*, 1993). Allelotyping of prostate carcinoma using restriction fragment length polymorphism (RFLP) showed allelic losses on the 8p, 10p, 10q, 16q and 18q arms (Carter *et al.*, 1990; Bergerheim *et al.*, 1991; Kunimi *et al.*, 1991; Bova *et al.*, 1993; Chang *et al.*, 1994).

Prostatic intraepithelial neoplasia (PIN) is characterized by cytological abnormality and proliferation of the normal luminal cell layer lining prostatic ducts and acini. PIN occurs more often in prostates with invasive carcinoma than in those without (reviewed by Brawer, 1992). In biopsy specimens containing high grade PIN, the patient usually develops clinically invasive cancer within a few years (Bostwick and Brawer, 1987; Weinstein and Epstein, 1992). In general, PIN lesions are considered to be the precursors of prostatic adenocarcinoma. DNA quantitation of isolated PIN lesions by flow cytometry (FCM) showed aneuploidy in about 40% of cases (Crissman *et al.*, 1993). As far as we know, no karyotyping data are available of PIN lesions. However, Macoska *et al.* (1993) found focal loss of chromosome Y in one of two PIN lesions by performing fluorescent in situ hybridization (FISH) analysis.

Cytogenetic analyses based on dissociated tissue eliminate the morphological architecture of the tissue. In this situation it is difficult to correlate specific chromosomal changes with histological characteristics of the source cells. In the last decade non-isotopic in situ hybridization (ISH) with (peri)centromeric chromosome specific DNA probes has emerged as a powerful tool for the discrimination of numerical chromosome changes in interphase cells of solid tumor specimens (Cremer *et al.*, 1988; van Dekken *et al.*, 1990a, b; Persons *et al.* 1993). This technique has recently been adapted for application to tissue sections, thereby allowing combined cytogenetic and histologic analysis (Hopman *et al.* 1991; van Dekken *et al.* 1992; Wolman *et al.*, 1992; Kim *et al.* 1993; Macoska *et al.*, 1993 ;van Dekken *et al.*, 1993; Krishnadath *et al.* 1994).

In this study we have applied ISH to paraffin-embedded tissue sections of 25 primary prostate adenocarcinomas with adjacent dysplasias (PIN). To our knowledge this is the largest panel of prostatic tumors and PIN lesions examined by this method. The following specific questions were addressed: 1) Can the reported numerical chromosomal changes in prostatic adenocarcinoma be confirmed? 2) Can new chromosomal aberrations be identified? 3) Do preneoplastic lesions adjacent to tumors contain (the same) cytogenetic aberrations? 4) Are specific chromosomal changes in prostatic adenocarcinoma associated with more aggressive tumor behavior? For this purpose, we used a set of relevant (peri)

centromeric alfoid DNA probes, specific for chromosomes 1, 7, 8, 10, 15 and Y. The centromeric probes for chromosomes 1 and 15 were added to this panel, since in solid cancers a gain of chromosome 1 is often seen (Atkin, 1986). Further, loss of chromosome 15 was seen by us in cancers of the bladder (Schervish and van Dekken, in preparation). In addition, ploidy status of the tumor was examined by DNA flow cytometry.

MATERIALS AND METHODS

Tissue Specimens

Routinely processed, formalin-fixed, paraffin-embedded materials, obtained between 1990 and 1992, from radical prostatectomies of 25 patients with primary prostatic adenocarcinoma were used for this study. Tumors were staged according to the TNM classification (Schröder *et al.* 1992) and graded according to the Gleason system (Gleason, 1992). The Gleason grading system recognizes five growth patterns with increasing loss of histological differentiation (Gleason, 1992). Forty-one Gleason areas were discriminated in our panel of 25 prostatic tumors. Seventeen tumors were accompanied by PIN lesions.

In Situ Hybridization

In situ hybridization (ISH) with the biotin-labeled DNA probe set, specific for chromosomes 1, 7, 8, 10, 15, and Y, was performed as described by van Dekken *et al.* (1992, 1993). Briefly, to facilitate DNA probe accessibility to the cellular DNA, sections were digested with 0.4% pepsin (Sigma, St. Louis, MO) in 0.2 M HCl at 37°C for 5-30 min (mean: 14 min). Cellular DNA was heat denatured for 2 minutes in 70% formamide in 2x SSC (pH 7.0), the chromosome specific repetitive DNA probes were denatured for 5 min at 70°C in a hybridization mixture containing: 1-2 µg/ml probe DNA, 500 µg/ml sonicated herring sperm DNA (Sigma), 0.1% Tween-20, 10 % dextran sulphate, and 60% formamide in 2x SSC at pH 7.0. The slides were then incubated overnight at 37°C in a moist chamber and subsequently washed. Histochemical detection of the biotinylated DNA probes was performed by the standard avidin-biotin complex (ABC) procedure and immunoperoxidase staining. Sections were counterstained with hematoxylin.

Evaluation of ISH results

The DNA probe set was analyzed for each prostate adenocarcinoma on consecutive 4 µm sections in a previously defined tumor area with a certain Gleason score. A section size of 4 µm was chosen after evaluating the degree of nuclear overlap (=countability) and section thickness. For each of the probes, 100 "intact" (=spherical) and non-overlapping 4 µm nuclear slices were counted by two independent investigators (100 nuclei each) and the number of solid diaminobenzidine (DAB) spots per nuclear fragment was scored (0, 1, 2, 3, 4, >4 spots per nuclear slice). The individual DNA probe spot distributions were then compared and totaled, when no significant counting differences between the investigators were found. In case a numerical aberration was detected, a third independent investigator was consulted. The probe spot distributions were statistically evaluated by means of the Kolmogorov-Smirnov test (Young, 1977). Underrepresentation of a specific chromosome was seen as a shift to the left of the DNA probe distribution, when compared with non-aberrant probe distributions. Conversely, gain of a specific chromosome was seen as a shift to the right. This method is described in detail in previous studies (van Dekken *et al.*, 1992; van Dekken *et al.*, 1993). Chromosome 1 was used as a measure for aneuploidy, since no isolated aberrations were found for this probe. Furthermore, in each case the non-aberrant

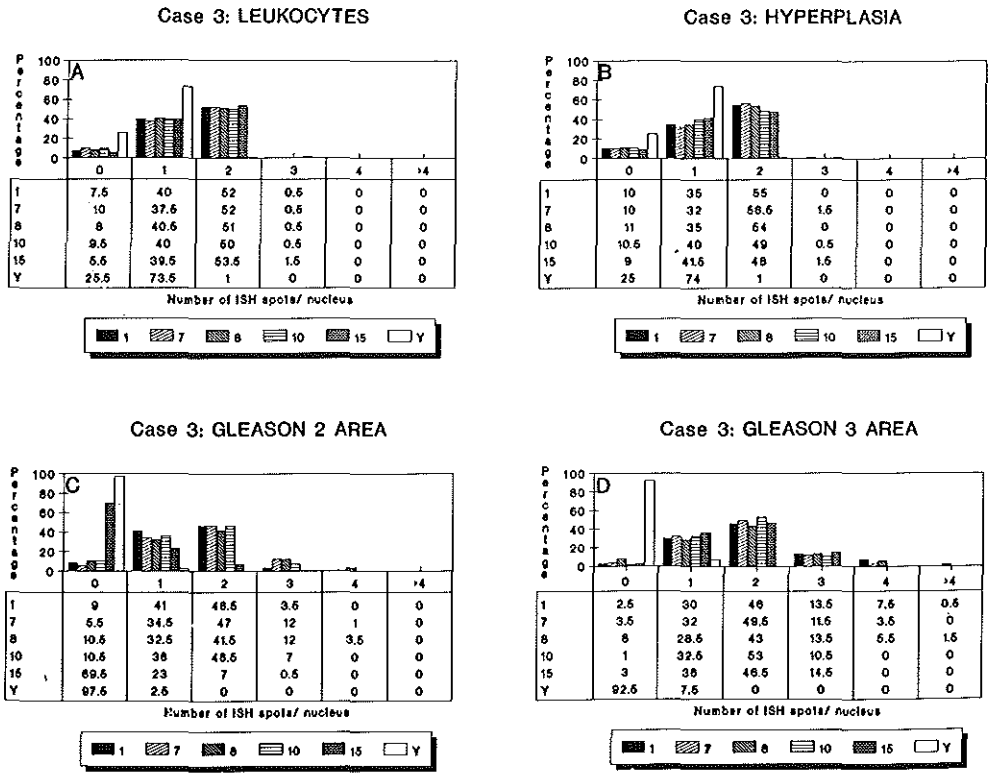


FIGURE 1.
DNA probe frequency distributions of the number of hybridization spots per nucleus for case 3 after ISH with the DNA probe set, specific for chromosomes 1, 7, 8, 10, 15, and Y, to 4 µm tissue sections. For all of the probes 100 spherical and non-overlapping nuclei were counted each by 2 independent investigators. The results were added and plotted as a percentage per probe. **A)** Leukocytes of patient 3 showing a diploid ISH profile. No aberrations are seen. **B)** BPH, also displaying the diploid ISH profile for all probes. **C)** Gleason 2 area of the tumor showing loss of the Y chromosome and loss of chromosome 15, indicated by a shift to the left of the DNA probe distribution. **D)** Gleason 3 area of this tumor revealing loss of the Y chromosome only.

probes revealed identical ploidy patterns for the Gleason areas.
On each tissue section leukocytes, benign prostatic hyperplasia (BPH), nerve cells, etc., served as internal controls to evaluate the quality of ISH and to detect probe polymorphisms. Internal controls (normal prostate glands: 13 cases; BPH: 4 cases; leukocytes: 13 cases; other cells: 5 cases) on the same tissue sections always showed a diploid pattern (van Dekken and Alers, 1993; Krishnadath *et al.*, 1994). The number of nuclei with a hyperdiploid spot number (likely artefacts) in these internal controls never exceeded 2.5%. This is illustrated by case 3. In Figures 1A and 1B the diploid probe spot pattern for 4 µm tissue sections is shown. Due to sectioning, the normal control cells

generally displayed 0 or 1 spot for the autosomes in 10% and 40% of nuclei, respectively. Moreover, in case 3 also chromosomal aberrations (loss of Y and loss of chromosome 15) and aneuploidy are demonstrated in the tumor areas (Fig. 1C, D). Despite the 4 µm sectioning artefact, which results in truncated nuclei, specific chromosome aberrations were detected and could be statistically evaluated. In contrast with true loss of chromosome 15, polymorphism for chromosome 15 might have been considered to be an aberration in four tumor specimens, if no internal controls had been examined. In these cases the alpha satellite DNA probe showed strong polymorphism in both tumor cells and control cells.

DNA Flow Cytometry

DNA content of the paraffin material was measured as described by Hedley *et al.* (1983). Three to five approximately 25-50 µm slices of Gleason-graded tumor cell areas were selectively cut out of the paraffin blocks. The lower boundaries were then examined for presence of tumor and pathologic grade. Correspondence between upper and lower boundary was seen in 93% of the tumor samples. Only in 3 of 41 (7%) areas the tumor area was not present at the lower boundary. Flow cytometry and analysis of the ethidium bromide (Sigma)-stained nuclei from these areas was performed using a Facscan (Becton Dickinson, Mountain View, CA). Tissue from a normal prostate served as a diploid control. A DNA index between 0.8 and 1.2 was considered diploid.

RESULTS

The results of ISH, histopathological examination (Gleason grading), staging, and DNA FCM are summarized in Table 1. ISH revealed numerical aberrations of at least one examined chromosome in 13 of 25 cases (52%): Loss of the Y chromosome and both loss and gain of chromosome 8 were the most common findings (20%), followed by loss of chromosomes 15 (16%) and 10 (12%), and gain of chromosome 7 (8%).

Loss of chromosome 8 was seen in two patients and gain of chromosome 8 in three patients. To illustrate heterogeneity, in case 4 loss of chromosome 8 was seen in the Gleason 3 area only (Fig. 2A; Table 1). In case 19 loss of the Y chromosome was seen in both Gleason areas (Fig. 2B). Loss of chromosome 15 was seen in three patients. In case 25 loss of chromosome 15 was seen in anaplastic areas (Fig. 2C). Loss of chromosome 10 was observed twice in cribriform growth patterns (cases 9, 13) and once in a high grade tumor (case 22; Fig. 2D, E). In the latter tumor a gain of chromosome 7 was seen in the Gleason 5 area (Fig. 2E, F). No aberrations of chromosome 1 were found. A lymph node metastasis of case 2, as well as the primary tumor, showed no chromosomal aberrations for this probe set. For all cases no chromosome abnormalities were seen in normal prostatic epithelium and BPH.

Generally, FCM data corresponded well with ISH ploidy (Spearman's rank correlation coefficient r_s=0.5219: P<0.02). In nine cases ISH revealed differences in ploidy within a tumor that were not detected by FCM. Seventy-three per cent of the 41 Gleason areas (80% of the 25 tumors) showed a varying rate of hyperdiploidy for chromosome 1, ranging from 2.5% to 42.5%. This rate of aneuploidy as

Table 1. Clinical data of patients and results of pathological examination, ISH, FCM

Case	Age (years)	Grade	Types[a]	Stage[b]	FCM[c]	ISH Aneuploidy[d]	Aberrations[e]
1	69	4	G2	pT3N0	D	±	-Y
2	61	5	G2	pT4N2[f]	D	+	
			G3			±	
3	58	5	G2	pT2N0	D	±	-Y,-15
			G3			++	-Y
4	66	5	G2	pT3N0	D	+	
			G3		A	+	-8
5	51	5	G2	pT2N0	T	+++	
			G3			±	
6	63	5	G2	pT2N0	D	ns	
			G3		T	ns	
7	60	6	G3c	pT2N0	T	+	
8	49	6	G3c	pT3N0	D	ns	-15
9	67	6	G3c	pT3N0	D	±	-10
10	63	6	G3	pT3N0	D	ns	
11	59	7	G3	pT3N0	D	ns	
			G4		T	+	
12	55	7	G3	pT3N0	T	ns	
			G4			+	
13	53	7	G3c	pT3N0	A	++	-10
			G4		T	+++	
14	59	7	G3	pT3N0	D	ns	
			G4		D	ns	
15	70	7	G3	pT3N0	D	ns	
			G4			+++	
16	57	7	G3c	pT4N0	T	+++	+8
			G4			±	
17	51	8	G3	pT3N0	T	+++	+7, +8
			G5		A	+++	
18	63	8	G3	pT4N0	D	ns	
			G5		D	ns	-Y
19	47	8	G3c	pT3N0	A	++	-Y
			G5			++	-Y
20	49	8	G4	pT3N1	T	+++	
21	64	9	G4	pT3N0	D	±	
			G5			±	
22	67	9	G4	pT4N0	D	+++	-10
			G5		T	+++	-10, +7, +8
23	69	10	G5	pT2N0	T	+++	-15
24	65	10	G5	pT3N0	D	++	
25	60	10	G5	pT3N0	T	+	-8, -15, -Y

[a] Dominant Gleason growth pattern (s).
[b] TNM classification: All tumors M0 (No distant metastasis).
[c] D(iploid): 4C peak ≤ 10%; T(etraploid): 4C peak > 10%; A(neuploid): other peak (non-4C).
[d] All control cells revealed < 2.5% hyperdiploid (> 2 spots) cells: ≤ 2.5% = ns (not significant); > 2.5%-5% = ±; 6-10% = +; 11-20% = ++; > 20% = +++.
[e] Lymph node metastasis of this tumor revealed the same hybridization pattern.
[f] $P < 0.01$ (Kolmogorov-Smirnov test).

detected by ISH increased with higher Gleason grades for both area and total score (Spearman's rank correlation coefficient r_s=0.3197 and r_s=0.4241, respectively: both P<0.05), whereas no statistically significant correlation was found between Gleason score and FCM ploidy.
In both low grade (Gleason score ≤ 6) and high grade (Gleason score ≥ 7)tumors approximately the same number of chromosomal aberrations were seen (Table 2). However, the type of chromosomal aberration seemed to differ between low and high grade tumors: Although loss of chromosome 15 and Y occurred in all Gleason patterns, gain of chromosome 7, alterations of chromosome 8 and loss of chromosome 10 were seen predominantly in the higher pathologic grades (Table 2).

Table 2. Gleason score and chromosomal aberrations determined by ISH[a]

	≤ 6 (n=10)	*≥ 7 (n=15)*
+7	-	2 (13%)
-8/+8	1 (10%)	4 (26%)
-10	1 (10%)	2 (13%)
-15	2 (20%)	2 (13%)
-Y	2 (20%)	3 (20%)

[a] The percentage of tumors within the Gleason subgroups is given in parentheses.

A comparable percentage of chromosomal aberrations was found in both tumors that were confined to the prostate (T2 tumors) and in tumors that invaded the prostatic capsule or other organs (T3 and T4 tumors; Table 3). Here also loss of chromosome 15 and Y was observed irrespective of stage, whereas loss of chromosome 10, gain of chromosome 7, and aberrations of chromosome 8 were noted in T3 and T4 tumors only (Table 3).

Table 3. TNM staging and chromosomal aberrations determined by ISH[a]

	T2[b] (n=5)	*T3-T4[c] (n=20)*
+7	-	2 (10%)
-8/+8	-	5 (25%)
-10	-	3 (15%)
-15	2 (40%)	2 (10%)
-Y	1 (20%)	4 (20%)

[a] The percentage of tumors within the TNM subgroups is given in parentheses.
[b] Tumor confined within the prostate.
[c] Tumor invades other organs and/or is fixed.

In 17 tumors (high grade) PIN lesions adjacent to the tumor cells were analyzed. No numerical aberrations were found of chromosomes 7, 8, 10 and 15 (Table 4;

Fig. 3A, B). In cases 3 and 19, however, loss of the Y chromosome was observed in both adenocarcinoma and PIN lesions (Table 4; Fig. 3C, D). In the other three cases with loss of the Y chromosome in tumor glands, no loss of Y was seen in the adjacent PIN lesion (Fig. 3E, F). Thirteen PIN lesions (76%) appeared to contain aneuploid cells (mean: 4.5%; Figs. 3G, H, 4). In the adjacent cancer cells a higher degree of aneuploidy was seen (mean: 8.4%). This aberrant ploidy status was not observed in normal cells and benign hyperplasia (BPH; see Materials and Methods section).

Table 4. Chromosomal aberrations in six PIN compared with numerical alterations in adjacent adenocarcinoma

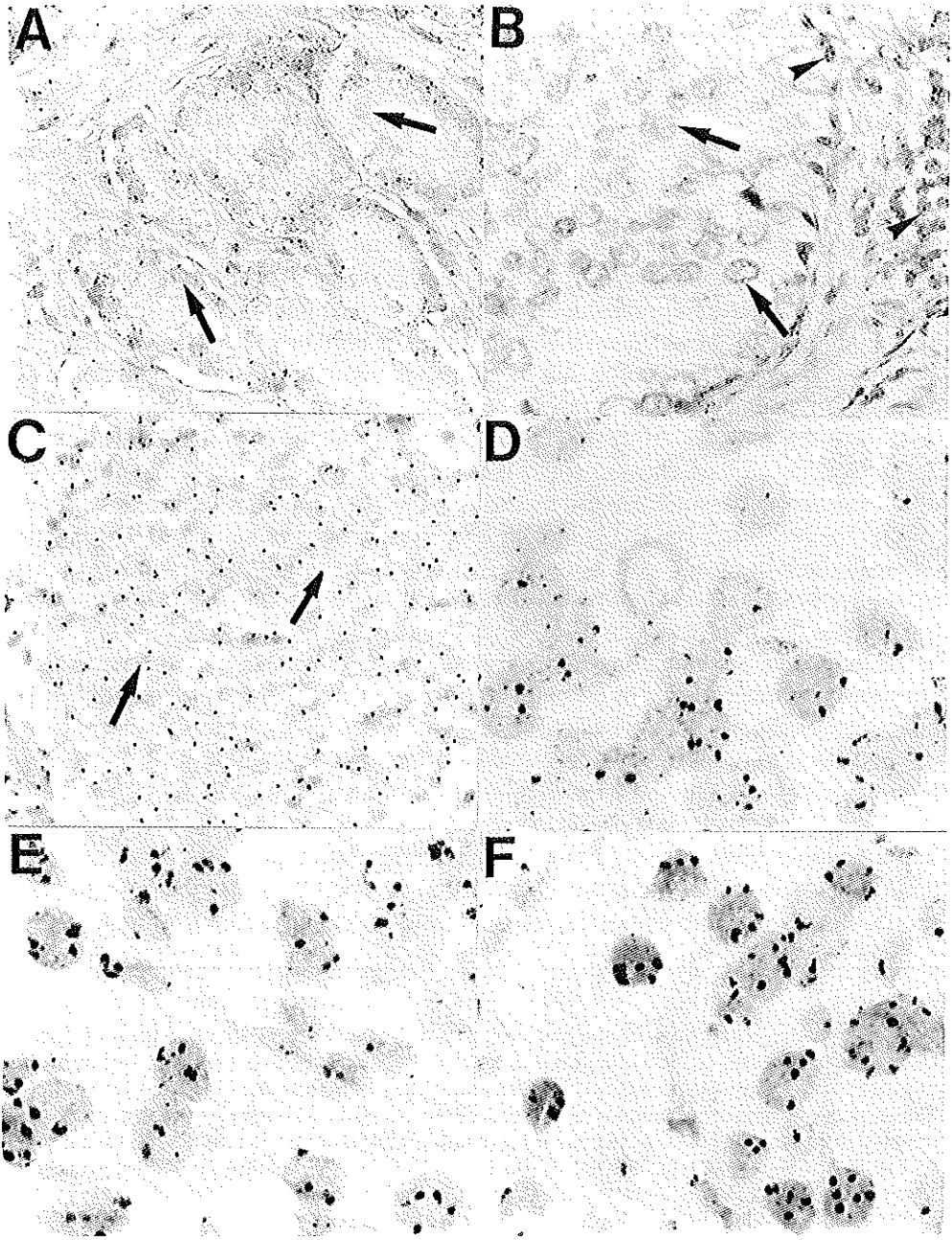
Case	*Gleason area*	*PIN*	*Adenocarcinoma*
1	G2	-	-Y
3	G2	-Y	-15, -Y
4	G3	-	-8
8	G3c	-	-15
9	G3c	-	-10
19	G5	-Y	-Y

* $P < 0.01$ (Kolmogorov-Smirnov test).

