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Technical Advance

Correlation of Microscopic Phenotype with Genotype in a Formalin-Fixed, Paraffin-Embedded Testicular Germ Cell Tumor with Universal DNA Amplification, Comparative Genomic Hybridization, and Interphase Cytogenetics

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We present a strategy for the evaluation of numerical copy number changes of DNA segments within a solid tumor genome that allows the correlation of microscopic phenotype with genotype in formalin-fixed, paraffin-embedded tumor material. Cells from a human testicular germ cell tumor and adjacent tissue areas with normal seminiferous tubules were selected separately from microscopically analyzed histological tissue sections, and DNA was extracted from the selected areas. After universal DNA amplification, the amplification products were subjected to comparative genomic hybridization. The results confirmed balanced chromosome copy numbers for the normal tissue area, although the analysis of the tumor tissue area revealed numerous gains and losses of chromosome segments. The comparative genomic hybridization results were used to select DNA probes for interphase cytogenetics on serial sections. We conclude that this technique allows the screening of selected tissue areas for numerical DNA alterations, thus enabling a direct phenotype-genotype comparison. (Am J Pathol 1995, 146:1332–1340)

Present knowledge about genetic alterations is still rudimentary for many solid tumors (for review see Ref. 1). As for decades tissue sections have been formalin fixed and embedded in paraffin for pathological analyses, methods to screen this material for genetic alterations would increase our knowledge about genotypes of solid tumors considerably and would allow a direct comparison with morphological features.

Interphase cytogenetics on paraffin sections yielded data on numerical and structural chromosome aberrations at the single cell level, thus allowing the assessment of different clones and their histopathological correlations[2,3] (for review see Ref. 4). In addition, the polymerase chain reaction (PCR) has provided the means to screen small numbers of histopathologically defined cells or even single cells for specific genetic changes, such as the presence, de-

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letion, and/or amplification of DNA markers.[5–11] Both interphase cytogenetics and PCR methods are very useful as region- or locus-specific methods, in particular in cases for which knowledge of relevant alterations at the chromosome or DNA level are already available for the tumor type in question. A survey for unknown genetic alterations by these methods is very labor intensive, as it requires the testing of a very large number of probes covering the whole genome and thus is not useful for routine applications.

Recently, comparative genomic hybridization (CGH)[12] was developed as a new and powerful approach to screen whole genomes for over- and underrepresented DNA sequences[12–22], (for review see Ref. 23). CGH has the principal advantage that genomic DNA from tumor tissue is sufficient to establish a copy number karyotype. We have combined this technique with degenerate oligonucleotide primed PCR (DOP-PCR)[24] and demonstrated that genomic DNA from a small number of cells and even from paraffin-embedded archival tissue can be analyzed.[15] Here we present a further development of this strategy. It consists of the analysis of serial sections from a tumor: one for the histological evaluation, one for which, according to the histological evaluation, a small number of cells from areas of interest can be selected for DNA extraction, DOP-PCR, and subsequent CGH, and one or several serial sections for interphase cytogenetics with DNA probes selected according to the CGH results. The feasibility of this approach is demonstrated exemplarily by using a case of a testicular seminoma, the most frequent type of human germ cell tumor.

Materials and Methods

Tissue Samples

Tumor tissue and adjacent testicular parenchyma with normal seminiferous tubules from a 41-year-old patient were investigated (Figure 1). The tumor was a pure and classical seminoma. The tissue was fixed in buffered formalin (4% phosphate-buffered saline) for 4 hours and embedded in paraffin. This fixation was shown previously to yield good results for fluorescence *in situ* hybridization studies on archival tissue

