tumor—1q21, 6q21, 7p22, 8p21, and 16p13—have also been found in previous cases to be preferentially involved (Fig. 3). It is tempting to interpret these breakpoint similarities as an indication that the clones detected in the present case do represent dividing tumor cells.

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Mapping of Chromosomal Gains and Losses in Prostate Cancer by Comparative Genomic Hybridization

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Comparative genomic hybridization (CGH) allows detection of chromosomal imbalances in whole genomes in a comprehensive manner. With this approach, ten cases of prostate cancer (seven primary tumors and three metastases) were analyzed. Frequent chromosomal gains detected by CGH involved chromosome arms 7q, 8q, 9q, and 16p, and chromosomes 20 and 22, as well as frequent losses of chromosome arms 16q and 18q, in at least three of the ten cases. Overrepresentation of chromosome arm 9q has not been described in published reports. The CGH data were compared with results of a loss of heterozygosity (LOH) study, in which complete allelotyping was performed in the same prostate tumors with 74 different polymorphic markers. In general, a high concordance between the CGH and LOH results was observed (92%). Tumors revealing discrepancies by CGH and LOH analysis were investigated further by interphase cytogenetics, and the resulting picture regarding the genomic alterations is discussed in detail. *Genes Chromosom Cancer 14:267–276 (1995)*

INTRODUCTION

Prostate cancer is one of the most common causes of cancer death among men in Europe and North America. The incidence of this carcinoma has been increasing, probably because of the improvement of diagnostic tools as well as the launching of screening programs. The clinical course of prostate cancer is highly variable and unpredictable. Despite improved methods for detecting small, localized tumors in young patients, the results of surgical treatment have been discouraging: 25–30% of the patients have a relapse within 2 years, indicating a need for earlier diagnosis. On the other hand, prostate tumors might also persist in a clinically silent form, as observed primarily in older men. Distinguishing silent cases from early stages of small aggressive tumors that can be cured by radical surgery could provide a basis for more adequate adjustments of treatment protocols (Renfer and Thompson, 1994).

Analysis of genetic alterations might provide criteria for an improved classification of prostate tumors. Cytogenetic studies of cell lines and cultured primary tumors revealed losses of chromosomes 1, 2, 5, and Y and gains of chromosomes 7, 14, 20, and 22 as recurrent aberrations (Atkin and Baker, 1985; Gibas et al., 1985; Brothman et al., 1990; Lundgren et al., 1992). However, chromosome banding analyses are often hampered by difficulties in preparing metaphase chromosomes from prostate tumor cells of sufficient quality and quantity. Furthermore, normal metaphase cells are frequently found. Analysis of chromosomal aberrations in interphase nuclei by fluorescence in situ hybridization (FISH) with selected probes revealed gains of chromosomes 7, 8, 17, and X (Bandyk et al., 1994; Baretton et al., 1994; Brothman et al., 1994; Brown et al., 1994; Visakorpi et al., 1994) and losses of 1, 8p, 10, 12, 17, and 18 (Baretton et al., 1994; Brothman et al., 1994; Matsuyama et al., 1994). The deletions described in interphase studies correlate by and large with the data obtained by loss of heterozygosity (LOH) analysis (Macoska et al., 1994; Matsuyama et al., 1994). LOH was also frequently found on chromosome arms 8p, 16q, and 18q, and on chromosome 10 (Carter et al., 1990; Kunimi et al., 1991). Within 8p, a region containing a putative tumor suppressor gene relevant to prostate cancer was narrowed down to 8p22 (Bergerheim et al., 1991a; Bova et al., 1993; MacGrogan et al., 1994). Further evidence that tumor suppressor genes play a critical role in prostate cancer was derived from microcell-mediated transfer of human chromosome 8 or 11 into rat prostate cancer cells, resulting in the suppression of the metastatic ability of the microcell hybrids (Ichikawa et al., 1994a,b).

In order to obtain a comprehensive picture of the chromosomal gains and losses in prostate tumors, we applied the new approach of comparative ge-

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TABLE 1. Clinicopathologic Data on the Ten Prostate Tumors

Pt. no.	Tumor type	Grade[a]	Stage[b]	%[c]	No. chromosomal imbalances (CGH)	FAL[d]
1	Primary tumor	W-M	$T_3N_0M_0$	80	0	0.043
2	Brain metastasis	P	$T_xN_xM_1$	90	15	0.304
3	Primary tumor	U	$T_3N_0M_0$	80	4	0.185
4	Primary tumor	W-M	$T_2N_0M_0$	50	4	0.000
5	Primary tumor	M	$T_2N_0M_0$	70	0	0.037
6	Primary tumor	M	$T_3N_0M_0$	85	8	0.091
7	Primary tumor	M-P	$T_3N_0M_0$	60	5	0.080
8	Primary tumor	M	$T_3N_0M_0$	50	1	0.000
9	Brain metastasis	M	$T_xN_xM_1$	50	8	0.127
10	Lymph node metastasis	M-P	$T_xN_1M_0$	90	6	0.192

