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BRIEF COMMUNICATION

Evaluation of Genetic Patterns in Different Tumor Areas of Intermediate-Grade Prostatic Adenocarcinomas by High-Resolution Genomic Array Analysis

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Prostate cancer is known for its highly heterogeneous histological appearance. Data concerning the cytogenetic content of areas with different histology are sparse. We have genetically evaluated 10 prostatic adenocarcinomas with intermediate histopathological grades (Gleason score 7) that showed two distinctive growth patterns with different pathologies, that is, Gleason grades 3 and 4 (G3 and G4). The G3 and G4 tumor specimens were taken from spatially separated regions within the cancer mass. Array-based comparative genomic hybridization (aCGH) was performed to obtain genotypes from the 10 pairs of G3 and G4 cancer areas. The cancer DNAs were retrieved from formalin-fixed and paraffin-embedded tissues allowing optimal recognition and selection of target cells. A genome-wide 2,400-element BAC array that provided high-resolution detection of both deletions and amplifications was used. In the 20 G3 and G4 areas, 252 genomic aberrations (88 gains, 164 deletions) were noted, of which 86 were concurrent in G3 and G4 areas (34% overlap). Ninety-five of the 252 alterations were defined by a single BAC clone (54 gains, 41 deletions). Overlapping changes were more frequent for deletions (46%) than for gains (13%). Frequent coinciding deletions ($\geq$ 20% of tumors) were seen on 8p (60%), 6q (30%), 1p (20%), 2q (20%), proximal 8q (20%), 10q (20%), 13q (20%), 16q (20%), and 18q (20%). A frequent overlapping gain ($\geq$ 20% of tumors) was detected on distal 13q (20%). The patterns of imbalance could be found to coincide in the G3 and G4 areas of the majority of cancers. Array-based CGH can be used as a tool for the evaluation of genetic patterns in prostate cancer. Supplementary material for this article can be found on the *Genes, Chromosomes and Cancer* website at http://www.interscience.wiley.com/jpages/1045–2257/suppmat/index.html.

Prostate cancer is the most commonly diagnosed male malignancy and the second-leading cause of cancer-related death in men in Western countries. Population-based PSA screening has resulted in a marked increase in the early detection of prostate cancer (Potosky et al., 1995; Schröder et al., 1998). Histopathologically, prostatic adenocarcinoma is marked by heterogeneity (Gleason, 1992; Ruijter et al., 1996; Hoedemaeker et al., 2000). Consequently, data are needed concerning the relation between morphologic and genetic heterogeneity.

Cytogenetic studies of prostatic adenocarcinoma have revealed trisomy of chromosome 7, del(7)(q22), del(8)(p21), del (10)(q24), and loss of the Y chromosome (Brothman et al., 1991; Lundgren et al., 1992). Loss of heterozygosity (LOH) analyses have shown frequent loss on chromosome arms 3p, 6q, 7q, 8p, 9p, 10pq, 13q, 16q, 17q, and 18q (Cooney et al., 1996; Cunningham et al., 1996; Vocke et al., 1996; Perinchery et al., 1999; Saric et al., 1999). Comparative genomic hybridization (CGH) analysis applied to a panel of both primary and recurrent tumors revealed losses of 8p and 13q in more than 30% of cases, whereas recurrent tumors showed gains of 8q and of chromosomes 7 and X, as well as loss of 8p in more than half of cases (Visakorpi et al., 1995). A CGH study of a panel of lymph node metastases showed loss of 8p, 10q,

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13q, 16q, and 17p, as well as gain of 1q, 3q, 8q, and 11p sequences in 50% or more of tumors (Cher et al., 1996). Some of these alterations could already be distinguished in the early stages of prostatic cancer (Alers et al., 2001). Fluorescence in situ hybridization (FISH) revealed numerical alterations of chromosomes 7, 8, 10, 16, 17, 18, X, and Y (Van Dekken et al., 1997; Brothman et al., 1999), as well as deletions and amplifications of specific chromosomal regions, for example, 8p22 (Macoska et al., 1994; Huang et al., 1996) and the MYC region on 8q24 (Jenkins et al., 1997; Bubendorf et al., 1999).

