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Chromosomal changes during development and progression of prostate adenocarcinomas

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Summary Chromosomal copy number changes were investigated in 16 prostate carcinomas, 12 prostatic intraepithelial neoplasias (PIN; 4 low-grade and 8 high-grade) adjacent to the invasive tumour areas, and 5 regional lymph node metastases. For this purpose, comparative genomic hybridization (CGH) was performed and a copy number karyotype for each histomorphological entity was created. CGH on microdissected cells from non-neoplastic glands was carried out on 3 different cases to demonstrate the reliability of the overall procedure. None of the non-neoplastic tissue samples revealed chromosome copy number changes. In PIN areas, chromosomal imbalances were detected on chromosomes 7, 8q, Xq (gains), and on 4q, 5q, 8p, 13q and 18q (losses). In the primary tumours, recurrent (at least 25% of cases) gains on chromosomes 12q and 15q, and losses on 2q, 4q, 5q, Xq, 13q and 18q became apparent. Losses on 8p and 6q as well as gains on 8q and of chromosome 7 were also detected at lower frequencies than previously reported. The pooled CGH data from the primary carcinomas revealed a novel region of chromosomal loss on 4q which is also frequently affected in other tumour entities like oesophageal adenocarcinomas and is supposed to harbour a new tumour suppressor gene. Gains on chromosome 9q and of chromosome 16 and loss on chromosome 13q were observed as common aberrations in metastases and primary tumours. These CGH results indicate an accumulation of chromosomal imbalances during the PIN–carcinoma–metastasis sequence and an early origin of tumour-specific aberrations in PIN areas.

Keywords: prostate carcinoma; prostatic intraepithelial neoplasia; tumour progression; comparative genomic hybridization

Comparative genomic hybridization (CGH) is a powerful tool for genome-wide screening of tumours for copy number changes of DNA sequences (Kallioniemi et al, 1992). Application of CGH and fluorescence in situ hybridization (FISH) to primary tumours of prostatic adenocarcinomas revealed consistent changes on chromosomes 7, 8p, 10, 13q, 16, 17 and 18q (Brothman et al, 1994; Macoska et al, 1994; Matsuyama et al, 1994; Joos et al, 1995; Qian et al, 1995; Visakorpi et al, 1995; Bova and Isaacs, 1996; Cher et al, 1996; Huang et al, 1996; Deubler et al, 1997). However, cytogenetic changes in prostatic intraepithelial neoplasias (PIN) which are considered as premalignant lesions and which are often present besides the invasive tumour are only poorly characterized cytogenetically (Alers et al, 1995; Qian et al, 1995; Zitzelsberger et al, 1998). Methodological improvements of approaches combining microdissection and CGH analysis (Kuukasjärvi et al, 1997) were prerequisites for the analysis of such early chromosomal aberrations in premalignant cells of other tumours like cervical carcinoma (Heselmeyer et al, 1996), breast cancer (Aubele et al, 2000b) and oral malignant lesions (Weber et al, 1998). Their application to premaligant lesions in prostate cancer has recently been described (Zitzelsberger et al, 1998; Kim et al, 1999), but for an improved understanding of mechanisms of tumour development and tumour progression more data on cytogenetic changes in thePIN–adenocarcinoma–metastasis sequence are needed.

The present study reports on the results of CGH analysis in 16 prostatic adenocarcinomas, 12 related PINs and 5 lymph node metastases. These investigations are aimed at a delineation of chromosome copy number changes in the non-neoplastic prostatic gland–PIN–invasive carcinoma–metastasis sequence.

