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Numerical chromosomal aberrations in prostate cancer: correlation with morphology and cell kinetics

Rolf-Peter Henke[1], Eva Krüger[1] *, Nebahat Ayhan[1], Dirk Hübner[2], and Peter Hammerer[2]

[1] Institute of Pathology, University of Hamburg, Hamburg, Germany
[2] Department of Urology, University of Hamburg, Hamburg, Germany

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Abstract. Eleven routinely processed radical prostatectomy specimens were studied for the presence of numerical chromosomal aberrations by means of in situ hybridization with nucleic acid probes specific for chromosomes 7, 10, 17, X, and Y. Cytogenetic information was correlated with morphology, tumour stage and volume as well as with cell kinetics, the latter being assessed by immunohistochemistry with antibodies raised against the proliferative cell nuclear antigen (PCNA) and against a formalin-resistant epitope of the Ki-67 antigen, MIB 1. In 5 of 11 cases, numerical aberrations of at least one chromosome were found. The cases with normal chromosome numbers were those with the smallest volumes of Gleason grade 4 and/or 5 tumour (mean 0.5 cm^3) and represented tumours restricted to the prostate. Tumours with aberrations in the number of detected chromosomes showed advanced stages and large volumes of high-grade tumour (mean 12.5 cm^3). All 4 tumours with positive surgical margins were recruited from a group with marked local heterogeneity in chromosome numbers. Immunostaining with MIB 1 and PCNA was most intense in areas of high-grade tumour and was positively correlated with the emergence of chromosomal aberrations. The data suggest that the appearance of numerical chromosomal aberrations in prostate cancer coincides with aggressive tumour behaviour and could be used as an additional prognostic marker.

Key words: Prostatic carcinoma – Interphase cytogenetics – In situ hybridization – Proliferative cell nuclear antigen – MIB 1

Introduction

Prostatic cancer is the most common malignancy in men in Western Europe and the United States and chromosomal aberrations have not been studied extensively in this tumour (reviewed by Sandberg 1992). A consistent primary cytogenetic change has yet to be identified. As in other epithelial malignancies, cytogenetic studies of prostate cancer by karyotyping of metaphase spreads are hampered by difficulties in obtaining analysable well-banded chromosome preparations. In addition, prostate cancers can be very heterogenous with divergent Gleason grades in adjacent areas of a single section. Therefore, cytogenetic studies on metaphase chromosomes, which have to be performed without direct morphological control, may be biased by over-representation of rapidly growing cell populations. Interphase cytogenetics (Cremer et al. 1986) is a means to by-pass these obstacles. This technique has been successfully applied to the detection of chromosomal abnormalities in several human malignancies (reviewed by Poddighe et al. 1992). While most of these studies, including one of prostate cancer (Van Dekken et al. 1990a), have been carried out on cytological material, the method has also become applicable to routinely processed tissue sections. In this respect, pioneering work has been done by Hopman and co-workers (1991).

The primary aim of our study was to study the distribution of numerical chromosomal aberrations in tissue sections of prostate cancer and the correlation of karyotypic abnormalities with morphological and prognostic hallmarks. In addition, proliferative activity was assessed by immunostaining with antibodies raised against the proliferative cell nuclear antigen (PCNA) and against a formalin-resistant epitope of the Ki-67 antigen, MIB 1.

Materials and methods

The tissues consisted of 11 formalin-fixed, radical prostatectomy specimens, sectioned according to the Stanford protocol (Stamey et al. 1988). After removal, the prostate was examined grossly, inked over its entire surface and fixed in formalin for at least 24 h. The apex was removed by a single transverse section perpendicular to the rectal surface as a single block measuring 5 mm in greatest vertical diameter and sectioned at 3-mm intervals in

Correspondence to: R.-P. Henke, Institute of Pathology, University of Hamburg, Martinistr. 52, W-2000 Hamburg 20, Germany

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Table 1. Clinical data of patients and results of pathological examination, in situ hybridization, and immunohistochemistry