[a]Histologic grading was performed according to the World Health Organization classification (Mostofi et al., 1980). Differentiation grade: W, well differentiated; M, moderately well differentiated; P, poorly differentiated; U, undifferentiated cancer.
[b]According to the TNM classification (Hermanek and Sobin, 1987).
[c]Numbers represent the percentages of tumor cells in the respective samples.
[d]FAL, frequency of allelic losses/frequences of informative loci in LOH analysis (Bergerheim et al., 1991b).

nomic hybridization (CGH; Kallioniemi et al., 1992). In contrast to chromosome banding analysis or interphase cytogenetics, this approach relies only on the availability of whole genomic DNA from a cell population and does not require the use of specific DNA probes. For CGH, tumor DNA and normal genomic reference DNA are cohybridized to normal metaphase chromosomes, under conditions suppressing signals from repetitive DNA sequences, and are detected with different fluorochromes (Kallioniemi et al., 1992, 1993; du Manoir et al., 1993; Joos et al., 1993; Speicher et al., 1993). The ratio of the fluorescence intensities along the chromosomes is calculated by use of dedicated software applications (du Manoir et al., 1995; Lundsteen et al., 1995; Piper et al., 1995). Over- and underrepresented chromosomal regions in the tumor genome are identified by increased or decreased fluorescence ratio values, respectively.

The CGH results obtained in the present study were compared with data obtained by FISH of suspended nuclei isolated from paraffin-embedded tumor samples. In addition, a detailed comparison of CGH and LOH data sets derived from the same tumor cases was performed.

MATERIALS AND METHODS

Tumor Specimens

Ten cases of prostate cancer, including seven primary tumors, two brain metastases (cases 2 and 9), and one lymph node metastasis (case 10), were analyzed. The samples were selected from tumors that were estimated to contain more than 50% of tumor cells by histologic examination. Clinicopathologic data of the tumors analyzed are summarized in Table 1. None of the patients received endocrine therapy prior to surgery.

Comparative Genomic Hybridization

For CGH, the preparation of metaphase chromosomes, labeling of probe DNA, and in situ hybridization were performed as described (Lichter et al., 1995). Genomic test DNA (isolated from tumor material) and control DNA (isolated from normal tissue—peripheral blood leukocytes or normal tissue adjacent to the tumor region) were obtained from the same patient. Test DNA was labeled with biotin and control DNA with digoxigenin, respectively. One microgram of test DNA, 1 μg of control DNA, and 80 μg of human Cot-1 DNA were coprecipitated in ethanol and dissolved in a 12 μl hybridization volume. After denaturation, probe and Cot-1 DNA were allowed to preanneal for 15 min and were hybridized to denatured metaphase chromosomes for 48 hr at 37°C. After posthybridization washes in 0.1 × SSC at 42°C, biotinylated DNA was detected by streptavidin-conjugated fluorescein isothiocyanate (FITC), and digoxigenin-labeled DNA by mouse-anti-digoxigenin antibody conjugated to rhodamine (TRITC). After counterstaining of the chromosomes with 4,6-diamino-2-phenylindole (DAPI), specimens were embedded in an antifade solution (Vectashield, Vector Laboratories, Burlingame, CA).

Image aquisition and processing were performed as described previously (du Manoir et al., 1995). Briefly, separate digitized images of FITC and rhodamine fluorescence were obtained with a cooled charged coupled device (CCD) camera (Photometrics, Tucson, AZ) connected to an epifluorescence microscope equipped with selective filter sets. Chromosomes were identified interactively on inverted DAPI images, and the ratio of FITC/rhodamine fluorescence intensities was calculated along each individual chromosome with dedicated software. Ratios obtained from eight to ten metaphase cells were averaged, and the resulting profile was plotted next to chromosomal ideograms. Examples are shown in Figures 1E and 3. The thresholds for over- and underrepresentation correspond to the values expected for monosomy or trisomy in 50% of diploid cells. In previous CGH studies, where the percentage of monosomic or trisomic cells was established by other techniques, these thresholds were shown to be valuable and robust criteria for the diagnosis of chromosomal imbalances (Schröck et al., 1994; Bentz et al., 1995a,b; du Manoir et al., 1995).

There are some chromosomal regions known to be critical in CGH analyses. In particular, the distal part of chromosome arm 1p and chromosome 19 have been shown to give unreliable results. Furthermore, heterochromatin blocks, such as centromeric regions or the distal long arm of the Y chromosome, often show a very low signal intensity due to the high supression by Cot-1 DNA. This results in gross ratio variations in spite of only small variations in the fluorescence intensity (Kallioniemi et al., 1994; du Manoir et al., 1995). Accordingly, these regions were not considered for CGH analysis.