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MATERIAL AND METHODS

Tissue samples

Formalin-fixed and paraffin-embedded tissue specimens of 16 adenocarcinomas of the prostate and of 5 related lymph node metastases were analysed. 12 intraepithelial neoplasias (4 low-grade and 8 high-grade PINs) adjacent to the primary tumours and non-neoplastic prostatic glands from 3 cases were additionally investigated. The histological classification and grading were performed on H&E-stained sections (Gleason and Mellinger, 1974; Sobin and Wittekind, 1997). Pathohistological data of cases are summarized in Table 1. Serial 5 μm sections of the tissue blocks were used for microdissection of tissue samples. For FISH analysis, consecutive 10 μm sections were analysed. Non-neoplastic glands, PINs and metastases were laser-microdissected and genomic DNA was amplified by DOP-PCR. DNA of extended areas of primary tumours was isolated from manually microdissected sections which provided sufficient DNA amounts for CGH without prior DOP amplification.

Laser-assisted microdissection

For microdissection, a laser microscope system (P.A.L.M., Wolfratshausen, Germany) was used consisting of a Zeiss Axiovert microscope (Zeiss, Jena, Germany), a pulsed UV-laser (wavelength 337 nm, maximum frequency: 20 pulses per second, pulse duration: 3 nanoseconds), and a computer-controlled micromanipulator. By means of the focussed UV-laser, unwanted cells or tissue areas, surrounding the cells of interest, were destroyed. Isolated cell compartments of 50 to 100 cells were subsequently collected. The cells were transferred into a sterile PCR reaction tube containing 20 µl laser buffer (100 mM Tris/HCl, pH 7.5, 100 µg ml^{-1} proteinase K). Microdissected probes were then heated for 3 h at 55˚C to allow proteolytic digestion and for 8 minutes at 100˚C to inactivate the proteinase K. Samples were stored at –20˚C until use.

DOP-PCR

DOP-PCR was performed according to a published procedure (Weber et al, 1998; Zitzelsberger et al, 1998). PCR reaction was carried out in a 50 µl reaction volume (3.5 mM $MgCl_2$, 50 mM KCl, 20 mM Tris/HCl, pH 8.4) containing the microdissected and pretreated cells in 20 µl laser buffer, 0.2 mM primer UW4B (5′-CCGACTCGAGNNNNNNATGTGG-3′), and 4 units Taq polymerase. After 40 PCR cycles (initial step for 10 minutes at 94˚C, 5 cycles with a low annealing temperature at 30˚C, 35 cycles with a high annealing temperature at 62˚C and a final extension step), the size of DNA fragments and DNA yields of each reaction were checked by agarose gel electrophoresis. DNA yields were additionally determined by fluorimetric measurements. To avoid contamination of PCR reactions, they were set up in a laminar flow using special aerosol resistant tips. PCR solutions were additionally checked for possible contaminations in PCR reactions without template DNA using gene-specific primers.

DNA extraction from paraffin-embedded tissue sections

For primary tumours, CGH was performed from paraffin-embedded tissue sections. The DNA was extracted according to standard procedures.

DNA labelling

Isolated whole genomic tumour DNA and DOP-PCR amplified samples were labelled with biotin-16-dUTP by standard nick translation. As reference DNA, SpectrumRed direct-labelled normal male total human genomic DNA (Vysis Inc, Downers Grove, IL) was used.

CGH and image analysis

Metaphase preparations for CGH analyses were obtained from peripheral lymphocytes of a healthy male donor according to standard procedures. CGH analysis was performed according to Kallioniemi et al (1992) and du Manoir et al (1993) with modifcations. 600 ng of biotin-16-dUTP labelled DNA and 600 ng of SpectrumRed direct-labelled normal male DNA were simultaneously hybridized with 25 µg unlabelled Cot-1 DNA (Life Technologies Inc, Grand Island, NY) to denatured lymphocyte metaphases for 3 days. Bound biotin-labelled DNA probes were detected by sequential incubations in Cy2-conjugated streptavidin/biotinylated anti-streptavidin (concentration: 10 µg ml^{-1} and 5 µg ml^{-1} in PNM-buffer consisting of PN-buffer plus 5% non-fat dry milk). Between each incubation step, slides were washed twice in PN-buffer (0.1 M sodium phosphate pH 8.0, 0.1% nonidet P-40). To obtain a fluorescence banding pattern, slides were stained with 4′,6′-diamidino-2-phenylindole (DAPI) at a concentration of 0.1 µg ml^{-1} in antifading solution. CGH images were captured by a black/white video CCD camera using on chip integration. The 3 colours were digitized consecutively with specific single colour filter combinations which were automatically changed on a Zeiss Axioplan2 microscope. For processing of captured images, a CGH analysis software from MetaSystems (MetaSystems, Altlussheim, Germany) was used. For one CGH analysis, 10 to 15 examples of each chromosome were measured after DAPI karyotyping of 5 to 10 metaphases. Average ratio profiles were then calculated after automatically scaling the profiles of individual homologous chromosomes of the same length. Average profiles were interpreted according to published criteria (Kallioniemi et al, 1994; Solinas-Toldo et al, 1996) using statistical confidence limits based on *t*-statistics.