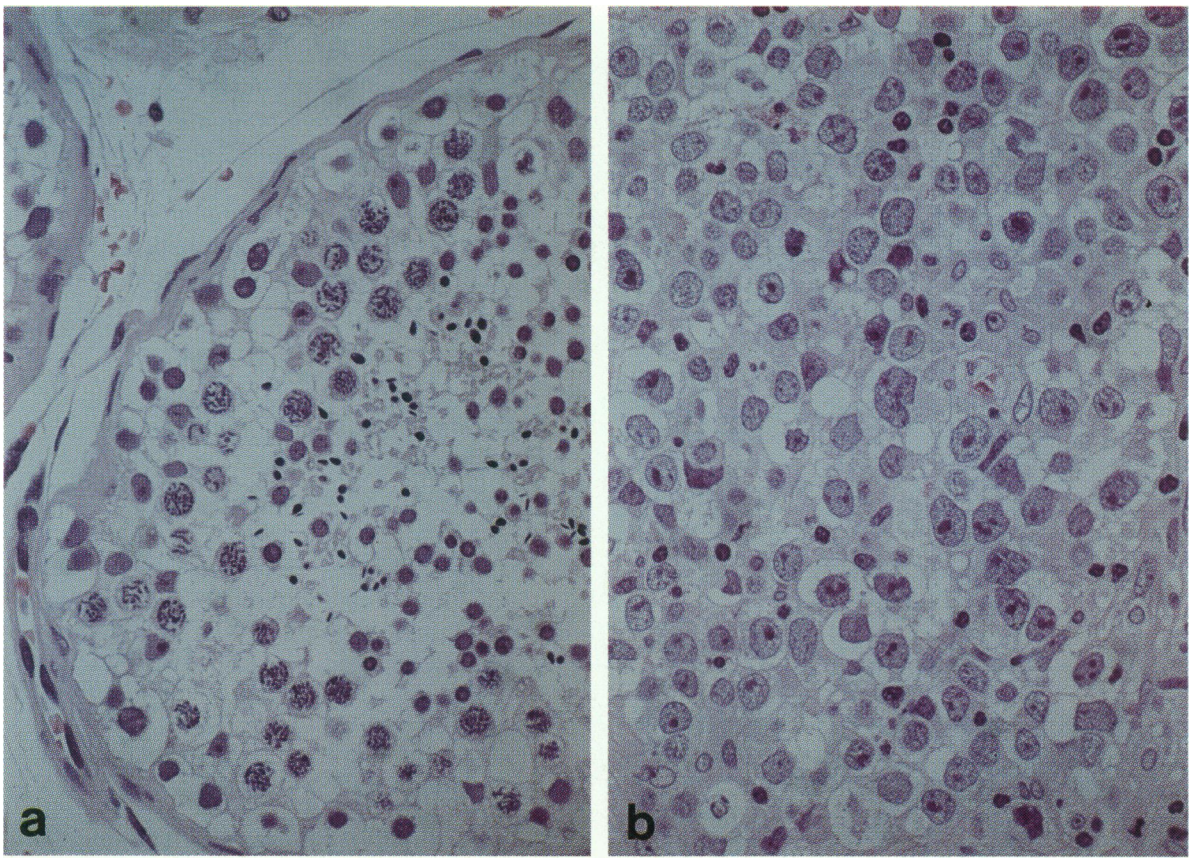

Figure 1. *Histological evaluation from one tissue section. Two areas were selected for subsequent molecular cytogenetic analysis (paraffin embedded, H&E, ×400).* a: *Area with seminiferous epithelium with intact spermatogenesis.* b: *Seminoma with typical cells; both cells and nuclei large with bright cytoplasm.*

material.[2,3] For our analysis several serial sections were prepared containing both normal testicular and tumor tissue. One section (2 μ) was stained with hematoxylin and eosin (H&E) for histological analysis. Areas of interest were identified on the H&E slide and the corresponding areas labeled on subsequent serial sections, 7–8 μ thick, for further analysis.

DNA Extraction

DNA was extracted as described previously.[15] Briefly, after dewaxing (3 × 10 minutes in xylol and 2 × 5 minutes in methanol), cells were dissected from labeled areas and collected in different Eppendorf tubes. The tissues were incubated overnight in 1 ml of 1 mol/L sodium thiocyanate. Formalin fixation is reversed in aqueous solutions,[6] but sodium thiocyanate, a strong protein-denaturing agent, which dissociates DNA-nucleohistone complexes,[25] was used in addition to improve the DNA yield from the archival tissue material. The tissue samples were washed in DNA isolation buffer (75 mmol/L NaCl, 25 mmol/L EDTA, 0.5% Tween 20), and an overnight digestion with proteinase K (10 mg/ml) was done. This was followed by phenol/chloroform DNA extraction and ethanol precipitation.

DOP-PCR

DOP-PCR was performed according to the protocol published by Telenius et al.[24] Briefly, PCR was done in a 50-μl reaction volume (2 mmol/L $MgCl_2$, 50 mmol/L KCl, 10 mmol/L Tris-HCl, pH 8.4, 10 μg/ml gelatin, 200 μmol/L of each dNTP, 1.7 μmol/L primer 6MW (5′-CCG ACT CGA GNN NNN NAT GTG G-3′), and 2.5 U of Taq polymerase) for 10 minutes at 93 C, followed by five cycles of 1 minute at 94 C, 1.5 minutes at 30 C, 3 minutes transition 30 to 72 C, followed by 35 cycles of 1 minute at 62 C and 3 minutes at 72 C, with an addition of 1 second per cycle to the extension step and a final extension of 10 minutes.