FISH to Interphase Nuclei

Five tumor cases (nos. 3, 4, 6, 7, and 10) were investigated further by interphase cytogenetics. Preparation of nuclei from tissue samples was performed as previously described (Heiden et al., 1991). Briefly, a 50 μm thick paraffin section adjacent to that used for histopathologic examination was analyzed. After deparaffinization and protease digestion, the suspension of nuclei was fixed in 70% ethanol and dropped on poly-L-lysine-coated slides.

The DNA probes used were the chromosome-specific centromeric repetitive DNA probes D9Z1 (chromosome 9), D8Z2 (chromosome 8), pMGB7 (chromosome 7), and pα10RP8 (chromosome 10). Two cosmid probes derived from chromosome arm 8p, lipoprotein lipase (LPL) (clone 114C11/ mapping to 8p22) and D8S7 (11E1/8p23), were kindly provided by S. Wood (Vancouver, Canada). Probe labeling, in situ hybridization, and signal evaluation were described in detail previously (Stilgenbauer et al., 1993; Matsuyama et al., 1994). Numerical chromosomal aberrations, as detected by the chromosome 8 centromeric probe, were diagnosed as monosomy, trisomy, or tetrasomy when the percentage of nuclei with one, three, or four signals, respectively, exceeded 10%. Deletions in chromosome arm 8p were diagnosed when more than 35% of the nuclei exhibited either one signal of the 8p cosmid probes (LPL or D8S7) and one signal of D8Z2 or fewer 8p cosmid signals compared with centromeric signals. Both the 10% and the 35% cut-off levels were set based on the upper limit (mean + 2 × SD) of decreased or increased fractions in benign tissue when the corresponding probes were used for FISH analysis (Matsuyama et al., 1994).

LOH Analysis

In previous studies by Kunimi et al. (1991) and Bergerheim et al. (1991a), complete allelotyping of 18 prostate tumors was performed. At least one polymorphic marker for each chromosomal arm was used, except for the short arms of the acrocentric chromosomes. Chromosome arm 8p and chromosomes 10 and 16 were analyzed with a higher density of markers, because these regions exhibit the highest frequency of LOH. Altogether, 74 polymorphic markers were used. The identical DNA preparations derived from ten of these tumors, selected for a high portion of tumor cells, were used for the present CGH study. Tumors 1–10 in the CGH analysis correspond to tumors 2, 3, 7, 10, 11, 12, 13, 14, 16, and 17, respectively, in the previous LOH study.

RESULTS

Comparative Genomic Hybridization

CGH was performed with ten prostate cancers, including seven primary tumors and three metastases. The analysis of one tumor (case 2) is illustrated in Figure 1, representing hybridization with tumor DNA (Fig. 1A) and control DNA (Fig. 1B), the ratio image indicating chromosomal gains and losses by applying a three-color look-up table (Fig. 1C), a copy number karyotype of the same metaphase spread (Fig. 1D), and the average ratio profile (Fig. 1E) of this tumor case.