Control experiments

DOP-PCR amplified DNA obtained from non-neoplastic prostatic glands with morphologically normal appearing prostatic epithelium was hybridized in 3 cases (Table 1) with non-amplified reference DNA (SpectrumRed™) to metaphase preparations. In these experiments no chromsomal changes were detected except for gains on chromosomes 1p34–36 and 19. Such regions of frequent artifactual appearance were excluded from further interpretation of data (see discussion). In addition, 4 cases of prostate carcinoma were comprehensively analysed using both DOP-PCR-amplified and non-amplified DNA. This comparison shows no significant differences for chromosomal changes detected by both methods. For additional control of the DOP-PCR approach microdissected normal epithelium present in tissues from other tumour entities like Barrett's adenocarcinoma (Walch et al, 2000) and ductal breast carcinoma (Aubele et al, 2000b) was investigated and revealed also CGH profiles without alterations.

Fluorescence in situ hybridization (FISH) analysis

FISH analyses with centromere and locus specific probes for c-myc (8q24), cyclin D1 (11q13), and HER-2/*neu* (17q11.2–q12) were performed to validate the CGH findings. Cases with copy number changes on chromosomes 8q, 11q, and 17q, known from the CGH experiments, were selected to validate these changes. Serial 10 µm sections of the tissue blocks were used for FISH analysis. Areas investigated correspond to those examined by CGH. Commercially available DNA probe kits were used for c-myc (Oncor, Gaithersburg, USA) and for centromere 8 (Oncor, Gaithersburg, USA), cyclin D1/centromere 11 (Vysis, Inc; Downers Grove; USA) and HER-2/*neu*/centromere 17 (Vysis, Inc; Downers Grove; USA). Signals from 150–200 tumour cell nuclei per specimen were counted using a confocal laser scanning microscope (Zeiss LSM 510). Nuclei from normal squamous epithelium or lymphocytes deposited separately on the same slide were used as controls for hybridization efficiency and specificity. The criteria established by Hopman et al (1988) were followed for signal enumeration. Amplification of the respective gene locus was considered for nuclei exhibiting at least twice as many locus-specific signals as centromere signals. More than two locus-specific signals accompanied by the same elevated number of centromere signals were considered to be indicative of polysomy. When the proportion of cells with nuclei

without any signal exceeded 20%, the procedure was regarded as insufficient and therefore repeated. For a detailed description of the FISH method used as well as for the evaluation by confocal laser scanning mircoscopy see Aubele et al (1997).

RESULTS

A summary of chromosomal imbalances detected in 16 adenocarcinomas, 12 PINs and 5 metastases is shown in Figure 1. 3 cases (Table 1) were also investigated for chromosome copy number changes in non-neoplastic prostatic glands for control; normal profiles were obtained in each of these samples.