DNA Probes for Interphase Cytogenetics

Two DNA probes were applied that recognize tandem repetitive sequences in the centromeric regions of chromosomes 3 (pa3.5)[26] and 7 (p7t1).[27] Band-specific YAC clones HTY3114 (mapped to 16p12), HTY3138 (10p15), 924H12 (12p13.1), 2734 (Xq25), and A168H4 (3p25) were amplified with Alu-PCR according to published protocols.[28]

DNA Labeling

DOP-PCR amplification products, normal male reference DNA, YAC clones, and chromosome-specific repetitive DNA probes were labeled with biotin-11-dUTP or digoxigenin-11-dUTP by nick translation.[29]

CGH and Image Analysis

CGH analysis was performed as described in detail elsewhere.[13,21] Image analysis was done with an epifluorescence microscope (Zeiss Axiophot) equipped with a cooled CCD camera (Photometrics, Tucson, AZ; Kodak 1400 chip). Chromosomes were identified by the fluorescence banding pattern obtained after 4′,6′-diamidino-2-phenylindole staining. Fluorescein/tetraethylrhodamine isothiocyanate pixel by pixel ratio images (Figure 2) were calculated as described.[15,21] A symmetrical look-up table was used for visualization of the different ratios in individual metaphase spreads.

To test for the consistency of values outside the normal range, fluorescein to tetraethylrhodamine isothiocyanate average ratio profiles were calculated from 10 metaphases (Figure 3).[21] The central line in the profiles represents the modal fluorescence ratio for each reference metaphase spread, and the left and the right lines correspond to the theoretical ratio values for a monosomy and trisomy, respectively, in 50% of the cell population. These thresholds correspond approximately to the fluorescence ratio values for the transition between red/blue and blue/green shown in Figure 2, a and b.

Pretreatment of Paraffin-Embedded Tissue Sections for Interphase Cytogenetics

Pretreatment of paraffin-embedded tissue sections was done as previously described.[2] Briefly, all sections were attached on microscope slides treated with aminoalkylsilane. Paraffin was removed by xylene and methanol for 5 minutes each. The slides were air dried and then treated with 0.2 N HCl for 20 minutes at room temperature. This was followed by a proteinase K digestion for 10 minutes at 37 C at a concentration of 50 μg of proteinase K/ml. After rinsing with phosphate-buffered saline, the slides were dehydrated over a graded series of ethanols (70, 90, and 99%).