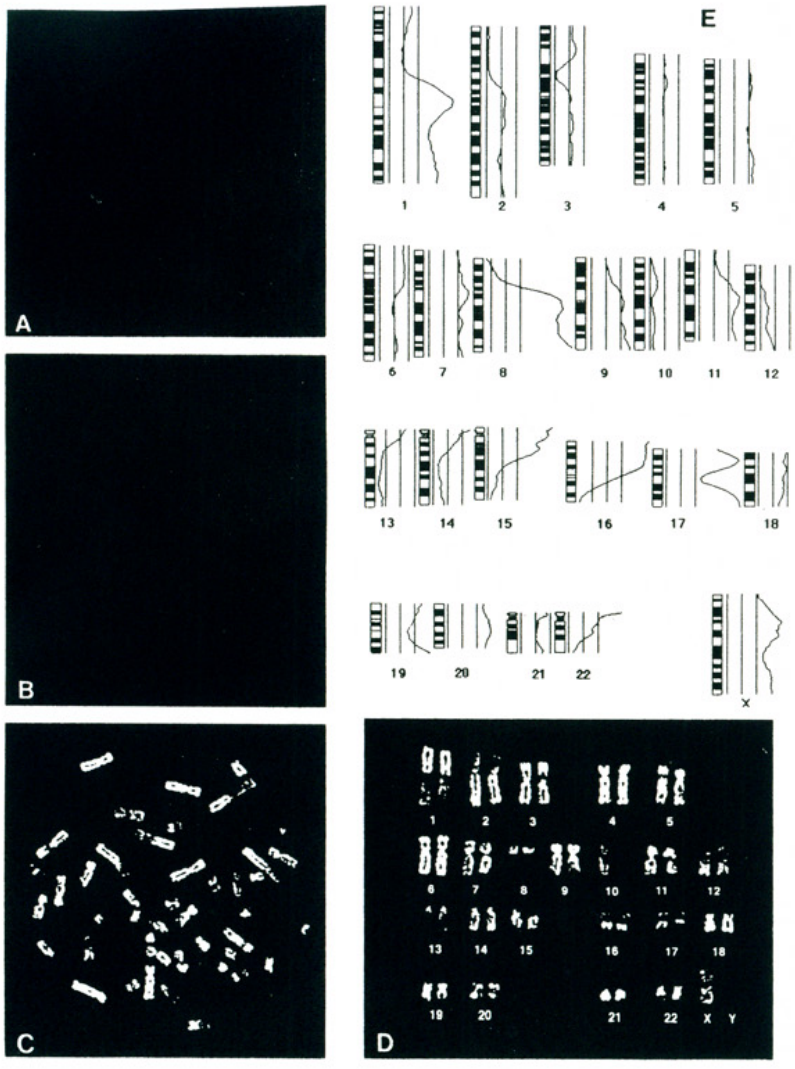

Figure 1. Example of the detection of chromosomal imbalances by CGH and digital image analysis of one prostate tumor case (case 2). **A:** Hybridization pattern of the biotin-labeled genomic tumor DNA detected by FITC. The image was acquired as a gray scale image with a cooled CCD camera and then digitally pseudocolored in green. **B:** Hybridization pattern of the control DNA labeled with digoxigenin and detected with rhodamine. The image was pseudocolored in red. Comparison of A and B reveals differences in the staining intensities among different chromosomes due to copy number changes within the tumor cells (gains of chromosome arms 1p, 8q, 9p, 11q, and 16p, and chromosomes 5, 7, 17, and 20, and losses of chromosome arms 2p, 8p, 12p, and 16q, and chromosomes 10 and 13). **C:** Applying a three-color look-up table to the ratio image of this metaphase cell allows visualization of chromosomal gains (green) and losses (red), as well as the balanced state of chromosomal copy number (white) in color. **D:** Chromosomes shown in C ordered in a "copy number karyogram." **E:** Ratios of the fluorescence intensities generated by tumor and control DNA were obtained from ten metaphase cells, and the average ratio profile was plotted along each single chromosome. The three vertical lines next to each ideogram indicate the balanced state of the chromosomal copy number (central line, ratio value 1.0) as well as the diagnostic thresholds (left line, ratio value 0.75; right line, ratio value 1.25) indicating chromosomal losses or gains, respectively. Shaded areas indicate heterochromatic chromosome regions in the centromeric and paracentromeric parts as well as on the short arms of the acrocentric chromosomes. These regions were excluded from evaluation (see Materials and Methods).

A summary of all chromosomal imbalances detected by CGH is shown in Figure 2. In eight of the ten tumors, at least one chromosomal imbalance was found. Overrepresented regions were observed about twice as often as underrepresented regions (35 versus 16 chromosomal regions). The most frequent imbalances were gains in the long arms of chromosome 7 and the X chromosome (each in four tumors); gains within 8q, 9q, 16p, 20, and 22 were each identified in three tumors, whereas chromosome arms 5p, 11q, 15q, and 17p showed overrepresentations in two tumors each. The chromosomal regions most frequently involved in losses of material were 16q and 18q (three cases) and 6q, 8p, 12p, and 13q (two cases). Overrepresentation of chromosome arm 9q has not yet been described as a recurrent chromosomal change in this tumor type.

The number of imbalances identified in each tumor is presented in Table 1. Metastases showed a higher number of imbalances (six or more); such a high number was only seen in one primary tumor of stage $T_3N_0M_0$. The frequency of allelic losses (FAL; Table 1) correlates in a similar way with tumor grade. These data are in agreement with a multistep tumor progression model with high accumulations of genetic alterations correlating with metastasizing tumors as described for colon (Fearon and Vogelstein, 1990) and prostate cancer (Visakorpi et al., 1995b).

Comparison of CGH and LOH Analysis

An extensive LOH analysis had been performed previously on the same DNA samples (Bergerheim et al., 1991a), allowing us to compare the results obtained with both approaches. For LOH analysis, 74 informative markers were used. This resulted in a total of 232 different chromosome arms with at least one informative locus. Markers on chromosome arm 1p and chromosome 19 are not included, because these regions were excluded from CGH evaluation (see above).

Figure 3 presents a comparison of all LOH identified (indicated as closed circles) with the average ratio profile of the corresponding chromosome. Furthermore, because chromosomes 8, 10, and 16 are most frequently found to be involved in LOH, these chromosomes are shown for all ten cases.