The average aberration frequency (± SEM) in the 12 PIN areas was 4.3 ± 1.1. In PIN areas, gains were detected on chromosomes 8q (42%), 7 (25%), 16p (25%), 17 (25%), 19 (33%), 20 (25%), whereas losses were found on 13q (25%). Additionally, gains on chromosomes Xq (17%), 12q (17%), 15q (17%), 22 (17%), 1p, 4p, 11q (one case each), as well as losses on chromosomes 4q (17%), 2p, 3p, 3q, 5q, 6q, 8p, 10q, 12q, 18q and the Y chromosome (one case each) became apparent at lower frequencies. The changes showed a distinct heterogenic distribution, however, all of them were detectable in the corresponding primary tumours as well.

An average of 8.5 ± 0.9 gains and losses was detected in the 16 primary tumours. The following aberrations were identified in at least 25% of tumours: gains on 1p33–36 (38%), 12q24 (25%), 15q23–24 (25%), 16p12–13 (69%), 17 (50%), 19 (75%), 20 (50%), 22 (56%) and losses on 2q32 (25%), 4q28 (25%), 5q21 (31%), Xq (25%), 13q22 (56%), 18q21–23 (25%). Losses on 8p and 6q as well as gains on chromosome 7 and 8q were detected at lower frequencies. However, these aberrations occurred in the corresponding primary tumours as well as in PINs and/or metastases (Fig. 1).

The 5 lymph node metastases showed changes affecting the same chromosomal regions in a similar frequency (mean 7.8 ± 1.2). Common aberrations to primary tumours were gains on chromosomes 9q and of chromosome 16 and loss on chromosome 13q.

CGH data were exemplary validated on selected cases (4778/92, 10673/91, 10844/91) using a FISH approach on consecutive 10 µm sections and subsequent laser scanning microscopy (Table 2). Additionally, LOH analysis for D8S137 locus on chromosome 8p was carried out for validation of 8p loss (Table 2). To verify chromosomal gains on 8q21–24 (4778/92 carcinoma and PIN), 11q13 (10673/91 carcinoma and PIN, 10844/91 carcinoma) and 17 (4778/92 carcinoma), locus-specific probes for c-myc (8q24), cyclin D1 (11q13) and HER-2/neu (17q11.2–q12) were applied together with the respective centromere probes. In case 4778/92 an amplification of the c-myc locus could be detected in carcinoma and PIN areas in addition to polysomy of chromosome 17 in carcinoma areas. Cases 10673/91 (carcinoma and PIN) and 10844/91 carcinoma revealed polysomy of chromosome 11 and amplification of the cyclin D1 locus in case 10844/91. No locus-specific amplifications could be detected for the HER-2/neu locus.

DISCUSSION

Prostate cancer development and progression is supposed to be driven by the accumulation of cytogenetic and molecular genetic alterations. At the histological level, PINs outside the invasive carcinoma are considered as premalignant lesions of prostate cancer (Bostwick and Brawer, 1987; Bonkhoff and Remberger, 1996). In the PIN–carcinoma–metastasis sequence, PIN areas are poorly characterized for chromosomal alterations because they appear as very small cell compartments which can be only studied utilizing either microdissection and subsequent molecular genetic techniques like CGH (Weber et al, 1998; Zitzelsberger et al, 1998; Kim et al, 1999) or FISH on paraffin sections (Alers et al, 1995; Qian et al, 1996; Jenkins et al, 1997). In this study, we were able to demonstrate cytogenetic changes in 5 lymph node metastases and 12 PIN areas from 6 different cases. Chromosomal imbalances occurring in primary tumours were basically consistent with changes in PINs and metastases, and affected the same chromosomal regions (Fig. 1).