A limited amount of data is available on the (cyto)genetic heterogeneity of prostate cancers. In several studies flow cytometry revealed a significant degree of variation in DNA ploidy within individual cases (Kucuk et al., 1993; O'Malley et al., 1993; Warzynski et al., 1995). Another flow cytometry study showed that foci with different ploidies were infrequent in early prostatic carcinomas (Shankey et al., 1995). Intratumoral heterogeneity has been distinguished by LOH analyses of multifocal cancers (Macintosh et al., 1998; Ruijter et al., 1999). Different patterns of allelic imbalance between multiple foci of preneoplastic lesions in the prostate also have been discerned (Bostwick et al., 1998). A mutation analysis of *TP53* showed heterogeneity in intratumor distribution of primary cancers (Mirchandi et al., 1995), whereas the *PTEN* gene displayed mutational heterogeneity among different metastatic sites (Suzuki et al., 1998). In situ hybridization with centromeric DNA probes has shown considerable heterogeneity within cases of prostatic adenocarcinoma (Henke et al., 1994; Alers et al., 1995a). A FISH study of a tissue microarray revealed significant differences in *HER2/neu* amplification and gain of chromosomes 7, 8, and 17 between the regions of Gleason grades 3 and 4 (Skacel et al., 2001), and a marked heterogeneity within different foci of a single prostatic carcinoma has been detected with CGH (Zitzelsberger et al., 1998).

Archival prostate cancer specimens were obtained between 1986 and 1994. Ten primary tumors from patients who underwent radical prostatectomy were included in this preliminary study. The tumors were pathologically staged according to the pTNM classification (Sobin and Wittekind, 2002) and graded according to the Gleason grading system (Gleason, 1992). For this study G3 and G4 cancer areas were selected from spatially separated, different parts of a generally large tumor mass, that is, they were not taken from different tumor foci of a multifocal prostatic adenocarcinoma. Isolation of DNA from the formalin-fixed, paraffin-embedded tumor material was performed as described by Alers et al. (1997). Briefly, the tissue blocks were counterstained in 4′,6-diamidino-2-phenylindole (DAPI) 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 needle coupled to the microscope. Lower boundaries were checked for the presence of tumor on 4 μm H&E-stained tissue sections. Isolation of DNA from the formalin-fixed, paraffin-embedded material was performed using the Puregene DNA isolation kit (Gentra Systems, Minneapolis, MN) in accordance with the manufacturer's instructions.

Genomic target DNA was isolated from bacterial cultures and arrayed as described previously (Pinkel et al., 1998), except chromium-coated slides were used (Nanofilm, Westlake Village, CA). Each array consisted of 2,460 BACs spotted in triplicate with an average resolution of approximately 1.4 Mb (Snijders et al., 2001). The clones on the array have already been mapped on the UCSC genome assembly (http://genome.ucsc.edu/index.html) and thus could be computationally linked to the underlying and annotated genome sequence.

Array-based CGH with the archival specimens was performed as described previously (Paris et al., 2003). In brief, 1 μg of each test and reference male genomic DNA (Promega, Madison, WI) was labeled by random priming using a Bioprime Labeling Kit (Invitrogen, Carlsbad, CA). The manufacturer's protocol was followed with the following concentration changes; 120 μM of dATP, dGTP, and dCTP, 30 μM of dTTP, and 40 μM of CY3-dUTP or CY5-dUTP (Amersham Pharmacia Biotech, Piscataway, NJ). Random DNA octamers served as the primers (Invitrogen, Carlsbad, CA). Unincorporated nucleotides were removed using a Qiagen QIAquick PCR Purification Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. DNA was eluted in 100 μl of EB buffer (provided with the kit). Labeled test and reference DNA samples were coprecipitated in the presence of Cot-1 DNA (Roche, Indianapolis, IN) with ethanol. The precipitated DNA was redissolved in a hybridization solution containing 50% formamide, 10% dextran sulfate, 2× SSC, 4% SDS, and 10 μg/μl yeast tRNA. The probes were denatured at 72°C for 10 min and then preannealed with a 1-hr incubation at 37°C. Each array was surrounded by a wall of rubber cement on the chromium-coated slide and then was cross-linked (Stratagene UV