DISCUSSION

Over 50% of the examined prostatic cancers showed numerical chromosomal aberrations. Aberrations of chromosome 8 and loss of the Y chromosome (both 20%) were the most common findings, followed by loss of chromosomes 15 (16%) and 10 (12%). Gain of chromosome 7 was seen in 8% of cases. No numerical changes of chromosome 1 were observed. Alterations of chromosome 8, loss as well as gain, were seen in five tumors. Classical cytogenetic analyses revealed

FIGURE 2.
A) ISH with the chromosome 8 specific probe to the Gleason 3 area of case 4, showing loss of chromosome 8 in the tumor nuclei (*arrows*). The ISH-related spots were visualized with immunoperoxidase/DAB (black); hematoxylin was used as a counterstain (gray). **B)** ISH with the chromosome Y specific probe to a Gleason 5 area of case 19 showing a complete loss of the Y chromosome in the tumor cells (*arrows*), while the basal and stromal cells carry this chromosome (*arrowheads*). **C)** ISH with the chromosome 15 specific probe to the Gleason 5 area of case 25 showing an underrepresentation of chromosome 15 in the cancer cell nuclei (*arrows*). The cells display only 1 or 0 spot. **D)** ISH with the chromosome 10 specific probe to the DNA tetraploid Gleason 5 area of case 22: An underrepresentation of chromosome 10 is noted, when compared with chromosome **1** in E. **E)** ISH with the chromosome 1 specific probe to the same area: A large number of aneuploid cells can be distinguished. **F)** ISH with the chromosome 7 specific probe to the Gleason 5 area of the same patient. An overrepresentation of chromosome 7 is seen, when compared with chromosome 1 (Fig. 2E). 40x objective was used in A-C; 100x objective was used in D-F.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E
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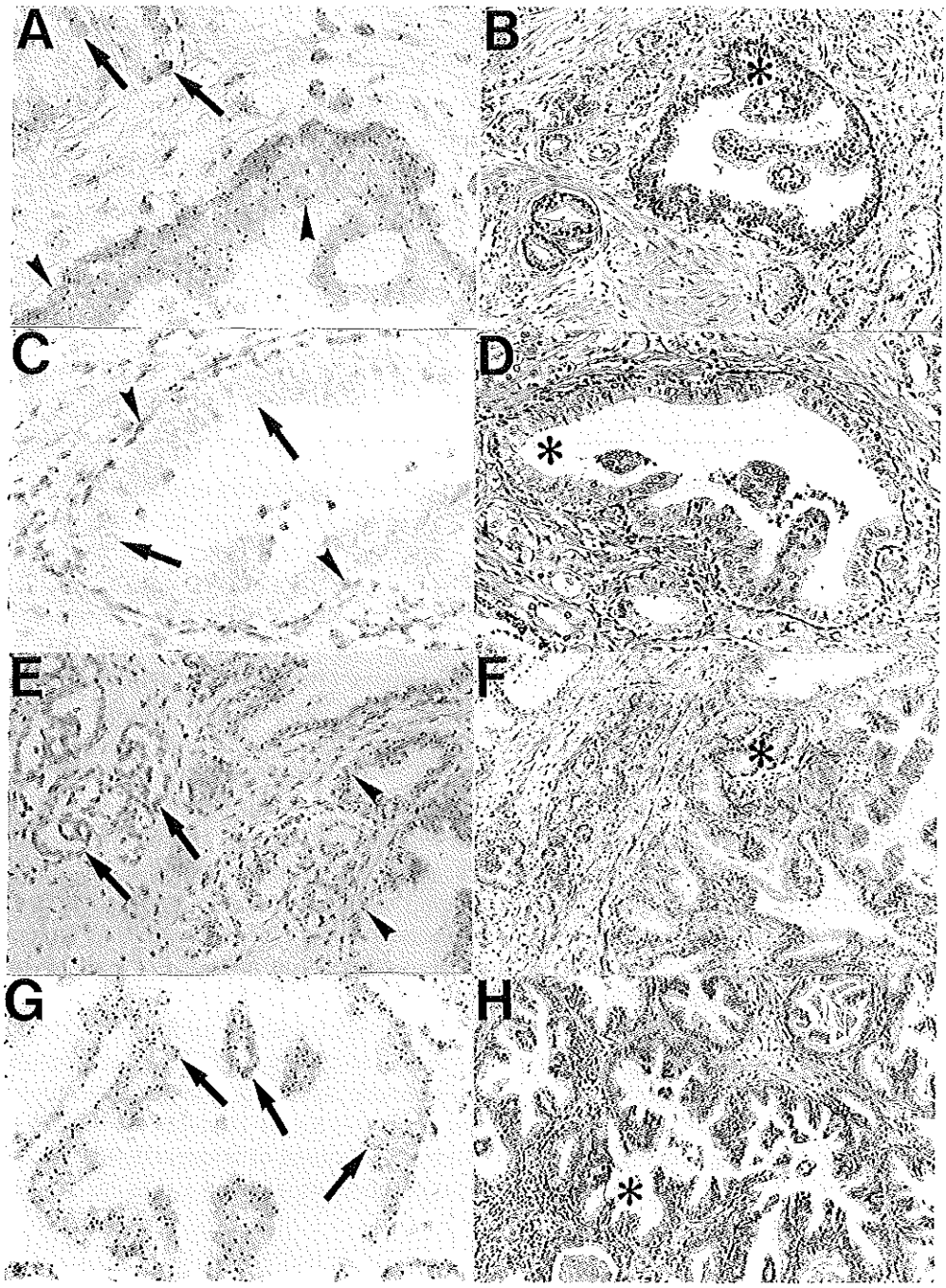
both monosomy 8 (Brothman *et al.*, 1990; Lundgren *et al.*, 1992a) and trisomy 8 (Micale *et al.* 1992). Gain of chromosome 8 was also demonstrated by FISH analysis (Macoska *et al.*, 1993; Micale *et al.*, 1993). In RFLP studies loss of alleles from the 8p region were seen in a high percentage of prostatic tumors. Loss of the Y chromosome in prostate cancer has been reported by karyotyping studies. For example, Lundgren *et al.* (1992a) found loss of the Y chromosome in 40% of the tumors. Loss of chromosomes Y and 10 was found by interphase cytogenetics on cytological material of a metastatic prostate carcinoma (van Dekken *et al.*, 1990a). In our study 16% of all tumors showed loss of chromosome 15. Chromosomal abnormalities of chromosome 15 in prostatic adenocarcinoma have not been reported previously in the cytogenetic literature. Loss of chromosome 10 was seen in 12% of the patients. In cytogenetic literature a del(10)(q24) has been reported and molecular studies showed allelic loss from the 10p and 10q arms. Monosomy of chromosome 10 was detected by FISH analysis in 2 tumors (van Dekken *et al.*, 1990a, Micale *et al.*, 1993). Gain of chromosome 7 occurred in 8% of the patients. Gain of chromosome 7 and a del(7)(q22) has been reported in prostate cancer. Recently, FISH analysis suggested that gain of chromosome 7 is associated with the progression of prostate cancer (Bandyk *et al.*, 1994).
In 17 prostatic precancerous lesions adjacent to tumor glands we did not find chromosomal aberrations of chromosomes 7, 8, 10 or 15, even if present in the cancer cells. This illustrates that PIN lesions are distinct entities, which are not created by ingrowth of tumor cells into normal prostatic glands. In two cases we found loss of the Y chromosome in both PIN and adjacent adenocarcinoma. These results suggest that loss of the Y chromosome is an early event in prostatic tumorigenesis. ISH analysis further revealed a high percentage of PIN lesions to be moderately aneuploid. Crissman *et al.* (1993) found 26% of 87 both low and high grade PIN lesions with coexisting carcinoma to be aneuploid by DNA quantitation.

FIGURE 3.
A) ISH with the centromere 8 specific probe to a PIN within a Gleason 3 area of case 4. Underrepresentation of chromosome 8 is seen in the tumor glands (*arrows*), but not in the adjacent PIN lesion (*arrowheads*). **B)** Corresponding hematoxylin and eosin (HE) stained tissue section. Region of interest is marked by an asterisk. **C)** ISH with the chromosome Y specific probe to a PIN lesion of case 19. Loss of chromosome Y is seen in the luminal cells of the PIN lesion (*arrows*), but not in the basal cells (*arrowheads*). **D)** Corresponding HE section of this PIN lesion. Asterisk marks part of this PIN lesion seen in C. **E)** ISH with the Y probe to a PIN lesion adjacent to the Gleason 2 area of case 1. Loss of the Y chromosome is seen in the tumor glands (*arrows*) but not in the PIN lesion (*arrowheads*). **F)** Corresponding HE section of the PIN area (asterisk) adjacent to tumor. **G)** ISH with the chromosome 1 specific probe to a PIN lesion of case 12. Several aneuploid nuclei can be distinguished (*arrows*). **H)** Corresponding HE section of this PIN lesion. Asterisk marks area depicted in G. A 40x objective was used in A, C, G, a 20x objective in D and E, and a 10x objective in B, F, H.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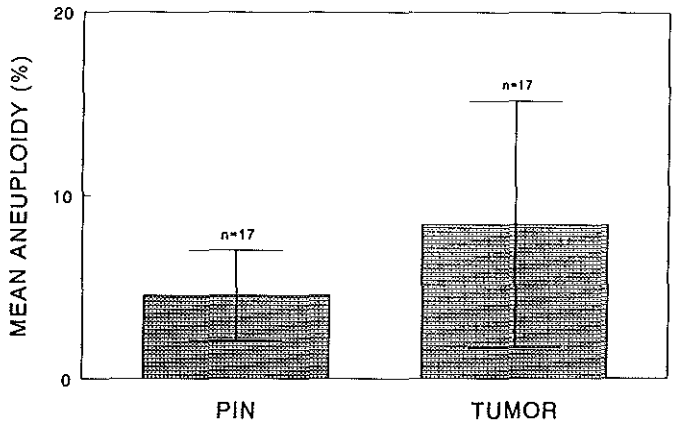

FIGURE 4.
Bar representation of the mean percentages of aneuploidy in prostatic tumors with adjacent PIN. An increase in the percentage of aneuploid cells can be seen in the adenocarcinoma.

In this study, however, half of the high grade PIN lesions showed aneuploidy.
Almost all chromosomal abnormalities occurred in subsets of tumor cells, irrespective of tumor grade. Genetic heterogeneity within a tumor is presumed to be important in the progression of a tumor to a highly malignant and metastatic state (Lundgren *et al.*, 1992b; Micale *et al.*, 1992). In our study, however, we observed genetic heterogeneity, i.e., subsets of tumor cells carrying a chromosomal abnormality, even in low grade, low stage tumors (Table 1). These cytogenetic growth patterns in prostate cancer will be described in detail in a separate paper (Alers *et al.*, in preparation). Furthermore, in case 19 we observed loss of the Y chromosome in the luminal cells, but not in basal cells in either PIN lesion or tumor cells (Figs. 3C, D, 2B, respectively). The basal cells are considered to contain the stem cells of the prostatic gland (Sell and Pierce, 1994). Thus, if chromosome Y loss is important in prostatic tumorigenesis, our results disagree with the concept of arrest of stem cell differentiation as a leading event in prostatic adenocarcinoma (Sell and Pierce, 1994). We have also seen loss of the Y chromosome in another precancerous lesion, i.e., dysplastic epithelium adjacent to adenocarcinoma of the esophagus (Krishnadath *et al.*, 1994).
In conclusion, interphase ISH to routinely processed paraffin sections of radical prostatectomies revealed genetic abnormalities in all grades and stages of prostatic tumors. It provides a tool to study the cytogenetic events during prostatic tumor progression, in which alterations of chromosomes 7, 8 and 10 might be related to more advanced cancers. In the latter tumors high degrees of aneuploidy/tetraploidy were found. In situ hybridization to histologic sections allowed us to distinguish

aneuploid cells already in the preneoplastic state. Moreover, the occurrence of loss of the Y chromosome in PIN lesions suggests that it is an early event, and a possible biomarker in prostatic tumorigenesis.

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CHAPTER 5

INTERPHASE CYTOGENETICS OF PROSTATIC TUMOR PROGRESSION: SPECIFIC CHROMOSOMAL ABNORMALITIES ARE INVOLVED IN METASTASIS TO THE BONE

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ABSTRACT

Only limited data are available on chromosomes specifically involved in the multistep tumorigenesis of prostate cancer. To investigate the cytogenetic status at different stages of prostatic tumor development, we have applied interphase in situ hybridization (ISH) with a set of (peri) centromeric DNA probes, specific for chromosomes 1, 7, 8, and Y, to routinely processed tissue sections of prostatic specimens of 75 different individuals. Our panel consisted of 16 normal/benign prostatic hyperplasia specimens (BPH), 23 primary, localized, prostatic tumors (N_0M_0 stage), 20 regional lymph node metastases (M_0 stage), and 16 distant metastases. Numerical aberrations of at least one chromosome were not observed in normal/BPH cases, but were present in localized tumors (39%), regional lymph node metastases (40%) and distant metastases (69%). Within the different pTNM groups we observed, in decreasing order of frequency: -Y, +8, -8, +7 in primary tumors; +8, +7, -Y, +Y, -8 in regional lymph node metastases; and +8, +7, +1, -Y, -8 in distant metastases. In primary tumors, the number of aberrant cases increased significantly with local tumor stage ($P<0.05$). A significant increase in gain of chromosome 8 was observed ($P<0.02$). Gain of chromosome 7 and/or 8 showed a significant increase with stage ($P<0.02$). Specific involvement of chromosome 8 was seen in bone metastases, but not in hematogenous metastases to other sites ($P=0.02$). Comparative genomic hybridization (CGH) analysis of these bone metastases disclosed centromere 8 gains as amplifications of the (whole) 8q arm, whereas centromeric loss appeared to be due to loss of 8p sequences. With progression towards metastatic disease an accumulation of genetic changes was seen as exemplified by gain of chromosome 1, which was solely observed in distant metastases. With tumor progression, gain of chromosomes 7 and/or 8 significantly increased ($P=0.03$), whereas the number of cases with aberrations of the Y chromosome did not change. Further, ploidy status determined by ISH revealed a significant increase in the number of aneuploid cases along with the pTNM stages ($P=0.04$). The data strongly suggest that: 1) Gain of chromosome 7 and/or 8 sequences are implicated in prostatic tumor progression. 2) In addition, gain of chromosome 8 sequences is related to local tumor growth. 3) Overrepresentation of 8q sequences, most likely by isochromosome 8q formation, is involved in metastatic spread to the bone. 4) Centromeric copy number changes, as detected by interphase ISH, might in some cases represent structural alterations, such as isochromosome 8q.

INTRODUCTION

Adenocarcinoma of the prostate is the most commonly diagnosed malignancy in males and the second leading cause of cancer death in men in Western countries. Its incidence is continuously rising, partially due to aging of the population (Carter and Coffey, 1990). Approximately half of the patients with clinically manifest prostate cancer will have extraprostatic disease at the time of diagnosis (Scardino *et al.*, 1992). These patients have a dismal prognosis with 10-year cancer-specific

survival rates of 10% and 40% for cases with distant metastases and regional lymph node metastases, respectively (Gervasi *et al.*, 1989; Scardino *et al.*, 1992). In contrast, in clinically localized tumors survival rates between 60-90% are found, dependent on the degree of local tumor invasion (Lerner *et al.*, 1991; Scardino *et al.*, 1992). The biological differences between those tumors prone to progress to life-threatening metastatic disease and those with little likelihood of causing morbidity and mortality and their clinical recognition are major goals of current prostate cancer research. Methods conventionally used to help predict the prognosis for patients with localized prostatic cancer include clinical staging and histopathological grading (Gittes, 1991; Gleason, 1992; Lieber *et al.*, 1995). The therapeutic strategy in individual cases, however, is difficult to design. Thus, there is a need for more markers that can serve as prognostic indicators.

The molecular genetic events responsible for the initiation and progression of prostate cancer remain largely unknown, although recently a major susceptibilty locus for familial forms of prostate cancer was linked to chromosome 1q24-q25 (Smith *et al.*, 1996). As with most types of human cancer, multiple genetic changes are thought to occur, involving both the inactivation of tumor suppressor genes and the activation of oncogenes (Isaacs *et al.*, 1995). Chromosomal aberrations encountered in prostatic adenocarcinoma include loss of the Y chromosome, trisomy of chromosome 7 and del(7)(q22), del(8)(p21), and del(10)(q24) (Brothman *et al.*, 1990; Lundgren *et al.*, 1992; Arps *et al.*, 1993). Prostate cancer allelotyping studies using RFLP and/or microsatellite markers have revealed frequent LOH on chromosome arms 7q, 8p, 10p, 10q, 13q, 16q, 17q, and 18q (Kunimi *et al.*, 1991; Bova *et al.*, 1993; Latil *et al.*, 1994; Trapman *et al.*, 1994; Gao *et al.*, 1995; Gray *et al.*, 1995; Takahashi *et al.*, 1995; Cooney *et al.*, 1996; Vocke *et al.*, 1996). Further, CGH analysis applied to tumor DNA of a panel of both primary and recurrent tumors revealed losses of 8p and 13q in over 30% of cases (Visakorpi *et al.*, 1995). Interestingly, local prostatic tumor recurrences showed gains of 8q, and of chromosomes X and 7, as well as loss of 8p in over half of cases. A recent CGH study (Cher *et al.*, 1996) performed on a panel of regional lymph node and bone metastases showed frequent gain of 8q, as well as loss of 8p, 10q, 13q, 16q, and 17p sequences.

Interphase (F)ISH to nuclear suspensions, touch preparations and paraffin sections of both prostatic tumors and precursor lesions (PIN) revealed numerical aberrations of chromosomes 7, 8, 10, 16, 17, 18, X and Y (Barreton *et al.*, 1994; Brown *et al.*, 1994; Alers *et al.*, 1995a, b, c), as well as loss of sequences in the 8p22 region (Macoska *et al.*, 1994; Matsuyama *et al.*, 1994). Furthermore, FISH studies of nuclear suspensions or touch imprints of mostly primary prostatic tumors suggested that alterations of chromosome 7 and/or 8 may be potential markers of poor prognosis in prostate cancer (Alcaraz *et al.*, 1994; Bandyk *et al.*, 1994; Takahashi *et al.*, 1994). These molecular genetic and interphase ISH studies have begun to

reveal chromosomal alterations occurring in primary and recurrent prostate cancer. However, a detailed knowledge regarding cytogenetic changes occurring during metastatic progression of prostate cancer is not available yet.
In the present study we have applied ISH to paraffin-embedded tissue sections of different patients in various stages of prostatic tumor development. Our tissue panel included 20 regional lymph node metastases and 16 distant metastases. To the best of our knowledge this is the largest collection of prostatic tumor metastases examined thus far. We addressed the following questions: 1) Is there a specific involvement of certain chromosomes, i.e., chromosomes 7 and/or 8, during prostatic tumor progression towards metastatic cancer? 2) Are certain (sub)stages, e.g., bone metastases, characterized by specific chromosomal abnormalities? And if so, what is the nature of these aberrations? 3) Is there an alteration in ploidy status of the tumors during tumor progression?

