

ORIGINAL INVESTIGATION

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Characterization of double minute chromosomes' DNA content in a human high grade astrocytoma cell line by using comparative genomic hybridization and fluorescence in situ hybridization

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Abstract The presence of double minute chromosomes (dmin) in cancer cells is known to be correlated with gene amplifications. In human high grade astrocytomas or glioblastomas, about 50% of cytogenetically characterized cases display dmin. G5 is a cell line which has been established from a human glioblastoma containing multiple dmin. In order to identify the DNA content of these dmin, three techniques were successively used: conventional cytogenetic analysis, comparative genomic hybridization (CGH), and fluorescent in situ hybridization (FISH). The karyotype of G5 cells showed numerical chromosome changes (hypertriploidy), several marker chromosomes, and multiple dmin. CGH experiments detected two strong DNA amplification areas located in 9p21-22 and 9p24, as well as an underrepresentation of chromosomes 6, 10, 11, 13, 14, and 18q. By using FISH with a chromosome 9-specific painting probe to metaphase chromosomes of the G5 cell line, dmin were shown to contain DNA sequences originating from chromosome 9. This study demonstrates the usefulness of a combination of classical karyotyping, CGH, and FISH to identify the chromosomal origin of amplified DNA sequences in dmin.

Introduction

Astrocytic tumors are the most common brain tumors in man (Chung and Seizinger 1992). The most aggressive subtype in adults is grade IV astrocytoma or glioblastoma. The mean survival time is less than 1 year, which is attributable to the lack of efficient therapeutic strategies.

A characteristic cytogenetic feature of glioblastomas is the frequent presence of double minute chromosomes (dmin) found in up to 50% of tumors (Bigner et al. 1988, 1991a). The presence of dmin in tumor cells had been shown to be associated with gene amplification. dmin often contain numerous copies of genes involved in cell proliferation, providing a selective advantage for in vitro and in vivo growth (Bigner et al. 1990b; Privitera et al. 1990). These chromosomal aberrations reflect genomic instability and a rather advanced stage of tumor progression (Bishop 1991). In human gliomas, the presence of dmin was shown to be correlated with a poor prognosis and an increased resistance to therapeutic intervention (Collins 1993). So, the identification of amplified genes localized in dmin should be of great interest in the study of the mechanisms which are involved in the oncogenesis of gliomas.

Molecular studies demonstrated the amplification of several genes in glioblastomas. The tumors show the amplification of the epidermal growth factor receptor gene (30–40%) and the amplification of other oncogenes such as MYC and MET (Bigner and Vogelstein 1990). The amplified sequences of these genes were found to be located on dmin by in situ hybridization on tumor metaphases (Muleris et al. 1994). Although specific molecular analyses provide a tool study gene amplifications with high resolution, they are focused on preselected genes and do not allow screening of the entire tumor genome for DNA amplification.

Recent comparative genomic hybridization (CGH) techniques provide a new method for detection and mapping the ensemble of changes in gene copy number in solid tumors (Kallioniemi et al. 1992, 1994a; du Manoir

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et al. 1993; Speicher et al. 1993; Schröck et al. 1994; Ried et al. 1996). The aim of this study was to develop a CGH-based technical procedure to characterize amplified DNA in dmin. A human glioblastoma cell line, named G5 (Girinski et al. 1990), containing multiple dmin has been used as an experimental model.

Our combined approach consisted of: (1) conventional cytogenetic analysis to show the presence of dmin and to detect chromosomal abnormalities; (2) CGH experiments to identify the chromosomal origin of the amplified DNA; and (3) fluorescence in situ hybridization to localize the amplified DNA in dmin.