Case	Age (years)	PSA (ng/ml)	p7	p10[a]	p17[a]	pX[a]	pY[a]	MIB 1[b] (cells/HPF)	PCNA[b] (% stained) cells	p53	Gleason	Stage	Margin	Volume (c^3)	$Vol_{GI \leq 4}$ (cm^3)
1	67	NI	2	2	2	1	1	25	25	-[c]	2+4=6	pT2cN0	neg	4.7	0.4
2	64	NI	2	2	2	1	1	15	10/75	–	3+2=5	pT2bN0	neg	1.4	0.0
3	60	72.0	3	2	2 (4)	2	2 (1)	20	10/20/50	+	4+5=9	pT4aNx	pos	23.9	23.9
4	64	7.5	2	2	2	1	1	5–7	75	–	3+2=5	pT2aN0	neg	0.3	0.0
5	64	66.9	3	2	3	2	2	20/50	75/10	–	4+3=7	pT3bN0	neg	12.5	8.8
6	68	6.2	2	2	2	2	2 (0)	10/25	25/50	–	4+3=7	pT3bN0	pos	6.7	3.3
7	62	11.3	2	2	2	1	1	25	20	–	3+2=5	pT2cN0	neg	4.1	0.0
8	67	7.0	2	2	2	1	1	7	20/80	–	4+3=7	pT2bN0	neg	2.9	2.3
9	61	6.9	5	3	3 (4)	2 (5)	0 (1, 2)	10	75	++	4+5=9	pT3cN0	pos	16.4	16.4
10	70	13.7	4	1 (3)	4 (5)	2 (1)	1 (2, 0)	25/10	75	–	4+5=9	pT3cNx	pos	12.9	10.3
11	64	35.0	2	2	2	1	1	15	75/25	–	3+2=5	pT2cN0	neg	14.6	0.3

PSA, Prostate specific antigen pre-operative level; NI, no information; HPF, high power field; $Vol_{GI \leq 4}$, volume of tumour with Gleason grade 4 and/or 5

[a] Numbers in parentheses denote hybridization results in a minor tumour fraction

[b] More than one number reflects a heterogenous staining pattern

[c] Immunoreactivity scored on a scale of – to ++

parasagittal planes. The seminal vesicles were amputated at the base and embedded complete. The bladder neck margin was removed as a single block of about 2 mm thickness and subsectioned parasagitally at 3-mm intervals. The remainder of the prostate was serially blocked at 3-mm intervals in transverse planes parallel to the apical section. If possible, whole mount 5-µm sections were prepared for routine staining with haematoxylin and eosin. If the prostate was too large for a 75 × 40 mm microscopic slide, the main portion was divided into right and left halves. The tumour grade was assessed by the Gleason method (Gleason 1977). For staging we used a forthcoming modification of the UICC classification that will take the place of the presently used 4th edition (H. Huland, personal communication). In short, stage T2 denotes tumours restricted to the prostate (2a, less than 50% of one side involved; 2b, more than 50% of one side involved; 2c, tumour on both sides). Stage T3 is used for growth beyond the capsule (3a, on one side; 3b, on both sides; 3c, with infiltration of the seminal vesicles), while stage T4 tumours show infiltration of neighbouring structures (4a, bladder neck and/or external sphincter and/or rectum; 4b, pelvic wall). The main difference to the 4th edition is that infiltration of the apex will no longer be on par with capsular perforation. The term "prostatic intraepithelial neoplasia" (PIN) was used as defined by Bostwick and Brawer (1987). All the cases and some clinical data are summarized in Table 1.

Tumour area was measured by using a digitizing tablet (Hitachi HDG 1212D), SigmaScan version 3.90 software (Jandel Scientific, Corte Madera, Calif., USA), and a personal computer based on a 80386DX microprocessor running at 40 MHz. The tumour volume was calculated by multiplication with the section thickness and a factor of 1.5 (as compensation for tissue shrinkage during fixation; Schmid and McNeal 1992). The volume of Gleason 4 and/or Gleason 5 portions of the tumours was determined separately by this procedure when distinct high-grade tumour areas were present or estimated, when high-grade tumour was mixed diffusely with lower graded tumour parts.

The following monoclonal antibodies were used. MIB 1 (Dianova, Hamburg, Germany) recognizes a formalin-resistant epitope of the Ki-67 antigen (Cattoretti et al. 1992) and was used in a dilution of 1:100. Antibodies against the PCNA (clone PC 10) were obtained from Medac (Hamburg, Germany) and diluted 1:20. Antibodies against the p53 antigen (clone DO-7) were supplied by Dako (Glostrup, Denmark) and diluted 1:100.