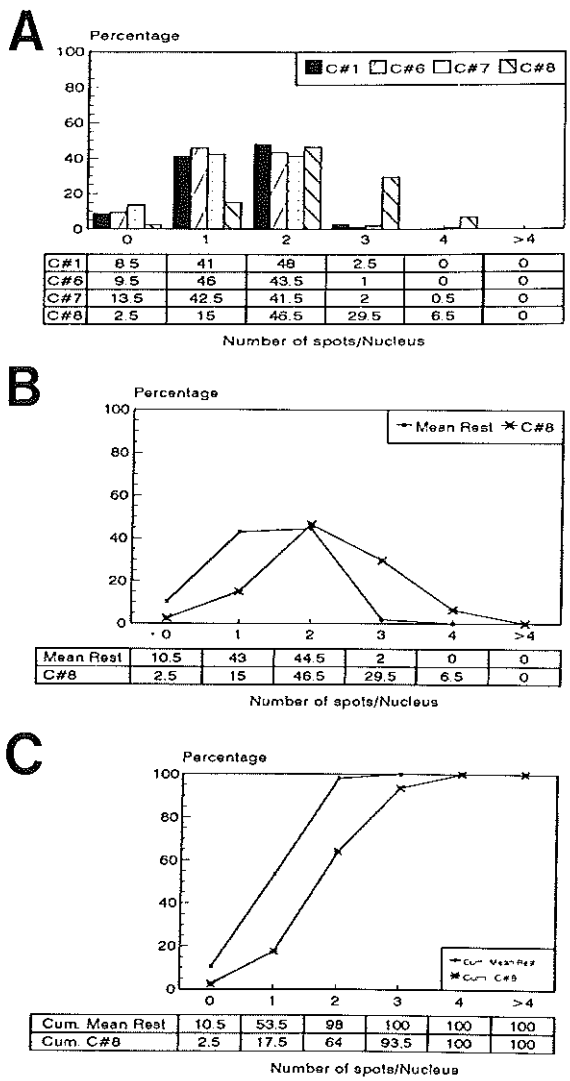
MATERIALS and METHODS

Patient Materials

Routinely processed formalin-fixed paraffin-embedded tissues from 75 different individuals, obtained between 1989 and 1996, were used for this study. Needle-biopsy specimens from 16 individuals with slightly elevated prostate specific antigen (PSA) levels, but without cancer, served as normal controls (Table 1). The mean age of the patients was 67 years (range 57-84 years). Twenty-three prostatic adenocarcinomas, which showed no lymph node involvement at the time of radical prostatectomy, served as a reference for clinically localized disease (Table 1). The mean age of the patients was 60 years (range 47-70 years). The tumors were pathologically staged according to the pTNM classification (Schröder *et al.*, 1992). The cases included five pT2 (tumor confined within the prostate), fifteen pT3 (tumor extends through the prostate capsule) and three pT4 tumors (tumor is fixed or invades adjacent structures other than seminal vesicles). The tumors were graded according to the Gleason grading system (Gleason *et al.*, 1992). The mean tumor Gleason score was G7 (range G4 to G10). The mean Gleason score per tumor stage was 6, 7, and 8 for stage pT2, pT3, and pT4 tumors, respectively. None of the patients received endocrine or radiation therapy prior to operation. Our set of regional metastases comprised of 20 pelvic lymph nodes with prostatic tumor metastasis, which were obtained prior to scheduled radical prostatectomy in most cases (Table 1). The mean age of the patients was 63 years (range 46-75 years). The regional metastases were staged pN1 (metastasis in a single lymph node, $\leq$ 2 cm in greatest dimension; 4 cases) or pN2 (metastasis in a single lymph node, $\leq$ 5 cm, or in multiple lymph nodes, none more than 5 cm in greatest dimension; 16 cases). None of the patients had distant metastasis at time of surgery. One of the patients had received endocrine therapy. Our panel of 16 distant metastases comprised of 3 distant peri-aortal lymph node metastases, 6 bone metastases and 7 metastases in other sites like brain (4 cases), liver (1 case), skin (1 case) and lung in one case (Table 1). Three of the samples were obtained at time of autopsy. The mean age of the patients was 65 years (range 41-78 years). Five of the patients with advanced disease had received endocrine treatment, four had received radiation therapy, and one patient had received a combination of both endocrine and radiation therapy.

Probe Set and Probe Labeling

All cases were analyzed with a probe set specific for chromosomes 1, 7, 8, and Y. A more



C#1	8.5	41	48	2.5	0	0
C#6	9.5	46	43.5	1	0	0
C#7	13.5	42.5	41.5	2	0.5	0
C#8	2.5	15	46.5	29.5	6.5	0

Mean Rest	10.5	43	44.5	2	0	0
C#8	2.5	15	46.5	29.5	6.5	0

Cum. Mean Rest	10.5	53.5	98	100	100	100
Cum. C#8	2.5	17.5	64	93.5	100	100

FIGURE 1.
Barhistograms (plus tables) illustrating statistical analysis of section ISH by the Kolmogorov-Smirnov test. **A)** Barhistogram displaying spot distributions for chromosomes 1, 6, 7, and 8 in a prostatic regional lymph node metastasis. Chromosomes 1, 6, and 7 display a non-aberrant profile. Chromosome 8 shows an overrepresentation. **B)** Same, line histogram for the mean of the non-aberrant probes (rest) compared to the chromosome 8 probe, which shows a shift to the right. **C)** Line histogram, cumulative: The maximum difference between the mean of the non-aberrant chromosomes and chromosome 8 is seen at 1 spot per nucleus, representing a P value of <0.01, if 100 cells are counted and a maximum difference of ≥16 is reached.

detailed description of the probes used is given elsewhere (Alers *et al.*, 1995b; Alers and van Dekken, 1996 and references within). Selection criteria were based on previous studies (Alers *et al.*, 1995a, b) and other literature data considering cytogenetic aberrations in prostatic tumors (Brothman *et al.*, 1990; Lundgren *et al.*, 1992; Arps *et al.*, 1993; Alcaraz *et al.*, 1994; Bandyk *et al.*, 1994; Takahashi *et al.*, 1994). The (peri) centromeric repetitive satellite DNA probes were labeled with biotin-14-dATP by nick translation of complete plasmid DNA according to the manufacturer's directions (BioNick kit, Gibco BRL, Gaithersburg, MD).

In Situ Hybridization (ISH)

ISH was performed on routine consecutive 4-µm tissue sections. Sections were mounted with distilled water on microscope glass slides coated with either aminoalkylsilane (Sigma, St. Louis, MO) or with a "para-tissuer pen" (ITK Diagnostics, Uithoorn, The Netherlands). Sections were baked overnight at 60°C for better adherence. The ISH procedure was performed as described earlier (van Dekken *et al.*, 1992; Alers *et al.*, 1995a, b). Briefly, after deparaffinization of the tissue sections in xylene, endogenous peroxidase activity was blocked with 0.3% H_2O_2 in methanol. To facilitate DNA probe accessibility to the cellular DNA, sections were digested with 0.4% pepsin (Sigma) in 0.2 M HCl at 37°C for 3-30 min (mean 10 min). Before applying the probe set, the optimal digestion time for each sample was determined by a pepsin time series (5, 10, 15, 20 min).

Cellular DNA was heat denatured for 2 min at 72°C in 70% formamide in 2x SSC (pH 7.0), followed by dehydration in graded ethanol series. Chromosome-specific repetitive DNA probes were denatured for 10 min at 72°C in a hybridization mixture containing 2 ng/µl probe DNA, 500 ng/µl sonicated herring sperm DNA (Sigma), 0.1% Tween-20, 10% dextran sulphate, and 60% formamide in 2x SSC at pH 7.0. Then, 15-30 µl of probe mixture was applied to the sections. The slides were incubated overnight at 37°C in a moist chamber. Tissue sections were washed in 60% formamide in 2xSSC (pH 7.0) at 42°C for 10-15 min, then in 2xSSC at 42°C for 10-15 min.

Histochemical detection was performed by immunoperoxidase staining. Slides were subsequently incubated for 30 min with mouse anti-biotin, biotin-labeled goat anti-mouse, and peroxidase-conjugated avidin-biotin complex (Dako, Glostrup, Denmark). The probe-related ISH signals were developed with diaminobenzidine (DAB; 0.5 g/l in 0.1 M PBS + imidazole with 0.05% H_2O_2) for 10 min. The signal was amplified with $CuSO_4$ (0.5% in 0.9% NaCl). Slides were counterstained with hematoxylin.

Evaluation of ISH Results

The DNA probe set was analyzed for each sample on consecutive 4-µm sections in a previously defined tissue area. On each tissue section leukocytes, stromal cells etc. served as internal controls to evaluate the quality of ISH. For each of the probes, 100 "intact" (i.e. spherical) and non-overlapping 4-µm nuclear slices were counted by two independent investigators simultaneously and the number of solid DAB spots per nuclear contour was scored (0, 1, 2, 3, 4, >4 spots/nuclear slice). The DNA spot-distributions of the two observers were then compared and averaged, when no significant counting differences between the investigators were found. In our series no discrepancies emerged using this approach.

In cases, which showed aberrations of one or more of the autosomal probes, a centromeric probe, specific for chromosome 6, was added to the probe set. In our study no aberrations of chromosome 6 were seen in those cases. Thus, at least two non-aberrant autosomal probes were used for analysis. A tumor was called aneuploid when the percentage of hyperdiploid tumor cells (>2 spots/nucleus) for the mean of the non-aberrant autosomal

probes was ≥2.5%. This cut-off value for aneuploidy was based on the mean rate of hyperdiploidy in both previous (Alers *et al.*, 1995a) and present panels of normal controls (n=33 in total). The number of nuclei with a hyperdiploid spot number (likely artifacts) in these controls never exceeded 2.5%. The mean rates of hyperdiploidy were well below this cut-off value (0.4% and 0.7%, respectively). In the primary prostatic tumors, the percentages of hyperdiploid cells in the two dominant Gleason patterns were averaged.

Statistical Analysis

Despite the 4 μm sectioning artifact, which results in truncated nuclei, specific chromosome aberrations were detected and could be statistically evaluated. The spot distributions of the different probes on consecutive tissue sections were evaluated statistically by means of the Kolmogorov-Smirnov test (Alers and van Dekken, 1996). This statistical test is very suitable for two-sided comparisons of histograms or other distributions. Underrepresentation of a specific chromosome was seen as a shift to the left of the DNA probe distribution, when compared with the mean of the non-aberrant probe distributions of the same (tumor) area on adjacent tissue sections. Conversely, gain of a specific chromosome was seen as a shift to the right. An aberrant probe spot distribution was considered to represent a numerical aberration, if the associated *P* value was <0.01. Following this approach, we were able to distinguish changes in ploidy status from individual chromosomal aberrations (i.e., gains or losses). An example is shown in Fig. 1.

The relation between two dichotomous parameters was investigated using Fisher's exact test. The relation between the presence of a characteristic, i.e. chromosomal aberration and staging (three or more ordered groups) was assessed using the χ^2 test for trend (Mantel, 1963). *P*=0.05 (two-sided) was considered the limit of significance.

Comparative Genomic Hybridization (CGH) of Archival Material

The same formalin-fixed, paraffin-embedded, tissue blocks, used for ISH analysis, were counterstained in 4,6-diamino-2-phenyl indole (DAPI; 0.1 µg/ml in distilled water) for 5 min. and placed under a fluorescence microscope, enabling a precise selection of the tumor area. Microdissection of the tumor areas was performed using a hollow bore coupled to the microscope (van Driel-Kulker *et al.*, 1986). Lower boundaries were checked for the presence of tumor on hematoxylin-eosin-stained tissue sections. Excised material was minced using a fine scalpel, deparaffinized in xylene and ethanol series and dried. Samples were digested in 1 ml of extraction buffer (10 mM Tris/HCl (pH 8.0), 100 mM NaCl, 25 mM EDTA, 0.5% SDS and 300 µg/ml Proteinase K) and incubated at 55°C for three to four days (Isola *et al.*, 1994). Fresh Proteinase K (300 µg/ml) was added every 24 hours. DNA was extracted with phenol-chloroform-isoamyl alcohol for at least four times and subsequently precipitated in ethanol according to standard protocols. DNA was treated with RNase (20 µg/µl in 2xSSC) for 1 hour at 37°C, precipitated and dissolved overnight in sterile water at 55°C. Concentration, purity, and molecular weight of the DNA was estimated using both UV spectrophotometry and ethidium bromide-stained agarose gels with control DNA series.

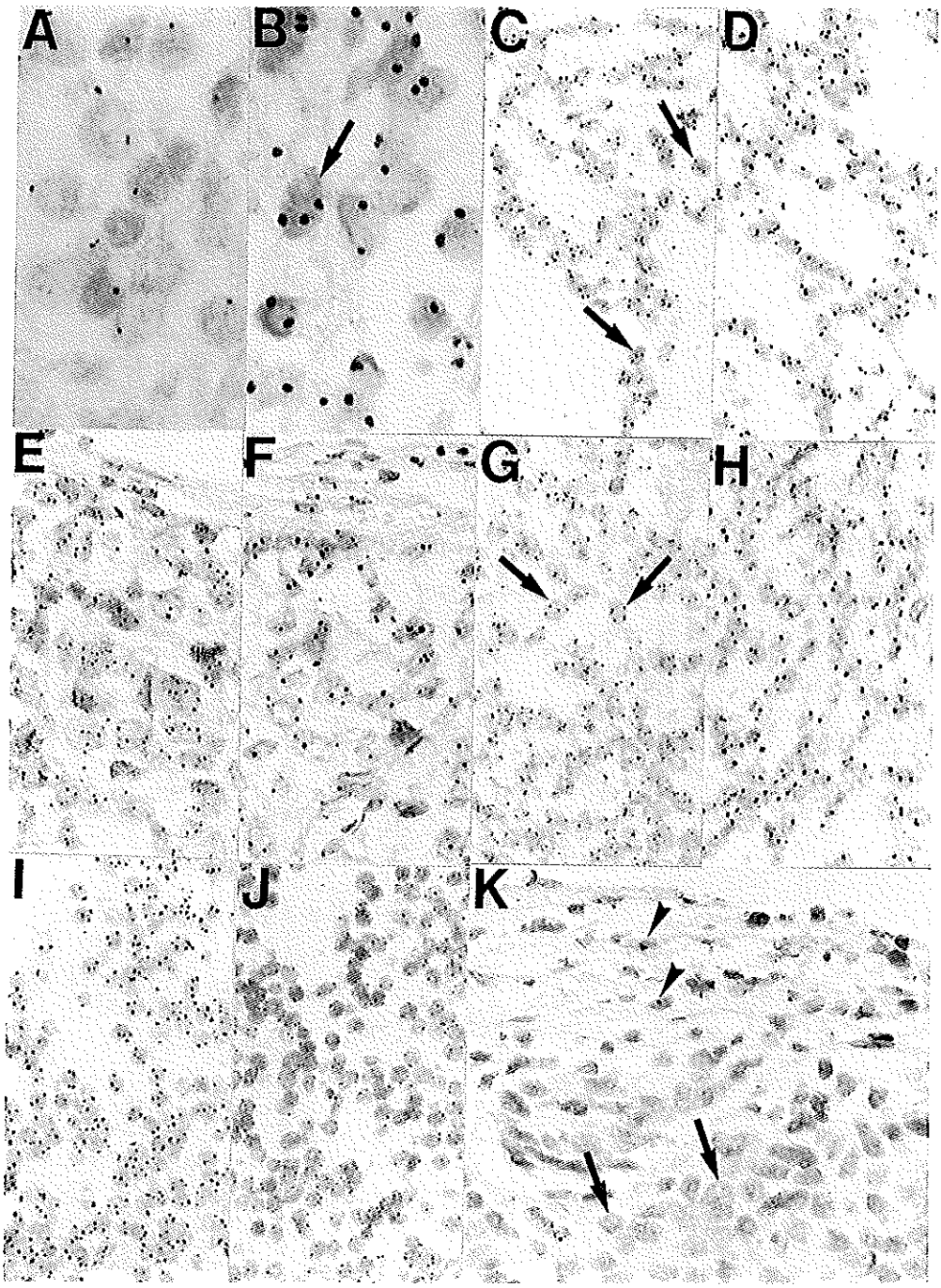
Tumor DNA was labeled with biotin by nick translation (Nick Translation System, Gibco BRL, Gaithersburg, MD). Likewise, male reference DNA (Promega, Madison, WI) was labeled with digoxigenin (Boehringer Mannheim, Indianapolis, IN) by nick translation. The reaction time and the amount of DNAse was adjusted to obtain a matching probe size for reference DNA and tumor DNA. Molecular weight of both tumor and reference DNA was checked by gel electrophoresis. Probe sizes were between 300 and 1.5 kb.

Labeled genomic DNAs were hybridized onto normal male lymphocyte metaphase preparations essentially as described by Kallioniemi *et al.*, (1992). In brief, equal amounts

Table 1. Results of ISH with a set of chromosome-specific repetitive DNA probes (1, 7, 8, Y) of 16 normal prostatic biopsy specimens, 23 primary localized prostatic tumors, 20 regional lymph node metastases and 16 distant metastases

	Normal/ BPH	*Adenocarcinoma*				*Lymph Node Metastasis*			*Distant Metastasis*			
		pT2	*pT3*	*pT4[a]*	*Total*	*pN1*	*pN2[b]*	*Total*	*Distant Lymph Node*	*Bone*	*Other Sites*	*Total*
Total	16	5	15	3	23	4	16	20	3	6	7	16
Number of aberrant patients (%)	0	1 (20)	5 (33)	3 (100)	9 (39)	3 (75)	5 (31)	8 (40)	3 (100)	4 (67)	4 (57)	11 (69)
Number of aberrations	0	1	7	4	12	7	5	12	5	5	6	16
Type of aberrations (%):												
+1	0	0	0	0	0	0	0	0	1 (33)	0	2 (29)	3 (19)
+7	0	0	1 (7)	1 (33)	2 (9)	2 (50)	0	2 (10)	2 (67)	0	2 (29)	4 (25)
+8	0	0	1 (7)	2 (67)	3 (13)	2 (50)	3 (19)	5 (25)	2 (67)	3 (50)	0	5 (31)
-8	0	0	2 (13)	0	2 (9)	1 (25)	0	1(5)	0	1 (17)	0	1 (6)
+Y	0	0	0	0	0	1 (25)	1 (6)	2 (10)	0	0	0	0
-Y	0	1 (20)	3 (20)	1 (33)	5 (22)	1 (25)	1 (6)	2 (10)	0	1 (17)	2 (29)	3 (19)
Aneuploid cases[c] (%)	0	4 (80)	12 (80)	2 (67)	18 (78)	3 (75)	15 (94)	18 (90)	3 (100)	6 (100)	7 (100)	16 (100)
Median % of hyperdiploid cells	0	15	6	22	7	6	8	11	5	14	16	15
Range of % of hyperdiploid cells	0-2	1-42	1-28	2-32	1-42	1-31	2-62	1-62	3-15	3-28	4-59	3-59

[a] Staging of localized prostatic adenocarcinoma (all N0M0 stage) according to the pathological TNM classification: pT2, tumor confined within the prostate; pT3, tumor extends through the prostatic capsule; pT4, tumor is fixed or invades adjacent structures other than seminal vesicles.
[b] Staging of regional lymph node metastases (all M0 stage): pN1, metastasis in a single lymph node, ≤ 2 cm in greatest dimension; pN2, metastasis in a single lymph node, ≤ 5 cm, or in multiple lymph nodes, none more than 5 cm in greatest dimension.
[c] Cases were defined aneuploid when the mean percentage of hyperdiploid nuclei of the non-aberrant autosomal probes amounted ≥2.5%. In cases with aberrations of autosomal DNA probe(s), ISH with an additional chromosome 6-specific DNA probe was employed for reference purposes.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(200 ng) of labeled tumor DNA and labeled reference DNA and 10 µg of unlabeled Cot-1 DNA were precipitated in ethanol. The pellet was dissolved in 10 µl of hybridization mixture (50% formamide, 0.1% Tween-20, and 10% dextran sulphate in 2x SSC at pH 7.0). Probes were denatured 10 min at 72°C immediately before applying onto the slides. Slides were denatured in 70% formamide, 2xSSC (pH 7.0) at 72°C for 2 min and dehydrated in graded ethanol series. Hybridization was done under sealed coverslips for 3 days at 37°C in a moist chamber. After hybridization, the slides were washed in 50% formamide in 2xSSC (pH 7.0) at 42°C for 10-15 min, then in 2xSSC at 42°C for 10-15 min. Fluorescent detection of the biotin- and digoxigenin labeled DNA probes was accomplished with avidin-fluorescein isothiocyanate and anti-digoxigenin rhodamine, respectively, for 1 hour at 37°C. Samples were counterstained with DAPI in anti-fade solution.
For image acquisition, an epifluorescent microscope (Leica DM, Rijswijk, The Netherlands) equipped with a cooled CCD camera (Photometrics, Tucson, AZ) and a triple band pass beam splitter and emission filters (P-1 filter set, Chroma Technology, Brattleborough, VT) was used. Grey level images of each of the three fluorochromes were collected and a three color image was built up by overlay of the three images in pseudo-colors selected to match the original color of the fluorochromes, using a algorithm implemented in SCIL-image (TNO, Delft, The Netherlands) on a Power Macintosh 8100. Image analysis was performed with the use of QUIPS XL software (version 2.0.3 Vysis Inc., Downers Grove, IL), using reversed DAPI banding to identify the chromosomes. For all the profiles in Fig. 5, losses of DNA sequences are defined as chromosomal regions where the mean green:red ratio is below 0.8, while gains are defined as chromosomal regions where the ratio is above 1.2. These threshold values were based on series of normal controls.