Common changes in the PIN–carcinoma–metastasis sequence became apparent and comprise losses on chromosomes 4q, 5q, 8p, 13q and gains on chromosomes 7, 8q, 12q and 15q. Losses on 2q and gains on 9/9q were only present in carcinoma and metastasis specimens and, thus, may indicate late events during tumorigenesis. Our data set on chromosomal changes in PIN areas provides clues that alterations reported as typical changes in prostate cancer

Table 1 Prostatic adenocarcinomas, PINs and lymph node metastases analysed

Case	Gleason Score	pTNM Classification[a]	Age	Tissue lesions analysed
15075/90	8	T3bN0	65	PT
635/91	6	T3bN1	64	PT, Met
5573/91	6	T3b N0	63	PT
5640/91	5	T3a N0	67	PT
9350/91	8	T2b N0	52	PT
14323/91	7	T2b N2	67	Met
15008/91	7	T3b N1	62	N, PIN high grade (3x), PT, Met
862/92	9	T4 N0	71	PIN high grade (2x), PT
1287/92	5	T3a N1	73	PIN low grade, PT, Met
4778/92	7	T3b N2	82	N, PIN low grade, PIN high grade, PT, Met
7757/92	4	T3a N0	65	PT
8039/92	9	T3b N0	66	PT
8385/93	3	T2a N0	71	N, PIN low grade (2x), PT
9971/93	8	T2a N0	61	PIN high grade (2x), PT
10601/93	5	T3a N0	67	PT
7632/94	5	T2a N0	55	PT
14624/94	6	T3a N0	70	PT

[a]UICC/TNM-Classification (Sobin and Wittekind 1997). N = Non-neoplastic prostatic glands; PIN = Prostatic intraepithelial neoplasia; PT = Primary tumour; Met = Lymph node metastasis.

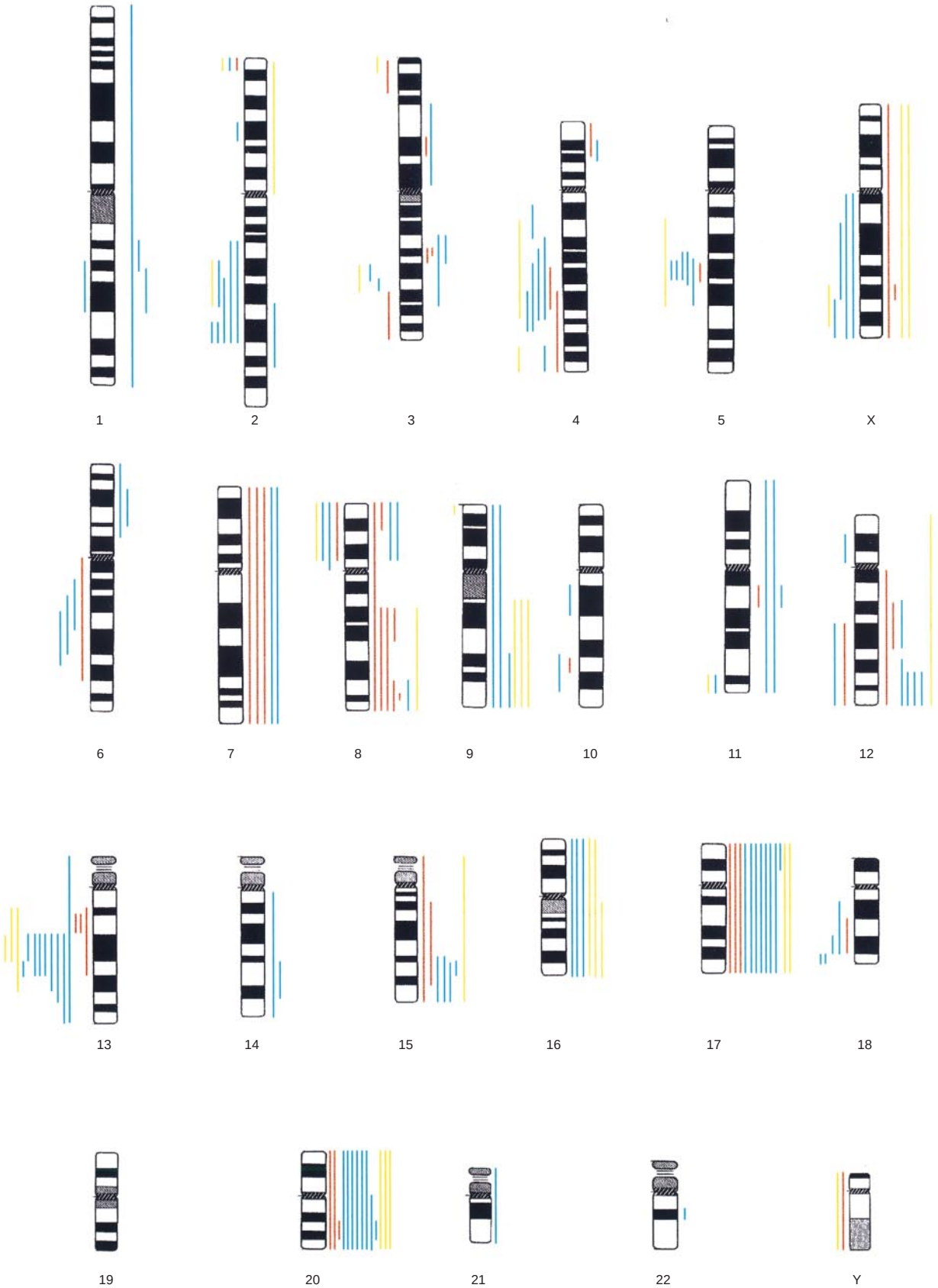
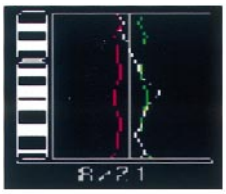
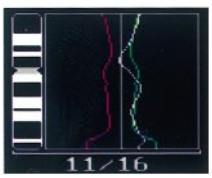