Results obtained by both approaches correspond in a high percentage of the chromosome arms (92%): in 88% (204/232), no LOH and no loss of chromosomal material as detected by CGH were found; in 4% (10/242) of the chromosome arms, LOH matched with a loss of chromosomal material by CGH (in Fig. 3, matching loci are summarized in subgroup I). For the remaining 18 chromosome arms, the results are discrepant. However, because some ratios are clearly shifted toward a diagnostic threshold (but not beyond the cut-off level), they are listed separately as a subgroup (II versus III in Fig. 3).

Interphase Analysis by FISH

In five cases (nos. 3, 4, 6, 7, and 10), sufficient tumor material was available for further cytological examination; therefore, aberrations of chromosome 8 could be analyzed in this subset of tumors by use of interphase cytogenetics (Table 2). The chromosome 8 centromere-specific probe D8Z2 was cohybridized with the cosmid probes representing LPL (in 8p22) and D8S7 (in 8p23), respectively.

As shown in Table 2, the mean number of chromosome 8 centromere signals was approximately 2 in three cases (nos. 4, 7, and 10), indicating disomy 8. In two of these cases (nos. 4 and 10), the mean number of interphase signals from other chromosomes (Table 2) was in agreement with a diploid status of the corresponding tumors. In cases 3 and 6, the mean numbers of chromosome 8 centromeres were 3.4 and 4.2, respectively. Because the CGH profiles indicate a balanced state for the corresponding chromosomes 8 (Fig. 3), these tumors are obviously polyploid. In case 6, the mean number of chromosome 9 signals suggests trisomy in at least a subpopulation of the specimen, which coincides with the profile of this chromosome in CGH (data not shown).

With regard to the short arm of chromosome 8,

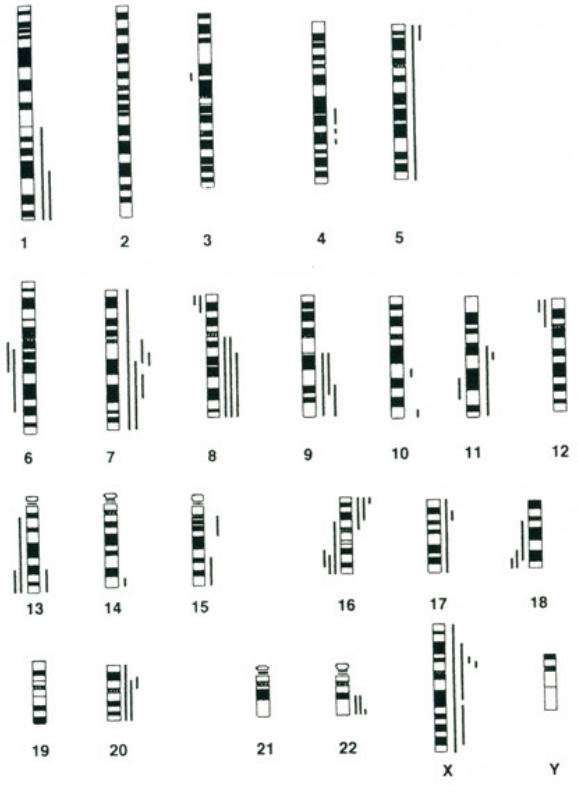

Figure 2. Summary of all chromosomal gains (vertical lines to the right of the chromosomal ideograms) and losses (vertical lines to the left of the chromosomal ideograms) detected by CGH in ten prostate cancers.

deletion of the LPL locus was found in all five tumors analyzed. In three of these, D8S7 was deleted as well. In one case, the deletion in 8p was confirmed by CGH, and in two tumors (nos. 3 and 7) the ratio values were suggestive of a deletion, but above the threshold of 0.75. In the remaining two cases (nos. 4 and 6), the deletion was not detected by CGH (Table 2). The explanation for these discrepancies and their impact on the application of CGH as a screening tool in prostate cancer are discussed below.

DISCUSSION

Up to now, knowledge on chromosomal aberrations in prostate cancer has been very limited. Accordingly, there are only a few candidate regions that are considered relevant to tumor pathogenesis, and candidate genes have not yet been defined.