Stratalinker, 2,600 × 100 μJ). The hybridization mixture (60 μl) was added to each array. A rubber gasket and a glass microscope slide fastened to the slide provided an enclosed chamber for the hybridization. A 48-hr hybridization at 37°C was carried out on a unidirectional tilting platform (3 RPM) within an incubator. Slides were washed for 15 min in 50% formamide, 2× SSC, pH 7.0, at 45°C; 2× SSC, 0.1% SDS for 20 min at 4°C; and once in 0.1 M sodium phosphate buffer, 0.1% NP40, pH 8, for 10 min. The array was counterstained with a 1 μg/μl DAPI solution.

A CCD camera equipped with filters for CY3, CY5, and DAPI was used to capture the array-based CGH images. The imaging setup and custom software are described elsewhere (Pinkel et al., 1998). Imaging processing was performed with SPOT version 1.2 and SPROC version 1.1.1 software packages (Jain et al., 2002). Log_2 ratios of chromosomal gains and losses were listed by an algorithm using a flexible symmetrical threshold based on the standard deviation of the data sets of the specimens, followed by a simple smoothing procedure. This resulted in an optimal sample-dependent detection of genomic alterations with little interference from "noise." Array validation was performed by hybridization with normal human female DNA against normal reference human male DNA (Paris et al., 2003). The female DNA was extracted from paraffin-embedded tissue; the male reference was the same in all experiments.

We investigated 10 prostatic adenocarcinomas with intermediate histopathological grades (Gleason score 7) that showed two dominant growth patterns: Gleason grades 3 and 4 (G3 and G4). We used the Gleason grading system because the classification of tumor histology is more detailed than in other grading systems for prostatic adenocarcinomas (Table 1). Array-based comparative genomic hybridization (Pinkel et al., 1998; Wessendorf et al., 2002; Paris et al., 2003) was performed to obtain genomewide genetic profiles from the 10 pairs of G3 and G4 cancer areas. The cancer DNAs were retrieved from formalin-fixed, paraffin-embedded tissues, allowing optimal selection of the G3 and G4 tumor growth patterns. A 2,400-element BAC array was used that provided high-resolution detection of both deletions and amplifications. In the 20 G3 and G4 areas, 252 genomic aberrations (88 gains, 164 deletions) were revealed, of which 86 were concurrent in G3 and G4 areas (34% overlap; Table 2). Ninety-five of the 252 alterations were defined by a single BAC clone (54 gains, 41 deletions). Overlapping changes were more frequent for deletions (46%) than for gains (13%). Frequent coinciding deletions (≥ 20% of tumors) were found on 8p (60%), 6q (30%), 1p (20%), 2q (20%), proximal 8q (20%), 10q (20%), 13q (20%), 16q (20%), and 18q (20%). A frequent overlapping gain (≥ 20% of tumors) was detected on distal 13q (20%). The patterns of imbalances were compared in both genomewide and chromosome-specific fashions. A high degree of genomewide overlap between G3 and G4 regions, suggesting a monoclonal origin, was detected in tumors 1, 3, 4, 5, 6, 7, and 9, whereas relatively little coincidence was seen between those areas in tumors 2, 8, and 10 (Table 2; examples in Fig. 1; complete data sets of tumors 1 and 9 are available as supplementary material on the *Genes Chromosomes & Cancer* website at http://www.interscience.wiley.com/jpages/1045–2257/suppmat/index.html). Few alterations were noted in tumors 2 and 8. The patterns of imbalance on specific chromosome arms often suggested a (clonal) relationship between the G3 and G4 cancer areas. Examples are the identical 8p loss and 10q loss profiles in both Gleason grades of tumors 6 and 7, respectively, confirming the genomewide patterns (Fig. 2). It should be noted, however, that our cancers were generally large, and overgrowth by a dominant clone might have obscured the presence of underlying clones.