Figure 1 Chromosomal gains and losses in 12 prostatic intraepithelial neoplasias (orange), 16 prostatic adenocarcinomas (blue) and 5 lymph node metastases (yellow). Gains are indicated on the right side, losses on the left side of ideograms

Table 2 Confirmation of CGH results with FISH and LOH analysis

Case	Representative CGH findings	FISH analysis[1] CEP 8 signals per cell[#] (range)	c-myc	FISH analysis[1] CEP 17 signals per cell[#] (range)	HER-2/*neu*	FISH analysis[1] CEP 11 signals per cell[#] (range)	cyclin D1	LOH analysis on 8p*
4778/92 CA	+8q21-24, –8p21-23	2.4 (1–3)	5.0 (2–6)	2.3 (1–3)	2.0 (1–3)	nd	nd	AL
4778/92 PIN	+8q21-24, –8p21-23	1.9 (1–3)	4.2 (2–4)	1.7 (1–3)	1.9 (1–3)	nd	nd	AL
								
10673/91 CA	+11q13, +17	nd	nd	3.8 (2–5)	3.4 (1–4)	3.1 (1–4)	2.9 (1–4)	nd
10673/91PIN	–	nd	nd	2.2 (1–3)	1.8 (1–3)	2.2 (1–4)	1.7 (1–4)	nd
								
10844/9 CA	+11q13-14, +17	nd	nd	3.9 (2–5)	3.6 (1–4)	2.7 (1–4)	3.2 (1–4)	AL
								

CA = carcinoma. PIN = prostatic intraepithelial neoplasia. CEP = centromeric DNA probe. AL = allelic loss. nd = not determined. * investigation of microsatellite locus D8S137. [1]A gene locus was classified as amplified if there were more than twice locus-specific signals than centromere signals (ratio > 2) per cell nucleus. More than two locus specific signals accompanied by the same number of centromere signals was considered to be indicative of polysomy of the respective chromosome (ratio 1:1). [#]Mean number of signals per cell. CGH profiles: average profiles for chromosomes 8, 11 and 17 are exemplary demonstrated. Below each idiogram, the respective chromosome number (left) as well as the number of homologous chromosomes included in the calculation of the profile (right) are indicated. The red/green ratio is displayed as a white line together with thresholds for loss (red line) and gain (green line). Thresholds are calculated as statistical confidence intervals by the CGH software.