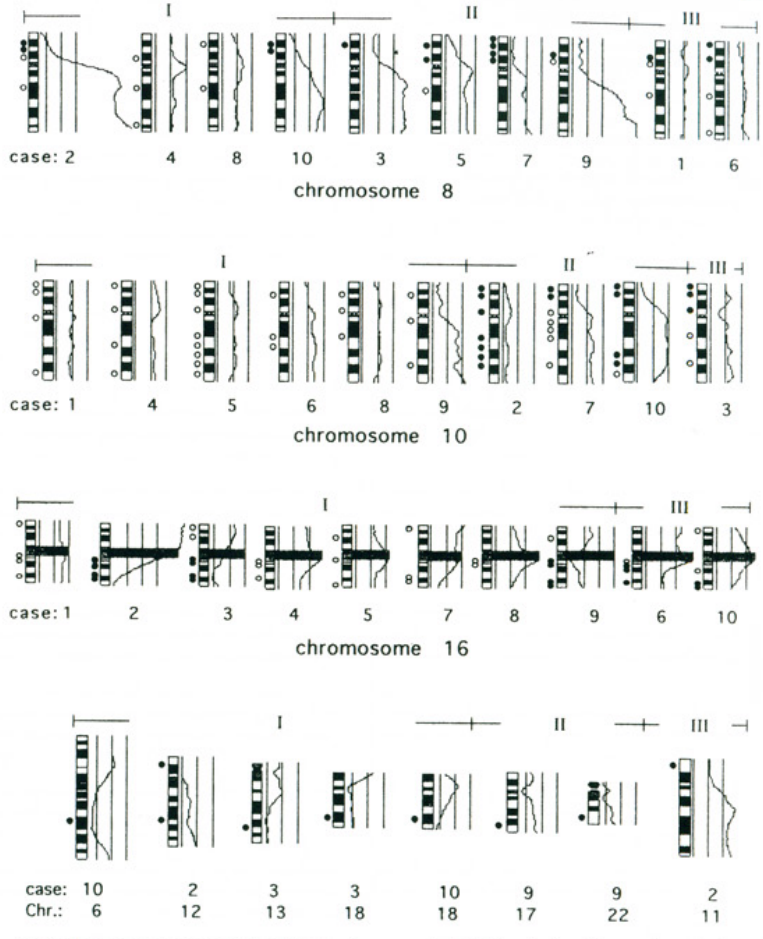

Figure 3. Examples of chromosomal and allelic imbalances as detected by CGH and LOH analysis. Results for all loci with LOH are shown. In addition, results regarding chromosomes 8, 10, and 16 are shown from all ten tumors analyzed. The positions of informative markers for the LOH analysis are indicated by circles to the left of the chromosome; closed circles indicate LOH. LOH on 8p, 10, and 16q was found in eight, four, and five tumors, respectively. Tumor cases are indicated by the numbers below the ideograms. Category I includes the cases in which LOH matches loss of chromosomal material as detected by CGH. In category II, LOH was accompanied by a decrease in the average ratio profile, which, however, did not exceed the diagnostic threshold, whereas in category III, LOH was not accompanied by a decrease in the ratio profile (for details, see Discussion). Two chromosomes had findings fitting two of the three categories (chromosome 10 in case 10 and chromosome 12 in case 2). They are listed on the basis of the respective findings on the short arms.

TABLE 2. Comparison of Interphase, LOH, and CGH Data

	FISH						LOH[b]				
	Mean no. signals/nucleus				Deletion of 8p[a]						
Pt. no.	D7Z1	D8Z2	D9Z1	D10Z1	LPL	D8S7	cen- PLAT	NEFL	LPL	D8S7 -tel	CGH[c] (results on chromosome arm 8p)
3		3.4			+ (54)	+ (66)	n.i.	n.i.	l.o.h.	n.i.	(+)
4	2.4	2.2	1.8	1.9	+ (52)	+ (54)	i	n.i.	n.i.	n.i.	−
6		4.2	2.5		+ (45)	− (27)	n.i.	l.o.h.	n.i.	l.o.h.	−
7		1.8			+ (39)	+ (47)	l.o.h.	l.o.h.	l.o.h.	l.o.h.	(+)
10		2.2	1.9	2.1	+ (43)	− (25)	n.i.	l.o.h.	l.o.h.	n.i.	+

[a] +, deletion; −, no deletion; the numbers in parentheses indicate the percentages of cells with deletions of chromosome arm 8p.
[b] i, informative; n.i., not informative; l.o.h., loss of heterozygosity.
[c] +, decrease in average ratio profile significant; (+), decrease in average ratio profile not significant; −, no decrease in average ratio profile.

This is mainly due to methodologic restrictions: chromosome banding analysis is hindered by the difficulty of culturing prostate epithelial cells, and FISH to interphase nuclei has largely been restricted to chromosome enumeration probes. Other molecular strategies, like the detection of LOH, are rather time-consuming because large numbers of sequences have to be analyzed for a comprehensive picture of allelic imbalances in a single tumor. CGH allows analysis of chromosomal imbalances in a comprehensive manner in a single experiment without the need to culture cells or the need for prior knowledge of the candidate regions to be investigated.

The regions most frequently involved in gains of material were chromosome arm 7q and the X chromosome (4/10 cases), as well as chromosome arms 8q, 9q and 16p, and chromosomes 20 and 22 (3/10 cases). The most frequent chromosomal losses were found on 16q and 18q (3/10 cases) as well as 6q, 8p, 12p, and 13q (2/10 cases). It should be noted that imbalances of chromosome arms 6q, 9q, and 16p have so far been identified as recurrent aberrations only by CGH.