The presence of genetic heterogeneity among Gleason grades 3 and 4 regions in intermediate-grade prostatic adenocarcinomas was studied by array-based CGH (Pinkel et al., 1998; Wessendorf et al., 2002; Paris et al., 2003). The G3 and G4

TABLE 1. Tumor–Patient Data

Tumor	Age[a]	Gleason score[b]	Gleason Grade 1	Gleason Grade 2	Stage[c]
1	61	7	3	4	4
2	70	7	3	4	4
3	69	7	4	3	3
4	65	7	4	3	3
5	59	7	3	4	3
6	61	7	4	3	3
7	70	7	3	4	4
8	59	7	4	3	3
9	74	7	3	4	3
10	72	7	3	4	3

[a]Patient age at operation.
[b]Pathological grade defined by Gleason score, which is the sum of the first-most and second-most predominant tumor growth patterns, Gleason grades 1 and 2, respectively.
[c]Pathological stage according to TNM classification of the UICC (2002). T3: extracapsular extension of the tumor or invasion of seminal vesicle(s): T4: tumor is fixed or invades adjacent structures other than seminal vesicles, for example, bladder or rectum. All tumors are N0M0.

TABLE 2. Overview of Genetic Changes (Overlapping Alterations in Bold)

Tumor	Threshold[b]	Loss	Gain
1G3	0.50 (142)	**1p21–p32**, 1q41–q42, 4p13–p14, 4p15.1–p15.3, 4q32.3–q34, **5q11.2–q12, 5q14.1–q21**, 5q33.2, 6q12–q13, **6q15–23.2, 8p12, 8p21.3–p23.1**, **8q11.2–q12.1**, 11p15.2, 12q24.2, 13q13–q14.1, 13q14.3, 13q21–q31, 14q23–q24, **16q11.2–q24**	2p22.1–p22.2, 11q12–q13.3, 15q26.1
1G4	0.44 (53)	**1p13–p32, 5q11–q13, 5q14.1–q21, 6q22.1–q22.3, 8p12, 8p21.3–p23.1, 8q11.2–q12.1**, 12p11.2–p13.1, **16q21, 16q23**	13q32.2
2G3	0.41 (27)	7q31.1, **8p21.2–p23.2**, 14q24.2, 18p11.3	7p21.1, 20q13.1[a]
2G4	0.47 (2)	**8p23.2[a]**	19q13.2[a]
3G3	0.30 (106)	1p31–p32[a], **2q21–q22, 2q23–q24.2**, 5q12, 5q21, 5q23[a], **6q12–q16.1, 6q21–q22.3, 13q13–q14.1, 13q14.3–q22**	4p16,[a] 5p15.3,[a] 6p21.3,[a] 7p11.2–p14, 7p22, 7q11.2, 7q32.3,[a] 7q36.1–qtel, 9q32–qtel, 10q26.1, 10q26.3–qtel, 11p15.5[a], **11q13**, 14q32.2–q32.3, 15q23–q24,[a] 16q24,[a] 17p12, 17q25, 22q13.1[a]
3G4	0.30 (160)	1q41–q43, **2q21–q24.1**, 3q24–q26.1, 5q34[a], **6q22[a]**, 7p11.2–p13, 8p23.2–p23.3, 8p23.2–p23.3, 10p15,[a] **13q12.3–q14.3, 13q21.3–q33.3**, Xp11.4[a]	8q21.3–q24.3, **11q12–q13.2**, 18p11.3[a], 19q13.2,[a]