RESULTS

Genetic Changes and Tumor Stage.

The results of ISH analysis of patients in different stages of prostatic tumor development are summarized in Table 1 and Fig. 2. None of the 16 cases of normal and hyperplastic prostatic tissues showed a numerical chromosomal aberration.

FIGURE 2.
A, B) ISH with the chromosome 8-specific probe to a stage pT3 primary prostatic adenocarcinoma, revealing loss of chromosome 8 in the tumor nuclei (A). The cells display only 0 or 1 spot. B, shows the same case with the chromosome 1-specific probe: Note that the mean number of spots is higher than in A. Also an aneuploid nucleus can be seen (*arrow*). **C, D)** Chromosome 8-specific ISH to a pN2 staged regional lymph node metastasis (C). Gain of chromosome 8 is seen, illustrated by many nuclei with 3 spots (*arrows*), when compared with chromosome 1 in D. **E, F)** ISH with the chromosome 8-specific probe to a bone metastasis of prostatic adenocarcinoma (E). Gain of chromosome 8 is seen, when compared with the chromosome 1-specific probe in F. **G, H)** Staining for chromosome 7 of a brain metastasis of prostatic adenocarcinoma (G). A gain of chromosome 7 is observed, visible as many nuclei with 3 or more spots/nucleus (*arrows*). The mean number of chromosome 1 spots is less than for chromosome 7 (H). **I, J)** Chromosome 1-specific ISH to a brain metastasis of prostatic adenocarcinoma (I). Gain of chromosome 1 is seen as many nuclei with three or more dots. Compare J: ISH with a chromosome 6-specific reference probe. **K)** ISH with the chromosome Y-specific probe to the same bone metastasis as in E and F. Loss of Y is seen in the tumor cell nuclei (*arrows*) but not in the stromal cells (*arrowheads*). Magnification: 361x except for A and B (880x).

Primary localized tumors: The 23 cases of primary prostatic adenocarcinomas revealed aberrations for chromosomes 7, 8, and Y in 9 cases (39%). The percentage of patients with numerical aberrations gradually increased ($P<0.05$) from 20% in stage pT2 up to 100% in stage pT4 tumors (Table 1; Fig. 3). This occurred independently of tumor grade. Some patients (three cases; 13%) showed more than one alteration at the time, especially in high staged tumors. The numerical aberrations observed were loss of the Y chromosome in five cases (22%), gain of chromosome 8 in three cases (13%), loss of chromosome 8 in two cases (9%; Fig. 2A, B), and gain of chromosome 7 in two cases (9%). Loss of chromosome 8 was seen in two pT3 cases only. A significant increase in aberrations of chromosome 8 ($P=0.04$) and, especially, in gain of chromosome 8 with increasing tumor stage was seen ($P<0.02$; Table 1; Fig. 3). Increase in gain of chromosome 7 with increasing tumor stage was observed but this prevalence did not reach statistical significance (Fig. 3). Combination of gain of chromosome 7 and/or gain of chromosome 8 showed a significant increase ($P<0.02$) from no involvement in stage pT2 tumors to 33% in stage pT4 tumors.

Regional lymph node metastases: In the set of 20 regional lymph node metastases, 8 patients (40%) displayed a numerical change. No significant difference in the percentage of aberrant cases was seen between stage pN1 and stage pN2 tumors. However, a subdivision in pN1 and pN2 stage lymph node metastases might not be useful, due to incomplete and selective sample collection. This is in concordance with epidemiological findings in which similar patterns of progression and cancer-specific mortality were found in pN1 to pN3 staged groups (Gervasi *et al.*, 1989). The numerical aberrations encountered in the regional lymph node metastases were as follows (Table 1): Gain of chromosome 8 in five cases (25%; Fig. 2C, D), gain of chromosome 7 in two cases (10%), loss of chromosome Y in two cases (10%), gain of chromosome Y in two cases (10%), and loss of chromosome 8 in one case (5%).

Distant metastases: In our panel of 16 distant metastases 11 tumors (69%) revealed numerical alterations for at least one of the chromosomes studied. No significant difference in the number of numerical aberrations between the different types of metastases was seen. In contrast to regional lymph node metastases, all of the distant para-aortic lymph node metastases showed aberrations (Table 1). The chromosomal changes detected in this panel were as follows: Gain of chromosome 8 in five cases (31%; Fig. 2E, F), gain of chromosome 7 in four cases (25%; Fig. 2G, H), gain of chromosome 1 in three cases (19%; Fig. 2I, J), loss of Y in three cases (19%; Fig. 2K), and loss of chromosome 8 in one case (6%). No alterations of chromosome 8 were seen in hematogenous metastases to other places than the bone marrow (bone metastases versus non-osseous hematogenous metastases; $P=0.02$).

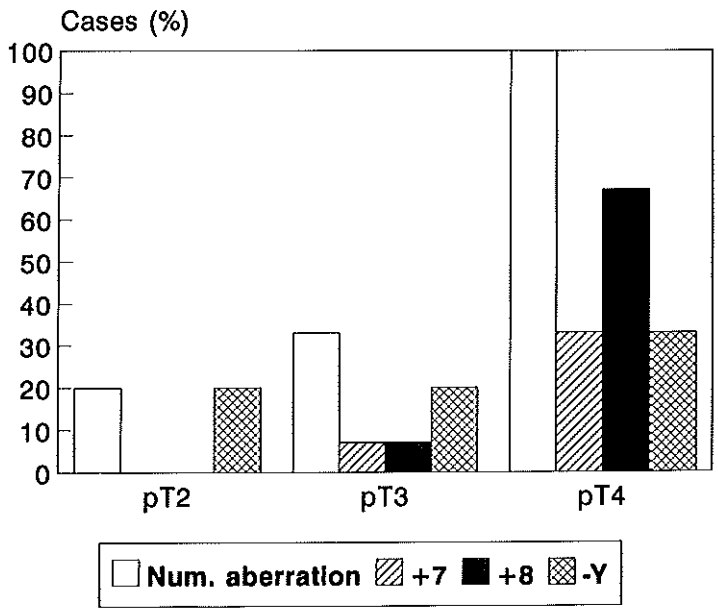

FIGURE 3.
Graphic representation of the results of ISH with a probe set specific for chromosome 1, 7, 8, and Y applied to pT2, pT3, and pT4 staged primary prostatic adenocarcinomas. A gradual increase in the percentage of cases with numerical aberrations is seen from pT2 to pT4 ($P<0.05$), as well as more frequent gain of chromosome 8 ($P<0.02$). Gain of chromosome 7 also occurs slightly more often in higher tumor stages, whereas loss of Y is seen in about equal percentages in both pT2, pT3 and pT4 staged tumors.

Genetic Changes and Tumor Progression

The chromosomal aberrations occurring during prostatic cancer progression to metastatic disease are summarized in Table 1 and Fig. 4. A tendency was observed for gain of chromosome 1 in distant metastases only ($P=0.06$ versus primary localized tumors, $P=0.08$ versus regional lymph node metastases; Fig. 4). Gain of chromosome 7 and gain of chromosome 8 were seen more frequently in metastases than in primary tumors, but this prevalence did not reach statistical significance. When cases with gain of chromosomes 7 and/or 8 were combined, this increased significantly with tumor progression ($P=0.03$) ranging from three cases (13%) in primary tumors, to six cases (30%) in regional lymph node metastases, and to seven cases (44%) in distant metastases (Fig. 4). In contrast, the number of cases with loss of chromosome 8 (Table 1), as well as alterations of the Y chromosome (both gains or losses) did not significantly change with tumor progression (Fig. 4).

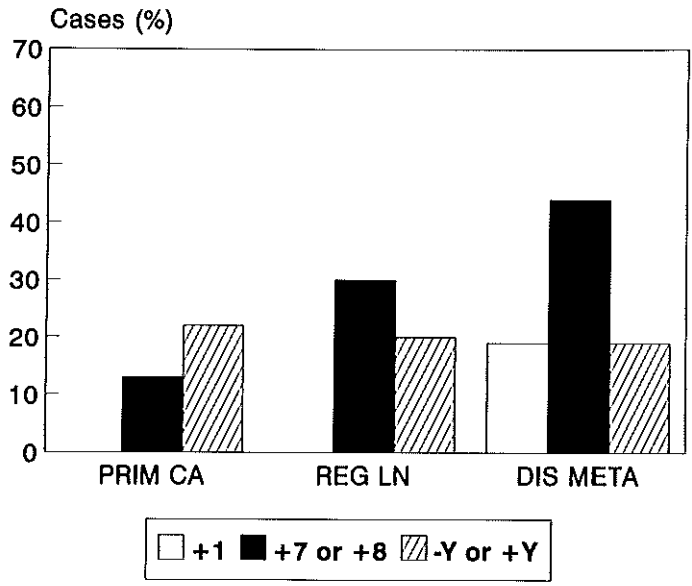

FIGURE 4.
Bar histogram, distinguishing markers for progression of prostatic cancer towards metastatic disease. Abbreviations used are: Primary, localized, prostatic cancer (PRIM CA); regional lymph node metastases (REG LN); distant metastases (DIS META). Gain of chromosome 1 solely occurs in distant metastases. Combination of cases with gain of chromosome 7 and/or 8 reveals a significant increase with progression to distant metastatic disease (P=0.03). In contrast, the percentage of cases with alterations of Y remained stable during tumor progression.

The results of analysis of the ploidy status in tumor progression are summarized in Table 1. The three groups, i.e., primary prostatic cancer, regional lymph node metastasis and distant metastasis showed a significant increase (P=0.04) in number of aneuploid (=hyperdiploid) cases along with tumor progression (Table 1).

CGH Analysis of Bone Metastases

CGH analysis was performed on four archival bone metastases to further study the alterations of chromosome 8 revealed by interphase ISH. Of three cases with centromeric gain of chromosome 8, CGH delineated amplification of the whole 8q arm in two cases (Fig. 5A, B), whereas the third sample showed gain of the 8q arm spanning from 8q13 to 8qter (Fig. 5C). Gain of the entire 8q arm combined with an increased spot number for the centromeric region of chromosome 8 is strongly suggestive for isochromosome i(8q) formation. Further, in two of these cases also

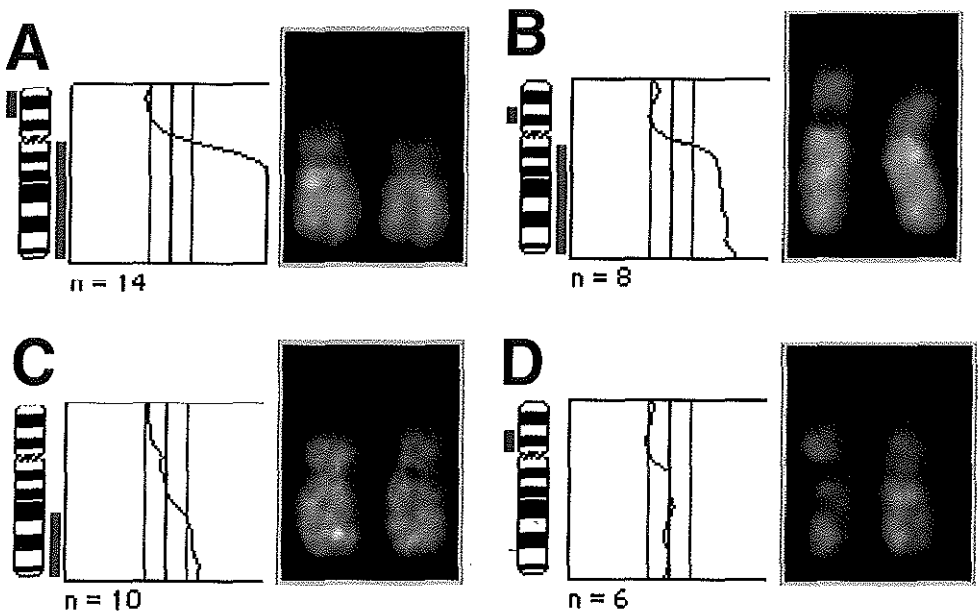

FIGURE 5.
Comparative genomic hybridization (CGH) of four archival, formalin-fixed and paraffin-embedded, bone metastases that showed gain (A-C) or loss (D) of the centromeric region of chromosome 8 by ISH analysis (Table 1). The chromosomal ideograms are shown along with the mean ratio profiles and the digitized fluorescent images of chromosome 8 (number of chromosomes analyzed varying from 6 to 14, lower/upper thresholds at ratios 0.80/1.20). Deleted regions are shown as a red bar on the left of the ideogram, gains are depicted in green along the right-side. **A)**. Same case as in Fig. 2E. CGH reveals gain of 8q sequences, involving the entire long arm. In Fig. 2E a gain of the centromeric region of chromosome 8 is seen. This strongly suggests the presence of an isochromosome of 8q (i(8q)). Concomitant loss of 8p sequences is seen in the 8p21-pter region. **B)** CGH analysis showing amplification of the entire 8q arm, as well as loss of 8p12-p21 sequences. **C)** Amplification of most of the 8q arm (8q13-qter region). **D)** Loss of 8p sequences (8pcen-p21), involving the centromeric region, is detected by CGH analysis.

loss of 8p sequences was found (8p21-pter and 8p12-p21 region: Fig. 5A and B, respectively). Loss of the centromeric region of chromosome 8 in one bone metastasis involved loss of the 8pcen-p21 region (Fig. 5D). Thus, all chromosome 8 aberrations resulted in overrepresentations of 8q sequences.

DISCUSSION

The aim of this study was to gain more insight in genetic changes at the chromosomal level during different stages of prostatic tumor development. In general, we observed a tendency for accumulation of genetic events in prostatic tumor metastases, as exemplified by gain of chromosome 1 in distant metastases only. Further, a significant increase was found in the frequency of gain of chromosomes 7 and/or 8. In contrast, the number of cases with alterations of Y (both loss or gain), as well as loss of chromosome 8, did not change significantly with tumor progression. A significant increase was seen in the percentage of aneuploid, i.e. hyperdiploid cases during tumor progression. Aneuploidy occurred more frequently in our prostate tumor metastases than has been described in DNA FCM studies, which have shown diploidy in 40 to 50% of metastases (Deitch and deVere White, 1992; Van den Ouden *et al.*, 1993). This higher percentage of hyperdiploid cases is most likely caused by the low cut-off value (2.5% hyperdiploid cells), as well as the selective analysis of cancer cells only by the ISH technique (Persons *et al.*, 1994; Alers *et al.*, 1995a, b; Alers and van Dekken, 1996).

Gain of chromosome 8 was the most frequent anomaly in metastatic tumor samples. Gain of chromosome 8q was seen in up to 85% of lymph node metastases and 89% of recurrent primary tumors by CGH analysis (Visakorpi *et al.*, 1995; Cher *et al.*, 1996). Further, gain of chromosome 8 was associated with high tumor grade (Takahashi *et al.*, 1994). Homozygous deletions and allelic loss of loci in the 8p12-p22 region are frequently reported in different stages of prostatic tumorigenesis (Bova *et al.*, 1993; Macoska *et al.*, 1994; Matsuyama *et al.*, 1994; Trapman *et al.*, 1994; Visakorpi *et al.*, 1995; Cher *et al.*, 1996; Vocke *et al.*, 1996), as well as in PIN (Emmert-Buck *et al.*, 1995). This suggests the presence of one or more putative tumor suppressor genes in this region important in the initiation and progression of prostatic adenocarcinoma. We observed both loss and gain of chromosome 8. Loss of chromosome 8 in a bone metastasis, as determined by ISH, was related to loss of 8p, when evaluated by CGH. Gain of chromosome 8 in bone metastases was disclosed by CGH as amplification of the (entire) long arm of 8. This overrepresentation of 8q sequences is likely caused by isochromosome i(8q) formation. i(8q) formation in prostate cancer has been suggested by others (Bova *et al.*, 1993; Cher *et al.*, 1994; Macoska *et al.*, 1994), however, it has never been substantiated. In addition, we observed i(8q) formation by CGH analysis and karyotyping in two prostate cancer cell lines, PC 133 and PC 346 (van Dekken *et al.*, in preparation). PC 133 is a prostatic tumor cell line derived from a bone metastasis (Noordzij *et al.*, 1996), whereas PC 346, derived from a primary prostatic tumor, displays rapid growth in athymic mice (Van Weerden *et al.*, 1996). Thus, both these cell lines show aggressive tumor behavior. Chromosome region 8q24 harbors the *c-myc* oncogene. Amplification of this region was detected in some cases of prostatic cancer and was shown to be correlated with adverse

prognosis (Van den Berg *et al.*, 1995; Jenkins *et al.*, 1997). However, CGH analysis of metastatic tumors showed that the region of most common gain was at 8q21.3, suggesting that other unknown oncogenes may reside in this region (Cher *et al.*, 1996).

Trisomy of chromosome 7 has been reported relatively frequently in prostatic cancer (Lundgren *et al.*, 1992; Arps *et al.*, 1993; Brown *et al.*, 1994; Alcaraz *et al.*, 1994; Bandyk *et al.*, 1994; Takahashi *et al.*, 1994). Gain of chromosome 7 was correlated with high local tumor stage and grade (Takahashi *et al.*, 1994), as well as with poor prognosis (Alcaraz *et al.*, 1994). Trisomy 7 is also a consistent aberration in bladder cancer and in this tumor type is strongly correlated with tumor grade and stage (Waldman *et al.*, 1991. Gain of chromosome regions 7p12-p21 and 7q11.3-q33 was seen by CGH analysis in up to 40% of the specimens from (lymph node) metastases (Cher *et al.*, 1996). Chromosome arm 7p contains the *erbB-1* oncogene. Further, the *c-met* oncogene, which maps to 7q31.1, a region that shows frequent LOH in prostatic cancer (Takahashi *et al.*, 1995), is expressed in the majority of both primary tumors and (lymph node) metastases (Pisters *et al.*, 1995). These findings suggest that gain of chromosome 7 may interfere with normal cell growth regulation and play a direct role in prostate cancer aggressiveness.