For immunohistochemistry serial 4-µm paraffin sections were adhered to silanized glass slides (Rentrop et al. 1986), air-dried at 40° C, and hydrated by incubations in xylene, acetone, a series of decreasing ethanol concentrations and demineralized water. The slides were placed in a plastic coplin jar, immersed in 10 mM citric acid monohydrate (pH 6) and boiled for 3 × 5 min in a microwave oven at 720 W (Shi et al. 1991). After each boiling step evaporated buffer solution was replaced. In order to block endogenous peroxidase activity the slides were incubated for 30 min in 0.03% hydrogen peroxide in phosphate buffered saline (PBS; 10.4 mM disodium hydrogen phosphate, 3.16 mM potassium dihydrogen phosphate, 150 mM sodium chloride, pH 7.6). Subsequently, the sections were treated with 1.5% normal horse serum in PBS for 30 min at room temperature, followed by incubation with the primary antibodies mentioned above for 30 min at 37° C. (Alternatively, an overnight incubation at 4° C can be used with comparable results.) Visualization of primary antibody was performed by means of the avidin-biotin peroxidase complex technique (Hsu et al. 1981), using a commercially available reagent kit (Vectastain Elite; Vector, Burlingame, Calif., USA).

The following biotinylated nucleic acid probes of the alphoid-satellite family were used: no. 7 (D7Z1), no. 10 (D10Z1), no. 17 (D17Z1), X (DXZ1), Y (DYZ3) (Oncor, Gaithersbury, Md., USA).

For in situ hybridization, 6-µm paraffin sections were adhered to silanized glass slides and air-dried at 60° C. Pretreatment of slides was performed according to Hopman et al. (1991) with minor modifications. Sections were dewaxed in xylene (2 × 10 min), rinsed in methanol (2 × 5 min), and air-dried. Subsequently, the sections were placed in a plastic coplin jar filled with 10 mM citric acid mono-hydrate, pH 6, and exposed for 2 × 5 min to microwaves in a household oven running at 720 W (this step is optional and seems to improve the accessibility of the target DNA and to shorten the proteolytic digestion time necessary), followed by a treatment with 1 M sodium thiocyanate (prewarmed in a microwave oven for 3 min at 240 W) for 10 min at 80° C. After washing with water, the sections were digested in pepsin (4 mg/ml in 0.2 M hydrochloric acid) at 37° C for 4–10 min (alternatively, 500 µg/ml proteinase K in 20 mM TRIS-HCl at a pH of 7.6, containing 2 mM calcium chloride can be used). After two washes for 5 min each in water, the slides were air-dried, and heated on a heating plate for 30 min at 80° C. Each section was covered with the following, freshly prepared hybridization solution: 65% deionized formamide, 2 × SSC (0.3 M sodium chloride, 0.03 M sodium citrate), 10% dextran sulphate, 1 µg/µl salmon sperm DNA, and 0.1 ng/µl biotinylated probe DNA. Sections were covered with cover slides and, after being sealed with rubber cement, were denatured by heating in a 78° C water bath for 10 min, followed by hybridization at 37° C overnight. After hybridization, coverslips were carefully removed

by floating the slides in 2 × SSC, followed by two 10 min washes in 2 × SSC, 50% formamide at 40 or 45° C, and three changes of PBS.

Labelled DNA was detected as follows. The slides were incubated with PBS containing 1.5% normal horse serum for 10 min at 37° C. The fluid was decanted and a monoclonal mouse anti-biotin antibody (Boehringer, Mannheim, Germany), diluted 1:100 in PBS was added for 30 min at 37° C. Detection of bound primary antibody was performed by using a kit based on the avidin-biotin-peroxidase complex technique (Vectastain Elite, Vector), keeping to the supplier's recommendations. The slides were counterstained with haemalaun, dehydrated in a series of alcohol followed by xylene, and permanently mounted in Eukitt (Kindler, Freiburg, Germany).

Specificity controls for immunohistochemistry were performed by incubating the slides with normal mouse serum. Specificity of the in situ hybridization experiments was scrutinized by omitting the denaturation step or the probe DNA. In addition, plausibility was checked on non-neoplastic tissue elements.