4G3	0.22 (224)	1q24–q31, 4p11–p13, 5q21–q31.1, **6q12–q22.3, 8p11.2–p23.1**, 8p23.2–p23.3, 10p12.3–p13, 10q22.3–q23.2, 15q23[a], 17p11–ptel	3q22–q26.3, 4q34[a], 5q33.2[a], 7q22–q31.1,[a] 10q26.2,[a] 13q14.1,[a] 13q21.3–q33.1, **13q34**, 15q15–q21.1
4G4	0.34 (32)	**6q12–q14, 6q16.3–q22.3, 8p12, 8p21.2–21.3**	**13q34**
5G3	0.23 (106)	**8p11.2–p12, 8p12–p23.1, 8p23.2–p23.3**, 10p12,[a] 10q21.3,[a] 10q23.1–q23.3, 10q25.1–q26.3, 14q22–q23[a], 17p12–p13.1, 17q25.1,[a] **18q12–q23**, 21q22.2–q22.3	13q32–q33.1, 13q34
5G4	0.34 (49)	**8p12–p21.2, 8p21.3–p23.1, 8p23.2–23.3**, 9q22.3–q31, 13q14.1, **18q12–q23**, 20p12.3–p13	11q12–q13.2
6G3	0.29 (31)	2p16–p21, **8p11.2–p12, 8p12–p22, 10q23**, 12q24.2,[a] 14q23, **16q23–q24**	8q24.2,[a] 11p11.2,[a] 13q32.2
6G4	0.27 (42)	5q11.2, **8p11.2,[a] 8p12–p22, 10q23,[a]** 11p15.2–p15.3, **16q22–q24**	7q22–q31.1,[a] 13q33, 16p13,[a] 16q21
7G3	0.32 (83)	3p21.3–p22, 3p24–p26, 3q26.2,[a] **10q23, 10q24–q26.3**, 11q23,[a] 13q13–q14.3, 13q21.3–q22, 17q24[a]	2q23.1,[a] 7q21.1,[a] 7q22–q31.1,[a] 7q31.2,[a] **8p21.3–p22**, 11p15.2–p15.4, 20p11–p12
7G4	0.47 (23)	**10q23, 10q24–q25.3, 10q25.3–q26.3**	**8p21.3–p22,[a]** 14q23.1,[a] 14q24.2,[a]
8G3	0.32 (7)	15q23,[a] 16p12–p13.1	11p15.3–p15.4, 13q32, 13q34[a]
8G4	0.33 (5)	21q21[a]	2q32.1,[a] 5p15.2–p15.3,[a] 8q24.1,[a] 13q21.3[a]
9G3	0.32 (260)	**1p36.1–p36.3, 2q21.3–q22.3**, 3p23–p25, 3p25–p26, 5q11.2–q13, 6q22.1–q22.3, **8p11.2–p23.3, 8q12.1–q13, 11q22.3–q23.2, 11q24, 12p12.3–p13.3**, 12q24.3, **13q12–q14.1**, 14q22.2–q32.1, 14q32.1–q32.2, 17q21.3,[a] **18q21–q23**	7q36.3,[a] 8q21.1–qtel, 11q12–q13.2, **13q32.2–qtel**, 15q25–qtel, 19p13.3,[a] Xp22.2[a]
9G4	0.36 (126)	**1p36.2–p36.3, 2q21.3–q22.3**, 6q14–q16.1, **8p11.2–p23.3, 8q11.2–q12.3**, 11q13.4,[a] **11q23.1–q23.2, 11q24**, 12p12.1,[a] **12p13.1,[a]** 12q21.1–q21.2, **13q12.3–q31**, 16p12–p13.1,[a] 16p13.2, 16q12.2,[a] 16q21,[a] 16q22–q23, 16q23–q24,[a] **18q12–q23**	**13q32,[a] 13q34**
10G3	0.40 (18)	5q14.1,[a] **10q23**, 16q21, 16q23–q24	1p36,[a] **2p21.1–p22.2,[a]** 5p13.3–p14,[a] 11q13.2–q13.3,[a] 15q26.1,[a] 19p13.3[a]
10G4	0.27 (144)	6q14–q21.1, **10q11.2–q25.3**, 10q26.1–q26.3, 11p11.2, 13q14.1–q14.3, 20p11.2–p13	2p23.3,[a] **2p21.1–p22.2,[a]** 5p15.2,[a] 8q21.1,[a] 13q32.2–q33.1, Xq24–q25