Gain of chromosome 1 was a new finding in our panel and was exclusively seen in distant metastatic tumors. Recently, a major susceptibility locus for familial prostate cancer was mapped to chromosome 1q24-q25 (Smith *et al.*, 1996). However, hereditary prostate cancer accounts for only 10% of all prostatic carcinomas and is predominantly seen in patients with an early age of onset. None of the metastases with gain of chromosome 1 were derived from young (< 60 years) patients. Further, the precise nature of the aberration on chromosome 1q remains to be unraveled. Gain of the chromosome 1q21-q42.3 region was seen in 52% of lymph node metastases by CGH analysis (Cher *et al.*, 1996). Karyotyping and ISH studies showed both loss and gain of (part of) chromosome 1 (Brothman *et al.*, 1990; Lundgren *et al.*, 1992; Arps *et al.*, 1993; Barreton *et al.*, 1994). Aberrations of chromosome 1 are common in both hematological malignancies and solid neoplasms (Atkin *et al.*, 1986). Mostly, trisomy 1 or duplication of the 1q arm are found. Further, chromosome 1 abnormalities occur relatively late in the course of disease (Atkin *et al.*, 1986).

Loss of the Y chromosome has been described to occur in up to 40% of cases of prostate cancer by both karyotyping and ISH studies (Lundgren *et al.*, 1992; Arps *et al.*, 1993; Barreton *et al.*, 1994; Alers *et al.*, 1995a, c). In a previous study of 17 PIN lesions adjacent to prostatic adenocarcinomas we found loss of Y in two cases, as well as in the corresponding tumor (Alers *et al.*, 1995a). These results suggest that loss of the Y chromosome is an early event in prostatic tumorigenesis. Currently, no specific genes involved in tumorigenesis are linked to the Y chromosome. Therefore, loss of Y may be the result of genomic instability in transformed cells. In

regional lymph node metastases, we also found gain of Y. Other ISH studies reported gains as well as losses for this chromosome (Alcaraz *et al.*, 1994; Barreton *et al.*, 1994; Brown *et al.*, 1994).
Prostatic tumors frequently metastasize to the bone. Interestingly, specific involvement of chromosome 8 was seen in bone metastases, but not in hematogenous metastases to other sites. In contrast, a tendency was observed for gain of chromosome 1 and/or 7 in non-osseous hematogenous metastases. Thus, one could speculate about the role of chromosome 8 in the development of micrometastases, that are present in the bone marrow of over 40% of patients with localized prostatic cancer at the time of diagnosis (Wood *et al.*, 1994). It suggests that tumors with alterations of chromosome 8 preferentially metastasize to bone, possibly via direct hematogenous routes.
In summary, gain of chromosome 7 and/or 8 sequences was related to local tumor progression. Moreover, we suggest that gain of chromosome 7 and/or 8 sequences is implicated in progression towards widespread metastatic disease. Hence, the term "progression markers" seems to be appropriate for these chromosomal alterations. They may reveal patients at risk for metastatic tumor behavior. Therefore, we propose a careful investigation of these biomarkers in follow-up studies (Alers *et al.*, submitted). As a result, these progression markers might serve to select patients who would benefit from additional therapeutic approaches.

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CHAPTER 6

LONGITUDINAL EVALUATION OF CYTOGENETIC ABERRATIONS IN PROSTATIC CANCER: TUMORS THAT RECUR IN TIME DISPLAY AN INTERMEDIATE GENETIC STATUS BETWEEN NON-PERSISTENT AND METASTATIC TUMORS

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ABSTRACT

Only limited data are available on chromosomes specifically involved in prostatic tumor progression. We have evaluated the cytogenetic status of primary prostatic carcinomas, local tumor recurrences and distant metastases, representing different time points in prostatic tumor progression. Therefore we applied interphase in situ hybridization (ISH) with a set of (peri) centromeric DNA probes, specific for chromosomes 1, 7, 8, and Y, to routinely processed tissue sections of 73 tumor specimens of 32 patients. Longitudinal evaluation was possible in 11 cases with local recurrence and 9 cases with distant metastases. The remaining 12 patients showed no evidence of local recurrence or distant metastasis after radical prostatectomy in follow-up (mean 58.5 months), and served as a reference. Numerical aberrations of at least one chromosome were found in 27% of the local recurrences and 56% of the distant metastases. We observed, in decreasing order of frequency +8, +7 and -Y in the recurrences, and +8, +7, -Y, +1 in the distant metastases. Evaluation of the corresponding primary tumor tissue of the recurrence group showed numerical aberrations in 45% of cases. The aberrations found were, in decreasing order of frequency, -Y, +7, and +8. In the concomitant primary tumor tissue of the distant metastasis group we detected numerical aberrations in 67% of cases. The aberrations most frequently encountered were +8, -Y, followed by +7. In four cases a concordance was found between primary tumor and its recurrence or distant metastasis. Discrepancies might have been caused by cytogenetic heterogeneity. Comparison of primary tumor tissue of reference, recurrence, and distant metastasis group showed a significant increase for the percentage of cases with numerical aberrations (P_{trend} =0.02). Likewise, a trend was seen for gain of chromosome 7 and/or 8 (P_{trend} <0.05). Also the number of DNA aneuploid tumors increased in these three different groups (P_{trend} =0.03). These data suggest that cancers, which recur in time display an intermediate position between tumors of disease-free patients and metastatic cancers.

INTRODUCTION

Adenocarcinoma of the prostate is the most commonly diagnosed malignancy among men and the second leading cause of cancer-related deaths in Western countries [1]. Approximately 30% of men with clinically manifest prostate cancer will have distant metastases at the time of diagnosis [2]. These patients have a dismal prognosis with a 10-year cancer-specific survival rate of only 10% [2]. In contrast, clinically localized tumors show survival rates between 60 and 90% [2-4]. However, after treatment of these tumors, mostly by radical prostatectomy, local recurrences and/or distant metastases can occur with an incidence of 10 to 40% within 5 years, and up to 60% within 10 years after treatment [4-7]. The highest incidence of recurrent disease is seen in those patients with large tumors that are not organ confined and whose surgical resection margins are positive for residual tumor after radical prostatectomy [4-7]. Patients that initially have only a local recurrence are less likely to die of prostate cancer than those with distant recurrent disease.

However, they still are at a substantial risk of eventually dying of prostate cancer [3].
The detection of biological differences between those tumors prone to progress to life-threatening metastatic disease, and those with little likelihood of causing morbidity and mortality, are major goals of current prostate cancer research. Staging and grading parameters are conventionally used to help predict the prognosis for patients with localized prostatic cancer [8-10]. The therapeutic strategy, however, is still difficult to adapt to the individual cases Hence, there is a need for other markers that can serve as prognostic indicators. The molecular genetic events responsible for the initiation and progression of prostate cancer remain largely unknown. Multiple genetic changes are thought to occur, involving both the inactivation of tumor suppressor genes and the activation of oncogenes [11]. Chromosomal aberrations encountered in prostatic adenocarcinoma include loss of the Y chromosome, trisomy of chromosome 7 and del(7)(q22), del(8)(p21), and del(10)(q24) [12-14]. Allelotyping studies using RFLP and/or microsatellite markers have revealed frequent (>30% of cases) LOH on chromosome arms 7q, 8p, 10pq, 13q, 16q, 17q, and 18q [15-23]. Further, CGH analysis of tumor DNA derived from a panel of both primary and androgen-independent recurrent tumors revealed losses of 8p and 13q in over 30% of cases [24]. Interestingly, recurrent prostatic tumors showed a higher total number of genetic changes than unrelated primary tumors. This was exemplified by gains of 8q, chromosome X and 7, as well as loss of 8p. A CGH study [25] performed on a panel of regional lymph node and bone metastases showed frequent gain of 8q, as well as loss of 8p, 10q, 13q, 16q, and 17p sequences in over half of cases. Interphase in situ hybridization (ISH) to nuclear suspensions, touch preparations and paraffin sections of both prostatic tumors and precursor lesions (PIN) revealed numerical aberrations of chromosomes 7, 8, 10, 16, 17, 18, X, and Y [26-30], as well as loss of sequences in the 8p22 region [31, 32]. Furthermore, fluorescent ISH (FISH) studies of nuclear suspensions or touch imprints of mostly primary prostatic tumors suggested that alterations of chromosome 7 and/or 8 may be prognostic markers predicting unfavorable outcome in prostate cancer [33-35]. Thus, molecular genetic and interphase ISH studies have begun to reveal chromosomal alterations in primary tumors and, to a lesser extent, in metastases and local recurrences of prostate cancer. However, a detailed longitudinal evaluation of cytogenetic changes, occurring in prostatic tumor recurrences or distant metastases of individual patients, has not yet been reported.
Therefore, we applied ISH to archival, paraffin-embedded, tissue sections of 73 samples of 32 patients at different time points of prostatic tumor progression. This included all tumor material available of 11 cases with local recurrences, and of 9 patients with distant metastases. A group of 12 patients that showed no evidence of local or distant recurrence after radical prostatectomy in follow-up served as a

reference. Main questions addressed were: 1) Is there a concordance in cytogenetic and ploidy status between primary tumor and its recurrence or distant metastasis? 2) Is there an accumulation of numerical chromosomal changes in the primary tumor material of patients with non-persistent, locally recurrent and metastatic disease, respectively? 3) Is there a specific involvement of certain chromosomes, i.e., gain of chromosome 7 and/or 8?

MATERIALS and METHODS

Tissue Specimens

Seventy-three routinely processed formalin-fixed paraffin-embedded tissue blocks, obtained between 1986 and 1996, from 32 individuals, were used for this study. Longitudinal evaluation of local recurrences was possible in 11 patients (Table 1). Our set of 30 tumor blocks consisted of 11 needle biopsy specimens of recurrences, 13 radical prostatectomy specimens of 11 patients, 4 needle biopsy specimens obtained prior to radical prostatectomy and 2 regional lymph node metastases. The mean time between radical prostatectomy and manifestation of local recurrence was 42 months (range 21 to 62 months). All patients showed elevated prostate-specific antigen (PSA) levels at time of recurrence (median 3 ng/ml; range 0.3 to 265 ng/ml). The mean age of the patients was 63 years (range 53 to 74 years) at time of radical prostatectomy and 67 years (range 56 to 77 years) at time of local recurrence. All tumors were pathologically staged according to the pTNM classification [36] and graded according to the modified MD Anderson Hospital grading system [9]. In this panel, significant differences were neither found between pathological stage and time to local recurrence, nor in the status of the surgical resection margins (free or not free from tumor) and time to recurrence. None of the patients showed any clinical manifestation of distant metastases at time of detection of the recurrence. None of the patients received adjuvant therapy after radical prostatectomy.

Longitudinal evaluation of distant metastases was possible in 9 patients (Table 2). A total number of 27 tissue blocks could be evaluated. This panel included 12 distant metastases of which 3 were distant lymph node metastases, 4 were bone metastases, and 5 were metastases to other sites like brain, liver, skin, lung and diaphragm (1 case each). Three regional lymph node metastases could also be retrieved. Seven of the samples were obtained at autopsy. Primary prostate material was available of all patients, i.e., biopsies, transurethral resection (TUR) and autopsy material. In 4 cases the primary prostate material was obtained previous to any clinical signs of distant metastatic disease. In the remaining 5 cases, primary tumor material was obtained once the tumor had already widely disseminated. This was partially due to the late clinical manifestation of prostatic cancer, i.e., a distant metastasis as first sign of disease. The mean age of the patients at time of distant metastasis sampling was 64 years (range 41 to 78 years). All patients showed high PSA levels at time of distant metastatic disease (median 87 ng/ml; range 12 ng/ml to 1425 ng/ml). Prior to evaluation of the distant metastases, 4 of the patients had received endocrine therapy and 2 had received a combination of endocrine and radiotherapy.

From a previously described panel [26] of 25 patients, who underwent radical prostatectomy in the period from 1990 to 1992, 12 cases (16 tissue blocks) were taken to serve as a reference. The selection criteria were as follows: A) Time of follow-up study > 42 months. B) No clinical evidence of local recurrence or distant metastases in follow-up. C) No detectable ($\leq$ 0.2 ng/ml) PSA levels at the last clinical visit, indicative of imminent local or distant recurrent disease [5]. The mean time of follow-up was 58.5 months (range 47-64 months). The median PSA level at last clinical visit was 0 ng/ml (range 0 to 0.2 ng/ml). The mean age

of the patients was 62 years (range 49-70 years) at time of radical prostatectomy (Table 3). In 5 cases the surgical resection margins were not free of tumor. One patient (case 12) had received anti-androgen therapy after surgery.

Probe Set and Probe Labeling
All cases were analyzed with a probe set, specific for chromosomes 1, 7, 8, and Y. A more detailed description of the probes used is given elsewhere [27, 37 and references within]. Selection criteria were based on previous studies [26, 27] and other literature data considering cytogenetic aberrations in prostatic tumors [12-14, 33-35]. The (peri) centromeric repetitive satellite DNA probes were labeled with biotin-14-dATP by nick translation of complete plasmid DNA according to the manufacturer's directions (BRL, Gaithersburg, MD). DNA probes were stored at -20°C.

In Situ Hybridization
In situ hybridization (ISH) was performed on routine consecutive 4 µm tissue sections. Sections were mounted with distilled water on microscope glass slides coated with either aminoalkylsilane (Sigma, St. Louis, MO) or with a "para-tissuer pen" (ITK diagnostics, Uithoorn, The Netherlands). Sections were baked overnight at 60°C for better adherence. The ISH procedure was performed as described earlier [26, 27, 38]. Briefly, after deparaffinization of the tissue sections in xylene, endogenous peroxidase activity was blocked with 0.3% H_2O_2 in methanol. To facilitate DNA probe accessibility to the cellular DNA, sections were digested with 0.4% pepsin (Sigma) in 0.2 M HCl at 37°C for 3-30 min (mean 9 min). Before applying the probe set, the optimal digestion time for each sample was determined by a pepsin time series (5, 10, 15, 20 min). Cellular DNA was heat denatured for at 72°C for 2 min in 70% formamide in 2x SSC (pH 7.0), followed by dehydration in graded ethanol series. Chromosome-specific repetitive DNA probes were denatured for 10 min at 72°C in a hybridization mixture containing 2 ng/μl probe DNA, 500 ng/μl sonicated herring sperm DNA (Sigma), 0.1% Tween-20, 10% dextran sulphate, and 60% formamide in 2x SSC at pH 7.0. Then, 15-30 μl of probe mixture was applied to the sections. The slides were incubated overnight at 37°C in a moist chamber. Tissue sections were washed in 60% formamide in 2xSSC (pH 7.0) at 42°C for 10-15 min, then in 2xSSC at 42°C for 10-15 min. Histochemical detection of the biotinylated DNA-probes was performed by the standard avidin-biotin complex (ABC) procedure and immunoperoxidase staining. Sections were counterstained with hematoxylin.

Evaluation of ISH Results
The DNA probe set was analyzed for each sample on consecutive 4 μm sections in a previously defined tissue area. On each tissues section leukocytes, stromal cells etc. served as internal controls to evaluate the quality of ISH. For each of the probes, 100 "intact" (i.e. spherical) and non-overlapping 4 μm nuclear slices were counted by two independent investigators and the number of solid DAB spots per nuclear contour was scored (0, 1, 2, 3, 4, >4 spots/nuclear slice). The DNA spot-distributions of the two observers were then compared and averaged, when no significant counting differences between the investigators were found. In our series no discrepancies emerged using this approach.
In cases, which showed aberrations of one or more of the autosomal probes, a centromeric probe, specific for chromosome 6, was added to the probe set. In our study no aberrations of chromosome 6 were seen in those cases. Thus, at least two non-aberrant autosomal probes were used for analysis. A tumor was called aneuploid when the percentage of hyperdiploid tumor cells (>2 spots/nucleus) for the mean of the non-aberrant autosomal

TABLE 1. Longitudinal evaluation of histopathological and ISH data (chromosomes 1, 7, 8, Y) of 11 patients with local recurrences, in the absence of distant metastases, after radical prostatectomy

Case	Time point (months)	Tissue studied	Grade[a]	Stage[b]	ISH Aneuploidy[c]	ISH Aberrations[d]
1	t=0	prostatic biopsy	M		+	
	t=4	radical prostatectomy	M	pT4aN1M0[e]	+++	
	t=42	recurrence biopsy	M		+++	
2	t=0	prostatic biopsy	M		±	+7
	t=1	radical prostatectomy	M	pT3cN1M0[f]	+	+7
	t=24	recurrence biopsy	M		+	
3	t=0	radical prostatectomy	M	pT2cN1M0	++++++	
	t=31	recurrence biopsy	M		+	
4	t=0	prostatic biopsy	M		++	+8
	t=1	radical prostatectomy	M	pT2cN0M0	+	
	t=63	recurrence biopsy	W		±	+8
5	t=0	radical prostatectomy	M	pT3bN0M0	++	
	t=62	recurrence biopsy	M		+	
6	t=0	radical prostatectomy	M	pT3aN0M0	+++++	
	t=62	recurrence biopsy	M		+	
7	t=0	prostatic biopsy	M		++++++	
	t=1	radical prostatectomy	M	pT4aN0M0	++++++	-Y
	t=22	recurrence biopsy	M		++++	
8	t=0	radical prostatectomy	M	pT2cN0M0	++	-Y
	t=46	recurrence biopsy	M		n.s	+8
9	t=0	radical prostatectomy	M	pT3cN0M0	+++	
	t=34	recurrence biopsy	P		+++++	
10	t=0	radical prostatectomy	M	pT4aN0M0	n.s.	+7,-Y
	t=30	recurrence biopsy	M		++	+7,-Y
11	t=0	radical prostatectomy	W	pT2cN0M0	+++++	
	t=54	recurrence biopsy	W		++	

[a]MD Anderson grading system: W, well differentiated; M, moderately differentiated; P, poorly differentiated.

[b]pTNM classification: pT1, tumor not palpable or visible; pT1a, ≤ 5%; pT1b, > 5%; pT1c, needle biopsy; pT2, tumor confined within the prostate; pT2a, ≤ 1/2 of lobe; pT2b, >1/2 of lobe; pT2c, both lobes; pT3, tumor extends through prostatic capsule; pT3a, unilateral; pT3b, bilateral; pT3c, seminal vesicles; pT4, tumor is fixed or invades structures other than seminal vesicles; pT4a, bladder neck, external sphincter or rectum; pT4b, levator muscles or fixed to pelvic wall; pN1, metastasis in a single lymph node, < 2cm in diameter; pN2, single lymph node > 2cm to 5 cm, or multiple ≤ 5 cm; pN3, lymph node > 5cm; M1a, metastasis in non-regional lymph nodes; M1b, bone; M1c, other sites.

[c]All control cells revealed < 2.5% hyperdiploid (> 2 spots) cells. Mean percentage of the non-aberrant probes: < 2.5%= n.s. (not significant); ≥ 2.5-5% = ±; 6-10% = +; 11-20% = ++; 21-30% = +++; 31-40% = ++++; 41%-50% = +++++; >50%= ++++++.

[d]P<0.01 (Kolmogorov- Smirnov test).

[e]Regional lymph node metastasis of this tumor showed the same hybridization pattern as the prostatic biopsy.

[f]Regional lymph node metastasis of this tumor also revealed gain of chromosome 7.

probes was ≥2.5%. This cut-off value for aneuploidy was based on the mean rate of hyperdiploidy in previous [26; Alers et al., submitted] panel of normal controls (n=33 in total). The number of nuclei with a hyperdiploid spot number (likely artifacts) in these controls never exceeded 2.5%. The mean rates of hyperdiploidy were well below this cut-off value (0.4% and 0.7%, respectively).

Statistical Analysis

Despite the 4 μm sectioning artifact, which results in truncated nuclei, specific chromosome aberrations were detected and could be statistically evaluated. The spot distributions of the different probes on consecutive tissue sections were evaluated statistically by means of the Kolmogorov-Smirnov test. This statistical test is very suitable for two-sided comparisons of histograms or other distributions. Underrepresentation of a specific chromosome was seen as a shift to the left of the DNA probe distribution, when compared with the mean of the non-aberrant probe distributions of the same (tumor) area on adjacent tissue sections. Conversely, gain of a specific chromosome was seen as a shift to the right. An aberrant probe spot distribution was considered to represent a numerical aberration, if the associated P value was <0.01. Following this approach, we were able to distinguish changes in ploidy status from individual chromosomal aberrations (i.e., gains or losses) [37].

Comparison of the rate of hyperdiploidy within groups was done using the Wilcoxon signed-ranks test. Comparison of the rate of hyperdiploidy and tumor grade between groups was performed using the Mann-Whitney-U test. Percentages between groups were compared using Fisher's exact test or the χ^2 test for trend if indicated [39]. P=0.05 (two-sided) was considered the limit of significance.