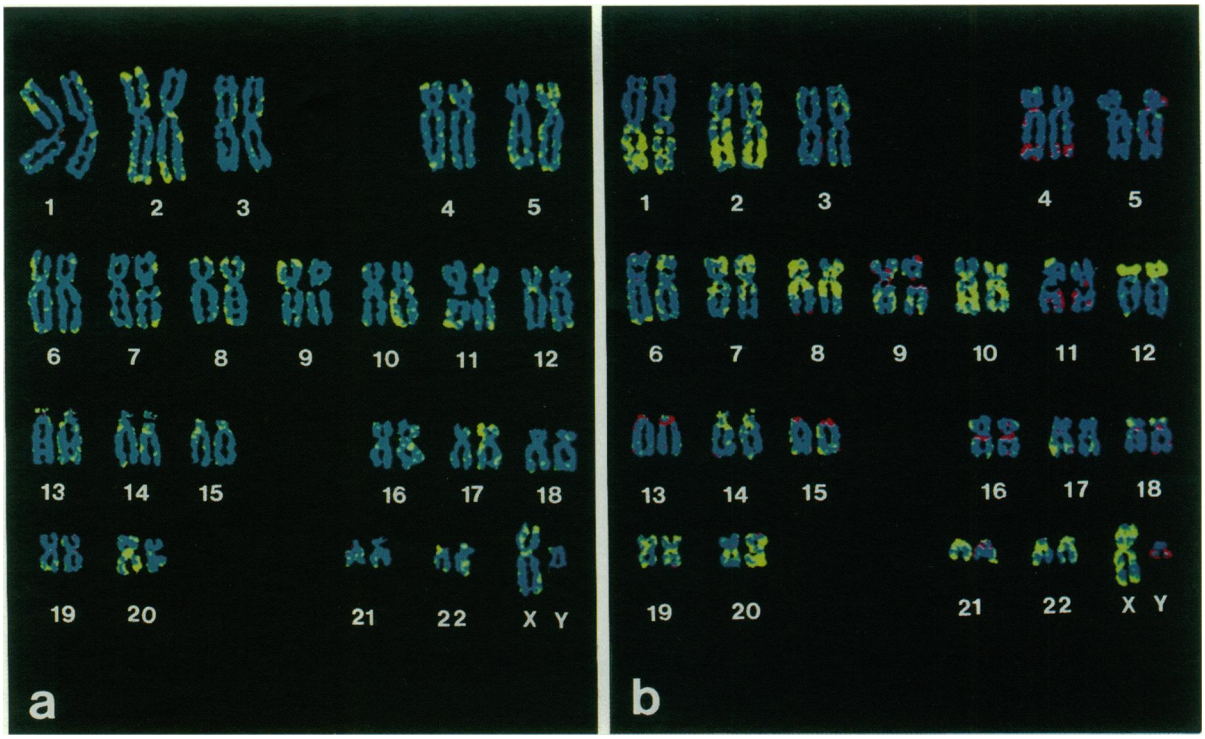

Figure 2. *Visualization of fluorescence ratio of a single metaphase by means of a look-up table. Chromosomes are arranged in a karyotype-like fashion. Blue color indicates the modal fluorescence ratio value. Green values suggest relative overrepresentation, red values relative underrepresentation of DNA sequences in the tumor genome.* **a:** *Fluorescence ratio karyotype of DNA from cells of the normal segment shown in Figure 1a. All chromosomes display the same fluorescence ratio values.* **b:** *Fluorescence ratio karyotype of DNA obtained from cells of the seminoma segment shown in Figure 1b. Overrepresentation of 12p, observed in seminoma and other germ cell tumors, is visible. Additional gains suggested from this analysis of a single metaphase include 1q, 2q, segments of chromosomes 7 and 8, chromosomes 10, 19, 20, 21, and 22, and the X chromosome.*

Confocal Laser Scanning Microscopy

Interphase cytogenetic evaluation was performed by using a confocal laser scanning microscope (Leica TCS 4D) equipped with an argon/krypton laser. The tissue samples were scanned at approximately 10 different levels with an average distance of 0.5 to 0.8 μ. In tumor regions with a very high density of nuclei, the counterstaining with propidium iodide did not allow the unequivocal delineation of the borders of the nuclei, making the assignment of signals to nuclei difficult or impossible. Therefore the evaluation was restricted to regions with a moderate nuclear density.

Results

DNA Extraction and DOP-PCR

An area with normal seminiferous epithelium (Figure 1a) and an area consisting of seminoma cells (Figure 1b) were identified and marked on parallel sections. The dissected areas for the tumor and the normal part covered an area of approximately 10 mm^2 each. As the thickness of the sections was 8 μ, the entire volume of the selected areas was less than 1 mm^3. The DNA was amplified by DOP-PCR.

CGH of the DOP-PCR Amplification Products

CGH was applied to test the DOP-PCR amplification products from the two tissue areas for relative copy number changes.

CGH performed with amplification products of DNA from the normal tissue area (Figure 1a) and genomic DNA from a normal male donor yielded balanced copy numbers for all chromosomes (Figures 2a and 3a).

In contrast, an overrepresentation of numerous chromosomes and chromosome segments was revealed by CGH of the amplification products from the tumor tissue area (Figures 1b, 2b, and 3b). The following regions were found to be overrepresented with fluorescence ratio values higher than the threshold as defined above: 2q22→qter, 8pter→8q21.3 with a large amplicon spanning 8q12→q21.3, chromosome 10, 12p, 20q, and the X chromosome.