[a]Single BAC clone alteration.
[b]Threshold level; for example, 0.5 defines gains above +0.5, losses below −0.5 (log2 scale); between parentheses is the number of aberrant BAC clones.

samples were collected from the dominant growth patterns in the main tumor mass. The aCGH was accomplished with a 2,400-element BAC array, rendering a genomic resolution of 1.4 Mb (Snijders et al., 2001). This density of BAC clones along the genome appeared sufficient for a high-resolution analysis of both genome-wide and chromosome-specific patterns. The number of overlapping alter-

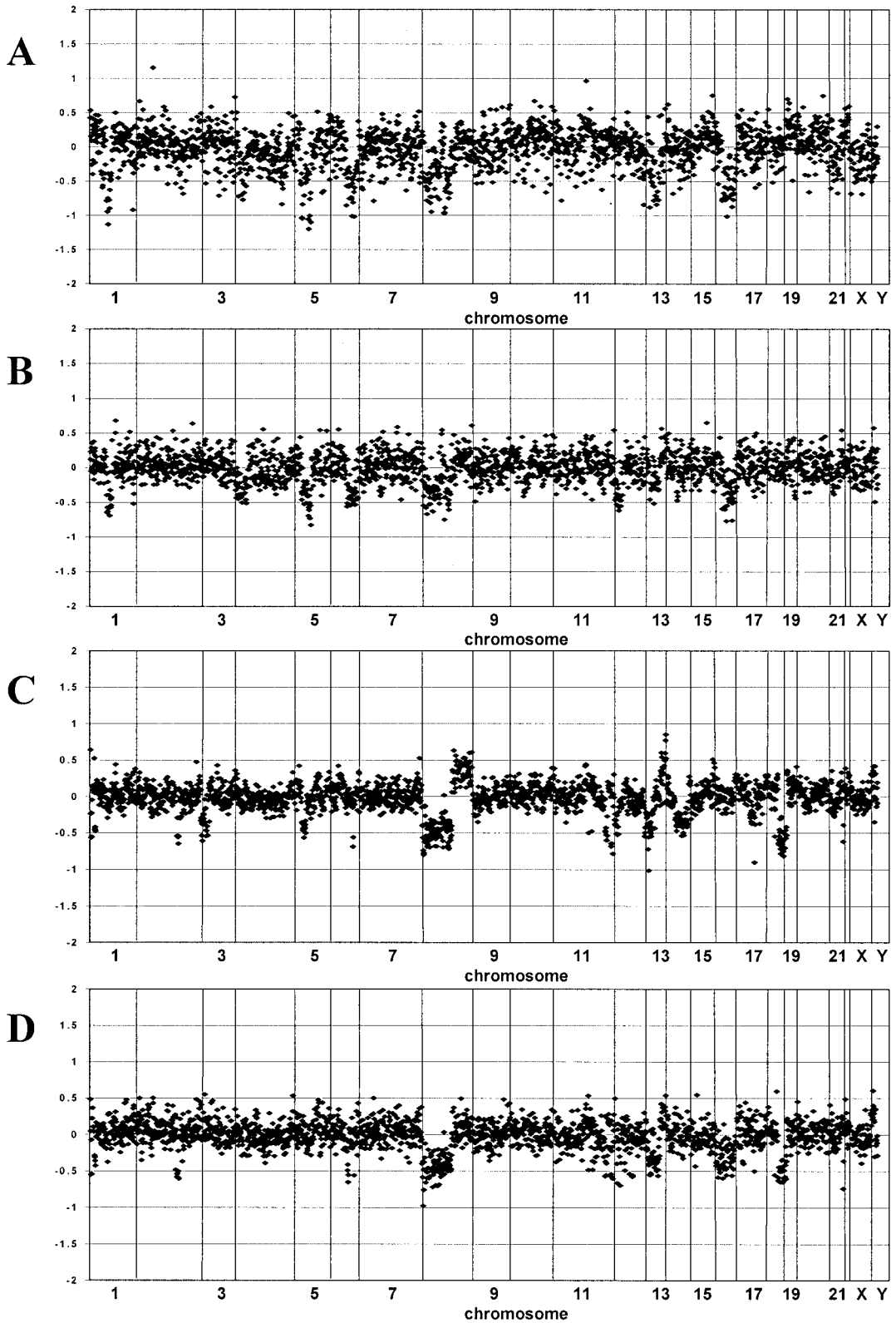

Figure 1. Array-based comparative genomic hybridization of G3 and G4 regions of prostatic adenocarcinomas. Log_2ratios along the genome are shown illustrating genomic imbalaces (raw data). A, B. G3 and G4 of tumor 1, respectively, depicting highly comparable profiles. C, D. G3 and G4 of tumor 9, respectively, also revealing comparable patterns. The complete data sets of tumors 1 and 9 are available as supplementary material on the *Genes Chromosomes & Cancer* website.