RESULTS

Longitudinal Genetic Evaluation of Local Recurrences

Our panel of local recurrences consisted of eleven patients who had undergone radical prostatectomy but showed a local recurrence in follow-up. Histopathological and ISH data are shown in Table 1. The mean time between radical prostatectomy and clinical manifestation of recurrence was 42 months (range 21 to 62 months). Genetic evaluation of primary prostatic tumor material was performed in both biopsy and/or radical prostatectomy specimen, if available, to minimize sampling artefacts due to cytogenetic heterogeneity. Following this approach, numerical aberrations were found in five primary tumor specimens (45%). Loss of the Y chromosome was the most common finding (27%), followed by gain of chromosome 7 (18%) and gain of chromosome 8 (9%). Numerical aberrations of at least one chromosome were found in three recurrences (27%): Gain of chromosome 8 was found in two cases (18%), gain of chromosome 7 and loss of Y in one case each (both 9%). In two patients a complete concordance was observed between the numerical aberrations occurring in the primary tumor tissue and its recurrence: In case 4 gain of chromosome 8 was seen in both the prostatic biopsy and its recurrence 62 months later (Fig. 1A-F); in case 10 gain of chromosome 7 and loss of Y were seen in both the radical prostatectomy specimen and its recurrence 30 months later (Fig. 1G-J).

TABLE 2. Longitudinal evaluation of 9 patients with distant metastases by means of ISH analysis (chromosomes 1, 7, 8, Y)

Case	*Time point (months)*	*Tissue studied*	*Grade*[a]	*Stage*[b]	*ISH Aneuploidy*[c]	*ISH Aberrations*[d]
1	t=0	prostatic biopsy	P	pTxN1M0	+	
	t=4	lung biopsy[e]	P	pTxNxM1c	++++++	
	t=42	prostatic biopsy[e]	P		+++++	
2	t=60	TUR[f]	P	pTxNxM1b	+	+8
	t=70	skin biopsy	P	pTxNxM1c	++++++	
3	t=0	prostatic biopsy	P	pTxN2M0[g]	+++	
	t=34	TUR	P	pTxNxM0	+++++	-Y
	t=37	liver biopsy[e]	P	pTxNxM1c	+	+1, -Y
		diaphragm biopsy[e]	P		++	
		distant lymph node biopsy[e]	P		+++	
4	t=0	prostatic biopsy	P	pTxNxM1c	++	
	t=23	bone biopsy	P		+++	
5	t=0	bone biopsy	P	pTxNxM1b	±	
	t=1	prostatic biopsy	M		++++	-Y
6	t=0	brain biopsy	P	pTxNxM1c	++++++	+7, -Y
	t=1	prostatic biopsy	P		+++++	-Y
7	t=0	bone biopsy	M	pTxNxM1b	±	+8
	t=1	prostatic biopsy	M		++	+8
8	t=0	radical prostatectomy	M	pT4aN2M0[h]	+++++	
	t=17	bone biopsy[e]	P	pTxNxM1c	++	+8
		distant lymph node biopsy[e]	P		+++++	+8
9	t=0	prostatic biopsy	M	pTxN2M0[i]	+	+8
	t=45	TUR	M	pTxNxM1b	++	+7,+8
	t=46	distant lymph node biopsy	M		±	+7,+8

[a] MD Anderson grading system: W, well differentiated; M, moderately differentiated; P, poorly differentiated.
[b] pTNM classification: px, pathological tumor staging unknown; for complete classification see Table 1.
[c] All control cells revealed < 2.5% hyperdiploid (> 2 spots) cells. Mean percentage of the non-aberrant probes: < 2.5%= ns (not significant); ≥ 2.5-5% = ±; 6-10% = +; 11-20% = ++; 21-30% = +++; 31-40% = ++++; 41-50% = +++++; >50% = ++++++.
[d] P<0.01 (Kolmogorov- Smirnov test).
[e] Material derived from autopsy.
[f] TUR: transurethral resection
[g] Regional lymph node metastasis displayed the same hybridization pattern as the prostatic biopsy.
[h] Regional lymph node metastasis also revealed gain of chromosome 8.
[i] Regional lymph node metastasis also showed gains of chromosome 7 and 8.

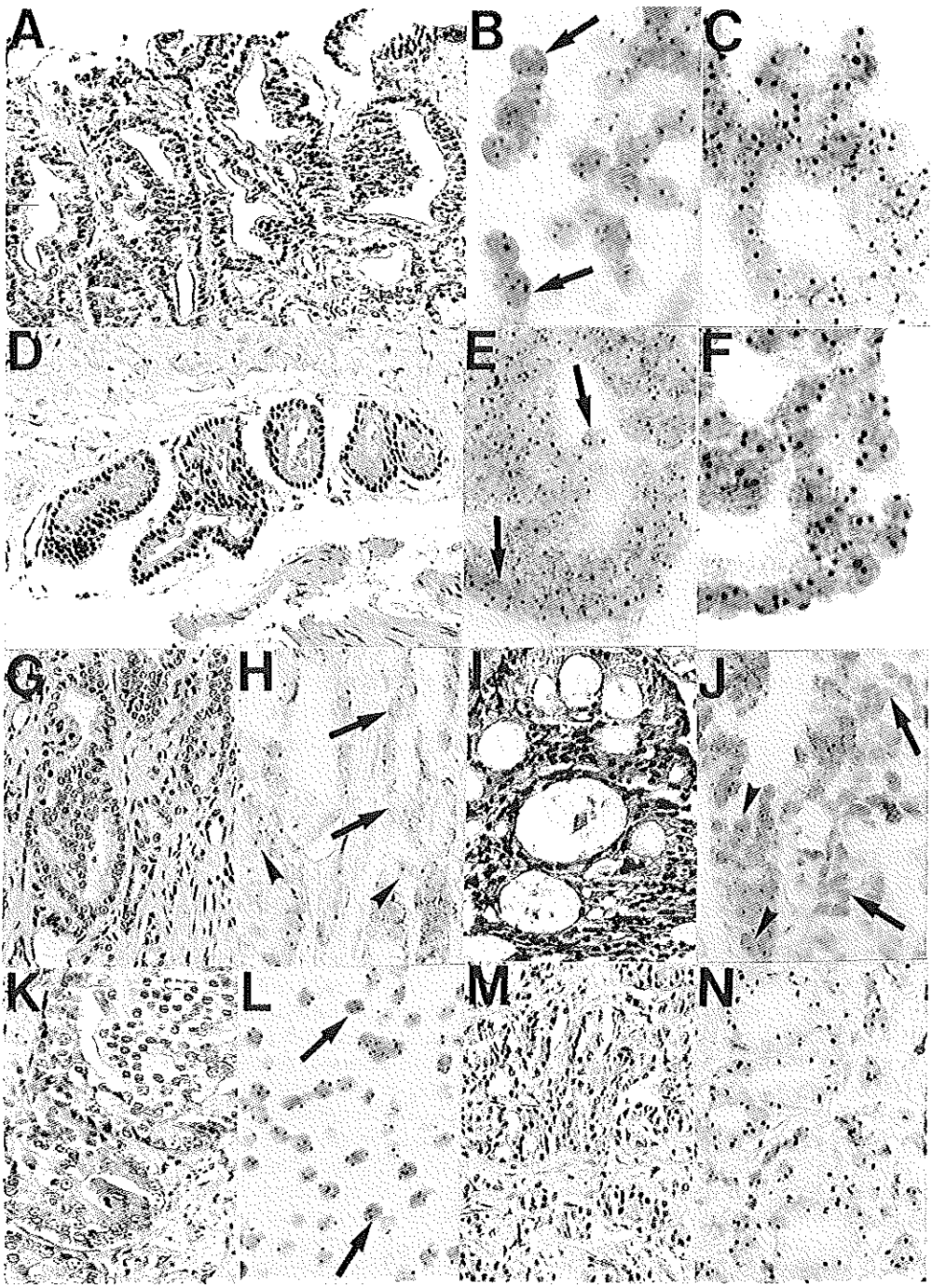
Ten of the recurrences were aneuploid (91%); the median level of hyperdiploidy was 10%. Also ten of the primary prostatic tumor samples appeared aneuploid (91%); the median level was 15%. On average the rate of hyperdiploidy was lower in the recurrences than in the primary tumors, however, this prevalence did not reach statistical significance (Table 1; Fig. 1K-N).

Longitudinal Genetic Evaluation of Distant Metastases

Our panel of distant metastases consisted of twelve metastases from nine patients. Histopathological and ISH data are summarized in Table 2. Primary prostatic material was derived from biopsies, TUR and autopsy material, taken before, during, or after the primary tumor had progressed towards metastatic disease. We detected numerical aberrations of at least one chromosome in primary prostatic material of six cases (67%). Gain of chromosome 8 and loss of Y were the most common findings (both 33%), followed by gain of chromosome 7 (11%). Numerical aberrations of at least one chromosome were seen in five cases with distant metastases (56%): Gain of chromosome 8 was the most common finding (33%), followed by gain of chromosome 7 and loss of Y (both 22%), and gain of chromosome 1 (11%). In one of the two cases in which more than one distant metastasis could be evaluated, concordance was found between a distant lymph node metastasis and a bone metastasis (case 8; Table 2). In case 3, however, loss of Y and gain of chromosome 1 were seen in the liver metastasis, but not in

FIGURE 1.

A-C) Hematoxylin and eosin (HE)-stained tissue section of the moderately differentiated prostatic biopsy of case 4 (A). Corresponding ISH with the chromosome 8-specific probe (B). Gain of chromosome 8 is seen, illustrated by many nuclei with 3 or more spots (*arrows*), when compared with chromosome 1 in C. The ISH-related spots were visualized with immunoperoxidase/DAB (black); hematoxylin was used as a counterstain (gray). **D-F)** HE section of the recurrence of the same tumor, 63 months later. Also in the recurrent tumor, gain of chromosome 8 is seen (E), when compared with the chromosome 1-specific probe in F. **G, H)** HE section of the moderately differentiated tumor of case 10 (G). Corresponding ISH with the chromosome Y-specific probe showing partial Y loss (H): Loss of Y can be seen in the majority of tumor nuclei (*arrows*), whereas some cancer cells still contain the Y chromosome (*arrowheads*). **I, J)** HE section of the tumor recurrence of case 10, occurring 30 months later (I). Partial loss of Y is also seen in the tumor recurrence (J), as displayed by tumor nuclei without (*arrows*) and with the Y chromosome (*arrowheads*). **K, L)** HE section of the moderately differentiated tumor of case 6 (K). Corresponding ISH with the chromosome 1-specific probe shows many aneuploid nuclei, a few are *arrowed* (L). **M, N)** HE section of the tumor recurrence of case 6, 62 months later (M). ISH with the probe specific for chromosome 1 displays a normal diploid spot-distribution (N). A 20x objective was used in A, D, G, I, K, and M; a 40x objective was used in H, J, L, N; a 100x objective was used in B, C, E, F.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metastases to other sites (Fig. 2A-D; Table 2). In four cases the aberrations found in the primary tumor material were in concordance with those observed in the distant metastases. In case 3 partial loss of Y was detected in the TUR material (Fig. 2E, F), whereas total loss of Y was found in the liver metastasis (Fig. 2A, B). However, the Y chromosome was retained in the other metastases (Fig. 2C, D), as well as in the initial prostatic biopsy (Table 2). In case 9, gain of chromosome 8 was seen in all specimens, whereas gain of chromosome 7 was found in TUR material and distant metastasis only (Fig. 2G-L).
Interestingly, a borderline gain of chromosome 7 was already present in the original biopsy. All distant metastases and primary prostatic specimens were aneuploid with median rates of hyperdiploid cells of 23% and 30%, respectively. No distinct patterns in the ploidy status of primary tumor material and its distant metastasis could be distinguished.

Comparison of Reference, Local Recurrence and Distant Metastasis Group
No significant differences were found with regard to numerical aberrations or the ploidy status in local recurrences versus distant metastases. Primary tumors eventually metastasizing or presenting with metastases were significantly less differentiated ($P<0.01$) than primary tumors that would recur in time.
A panel of twelve patients who had undergone radical prostatectomy without clinical or biochemical evidence (blood PSA levels) of local recurrent disease or distant metastasis in follow-up (mean time 58.5 months; range 47 to 64 months), served as a reference. Histopathological and ISH data are shown in Table 3. The grade and stage of the primary tumor tissue of the reference group did not differ significantly from that of the local recurrence group. The grade of primary tumor tissue of the distant metastasis group was significantly less differentiated ($P<0.01$). In primary

FIGURE 2.
A, B) HE section of the liver metastasis of case 3 (A; T= tumor; LI=Liver). ISH with the Y-specific probe shows total loss of Y in the tumor nuclei (*arrows*), but not in the liver cells (*arrowheads*; B). **C, D)** HE section of the distant lymph node metastasis of the same case (C; T=tumor; LY= lymphocytes). ISH reveals presence of the Y chromosome in both the tumor cell nuclei (*arrows*), as well as in the surrounding lymphocytes (*arrowheads*; D). **E, F)** HE section of the poorly differentiated primary tumor obtained by transurethral resection of the same case (E). Corresponding ISH with the chromosome Y-specific probe (F). Partial loss of Y is seen as illustrated by nuclei without (*arrows*), and with the Y chromosome (*arrowheads*). **G-I)** HE section of the moderately differentiated primary tumor material of case 9, obtained by transurethral resection (G). Corresponding ISH with the chromosome 7 specific probe (H). Gain of chromosome 7 is seen, visible as many nuclei displaying 3 or more dots (*arrows*), when compared to the chromosome 1-specific probe in I. **J-L)** HE section of the distant lymph node metastasis of the same case (J). ISH with the chromosome 7-specific probe shows many nuclei with three or more dots (*arrows;* K). L: ISH with the chromosome 1-specific probe. Most nuclei display two dots. A 20x objective was used in A, C, E, G, and J; a 40x objective in the other microphotographs.

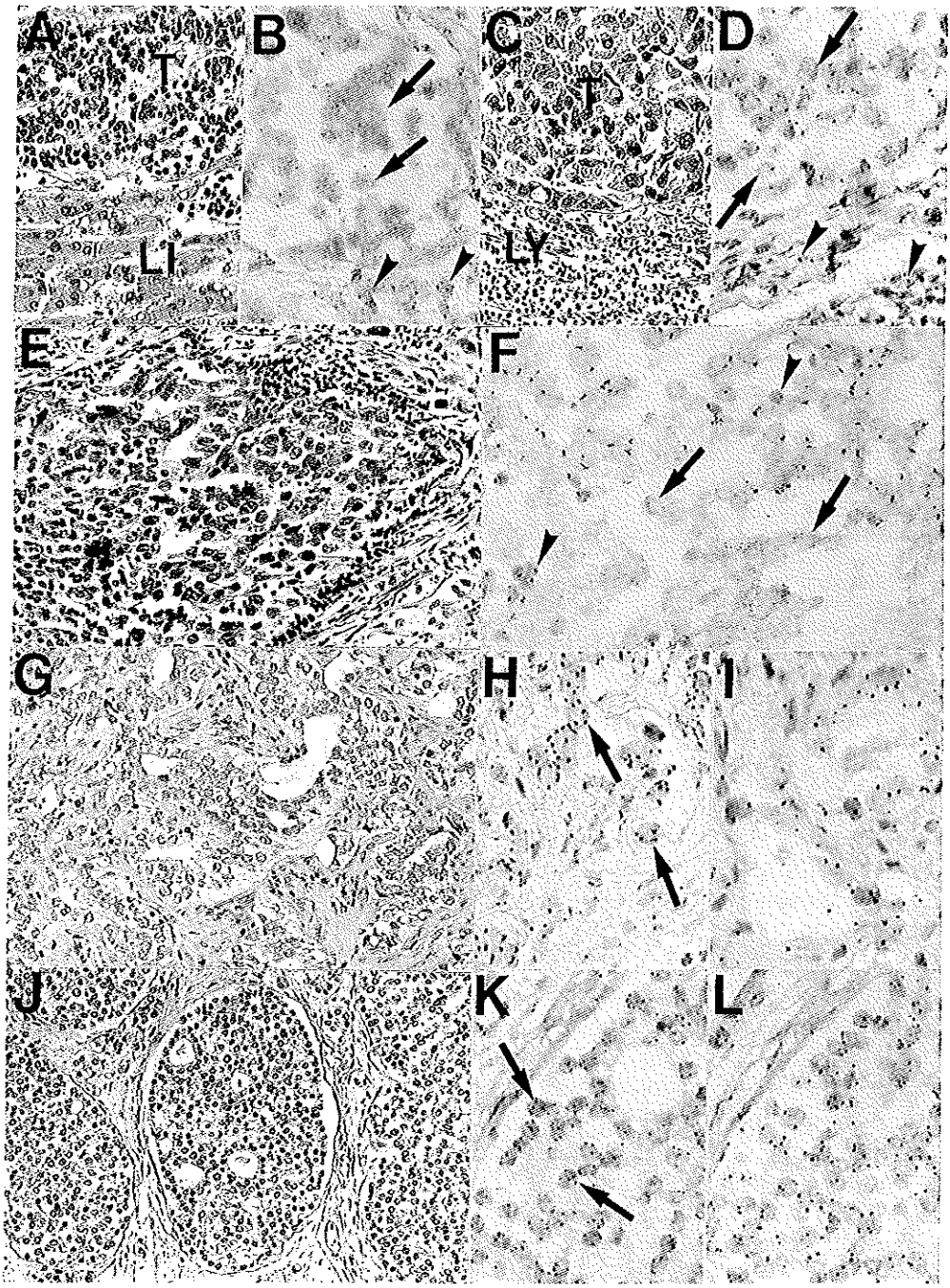
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Table 3. ISH analysis (chromosomes 1, 7, 8, Y) of 12 patients with no evidence of recurrence or distant metastases after radical prostatectomy

Case	Follow-up (months)	Grade[a]	Stage[b]	ISH Aneuploidy[c]	ISH Aberrations[d]
1	60	W	pT3aN0M0	±	-Y
2	63	W	pT3bN0M0	+	-8
3	59	W	pT2cN0M0	++	
4	52	W	pT2cN0M0	n.s.	
5	63	M	pT2cN0M0	+	
6	64	M	pT3aN0M0	n.s.	
7	55	M	pT3aN0M0	±	
8	47	M	pT3aN0M0	n.s.	
9	63	M	pT3bN0M0	±	
10	50	M	pT3bN0M0	n.s.	
11	62	M	pT3aN0M0	++	
12	64	P	pT3cN0M0	++	

[a]MD Anderson grading system: W, well differentiated; M, moderately differentiated; P, poorly differentiated.
[b] pTNM classification: for complete classification see Table 1.
[c] All control cells revealed < 2.5% hyperdiploid (> 2 spots) cells. Mean percentage of hyperdiploid nuclei of the non-aberrant autosomal probes < 2.5%= ns (not significant); ≥2.5-5%= ±; 6-10% = +; 11-20%= ++.
[d] P<0.01 (Kolmogorov- Smirnov test).

tumor material the percentage of patients with numerical chromosomal changes increased from 17% in primary tumor tissue of the reference group, to 45% in primary tumor of the local recurrence group, and to 67% in primary tumor material of the distant metastasis group (P_{trend} =0.02; Fig. 3). A significant increase (P_{trend} <0.05) was seen for gain of chromosome 7 and/or 8 in primary tumor tissue from the reference group (0%), to the recurrence group (27%), to the distant metastasis group (33%; Fig. 3). In contrast, loss of Y did not show significantly different patterns. Further, a significant increase (P_{trend} =0.03) was also observed in the number of aneuploid cases in primary tumor tissue of the three different groups (range 67% to 100%; Fig. 3). The median number of hyperdiploid cells appeared higher in primary tumors of the recurrence group (15%) than in those of the reference group (4%; P=0.01). This difference was even more prominent in primary prostatic tissue of the distant metastasis group (30%) versus the reference group (P<0.001).