Statistical calculations were performed by using a statistical software (Instat; GraphPad, San Diego, Calif., USA). The Mann-Whitney Test (MWT) was used as a non-parametric test for unpaired samples. Contingency tables were calculated with Fisher's Exact Test (FET). All *P*-values given are two-tailed.

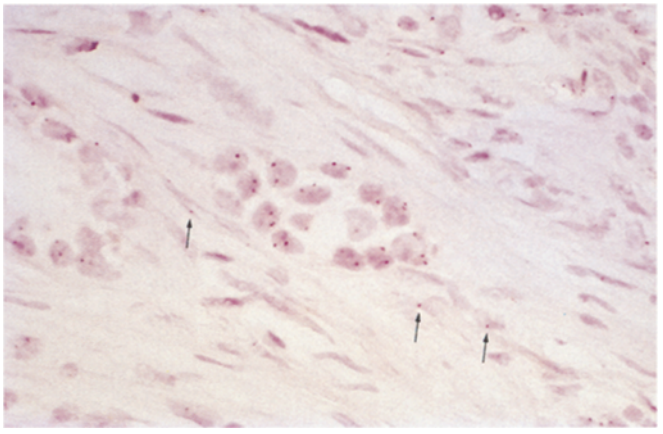

Fig. 1. In situ hybridization with a DNA probe specific for the X chromosome showing two signals (centre field), thus unmasking a disomy for this chromosome in tumour glands with tubular differentiation. *Arrows* point to non-neoplastic stroma cells with one hybridization spot. Case 9, × 500

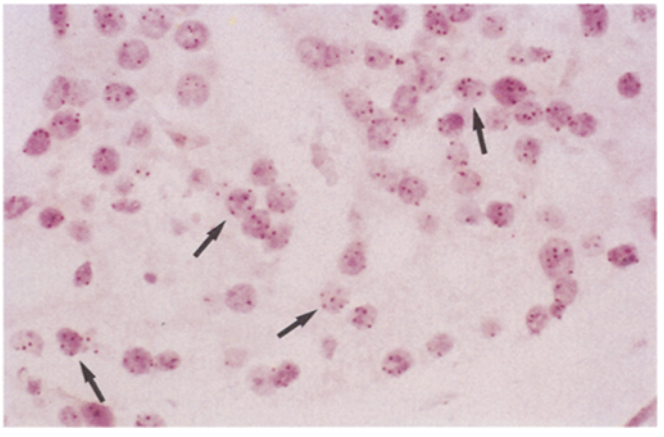

Fig. 2. Same case and DNA probe as in Fig. 1. In this cribriform differentiated area of the tumour, a pentasomy can be detected (*arrows*, not all dots in plane of focus). × 500

Results

The results of conventional histological examination (including grading, staging, and the assessment of the specimens' margins) as well as the tumour volumes are summarized in Table 1. One of the tumours (case 5) showed a 50% proportion of papillary carcinoma of ductal type.

In situ hybridization with a set of five chromosome-specific nucleic acid probes revealed numerical aberrations of at least one chromosome in 5 of 11 cases. It can be seen from Table 1 that the cases with normal chromosome numbers are those with tumour volumes at the lower end of the series (mean volume 4.7 cm^3 vs 14.5 cm^3 for tumours with numerical chromosomal aberrations: $P=0.0303$, MWT), especially when the quantity of Gleason grades 4 and/or 5 is considered separately. The 6 cases with normal chromosome numbers are those with the smallest Gleason 4 and/or 5 volumes (mean 0.5 cm^3 vs 12.5 cm^3; $P=0.0043$, MWT), only 1 case (no. 8) having more than 0.5 cm^3 high-grade tumour volume. Likewise, these cases represented those with tumours restricted to the prostate and without penetration of the capsule or infiltration of the seminal vesicles, i.e. those with pT2 stages according to the UICC classification ($P=0.00216$, FET).

In 4 of the cases, the number of chromosomes detected varied in different areas of the tumour. These variations corresponded for the most part with changes in histological differentiation, for example in case 9, the X-chromosome, while being disomic in most parts of the tumour, showed a pentasomy in all cribriform differentiated tumour areas (Figs. 1, 2). This feature was not completely uniform. In case 3, di- and tetrasomy for chromosome 17 was found in cribriform areas as well as in fields of the tumour with a clear cell aspect.