The data regarding underrepresentation of chromosomal regions are in good agreement with two recently published CGH analyses on primary prostate carcinomas (Cher et al., 1994; Visakorpi et al., 1995a) despite the smaller number of cases in the present study. The only differences are as follows: 1) loss of chromosome arm 12p is not described in the other studies; and 2) losses of 16p and 17 are described in the other studies but were not found during the present analysis.

In the present study, gains were found about twice as often as losses. This is in clear contrast to the previously published CGH data showing chromosomal losses as the predominant imbalance. Furthermore, chromosome arms 15q, 20q, and 22q were previously not found to be overrepresented, and gains of 7q, 9q, and X were not recognized as recurrent imbalances by CGH in primary prostate tumors. However, with regard to 7q and X, our data, obtained mainly from advanced prostate tumors, resemble the imbalances found by CGH in tumors of hormonally treated patients (Visakorpi et al., 1995a). Thus, discrepancies between the different CGH analyses could be due to selection of the study material. However, increased copy numbers of chromosomes 7, 20, 22, and X were frequently observed in studies applying chromosome banding and interphase cytogenetics to tumors of various stages (Brothman et al., 1990; Sandberg, 1992; Visakorpi et al., 1994).

Comparison of the results from a previous LOH study with the same prostate tumors showed a high concordance (92%) with the CGH data. This percentage is similar to the data obtained in two very recent studies, with 88% (Cher et al., 1994) and 76% (Visakorpi et al., 1995a) concordance, comparing 322 and 37 informative loci, respectively. In these studies, the chromosome regions investigated were selected based on prior knowledge of LOH commonly occurring in prostate cancer. Thus, the higher concordance described in the present study can be attributed to a higher frequency of analyzed loci with no LOH and no loss of material detected by CGH.

CGH allows detection of loss of material, but not the loss of alleles, and therefore discrepancies between CGH and LOH data are expected. The discrepancies found in this study can be classified in two categories: in 18 of the 28 regions for which LOH was established, the ratio profile either did not exceed the diagnostic threshold (11 regions; see category II in Fig. 3) or showed no obvious decrease from the central value (7 regions; see category III and chromosome arm 10q in case 10 in Fig. 3). However, among the 11 regions in category II there are 5 (chromosome arm 8p of cases 5 and 7 and chromosome 10 of cases 2, 7, and 10) that would be diagnosed as loss of material when a threshold of 0.8 (instead of 0.75) is applied, a cut-off level frequently used in other CGH studies (see, e.g., Cher et al., 1994), or an even less stringent cut-off level of 0.85 (Visakorpi et al., 1995a).

There are three possible explanations for these discrepancies: 1) uniparental disomy, a result of allelic but not of copy number imbalance; 2) limited spatial resolution of the current CGH protocol (i.e., small deletions detectable by the polymorphic marker are not detected by CGH) that has been described to be in the range of 10–20 MB (Kallioniemi et al., 1994; Bentz et al., 1995b) or 20–30 cM (Cher et al., 1994); or 3) limited resolution of CGH regarding small copy number changes (i.e., when small copy number changes are present only in a subpopulation of the analyzed cells) (Kallioniemi et al., 1994; du Manoir et al., 1995). The last explanation is probably responsible for the cases listed under category II, whereas the cases in category III could be due to any one of the three possible explanations.

To elucidate the possible explanations for discrepant results further, we investigated a subset of five tumors by means of interphase cytogenetics regarding chromosome 8 (Table 2). The results of three cases were discordant with the data obtained by CGH.

In case 4, interphase analysis demonstrated a deletion of the region defined by the LPL and D8S7 sequences, whereas CGH did not detect any loss of the corresponding region. Because the data obtained with four chromosome enumeration probes indicate diploidy of the tumor cell population, the most likely explanation for the discrepant result is a small deletion that is missed because of the limited spatial resolution of CGH (see above). Another possible explanation is that the interphase analysis was carried out with a different part of the tumor exhibiting a different clonal composition. This constitutes a general problem when interphase analysis is combined with CGH in solid tumors, in particular when non-consecutive tissue sections are used.