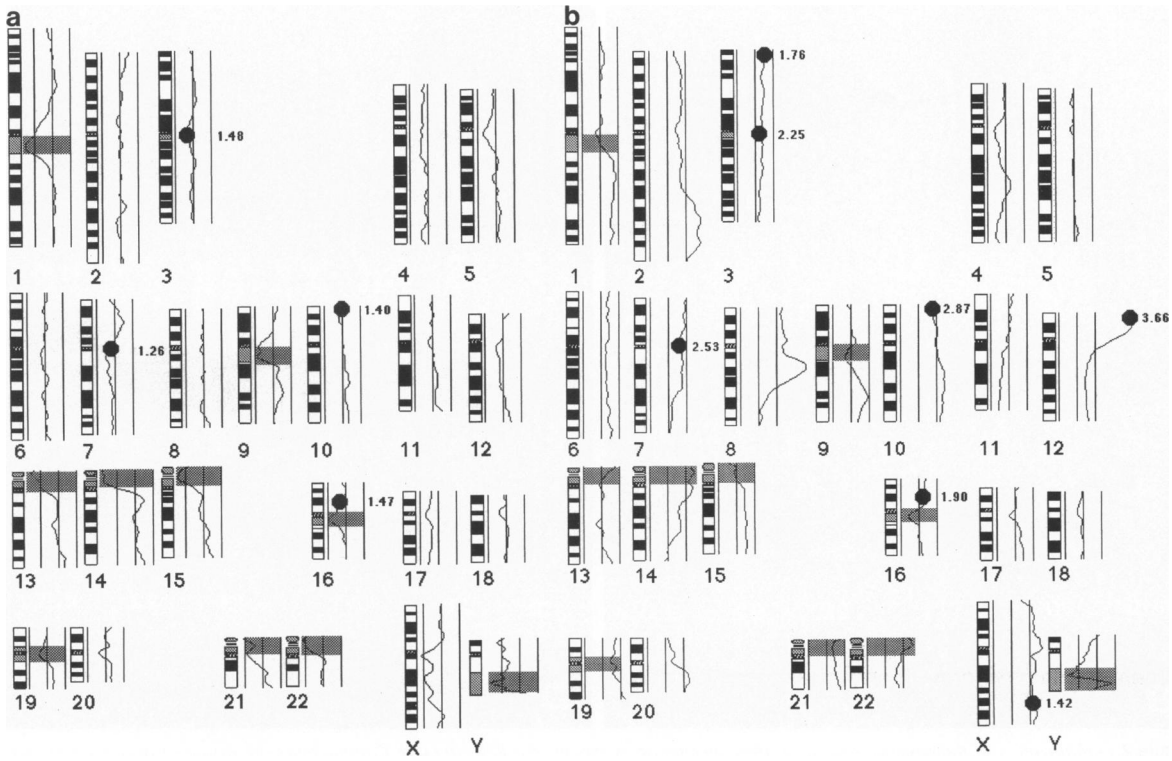

Figure 3. *Average ratio profiles calculated from 10 metaphase spreads. The gray shaded boxes represent chromosomal regions rich in heterochromatin that cannot be interpreted because of the abundance of highly repetitive DNA sequences. The three vertical lines on the right side of the chromosome idiograms represent different values of the fluorescence ratio between the tumor and the normal DNA. The central line represents the modal fluorescence ratio value, the right line corresponds to the theoretical ratio value for a trisomy in 50% of the cell population, and the left line represents the theoretical ratio value for a monosomy in 50% of cells.* a: *CGH average ratio profile of DNA from the normal seminiferous epithelium. No genetic imbalances are suggested.* b: *CGH average ratio profile of DNA from the seminoma, calculated from 14 different CGH metaphase spreads. Chromosomal gains include 2q22→qter, 8pter→8q21.3 (note the amplicon spanning 8q12→q21.3); chromosome 10, 12p, 20q, and the X chromosome. Additional gains with lesser ratio shift are observed for 1q, 2pter→q22, 7pter→7q31, 9q22→qter, 12q, 14q11.2→q24, and chromosomes 15, 19, 21, and 22. The ratio values for 11q are decreased.*

Some chromosome regions demonstrated elevated fluorescence ratio values but not beyond the threshold. These regions were 1q, 2pter→q22, 7pter→7q31, 9q22→qter, 12q, 14q11.2→q24, and chromosomes 15, 19, 21, and 22. 11q showed a decreased fluorescence intensity ratio value.

The central line of the ratio images represents the modal fluorescence intensity ratio value.[21] If the test DNA comprises complete chromosome sets from haploid, diploid, triploid etc. cells or any mixtures thereof, ratio profiles for each chromosome would be expected at the central line. Thus, CGH detects relative differences in copy numbers of individual chromosomes or chromosome segments but not differences in the ploidy levels between tumor and reference genomic DNA.