All of the tumours with positive surgical margin showed aneuploid chromosome numbers ($P=0.01515$, FET). Two of the 4 tumours with local variation in the number of chromosome counts showed very complex numerical aberrations with pentasomies found not infrequently. It is evident from Table 1, that 3 of the 4 tumours with chromosomal heterogeneity represent those with invasion in seminal vesicles and beyond, and those with the largest tumour volumes, especially when the amount of Gleason grade 4 and higher is considered.

In 3 of the cases, supernumerary chromosomes were detected in nuclei of PIN. Interestingly, these cases showed normal chromosome numbers in well-differentiated tubular areas of invasive carcinoma. In none of the specimens were numerical aberrations found in stromal or inflammatory cells, areas of squamous metaplasia, or hyperplasia.

In 2 of the cases, p53 antigen was detected by immunohistochemistry (Table 1). These cases represented those with the highest tumour volumes of our series and displayed a marked derangement in the number of chromosomes 17.

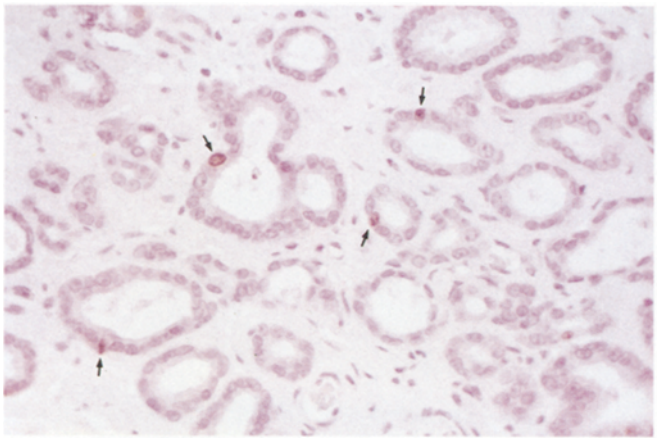

Fig. 3. Immunohistochemistry with MIB 1 reveals labelled nuclei in a small minority of cells (*arrows*) in well-differentiated tumour acini of case 4. In this case, no numerical chromosomal aberrations were detected. ABC, ×250

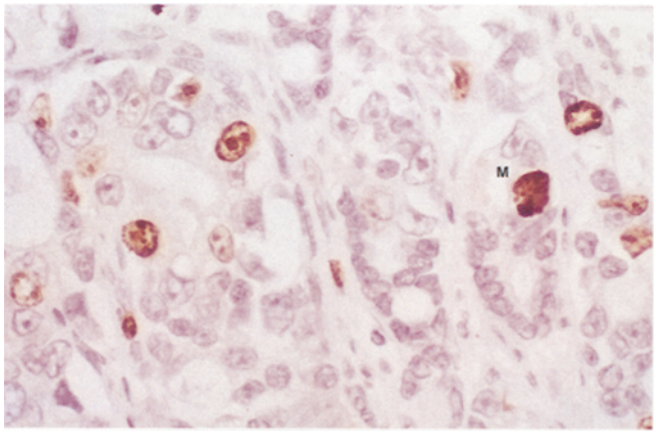

Fig. 4. Same antibody as in Fig. 3. In this area of case 5, the tumour consists of irregular spaced and shaped glands. About ten nuclei are stained with varying intensity. This tumour displayed numerical chromosomal aberrations. M, Mitosis, ABC, ×500

Immunohistochemistry with MIB 1 showed varying numbers of stained nuclei in different types of tissue. In atrophic and in hyperplastic glands nuclei were decorated only rarely (up to 3/high power field, HPF). In fields of basal cell hyperplasia the number of stained nuclei resembled that found in low-grade carcinoma (up to 15/HPF, Fig. 3). The highest proportion of labelled nuclei was noted in the papillary prostatic duct carcinoma of case 5 (up to 50/HPF). In the same case, tumour areas with well-circumscribed cribriform nests of Gleason pattern 3 or built of irregularly shaped acini showed up to 20 stained nuclei per HPF (Fig. 4). Also, glands or cells in the invasion zone were frequently labelled to a higher degree than those of identical differentiation in the more central areas of the tumours. With PCNA, the staining pattern was nearly identical, but the number of labelled nuclei was higher, reaching 80% in some tumour areas. Basal cells were stained in hyperplastic glands as well as in basal cell hyperplasia. In lesions of PIN grades 1 and 2, polarization was disturbed but not abolished and nuclei were decorated basally and in the midzone of the stratifed epithelium.