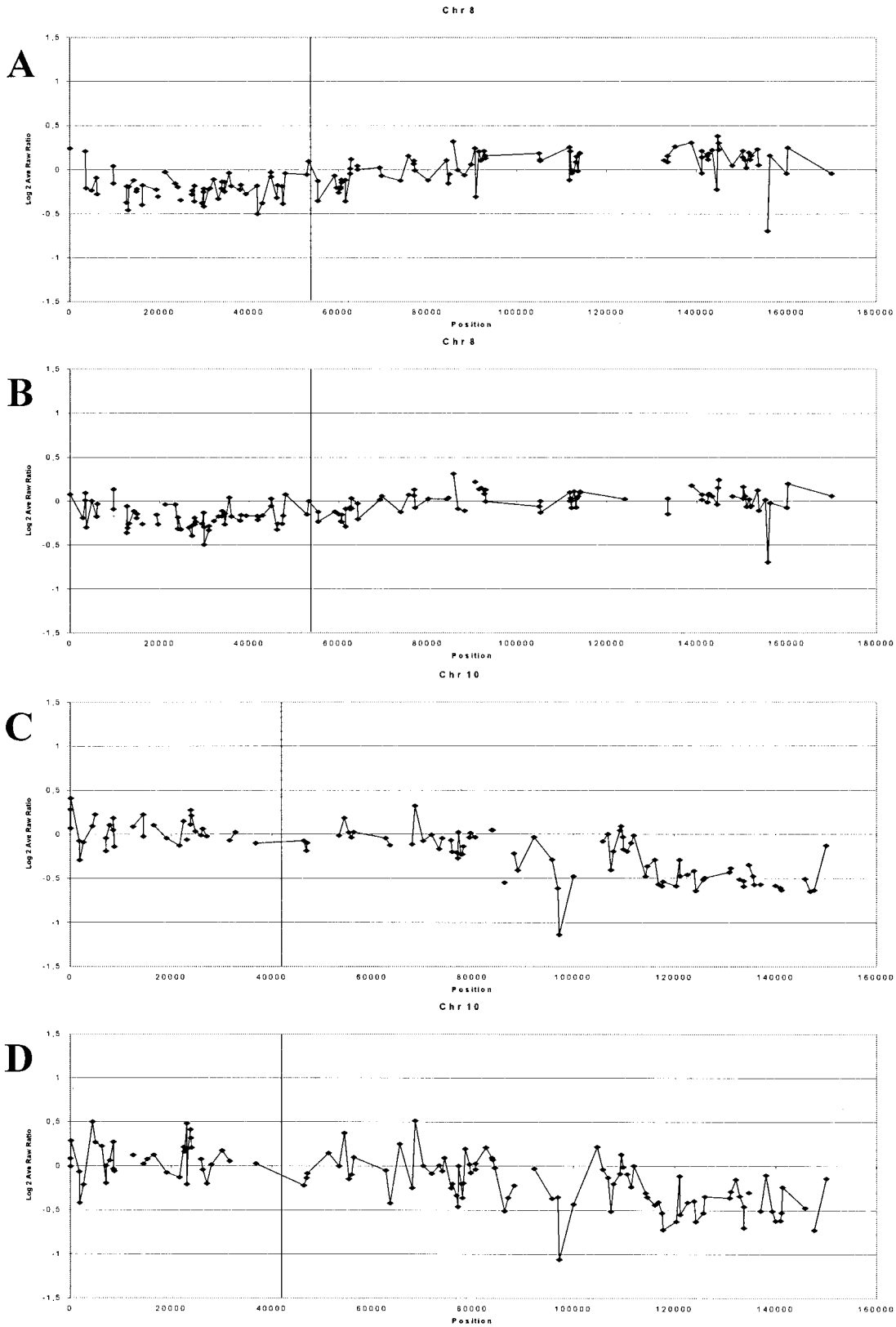

Figure 2. Array-based CGH of selected chromosomes shown as $\log_2$ratios (raw data). A, B. G3 and G4 of tumor 6 illustrating the same pattern of loss of chromosome 8p. C, D. Tumor 7, loss on 10q in both G3 and G4 being highest in the q23-PTEN region.

ations in G3 and G4 areas was found to be substantial. Evaluation of the global patterns of imbalances revealed a high level of concordance in the majority of tumors, especially for deletions. The latter phenomenon might be the result of the smaller number of single BAC clone deletions than of gains. Focal changes involving single BAC clones could be due to clone mismapping or inadequate hybridization. On the other hand, it could be a reflection of genetic instability. We used a filter for removal of noise (illustrated by the exclusion of the single BAC "loss" on distal 8q in Fig. 2A and B), but the presence of artifacts cannot be fully excluded. In our series, genetic heterogeneity can be seen in the G3 and G4 regions of individual cases. However, the high percentage of overlapping changes in the same cases strongly suggests a clonal relation. This is in agreement with Nowell's concept of genetic divergence during the clonal evolution of cancer (Nowell, 1976). The issue of clonality in prostatic adenocarcinoma is related to its presumed premalignant stage, that is, prostatic intraepithelial neoplasia (PIN). PIN lesions are found more frequently in cancerous than in noncancerous prostates, often in a multifocal fashion (Bostwick, 1995). Thus, a multifocal and polyclonal origin of prostate cancer is possible. An LOH study revealed heterogeneity of multiple foci of PIN lesions in the same cancerous prostate (Bostwick et al., 1998). On the other hand, in the same study, a similar pattern of allelic imbalance for at least one marker was detected in 21 of 22 matched PIN and adenocarcinoma foci. Similar observations were reported in an in situ hybridization investigation of prostatic carcinomas and adjacent PIN lesions (Alers et al., 1995b).