Testis tissue represents a case in point for which neither the cells of the normal seminiferous epithelium nor the seminoma cells must have a diploid chromosome set. During the course of spermatogenesis, some cells of the normal seminiferous epithelium have a diploid chromosome set, whereas others are haploid. The vast majority of seminomas investigated had chromosome counts in the triploid/tetraploid range.[30] These differences in the ploidy of the individual cells do not affect the balanced fluorescence ratio detected by comparison with reference DNA prepared from diploid somatic cells.[21]

Interphase Cytogenetics

Interphase cytogenetics was therefore applied to obtain information on the ploidy level of the seminoma studied. Three DNA probes were chosen that, according to the CGH analysis, hybridize to chromosome regions present in apparently balanced copy numbers, ie, an alphoid probe specific for the centromeric DNA of chromosome 3 (pa3.5) and YACs specific for bands 16p12 (HTY3114) and 3p25 (A168H4), respectively. Four other DNA probes, ie, an alphoid probe for the centromeric DNA of chromosome 7 (p7t1) and band-specific YACs for 10p15 (HTY3138), 12p13.1 (924H12), and Xq25 (2734),

were applied to delineate chromosome regions for which CGH had indicated increased ratio values. Scoring criteria were as outlined by Hopman et al.[25]

The results of interphase cytogenetics are summarized in Tables 1 and 2. For comparison with ratio profiles, average signal numbers are indicated in Figure 3 at the respective chromosomal sites.

Several features were interesting. The normal tissue consisted basically of cells with one or two signals for each probe. Usually, cells with one signal were observed only within the seminiferous tubules. Within seminiferous tubules, cells with two signals were observed at the tubule periphery and interpreted as spermatogonia or early stages of meiotic prophase before homologous pairing. Closer to the center of the tubule, the vast majority of cells presented only one signal and corresponded to spermatids.

Within the tumor tissue, DNA probes chosen for DNA segments close to the modal fluorescence ratio value showed mostly approximately two signals per cell (Figure 4b). For the DNA probes labeling the centromere of chromosome 7 and chromosome band 10p15, a large fraction of nuclei with three signals was observed (44 and 64%, respectively). The chromosome band-specific YAC for Xq25 yielded two signals in the majority of tumor cells (Figure 4a). YAC 924H12, specific for 12p13.1, yielded the highest signal per cell number of all DNA probes used. Most cells demonstrated more than two signals and, in a large number of cells, even six signals were observed (Figure 4, c and d).

Discussion

In this study we demonstrate the feasibility of a strategy for the CGH analysis of selected areas from paraffin-embedded, formalin-fixed tissue sections. After examination of a stained (H&E) section, areas of interest were dissected, DNA was extracted and, after universal DNA amplification with DOP-PCR, analyzed by CGH. DNA probes were selected according to the CGH results and interphase cytogenetics performed on serial sections. This strategy opens new avenues for molecular pathology. It enables the pathologist to establish copy number karyotypes from selected tissue areas with abnormal cells and compare them with other areas that may exhibit distinctly different histopathological features or, for control purposes, with areas of apparently normal tissue.

In tissue sections routinely prepared for histopathological diagnosis, many nuclei are cut. Because of random chromatin losses in the population of cut nuclei, copy number karyotypes should not be affected in cases in which thousands of cut nuclei are pooled for DNA amplification. Such an effect, however, needs to be taken into account if only a few or, in case of a further improvement of this method, even single nuclei are used to establish a copy number karyotype. In such cases, it is essential that CGH is performed with DNA amplified from intact nuclei.

In the example presented here, two areas, each comprising a few thousand cells, were selected from different parts of one 8-μ section. Thresholds used in this study were defined to detect copy number changes present in at least 50% of the cells pooled for DOP-PCR.[21] Although the fluorescence ratio profile established for the tumor area indicated numerous gains but no losses of genetic material (Figures 2b and 3b), normal seminiferous epithelium showed no indication for chromosomal imbalances (Figures 2a and 3a).

Previously, we did detailed comparisons of CGH results with DNA extracted from archival material after DOP-PCR with established technologies, such as cytogenetics and Southern blot analysis.[15] Hence, the CGH results should reflect accurately the relative copy numbers of DNA segments in the tumor and normal tissue, respectively. To control further the accuracy of our approach, interphase cytogenetics was used. In addition, interphase cytogenetics allows the establishment of the exact degree of ploidy and gives information on the single cell level.