(losses on 8p, 13q, gains on 7, 8q) have a very early origin in PIN. Thus, a subset of PIN areas, irrespective of their differentiation into low- or high-grade PIN, exhibits a number of aberrations similar to invasive carcinoma. These CGH findings on PIN areas confirm for many of the chromosomal alterations earlier studies which investigated corresponding loci either with LOH (Macintosh et al, 1998; Saric et al, 1999) or FISH analysis (Alers et al, 1995; Qian et al, 1996). These data provide evidence for the biological significance of PINs and support the assumption that they represent premalignant lesions of prostate cancer. It might be therefore of prognostic value to survey PIN areas for their chromosomal aberrations.

The most frequent losses (25–56%) in the primary tumours were found on 13q22, 5q21, 2q32, 18q21–23, 4q28 and Xq; most common regions (25–75%) of chromosomal gains were detected on 12q and 15q (Fig. 1). Frequently observed gains on chromosomes 1p, 16p and of whole chromosomes 19 and 22 were not taken into account for the interpretation of data because they are known to represent frequently artifactual results in CGH analysis (Kallioniemi et al, 1994; Lichter et al, 1995; Weber et al, 1998). Frequently occurring gains of whole chromosomes 16, 17 and 20 reflect the aneuploid karyotype of the tumours. The frequent finding of gain on chromosome 12q is in good agreement with a recent publication (Sattler et al, 1999) reporting on frequent copy number gains in human prostate cancer. Losses on 8p and 6q as well as gains on 8q and of chromosome 7, which were considered to be typical aberrations for prostate cancer (for review see Bova and Isaacs, 1996), were also detected in primary tumours investigated in this study, but at a lower frequency. This fact can be partly explained by the smaller number of cases in our study compared to the literature data. The use of laser-ablation of unwanted cells surrounding the carcinoma area prior to CGH analysis might be a second reason for the difference between our and published data because stromal tissue, normal prostatic glands and PIN areas are removed before DNA extraction with this approach. A third difference to most of published prostate cancer cases is the fact that the majority of our cases do not represent advanced stages. Only 5 of 17 cases (29%; Table 1) are metastasizing cancers which might influence the cytogenetic results. With respect to the reported extensive genetic heterogeneity in prostate cancers (Qian et al, 1996; Macintosh et al, 1998) it is not surprising that changes detected in PIN areas coincidentially resemble more to 'typical' published aberrations in prostate cancers than the corresponding primary tumours. Altogether, our findings in primary carcinomas are confirmatory for losses on 13q, 18q, 5q, 2q and gains on 7, 8q.

In addition to these known hotspots of chromosomal copy number changes in prostatic adenocarcinoma, a novel region of chromosomal loss on 4q could be detected in our subset of cases. Deletions on 4q are frequent events in other tumour entities such as lung tumours (Petersen et al, 1997), renal carcinoma (Jiang et al, 1998), papillary bladder cancer (Simon et al, 1998), and appear to play a crucial role during aggressive progression of hepatocellular carcinoma (Piao et al, 1998). It is postulated that several, yet unidentified, putative tumour suppressor genes may be located on 4q (Hammoud et al, 1996). Although for other known altered chromosomal regions in prostate cancer (8p, 13q, 8q) candidate tumour suppressor genes and oncogenes have already been identified (multiple novel genes on the short arm of chromosome 8, *RB* gene on 13q14, *c-MYC* gene on 8q24), the identification of candidate genes in further chromosomal regions affected is still in its initial stage (Bova and Isaacs, 1996).

In summary, CGH analysis of 16 adenocarcinomas of the prostate revealed a series of known chromosomal imbalances in addition to a novel described loss of DNA sequences on chromosome 4q. Investigation of related PINs and lymph node metastases demonstrated an accumulation of chromosomal imbalances during cancer development and progression and an early origin of tumour-specific aberrations in PIN areas.

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