We studied intermediate-grade (Gleason score 7) prostatic cancers and found a relatively low level of heterogeneity within the 10 pairs of G3 and G4 regions. Heterogeneity might have been higher when tumor areas with stronger differences in histopathology were investigated, for example, G2 versus G5. However, the vast majority of Gleason score 7 cancers predominantly had growth patterns 3 and 4. Our low-level heterogeneity seems to contradict the (sparse) literature on the subject. DNA flow-cytometric studies showed high levels of intratumor heterogeneity of ploidy (Kucuk et al., 1993; O'Malley et al., 1993; Warzynski et al., 1995). However, this concerns variations in cellular DNA content, which is basically independent of heterogeneity at the chromosomal level. Intratumoral genetic heterogeneity was reported for LOH of 8p (Macintosh et al., 1998), whereas Ruijter et al. (1999) detected both concordant and discordant changes in allelic imbalance of multifocal prostate cancer lesions. However, a drawback of these investigations was the low number of genetic targets, making it difficult to differentiate between divergence within clonal evolution and the real polyclonal origin of tumors. This issue also relates to in situ hybridization (Henke et al., 1994; Skacel et al., 2001) and gene mutation (Mirchandani et al., 1995; Suzuki et al., 1998) studies in which only a few genetic loci were included. Our study demonstrates that a detailed genomewide approach is required to answer questions concerning genetic variations in cancer.

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