In individual cases, areas stained with MIB 1 and PCNA corresponded with areas in which numerical aberrations could be seen. Due to differences between cases in the extent of labelling, the maximum scores of cell staining could not be correlated significantly with the appearance of numerical aberrations.

Discussion

The data of the present study suggest that the appearance of detectable chromosomal aberrations in prostatic cancer is correlated with a shift of histological differentiation towards the higher grade end of the morphological spectrum. By performing karyotyping of short-term cultures of prostatic tumours Brothman and co-workers (1991) found cytogenetically abnormal clonal populations in only 5 of 20 cases and arrived at a similar conclusion, proposing that early-stage prostatic cancers contain a submicroscopic change that cannot be detected using standard cytogenetic procedures. A comparable yield of clonal abnormalities was described in another recently published paper. In a series of 57 tumours, Lundgren et al. (1992a) detected clonal karyotypic abnormalities in 15 carcinomas, nonclonal aberrations in 18 cases and normal karyotypes in 24 tumours. Just as we showed in our series, clonal aberrations were found in locally advanced or metastatic tumours for the most part. This finding was consistent with earlier reports (Atkin and Baker 1985; Gibas et al. 1985; Brothman et al. 1989, 1990). Obviously, the cytogenetic abnormalities of early-stage, low-grade prostatic cancer are too subtle to be detected by conventional cytogenetics as well as by interphase cytogenetics with the panel of probes we used.

Numerical chromosomal alterations represent rather coarse alterations of the enome and, in our investigation, were restricted to advanced cases which showed growth beyond the capsule. A similar finding was reported recently by Micale and colleagues (1992) who, by karyotyping 62 primary prostatic carcinomas, found clonal aberrations confined to tumours in advanced stages. These authors suspected heterogeneity of prostate cancers in vivo to be the reason for the co-existence of clonally aberrant, nonclonal aberrant, and normal diploid cells in culture. This supposed karyotypic heterogeneity of prostate cancer could now be confirmed by our cytogenetic study of nonmitotic cells in intact histological environs.

In our study, the frequency of numerical changes was nearly identical for all chromosomes, with only a slight preponderance of changes in the number of sex chromosomes. In the paper of Brothman et al. (1991) loss of the Y chromosome in one cell strain out of five and gain of this chromosome in another was described. Lundgren et al. (1992a) found losses of the Y chromosome in 6 of their 15 tumours with clonal aberrations. Loss of the Y chromosome was also found in one case studied by Van Dekken et al. (1990a) by interphase cytogenetics on cytological material. Based on these data,

the possibility that changes in the number of Y chromosomes could be characteristic for prostate cancer was discussed. However, the Y chromosome is very small and the number of included genes that may be of importance in tumour initiation and promotion is necessarily limited. Loss of the Y chromosome may be a nonspecific event and has been described frequently in other tumours, for example, in gastric carcinomas (Van Dekken et al. 1990b) or chronic nonlymphocytic laeukemia (reviewed by Heim and Mitelman 1987).

An interesting feature is the numerical chromosomal instability found in areas of intra-epithelial neoplasia, while on the same section adjacent areas of well-differentiated carcinoma showed normal chromosome numbers. This finding would support the concept that PIN and related lesions are biological precursors of prostatic cancer (Kastendieck 1980; McNeal and Bostwick 1986; Bostwick and Brawer 1987; for review see Brawer 1992). Against the background of our observation we would suggest that intra-epithelial neoplasia can progress directly into tumour with cribriform or higher-grade appearance, skipping the stage of well-differentiated tubular formations. Looking at the histological aspect of higher-grade dysplasias with their fusing papillary processes giving rise to transluminal bridging, this possibility does not seem to be far-fetched.