The results of interphase cytogenetics are compatible with the assumption that tumor cells in this seminoma were mostly hypotriploid. Three probes applied for apparently balanced regions with a fluorescence

Table 1. *Summary of Interphase Cytogenetics Results for the Normal Seminiferous Tubules*

DNA probe	Probe location	Signals per cell							Average signal number
		0	1	2	3	4	5	6	
pa3.5	3 centromere		15	17	2				1.48
A168H4	3p25	11	69	16					1.18
p7t1	7 centromere		20	7					1.26
HTY3138	10p15		29	20	1				1.40
924H12	12p13.1	11	70	13					1.16
HTY3114	16p12		25	22					1.47
2734	Xq25	68	31						0.36

Table 2. *Summary of Interphase Cytogenetics Results for the Seminoma Cells*

DNA probe	Probe location	Signals per cell							Average signal #
		0	1	2	3	4	5	6	
pa3.5	3 centromere		1	23	5	2			2.25
A168H4	3p25	12	38	117	8				1.76
p7t1	7 centromere		3	12	14	3			2.53
HTY3138	10p15		1	6	20	4			2.87
924H12	12p13.1	3	10	23	21	11	15	28	3.66
HTY3114	16p12		4	23	2				1.90
2734	Xq25	13	41	62					1.42