DISCUSSION

The aim of the present study was twofold: On the one hand we investigated chromosomal abnormalities and ploidy status of recurrences and distant

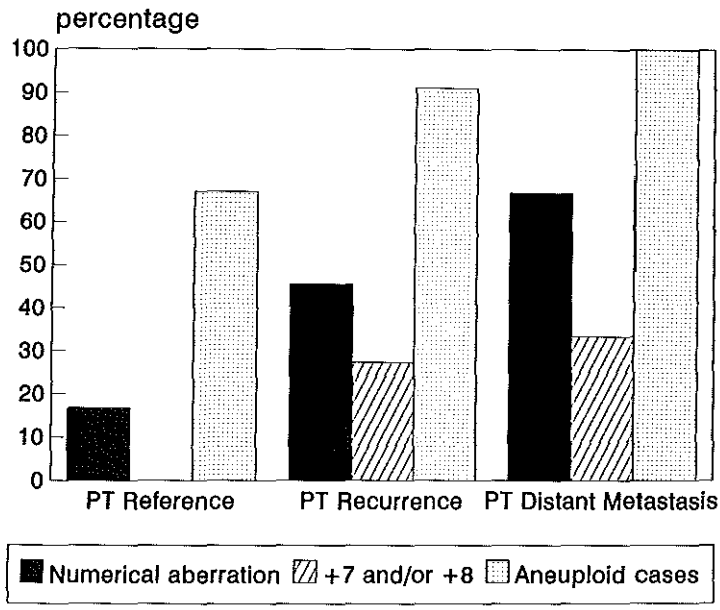

FIGURE 3.
Graphic representation of the results of ISH with a probe set specific for chromosome 1, 7, 8, and Y to primary tumor material (PT) of three groups of prostatic cancer patients, i.e., a reference group of patients free of disease in follow-up, patients with locally recurrent tumors, and patients with distant metastases. A gradual increase in the percentage of cases with numerical aberrations is seen in the primary tumor material (P_{trend} =0.02). Combination of cases with gain of chromosome 7 and/or 8 reveals a significant increase (P_{trend} <0.05), going from reference group, to recurrence group, to distant metastasis group. Also the number of aneuploid cases increases significantly along these three groups (P_{trend} =0.03).

metastases of prostatic adenocarcinoma longitudinally. On the other hand we examined the genetic composition of the primary tumor material of three groups of patients with different clinical status. These included a group of patients without evidence of recurrence or distant metastases after treatment in follow-up (reference group), a group of patients with recurrent disease, and a group of patients which (had) developed distant metastases.

In our panel of recurrences and distant metastases gain of chromosome 8 was the most common finding, occurring in 18% and 33% of recurrences and distant metastases, respectively. CGH analysis of androgen-resistant recurrent prostate cancers, regional lymph node and bone metastases demonstrated gain of the entire

8q arm, either alone or in association with 8p loss, in over 80% of cases [24, 25]. In several cases, CGH and/or microsatellite analysis showed simultaneous p arm deletion and q arm gain [18, 32, 40]. This might be caused by isochromosome i(8q) formation. We identified the overrepresentation of chromosome 8 in the bone metastases of cases 7 and 8 as an amplification of the complete long arm of chromosome 8 by CGH analysis (Alers *et al.*, submitted). This combination of whole arm amplification detected by CGH, and gain of centromeric ISH spot numbers, is very suggestive for i(8q) formation. Previously, we distinguished i(8q) formation by CGH analysis in two prostate cancer cell lines, PC 133 and PC 346. Both PC 133, a cell line derived from a bone metastasis [41], and PC 346, show aggressive tumor behavior [42]. Gain of chromosome 8q sequences and/or i(8q) formation has also been reported in other cancers. In breast tumors CGH revealed gain of 8q sequences in about half of the cases, and it was significantly associated with disease recurrence [43]. In most cases gain of 8q occurred simultaneously with loss of 8p sequences, suggestive of i(8q) formation. In uveal melanoma i(8q) formation is a common characteristic as detected by conventional methods [44]. In hepatocellular carcinoma a 2 to 6 fold multiplication of (parts of) the 8q arm was reported in 41% of cases [45]. Altogether, multiple known (e.g., *c-myc*) and unknown genes residing on the long arm of chromosome 8 might be involved in tumor progression of prostate and other cancers.

Gain of chromosome 7 was observed in one recurrence and in two distant metastases, which constituted the second most frequent anomaly in our panel. Trisomy of chromosome 7 is a relatively frequent aberration in prostatic cancer [13, 14, 30, 33-35]. Gain of chromosome 7 was found in more than half of the recurrent prostate cancers, but very infrequently in primary tumors, by means of CGH analysis [24]. Both gain of the entire chromosome 7 was seen, as well as partial gains with the minimal overlapping region residing at 7p13. Likewise, gain of chromosome regions 7p12-p21 and 7q11.3-q33 was seen in up to 40% of the specimens from (lymph node) metastases [25]. Gain of chromosome 7 has also been reported as a consistent anomaly in other solid tumors, such as bladder, brain, colon and kidney [e.g., 46]. Further, several studies have implicated the presence of genes involved in invasion and metastasis on chromosome 7 [e.g., 47]. Comparison of pairs of primary tumors and their recurrences revealed a concordance of chromosomal aberrations in two patients (cases 4 and 10). Discordance between primary and recurrent tumor was seen in three cases. E.g., in case 8, we detected loss of Y in the primary tumor, but gain of chromosome 8 in the recurrence. This discrepancy might have been caused by cytogenetic heterogeneity of the primary tumor, which is a prominent feature of prostatic cancer [48, 49]. Our and other data suggest that local tumor recurrences arise from local residual tumor cells after radical prostatectomy [4-7]. Apparently, these cells had the appropriate genetic background, i.a., gain of chromosome 7 or 8 sequences, to

exhibit clonal expansion into a tumor recurrence [50].
In our panel, concordance of genetic aberrations in primary tumor material and concomitant distant metastases was found in two patients (cases 7 and 9). Furthermore, in three patients the regional lymph node metastases could be evaluated, and these shared the genetic status of the corresponding distant (lymph node) metastases in all cases. (Partial) dissimilarity between primary tumor material and distant metastasis was observed in five cases. This is in agreement with (limited) data on pairs of primaries and their metastases, of which some show concordance and others discordance between primary tumor and its (regional) metastasis [51, 52]. This inconsistency might be caused by the multifocal nature of prostatic cancer and therefore incomplete sampling of prostatic tumor material, which could be circumvented by whole mount analysis [49, 51]. In three cases additional chromosomal alterations were detected in the distant metastases, as compared to the primary tumors. E.g., in case 6, both primary tumor and its metastasis to the brain showed loss of Y, whereas gain of chromosome 7 was solely found in the metastasis. Likewise, loss of Y was seen in both the liver metastasis and the TUR specimen of case 3, whereas gain of chromosome 1 was detected in the liver metastasis only. This is in agreement with other reports, in which accumulation of genetic abnormalities was seen in (distant) prostatic tumor metastases [Alers *et al.*., submitted; 19, 25, 35]. These observations can be explained by either the cytogenetic heterogeneity of the primary tumor material which obscured subclones with the above mentioned characteristics or, more likely, by genetic instability of metastatic tumor cells [50, 53].
Comparison of primary tumor tissue of reference, local recurrence, and distant metastasis group showed a significant trend for the percentage of cases with numerical aberrations. This suggests that primary tumor cells with multiple genetic anomalies are more capable to metastasize [53]. Likewise an increase was seen in the number of primary tumors with gain of chromosome 7 and/or 8 along these three groups. This is in concordance with other findings, which suggest that alterations of chromosome 7 and/or 8 are potential biomarkers of progression in prostate cancer [Alers *et al.*, submitted; 33-35]. Further, the number of aneuploid patients, as well as the median number of aneuploid cells increased in these different groups. It has been reported that DNA aneuploidy is associated with post-prostatectomy disease recurrence, as well as the occurrence of distant metastasis [10, 54].
In conclusion, this retrospective study sheds more light on the genetic changes occurring in individual patients with tumor recurrences and distant metastases. Furthermore, these data suggest a cytogenetic trend in the primary tumors with respect to aggressive behavior: Tumors that will recur in time display an intermediate role. This phenomenon is further illustrated by accumulation of genetic changes, most importantly, gains of chromosome 7 and/or 8. It is in view with

epidemiological studies reporting that patients with local recurrences are at intermediate risk of eventually dying of prostatic cancer compared to patients with non-persistent disease, and patients with distant metastases, respectively. The presence of the biomarkers stated above in the primary tumor may be considered as risk factors for the development of recurrences and/or distant metastases. Therefore, careful evaluation of the genetic status of the primary tumor specimens may render valuable prognostic parameters, which can help to develop treatment strategies in individual cases.

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CHAPTER 7

GENERAL DISCUSSION

In recent years, interphase in situ hybridization (ISH) has been established as a valuable tool for the detection of chromosomal aberrations and aneuploidy in a variety of human solid tumors (Alers and van Dekken, 1996). ISH applied to nuclear suspensions is very suitable for the cytogenetic analysis of homogeneous tumor specimens. However, for the evaluation of prostatic adenocarcinoma it is less applicable, because of its highly heterogeneous histology. Therefore, in this study we have applied interphase ISH to archival tissue sections, permitting a combination of cytogenetic and histopathological analysis. After validation of the method (Chapter 2), this approach was used to address the following questions: 1) Which chromosomal aberrations occur in different stages of the prostatic cancer spectrum, i.e., precancerous lesions, organ-confined tumors, regionally advanced tumors, metastatic tumors and tumor recurrences? 2) Are specific chromosomes involved in prostatic tumor progression towards metastatic disease? 3) Are the same chromosomes also implicated in progression towards local recurrence and distant metastatic disease in individual patients? 4) What is the ploidy status of the tumors, representing different stages of prostatic tumor development? 5) Does the histological heterogeneity of prostate cancer reflect a cytogenetical heterogeneity? These questions will be answered briefly hereafter and future perspectives will be mentioned.

7.1 Cytogenetic Aberrations in Prostatic Tumorigenesis and Tumor Progression

High-grade PIN is the most likely precursor of prostatic adenocarcinoma (Chapter 1). Only limited information exists concerning genetic changes in PIN, partially due to its focal nature that can only be recognized in a histological setting. Loss of 8p sequences was noted in several PIN lesions, indicating a possible initiating role of one or more tumor-suppressor genes at 8p in prostatic tumorigenesis (e.g., Emmert-Buck *et al.*, 1995). Further, aneuploidy was reported in over half of high grade PIN lesions by image cytometry (e.g., Crissman *et al.*, 1993). In Chapter 4, we describe interphase ISH on 17 high grade PIN lesions adjacent to invasive carcinoma. We observed loss of Y in both PIN and surrounding carcinoma in two cases. In one PIN lesion we found loss of Y in the luminal, but not in the basal cell layer. This finding supports the idea that prostatic adenocarcinomas may have a secretory luminal origin (Chapter 1). Further, in four other PIN lesions no chromosomal aberrations were found, in contrast to adjacent carcinoma cells. Most PIN lesions were moderately aneuploid. No correlation was found between the rate of aneuploidy of PIN lesion and adjacent carcinoma. These data indicate that PIN lesions are distinct entities, which are not created by ingrowth of tumor cells into normal prostatic glands.

In Chapter 5 the genetic status in different stages of prostatic tumor development was determined. In primary tumors the number of cases with numerical changes increased significantly with local tumor stage, as did the number of cases with gain of chromosome 7 and/or 8. Further, with progression towards metastatic disease an

accumulation of genetic changes, most importantly gain of chromosome 7 and/or 8, as well as aneuploidy, was seen. Interestingly, we identified specific involvement of chromosome 8 aberrations in bone metastases, but not in hematogenous metastases to other sites. This suggests that tumors with alterations of chromosome 8, the most heavily committed chromosome in prostate cancer (Cunningham *et al.*, 1996), preferentially metastasize to bone, possibly via direct hematogenous routes. Indeed, micrometastases in bone can already be present in early stages of prostatic disease (Wood *et al.*, 1994). The nature of the alterations of chromosome 8 was further investigated by CGH analysis. Gains of chromosome 8 copy number were identified as 8q amplifications. These findings, in combination with gain of centromeric ISH spot numbers, are very suggestive for isochromosome 8q (i(8q)) formation. Additionally, in two cases gain of the whole 8q arm was accompanied by 8p loss.

Evaluation of primary tumor material of three groups of patients, i.e., patients with no evidence of local recurrence or distant metastases, patients with local recurrences, and patients who (had) developed distant metastases, is presented in Chapter 6: A significant increase was seen for the percentage of cases with numerical aberrations from reference group, to local recurrence group, to distant metastases group. Likewise a significant trend was seen for gain of chromosome 7 and/or 8. Also the rate of DNA aneuploidy increased along these three different groups.

Our findings are in agreement with those reported in literature, in which (partial) gains of chromosomes 7 and/or 8 are reported as potential prognostic markers in prostate cancer, associated with high tumor grade and/or stage, as well as frequently present in metastases and hormone-refractory cancers (e.g., Takahashi *et al.*, 1994; Visakorpi *et al.*, 1995; Cher *et al.*, 1996). Therefore, they seem to be late events in prostatic tumorigenesis. Several studies have implicated the presence of genes involved in invasion and metastasis on chromosome 7 (e.g., Collard *et al.*, 1987). We found 8q gain in bone metastases of prostatic adenocarcinoma (Chapter 5). Gain of chromosome 8q sequences has also been reported in other cancers, such as breast tumors, in which it is associated with disease recurrence (Isola *et al.*, 1995). Multiple known (e.g., *c-myc*) and unknown genes residing on the long arm of chromosome 8 might be involved in tumor progression and metastatic behavior of prostate and other cancers.

In conclusion, ISH applied to tissue sections revealed cytogenetic changes in both prostatic cancer and precursor lesions. Gains of chromosomes 7 and, especially, of chromosome 8, the latter possibly representing i(8q) formation, appear to be related to local tumor progression and local recurrence, as well as progression towards metastatic disease. The name "progression markers" seems to be appropriate for these chromosomal alterations and careful evaluation of these biomarkers in primary tumor specimens may render valuable prognostic parameters, which might

serve to select individual patients who would benefit from additional therapeutic approaches. Further, the abnormalities in PIN lesions are more likely to be structural than numerical. Genome-wide screening for regional losses and amplifications by CGH analysis on micro-dissected archival PIN lesions and other early manifestations of prostatic adenocarcinoma, such as incidental, stage T1a cancers, is required. In cases concerning early cancers, these studies may yield better criteria for active or passive treatment policies.

7.2 Cytogenetic Heterogeneity in Prostatic Adenocarcinoma

Prostatic cancer has long been recognized for its multifocal and highly heterogenous histological appearance. Foci within a prostate often show varying degrees of differentiation, and this phenotypic heterogeneity may reflect genetic heterogeneity. ISH applied to archival tissue sections disclosed intratumoral cytogenetic heterogeneity for both chromosomal aberrations, as well as ploidy status, at three different histological levels, i.e. interregionally (between different Gleason graded areas), intraregionally (within one Gleason area), and intraglandularly (within one tumor gland; Chapters 3, 4). Further, analysis of pairs of primary tumors and their recurrences or distant metastases showed both concordances and discordances in the occurrence of chromosomal abnormalities, as well as in ploidy status (Chapter 6). The latter observations can be explained by either the cytogenetic heterogeneity of the primary tumor material, wherein undetected subclones had the proper characteristics to display aggressive tumor behavior, or by the increased genetic lability of metastatic tumor cells (Nowell, 1976).

There are two main ways to explain intratumoral heterogeneity in prostatic adenocarcinoma. 1) It could be regarded as the heterogeneity of different tumor sublines derived from a common precursor cell. 2) It could be a sign for a polyclonal origin of prostate cancer. Cytogenetic aberrations detected by interphase ISH with chromosome-specific repetitive DNA probes likely represent relatively late events in prostatic tumorigenesis, reflecting either secondary changes specifically involved in tumor progression (e.g., gain of chromosome 7 and/or 8), or unspecific cytogenetic changes due to an increased genetic instability (e.g., loss of Y). These data seem to be in view with the first assumption.

Prostate cancer and PIN frequently have a multifocal appearance located in the peripheral zone (Bostwick, 1995). Whether these multifocal tumors represent multicentric origins is presently not clear (Miller and Cygan, 1994). In this respect, Emmert-Buck *et al.* (1995) showed that near 80% of cases with multiple foci of PIN showed differences in allelic loss profiles on chromosome 8p among the PIN foci, suggesting that these lesions arise independently and multifocally. These observations appear to be in line with a polyclonal origin of prostate cancer.

In conclusion, multifocality and genotypic and phenotypic intratumoral heterogeneity

are prominent features of prostatic adenocarcinoma. This intratumoral heterogeneity, combined with the multifocal nature of prostatic cancer, indicates that a single cytogenetically or flow cytometrically analyzed tumor biopsy might not be representative for a given carcinoma. The biology and the clinical implications of intratumoral heterogeneity remain to be further unraveled.

7.3 Prospects in Interphase Cytogenetics

The evaluation of chromosomal aberrations by means of ISH with chromosome-specific repetitive (peri)centromeric DNA probes is currently at a reliable level. Genome-wide screening of tumors, made possible by the CGH technology, provides us with a vast amount of information concerning region-specific imbalances. It may be important, especially in prostatic cancer, to relate the genetic aberrations found to histopathological characteristics, i.e. growth patterns and intratumoral genetic heterogeneity. Presently, the resolution of CGH is approximately 2 Mb at best. Therefore, fine mapping by ISH with region-specific probes, combined with LOH studies, is necessary to define regions of DNA loss more precisely. Thus, a combination of CGH analysis and interphase ISH with target-specific probes applied to tissue sections will be of great importance. FISH is capable of distinguishing structural changes in fresh preparations of metaphase and interphase nuclei with cosmid and yeast artificial chromosome (YAC) DNA probes. In contrast, interphase cytogenetics with regional DNA probes applied to tissue sections would benefit from a higher detection efficiency. It would make this application of the technique more useful for routine-oriented purposes. The type of DNA probe is also important. For instance, so-called P1-clones are increasingly being used for ISH purposes since they can carry large DNA inserts of 100 kb (in comparison, cosmids can harbor inserts of around 40 kb). The availability of these and other large inserts containing DNA probes is improving rapidly (Monaco and Larin, 1994). Further, signal amplification is needed for the visualization of these small probes on archival tissue sections. As discussed before (Chapter 1), the CARD procedure may be a possible breakthrough in this field. We have successfully used this amplification method to detect loss of 8p21.3 sequences with a cosmid probe in a bone metastasis of prostate cancer, which showed loss of 8p sequences by CGH analysis (Fig. 1). However, at present its reproducibility for screening panels of archival specimens with target-specific probes is insufficient. Finally, developments in computer-assisted evaluation of ISH spot signals are to be expected. ISH-spot counting is very suitable for (semi)automatic approaches, which would certainly improve its use in a clinical setting. At present, several groups, including commercial manufacturers, have developed ISH-spot counting systems for image analysis of solid tumor specimens. Most systems are dedicated to nuclear suspensions, since these are more readily accessible for automation purposes. Accurate automated analysis of ISH signals on tissue sections is also

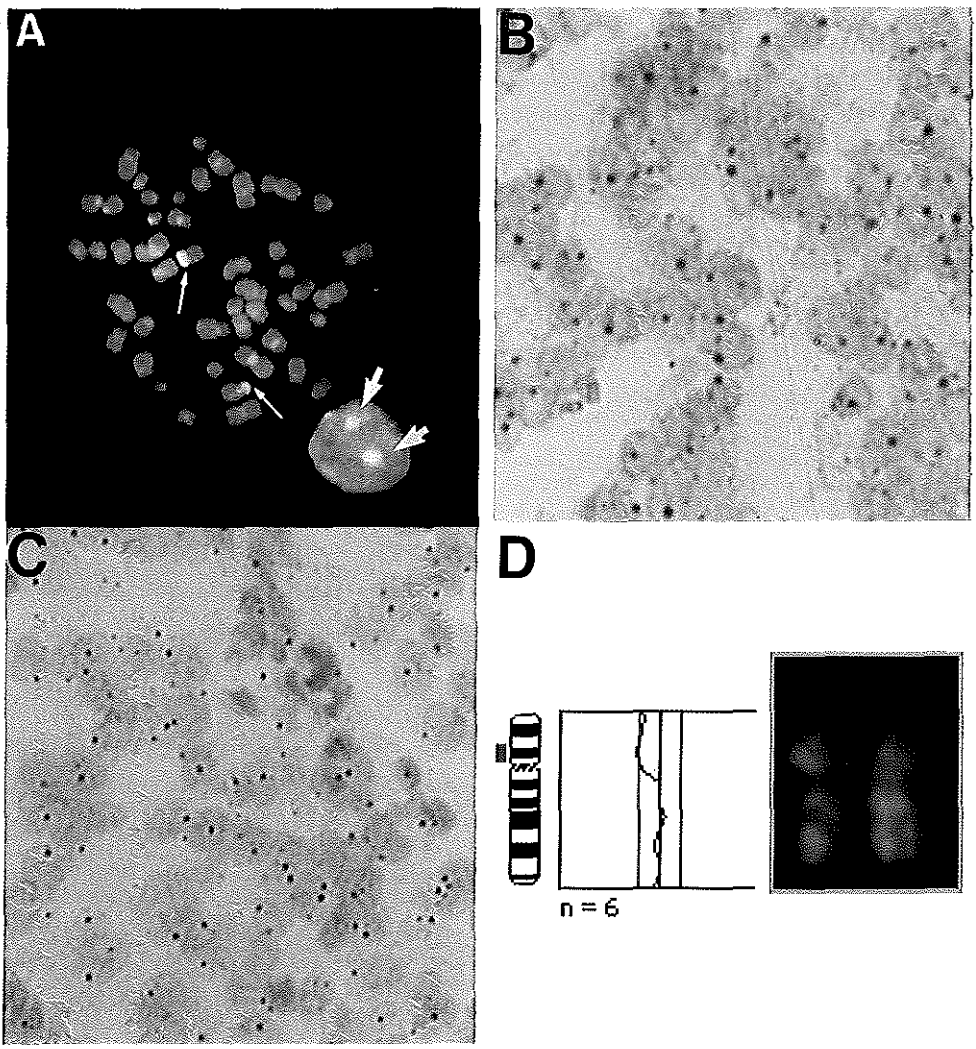

FIGURE 1.
Histogenetic analysis of prostate cancer by a combination of interphase ISH and CGH. **A)** FISH to a normal metaphase spread with a biotin-labeled cosmid DNA probe specific for the 8p21.3 region. ISH signals can be seen in both metaphase spread (*arrows*) and interphase nucleus (*arrowheads*), due to amplification with the CARD procedure. **B)** Section ISH with the same probe to a bone metastasis of prostatic cancer using the CARD amplification procedure. Most cancer cells display one ISH spot for this DNA probe indicating loss of one copy of this chromosome segment. Without CARD amplification cosmid signals could not be visualized. **C)** The adjacent tissue section shows also loss of the centromeric region of chromosome 8. **D)** The results were in concordance with CGH of DNA, derived from the same archival tissue, revealing loss of 8p sequences. Microphotographs A and D were taken from a video screen; magnification of B and C: 750x.

feasible but more complicated (Krijtenburg *et al.*, 1996). It is clear that such procedures will make interphase cytogenetics more generally applicable. It will also increase its clinical utility, since automation will facilitate objective and standard evaluation protocols.