In cases 3 and 6, interphase cytogenetics revealed deletions within 8p that were confirmed by LOH data. The extension of LOH, at least in case 6, argues against a small deletion that could be missed by CGH. Interestingly, the mean number of the chromosome 8 centromere probe together with the fluorescence ratios for these cases indicated polyploidy of the tumors (see above). Therefore, it is more difficult to detect small changes in copy number, as expressed, for example, by deletion in one of three or one of four chromosome copies (corresponding to theoretical ratios of 0.67 and 0.75 for a homogeneous cell population, respectively). Thus, the discrepancies in cases 3 and 6 could be explained by deletions within 8p in less than half of the chromosome 8 copies per cell. Such a distribution was observed by dual color FISH to interphase nuclei cohybridizing the chromosome 8 centromere probe with the LPL or the D8S7 cosmid probes. Furthermore, small copy number changes are in agreement with the data from LOH analysis, because in case 6, LOH of NEFL and D8S7 was defined by a decrease of only approximately 30% of the signal intensity on the hybridized Southern blot (data not shown).

The potential for detecting imbalances of chromosomal material by CGH in polyploid tumors is severely impaired in cases where gains or losses are present in less than half of the chromosome copies per cell. Even when the specimens are preselected for containing > 50% tumor cells, such a constellation could prevent the detection of relevant chromosomal aberrations. Since high portions of normal tissue and polyploidy of tumor cells are frequently encountered in prostate carcinoma, it seems advisable to perform an independent assessment of the ploidy status of prostate tumor specimens, as, for example, by flow cytometry or appropriate interphase analysis. In the case of polyploidy, diagnostic thresholds for CGH profiles should be considered carefully, and interphase analysis might have to be extended.

In conclusion, LOH analysis, CGH, and interphase cytogenetics represent complementary approaches that, when employed in combination, could greatly facilitate the comprehensive analysis of chromosomal imbalances in solid tumors.

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Germline Mutations in the *RB1* Gene in Patients With Hereditary Retinoblastoma

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We have analyzed the 27 exons and the promoter region of the *RB1* gene in familial or sporadic bilateral retinoblastoma by using single-strand conformation polymorphism analysis. For improvement over previous studies, a new set of primers has been designed, which allow for amplification of the coding and splicing sequences only. The positioning of the polymerase chain reaction (PCR) primers was such that the resulting PCR products were of different sizes, which enabled us to analyze two different exons simultaneously and still distinguish between the banding profiles for both (biplex analysis). By using this approach, we were able to identify mutations in 22 new patients, but the overall efficiency of the procedure when we used a single-pass regimen was only 48%. The mutations were small insertions and deletions and point mutations in roughly equal proportions.

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INTRODUCTION

The identification of mutations in the *RB1* gene in patients with retinoblastoma (Rb) is becoming essential for genetic screening and counseling for this hereditary pediatric eye tumor. Only 10–12% of Rb patients have a family history of tumors, and, for them, genetic screening with intragenic polymorphic DNA markers is already very effective (Wiggs et al., 1988; Scheffer et al., 1989; Onadim et al., 1990). However, for the remaining 85–90% of patients, identifying the causative mutation is the only means of determining whether their first-born children have inherited the predisposing mutation.

Identifying mutations in Rb patients also has a very important advantage for all family members; it unequivocally identifies those individuals who have not inherited the mutation. This means that they do not need repeated ophthalmologic examination in the early part of their lives. This is particularly relevant to those families among whom there is evidence of incomplete penetrance of the *RB1* gene, manifesting as unaffected mutant-gene carriers.

The ability to identify causative mutations in Rb patients has improved dramatically since 1989 with the publication of the exon-intron structure of the *RB1* gene (McGee et al., 1989). This information meant that polymerase chain reaction (PCR) amplification of each of the 27 exons of *RB1* was possible, and direct sequencing of these products successfully identified mutations (Yandell et al., 1989). Sequencing of the individual exons, however, was labor-intensive, and prescreening methods, such as single-stand conformation polymorphism (SSCP; Orita et al., 1989) and denaturation gradient gel electrophoresis (DGGE; Travstman et al., 1990), have been incorporated successfully into mutation-detection procedures. As a result of these developments, several reports have emerged demonstrating that the vast majority of hereditary Rb cases carry germline mutations that either generate premature stop codons or affect the correct splicing of the gene (Dunn et al., 1989; Yandell et al., 1989; Hogg et al., 1992; Blanquet et al., 1993; Onadim et al., 1993; Shimizu et al., 1994). Studies of tumor DNA samples confirmed that loss of function of both copies of the gene results in tumorigenesis (Dunn et al., 1989; Hogg et al., 1993; Kato et al., 1994; Su et al., 1995).

Although the technology is now in place for screening of large numbers of Rb patients, many centers specializing in the treatment and counseling of Rb families face a large backlog of patients, and methods for improving the efficiency of screening should be developed. In our analysis of mutations in Rb tumors, we noticed (Hogg et al., 1993) that C → T transitions in CGA-arginine codons, which generated TGA stop codons directly, were a common type of mutation. There are 14 CGA_{arg} codons in the *RB1* gene, and, in a study of 113 patients, we demonstrated that 15–18% of mutations were at one of the 14 CGA sites (Cowell

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