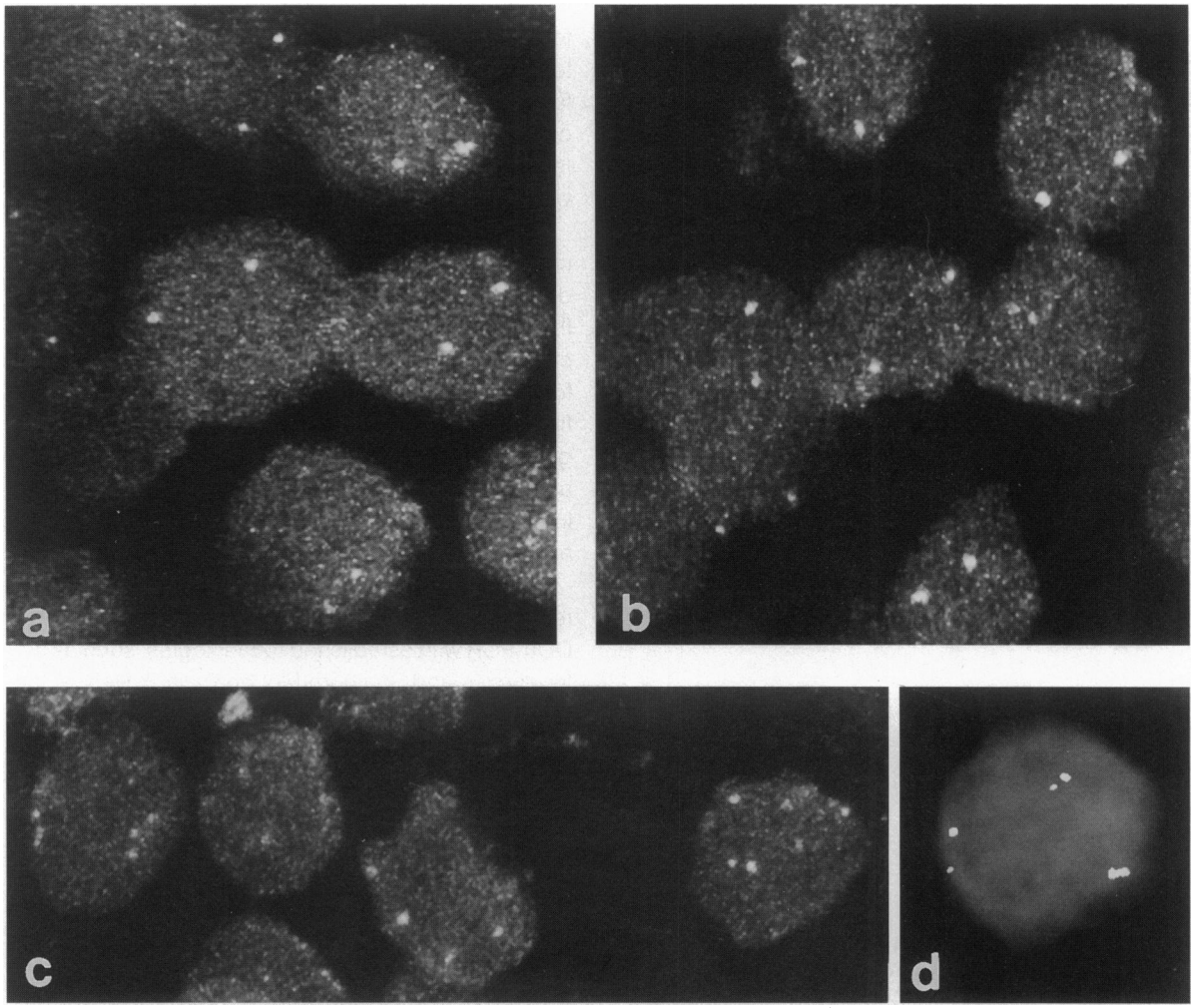

Figure 4. *Paraffin sections (7 to 8 μ) of formalin-fixed seminoma cells after* in situ *hybridization with several YAC clones.* **a** *to* **c:** *Images obtained with a laser scanning microscope.* **a:** *Hybridization with YAC clone 2734 mapped to Xq25. Most seminoma cells show two signals.* **b:** *YAC clone A168H4 (3p25) yields two signals in the majority of seminoma cells.* **c:** *Hybridization pattern of YAC clone 924H12 (12p13.1) on seminoma cells. Three to six signals are visible.* **d:** *Single seminoma cell after hybridization with the same chromosome band 12p13.1-specific YAC clone 924H12. This image was obtained with a CCD camera for better visualization of the hybridization pattern observed in many nuclei. Two signals were often co-localized as shown, suggesting the presence of an isochromosome i(12p).*

ratio close to the central line, namely 3c, 3p25, and 16p12, revealed a majority of nuclei with two signals, although a few nuclei clearly exhibiting three signals may suggest a small cell population trisomic for these regions as well. Two probes for regions with fluorescence ratios close to the upper threshold, ie, 7c and 10p15, yielded a large fraction of nuclei with three copies. Notably, reliable fluorescence ratios are difficult to obtain for the telomeric regions of chromosomes. The ratio profile established for chromosome 10 was above the upper threshold except for the distal end of the short arm. The combined data of CGH

and interphase cytogenetics therefore indicate a trisomy 10 in the majority of tumor cells.

In addition, interphase cytogenetics clearly demonstrated a duplication of chromosome X in the majority of tumor cells. The overrepresentation of 12p seen with CGH is a well known hallmark alteration in germ cell tumors.[31] Corresponding to the CGH results, the band-specific YAC for 12p13.1 yielded elevated signals per cell number in most tumor cells. Large fractions of cells showed three to six signals, which might indicate a considerable heterogeneity within this tumor.

Two restrictions should be kept in mind for a proper interpretation of interphase counts. First, when many tumor nuclei were cut, the resulting interphase counts were shifted to smaller numbers, ie, in case of a pseudotriploid tumor, many nuclei would have yielded only two instead of three signals. Although cut nuclei should not be included in the evaluation,[25] they cannot always unequivocally be identified, even when a laser scanning microscope for the three-dimensional evaluation is used. Secondly, a comparison of interphase counts obtained with centromeric heterochromatin is problematic, because hybridization signals from tumor and normal reference DNA are suppressed over repetitive segments by an excess of Cot-1 DNA, which is routinely applied in CGH experiments. In this particular experiment, the ratio values at centromeric regions of chromosomes 3 and 7 were similar to the profiles obtained for adjacent chromosome bands, and it seems reasonable to assume that the counts obtained with the centromere-specific probes provided a reliable assessment for copy numbers of the adjacent chromosome region as well. Interphase counts with DNA probes obtained in nuclei from apparently normal seminiferous tubules with few exceptions showed either one or two signals per nucleus. It is unlikely that the second signal was simply lost from many nuclei as a result of nuclear cuts, as the thickness of the sections used for interphase cytogenetics should have been sufficient to preserve the majority of nuclei intact. More likely, a single signal is indicative for cells with a haploid chromosome set. The good correlation obtained between fluorescence ratio values and interphase counts supports the reliability of the CGH technique even for cases in which minute amounts of test DNA are amplified by DOP-PCR.

The ratio profiles obtained for the tumor tissue revealed a number of chromosomes or chromosome parts for which the ratios were clearly elevated above the upper threshold. Other DNA segments, eg, the long arm of chromosome 1, demonstrated elevated ratio values that did not exceed the threshold. Such moderately increased ratios are suggestive for subclones carrying the respective trisomies in less than 50% of the cells.

The approach described here offers new opportunities to study genomic variations in germ cell and other neoplasia in detail. In particular, the study of early tumor stages that often present as small lesions is promising and can help to elucidate the first genetic changes in the multistep genesis of solid tumors.

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