7.4 References

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SUMMARY

At present prostatic cancer is the second leading cause of cancer-related death in Western countries, including The Netherlands. The clinical course of prostate cancer is highly variable and unpredictable. Present methods of assessing the prognosis for patients with prostate cancer include clinical and pathological staging and histopathological grading. Unfortunately, these methods fail to provide consistent predictive information regarding the clinical outcome and the therapeutic strategy of individual cases, particularly in tumors confined to the prostate. Hence, there is a need to identify characteristics of prostate cancer cells that would help in defining biological aggressiveness of individual tumors and guide the choice of therapy. An understanding of prostate cancer cytogenetics during tumor initiation and tumor progression might provide such information.

This thesis describes the detection of cytogenetic aberrations and changes in ploidy occurring in prostatic tumorigenesis and prostatic tumor progression by means of interphase *in situ* hybridization applied to archival tissue sections. This procedure allows a combined cytogenetic and histologic analysis.

In the first part of Chapter 1 an overview is given on the histomorphological and functional aspects of the normal human prostate. Secondly, the epidemiological and clinicopathological features of prostatic adenocarcinomas are discussed, as well as putative precancerous lesions, i.e., prostatic intraepithelial neoplasia (PIN). Further, the state of the art of (cyto)genetic aberrations occurring in prostatic cancer is presented, as detected by molecular methods and cytogenetics including, ISH studies. The third part of this chapter describes the development and methodology of non-isotopic *in situ* hybridization (ISH) as a powerful tool for the detection of cytogenetic alterations in human solid tumors.

Chapter 2 describes a comparative study of interphase ISH to routinely processed, paraffin-embedded, 4 µm tissue sections and nuclear suspensions from eight prostatic adenocarcinomas. Nuclear suspensions were prepared from the same tumor areas to evaluate differences of ISH to truncated vs. whole nuclei. DNA probes specific for the centromeres of chromosomes 1, 7, 8, 10, and Y were used for the detection of numerical aberrations and aneuploidy. It was concluded that both section and suspension ISH were able to accurately detect aneuploidy and numerical aberrations occurring in larger histological areas. However, section ISH was also capable of revealing (small) focal cytogenetic abnormalities, due to precise analysis of only target cells. Focal abnormalities were not detected by suspension ISH, probably due to an admixture of non-aberrant tumor cells and stromal elements. Further, ploidy patterns as assessed by both methods were in concordance with DNA flow cytometry (FCM).

Prostatic cancer is known for its highly heterogenous histological appearance. In Chapter 3 twenty-five prostatic adenocarcinomas were studied for the presence of

intratumoral cytogenetic heterogeneity by means of ISH to archival tissue sections. ISH with a chromosome Y-specific probe provided a model to investigate patterns of chromosomal heterogeneity within and between different pathological Gleason grades. Heterogeneity with respect to ploidy of the tumor was examined by ISH with a chromosome 1-specific probe. Cytogenetic heterogeneity at the (Y) chromosomal level was observed between areas with different Gleason grades (interregional heterogeneity), within one area (intraregional heterogeneity), and even within single tumor glands (intraglandular heterogeneity). The different patterns of chromosomal heterogeneity were seen in all tumor grades and stages. Differences in ploidy status were also found following the aforementioned histological patterns, again, in all grades and stages. Intraglandular heterogeneity was most frequently seen. In contrast to current views on clonality, suggesting regional separation of subclones with different DNA content, this chapter demonstrates that these subclones can be interspersed.

In Chapter 4 twenty-five prostatic carcinomas derived from radical prostatectomy, as well as seventeen adjacent PINs, were screened for the presence of numerical chromosome changes by means of ISH to archival tissue sections. To this end a probe set specific for the centromeric regions of chromosomes 1, 7, 8, 10, 15, and Y was used. Numerical aberrations were seen in 52% of prostatic adenocarcinomas. Alterations of chromosome 8 and loss of Y were the most frequent findings (each 20%), followed by loss of chromosomes 15 (16%) and 10 (12%). Gain of chromosome 7 was seen in 8% of cases. No aberrations of chromosomes 7, 8, 10, and 15 were found in the adjacent PIN lesions, whereas loss of the Y chromosome occurred in both PIN and tumor glands in two cases. Also (low) level aneuploidy was observed in most PIN lesions. Ploidy of the carcinomas as assessed by ISH correlated well with ploidy measured by DNA FCM. Thus, ISH analysis revealed chromosomal aberrations and aneuploidy in precancerous lesions, as well as in the majority of carcinomas.

In Chapter 5 the genetic status in different stages of prostatic tumor development was determined by applying ISH with a probe set specific for chromosome 1, 7, 8, and Y to a panel of 23 primary localized prostatic tumors, 20 regional lymph node metastases, and 16 distant metastases. All samples were from different patients. In primary tumors the number of aberrant cases increased significantly with local tumor stage, as did the number of cases with gain of chromosome 8. With progression towards metastatic disease an accumulation of genetic changes and aneuploidy was seen. Gain of chromosome 7 and/or 8 increased significantly along with tumor progression, whereas the number of cases with aberrations of Y did not change. Furthermore, specific involvement of chromosome 8 was seen in bone metastases, but not in hematogenous metastases to other sites. The nature of the alterations of chromosome 8 in bone metastases was further examined by comparative genomic hybridization (CGH) analysis of the same archival material.

The combined CGH and ISH evaluation suggested the presence of isochromosome 8q formation, a possible progression marker in prostatic cancer.
Chapter 6 deals with a longitudinal evaluation of the cytogenetic status of 11 patients with local tumor recurrence, and 9 patients with distant metastases. A group of 12 cases, which showed no evidence of local recurrence or distant metastases after radical prostatectomy in follow up (mean 58.5 months), served as a reference. In its totality 73 specimens were evaluated with a probe set specific for chromosomes 1, 7, 8, and Y. Gain of chromosome 8 was the most frequent alteration in both recurrences and distant metastases, whereas gain of chromosome 8, as well as loss of Y and gain of chromosome 7 were the most common alterations in the concomitant primary tumor tissue of these cases. In four aberrant cases a concordance was found between primary tumor and its recurrence or distant metastasis. Discrepancies might have been caused by cytogenetic heterogeneity. Further, comparison of primary tumor tissue of reference, recurrence, and distant metastasis group showed a significant increase for the percentage of cases with numerical aberrations. Likewise, a trend was seen for gain of chromosome 7 and/or 8. Also the rate of aneuploidy increased in these three different groups. These data suggest that tumors that recur in time display an intermediate position between tumors of disease-free patients and metastatic cancers.
The general discussion (Chapter 7) emphasizes and discusses the most important findings of these studies in a wider context, and possible future research directions in this field are given.
Finally, it is concluded that interphase ISH with chromosome-specific DNA probes applied to tissue sections can serve as a valuable cytogenetic tool for the determination of chromosomal aberrations and ploidy status during prostatic tumor development and progression. Loss of the Y chromosome, as well as aneuploidy are already present in PIN lesions, indicating that these changes may be early events in prostatic tumorigenesis. With tumor progression an accumulation of genetic changes and aneuploidy is seen. Particularly, gain of chromosome 7 and/or 8 appears to be related to local tumor progression and local recurrence, as well as progression towards widespread metastatic disease. Hence the name "progression markers" seems to be appropriate for these chromosomal alterations. Evaluation of these and other genetic biomarkers in primary tumor specimens may render valuable prognostic parameters, which can help to develop treatment strategies in individual patients.

SAMENVATTING

Prostaatkanker is op dit moment de op een na belangrijkste oorzaak van kankersterfte in Westerse landen, inclusief Nederland. Het klinische verloop van prostaatkanker is zeer variabel en moeilijk te voorspellen. De huidige methoden om de prognose voor patiënten met prostaatkanker te bepalen, omvatten klinische en pathologische stagering en histopathologische gradering. Helaas geven deze methoden geen eenduidige informatie omtrent het te verwachten klinische verloop en de te volgen behandeling in individuele gevallen, vooral in patiënten met tumoren die nog niet uitgezaaid zijn. Het is dus noodzakelijk om die eigenschappen van prostaatkanker cellen te bepalen, die de biologische agressiviteit van individuele tumoren kunnen aangeven en tevens tot hulp zijn bij de keuze van de te volgen behandeling. Onderzoek naar de cytogenetische veranderingen die optreden tijdens zowel het ontstaan van prostaatkanker als de progressie van prostaatkanker, kan hieraan een belangrijke bijdrage leveren.
Dit proefschrift beschrijft de detectie van cytogenetische afwijkingen en veranderingen in de ploidie die optreden tijdens het onstaan en de progressie van prostaattumoren met behulp van de interfase *in situ* hybridisatietechniek, toegepast op formaline-gefixeerde, in paraffine-ingebedde dunne weefselcoupes.
In Hoofdstuk 1 wordt allereerst een overzicht gegeven van de histomorfologische en functionele aspecten van de normale prostaat in de mens. Voorts worden de epidemiologische en klinischpathologische eigenschappen van prostaatkanker besproken, alsook die van de mogelijke voorloper laesie, prostaat intraepitheliale neoplasie (PIN). Tevens wordt een overzicht gegeven van de tot op heden bekende (cyto)genetische afwijkingen voorkomend in prostaatkanker, die ontdekt zijn met behulp van moleculaire en cytogenetische technieken, inclusief *in situ* hybridisatie. Verder beschrijft dit hoofdstuk de ontwikkeling en de methodologie van de niet-radioactieve *in situ* hybridisatietechniek tot een waardevolle methode om cytogenetische afwijkingen in humane solide tumoren te detecteren.
Hoofdstuk 2 beschrijft een vergelijkende studie van interfase ISH toegepast op routine behandelde 4 μm dunne paraffinecoupes en kernsuspensies van acht adenocarcinomen van de prostaat. De kernsuspensies werden geïsoleerd uit dezelfde tumorgebieden om verschillen te kunnen bepalen tussen doorgesneden kernen en hele kernen. DNA probes die specifiek zijn voor de centromeren van chromosomen 1, 7, 8, 10, en Y werden gebruikt om numerieke afwijkingen en aneuploidie te kunnen bepalen. In conclusie, ISH toegepast op zowel coupes als op kernsuspensies was in staat om accuraat aneuploidie en numerieke afwijkingen te bepalen, die in grotere tumorgebieden voorkwamen. Daarentegen was ISH toegepast op coupes ook in staat om kleine focaal voorkomende afwijkingen te detecteren, doordat alleen de tumorcellen werden geanalyseerd. Deze focale afwijkingen werden niet ontdekt met behulp van ISH toegepast op kernsuspensies,

waarschijnlijk door de bijmenging van niet afwijkende tumorcellen en stromale cellen. Voorts kwamen de patronen van ploidie gevonden met behulp van ISH op coupes en kernsuspensies overeen met die zoals bepaald met behulp van DNA flowcytometrie.
Prostaatkanker staat bekend om zijn uitermate heterogene histologie. In Hoofdstuk 3 werden 25 adenocarcinomen van de prostaat bestudeerd op de aanwezigheid van intratumorale heterogeniteit met behulp van ISH toegepast op paraffinecoupes. ISH met de probe specifiek voor het Y-chromosoom verschafte een model om patronen van chromosomale heterogeniteit te bestuderen binnenin en tussen verschillende Gleason graderingsgebieden. Heterogeniteit voor ploidie werd onderzocht met behulp van ISH met de probe specifiek voor chromosoom 1. Cytogenetische heterogeniteit voor afwijkingen van het Y-chromosoom werd gevonden tussen verschillende Gleason gebieden (interregionale heterogeniteit), binnenin een Gleason gebied (intraregionale heterogeniteit), en zelfs binnen een tumorbuisje (intraglandulaire heterogeniteit). Deze verschillende patronen van heterogeniteit werden gezien in alle differentiatiegraden en alle stadia van prostaattumoren. Verschillen in de ploidie-status werden ook gevonden in de hierboven beschreven patronen. Intraglandulaire heterogeniteit werd het meest frequent waargenomen. In tegenstelling tot de huidige inzichten omtrent clonaliteit, waarbij een regionale scheiding van subclonen met verschillende DNA compositie wordt verondersteld, laat deze studie zien dat deze subclonen met elkaar verweven kunnen zijn.
In Hoofdstuk 4 wordt de screening van 25 primaire prostaatcarcinomen en 17 naast gelegen PIN-laesies, afkomstig van radicale prostatectomieëen, op de aanwezigheid van numerieke chromosomale afwijkingen met behulp van ISH op paraffinecoupes beschreven. Hiertoe werd een probe-set specifiek voor de centromeerregionen van chromosomen 1, 7, 8, 10, 15, en Y gebruikt. Numerieke afwijkingen werden gevonden in 52% van de prostaatcarcinomen. Afwijkingen van chromosoom 8 en verlies van het Y chromosoom werden het meest frequent aangetroffen (beide 20%), gevolgd door verlies van chromosomen 15 (16%) en 10 (12%). Overrepresentatie van chromosoom 7 werd gezien in 8% van de gevallen. In de nabij gelegen PIN-laesies werden geen afwijkingen van chromosomen 7, 8, 10, en 15 gevonden, terwijl verlies van het Y-chromosoom werd gezien in zowel tumor als PIN in twee tumoren. De meeste PIN-laesies waren ook in geringe mate aneuploid. De ploidie van de carcinomen bepaald met behulp van ISH kwam goed overeen met die gemeten met behulp van DNA flowcytometrie. In conclusie, ISH analyse toonde chromosomale afwijkingen en aneuploidie aan in zowel de PIN-laesies als in de meerderheid van de carcinomen.
In Hoofdstuk 5 werd de genetische status bepaald in verschillende stadia van prostaattumorontwikkeling met behulp van ISH met een probe-set specifiek voor chromosoom 1, 7, 8, en Y. Het panel bestond ondermeer uit 23 primaire

gelocaliseerde prostaattumoren, 20 regionale lymfekliermetastasen, en 16 metastasen-op-afstand. Al het materiaal was afkomstig van verschillende patiënten. In primaire tumoren nam zowel het aantal gevallen met afwijkingen als het aantal gevallen met overrepresentatie van chromosoom 8 significant toe met het locaal tumorstadium. In metastases werd een accumulatie van genetische afwijkingen en aneuploidie gezien. Overrepresentatie van chromosoom 7 en/of 8 nam significant toe met de tumorprogressie, terwijl het aantal gevallen met afwijkingen van het Y-chromosoom nauwelijks veranderde. Voorts werden afwijkingen van chromosoom 8 specifiek gevonden in metastasen naar het bot, maar niet in andere hematogene metastasen. De aard van deze afwijkingen werd verder bestudeerd met behulp van vergelijkende genomische hybridisatie (CGH) analyse van hetzelfde paraffinemateriaal. De combinatie van CGH en ISH gegevens wijst op de mogelijke vorming van een isochromosoom van 8q, dat dus een progressiemarker voor prostaatkanker kan zijn.

Hoofdstuk 6 gaat over de longitudinale evaluatie van de cytogenetische status van 11 patiënten met een locaal recidief, en 9 patiënten met metastasen-op-afstand. Een groep van 12 gevallen, die geen locaal recidief of metastase op afstand hadden gekregen in de jaren (gemiddeld 58.5 maand) volgend op een radicale prostatectomie, diende als referentie. Totaal werden 73 weefselspecimens bestudeerd met een probe-set specifiek voor chromosoom 1, 7, 8, en Y. Overrepresentatie van chromosoom 8 was de meest voorkomende afwijking in zowel de recidieven als de metastasen op afstand, terwijl overrepresentatie van chromosoom 8, verlies van Y, en overrepresentatie van chromosoom 7 de meest frequente afwijkingen waren in het corresponderende primaire tumorweefsel van deze patiënten. In vier gevallen werd een overeenkomst gevonden tussen de afwijkingen in de primaire tumor en het recidief of metastase-op-afstand. Verschillen tussen de primaire tumor en recidief of metastasen werden waarschijnlijk veroorzaakt door cytogenetische heterogeniteit. Voorts toonde een vergelijking tussen primair tumorweefsel van de referentie, locaal recidief en metastase-op-afstand groep de significante toename aan van het aantal gevallen met numerieke afwijkingen. Tevens werd er ook een trend gezien voor het aantal patiënten met overrepresentatie van chromosoom 7 en/of 8. Ook de mate van aneuploidie nam toe met deze drie groepen. Deze gegevens suggereren dat tumoren, die mettertijd een locaal recidief vormen een tussenpositie innemen tussen tumoren van genezen verklaarde patiënten en patiënten die metastasen krijgen of hebben gekregen.

De algemene discussie (Hoofdstuk 7) benadrukt en bediscussieert nog eens de meest belangrijke bevindingen van deze studie in een breder verband. Tevens worden mogelijke toekomstige onderzoeksrichtingen in dit gebied aangegeven.

In conclusie, interfase ISH met chromosoom-specifieke DNA-probes toegepast op weefselcoupes kan dienen als een waardevolle methode om chromosomale

afwijkingen en ploidie-status tijdens het onstaan en de progressie van prostaatkanker te bepalen. Verlies van het Y-chromosoom en aneuploidie zijn reeds aanwezig in PIN laesies, wat erop wijst dat deze veranderingen waarschijnlijk vroeg optredende gebeurtenissen zijn in de ontwikkeling van prostaatkanker. Tijdens tumorprogressie zien we een accumulatie van genetische afwijkingen en aneuploidie. Vooral overrepresentatie van chromosoom 7 en/of 8 lijkt gerelateerd te zijn aan locale tumorprogressie en locale recidieven, alsook aan progressie tot metastasen-op-afstand. De term "progressie markers" voor deze chromosomale afwijkingen lijkt dus op zijn plaats. Het zorgvuldig bestuderen van deze en andere gentische biomarkers in primaire tumoren kan misschien waardevolle prognostische parameters opleveren, die weer van dienst kunnen zijn bij het bepalen van de te volgen behandeling in individuele patiënten.

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