Case 11 shows some special features. Although displaying a large tumour volume and involving both lobes, the proportion of high-grade cancer is minimal and neither perforation of the capsule nor chromosomal abnormalities were detected. However, from whole mount sections it was evident that this tumour, in contrast to the others in our series, spared the peripheral zone and grew mainly in the central periurethral portions of the gland, probably representing unusually large transition zone cancer.

Next to chromosomes 7, 10, and Y, for which aberrations had been described in the afore-mentioned cytogenetic studies, we used a probe for chromosome 17 because genes such as p53 and c-*erb*B-2 which might be involved in the pathogenesis of a whole string of human cancers are mapped to this chromosome. Immunohistochemical detectable levels of p53 in prostate cancer were described recently by Mellon and co-workers (1992) in 5 of 29 cases. All of these 5 cases were poorly differentiated, but no significant relation between the tumour grade and stage and p53 detection existed. The p53 gene is mapped to the short arm of chromosome 17, at position 17p13.1 (Miller et al. 1986) and in this context our finding that 2 cases with a markedly deranged pattern in the number of chromosomes 17 are positive for p53 is an interesting observation. But it is not more at the present time. Using DNA probes for the centromeric regions only, as we did, nothing can be said about the more distal regions of the chromosomes and we have no information about eventual structural aberrations as deletions or translocations affecting the short arm of chromosome 17. Speculations about the mechanism which leads to increased amounts of p53 in our cases are also beyond the scope of this study. Furthermore, the expression of the c-*erb*B-2 protein, whose gene also is located on chromosome 17, at 17q21 (Fukushige et al. 1986), seems to be unrelated to the ploidy state of this chromosome in gastric and prostatic cancer (unpublished data). This shows that an increased number of chromosomes does not necessarily lead to increased expression of its genes.

All tumours showed positive staining with the antibodies MIB 1 and PCNA, although to a varying degree. In this respect, the enhancement of antigen retrieval by exposure to microwaves (Shi et al. 1991; see Kok and Boon 1992 for a review on the use of microwaves in histopathology) can be strongly recommended. As a rule, areas of tumour with chromosomal aberrations showed a high proliferative activity, as assessed by immunohistology with PCNA and MIB 1, which is not too surprising for these higher-grade lesions. But this feature could be reproduced only in individual cases. No significant correlation was found between the appearance of numerical aberrations and the maximum score of labelled cells when a comparison of cases was performed. Also, it should be noted that these antibodies can stain a given tumour quite heterogeneously. Consequently, significant areas of tumour should be evaluated before drawing any conclusions. Similar findings concerning heterogeneity of proliferative activity measured by PCNA reactivity were published recently in a study of lung cancer (Carey et al. 1992).

The presence of a relatively large number of cases without detectable numerical chromosomal aberration and the correlation of numerical changes with differentiation is a peculiar feature of prostate cancer. This situation is different from other carcinomas (e.g. gastric cancer, Van Dekken et al. 1990b; own unpublished observation) and underlines the special biology of prostate cancer. Utilization of probes specific for loci as 7q22 and 10q24, which displayed structural aberrations in the series of Lundgren et al. (1992a), would be necessary for a further dissection of chromosomal structure in prostate cancer and to determine the frequency and meaning of these changes.

From this and other studies it appears that morphologically detectable chromosomal aberrations in prostatic cancer represent rather coarse changes in an already advanced process and are far from being causal events. While this is true, the possibility exists that they may be used as markers of aggressive tumour behaviour. When the tumour material of Lundgren et al. (1992a) was used to evaluate the additional prognostic significance of chromosomal changes in excess of that attributable to prognostic factors such as tumour stage, grade, acid and alkaline phosphatases, and performance status (Lundgren et al. 1992b), an association between the presence of clonal chromosomal aberrations and an unfavourable clinical outcome was apparent.

In the present study, the occurrence and degree of numerical chromosomal aberrations correlated very well with tumour grade, stage, volume, and the presence of positive surgical margins; all well-known determinants of prognosis. Of course, results obtained on small uneven stratified patient groups have to be interpreted with great caution. Whether interphase cytogenetics could

help to define an independent prognostic factor remains to be seen in a study involving more patients and sufficient time for evaluation of clinical outcome.

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