Materials and methods

Establishment and culture of glioblastoma cells

The cell line G5 was established from a grade IV astrocytoma of a 63-year-old woman (Girinski et al. 1990). The cell line was initiated by dispersion of minced tumor tissue, followed by culture in F12 medium supplemented with L-glutamine, antibiotics (10 IU/ml, penicillin, 100 mg/ml streptomycin) and 15% heat inactivated fetal calf serum. The cells were cultivated in Falcon flasks at 37°C with 5% CO₂. The G5 cell line has been passaged at least 76 times and is therefore established and considered "permanent", indefinitely propagatable in vitro according to standards of the American Tissue Culture Association.

Cytogenetic analysis

Cultured G5 cells were exposed to colcemid added at a final concentration of 0.1 mg/ml for 17 h. After trypsin treatment, the cells were resuspended in a hypotonic solution (sterile water/fetal calf serum, 7:1 v/v) and then incubated at 37°C for 30 min and fixed at least 3 times in methanol/glacial acetic acid (3:1 v/v). Metaphase spreads were obtained by dropping a cell suspension on cooled and wet slides. The slides were stained by G- and R-banding techniques for karyotyping and by routine methods to detect dmin.

Fluorescence in situ hybridization

Fluorescence in situ hybridization (FISH) was performed as described elsewhere (Ried et al. 1992). Briefly, 300 ng of a biotinylated chromosome 9-specific plasmid library was hybridized to metaphase chromosome preparations from the cell line G5. Probe sequences were detected with avidin conjugated to Cy3 (Jackson Immuno Research Laboratories). Images were acquired using a Zeiss Axiophot epifluorescence microscope equipped with a cooled charge-coupled device (CCD) camera (Photometrics). Digital imaging microscopy was performed as previously described in detail (Ried et al. 1992). Photographs were taken directly from the computer screen. Conventional epifluorescence microscopy was performed using a Leica DM RBE microscope.

CGH analysis

CGH analysis was performed according to a previously described protocol (du Manoir et al. 1993) with minor modifications. DNA was extracted from G5 cells and from a healthy male donor following standard protocols (Sambrook et al. 1989). Tumor and normal DNA were labeled in standard nick-translation reactions by biotin-16-dUTP and digoxigenin-11-dUTP (Boehringer Mannheim), respectively. The DNase I concentration in the labeling reaction was adjusted in order to obtain an average fragment size of 500–1000 bp.

For CGH, 200 ng of digoxigenin-labeled normal DNA and 200 ng of biotin-labeled tumor DNA were ethanol-precipitated in the presence of 10 mg of salmon sperm DNA and 30 mg of the *Cot1* fraction of human DNA (Gibco, BRL). This excess of *Cot1* is required to prevent the ubiquitous hybridization of interspersed repetitive sequences contained in the tumor and normal DNA probes. The probe mixture was dried and resuspended in 10 ml of hybridization solution (50% formamide, 2 × SSC, 10% dextran sulfate). The probe was denatured at 76°C for 5 min, and allowed to preanneal at 37°C for 60 min. Reference metaphase spreads were prepared following standard procedures from peripheral blood lymphocytes of a healthy donor (46, XX). The chromosome preparations were denatured separately at 80°C in 70% deionized formamide, 2 × SSC for 2 min, and dehydrated through an ethanol series (70, 90, 100%). The probe mixture was then applied to the denatured metaphase chromosomes under a coverslip (18 mm²), sealed with rubber cement, and hybridized for 4 days at 37°C.

Posthybridization steps were performed as described (Ried et al. 1992). The biotinylated sequences were detected with fluorescein isothiocyanate (FITC) conjugated to avidin (Vector Laboratories). Probe sequences haptenized with digoxigenin were visualized with anti-digoxigenin Fab fragments conjugated to rhodamine. Chromosomes were counterstained with 4', 6-diamidino-2-phenylindole (DAPI) and embedded in glycerol containing an antifading agent (DABCO, Sigma Chemicals) to reduce photobleaching.

Fluorescence was detected using a Zeiss Axiophot or a Leica DM RBE epifluorescence microscope. The fluorescence banding pattern obtained after DAPI staining was used for chromosome identification. FITC and TRITC fluorescence, specific for the tumor and the control genome, respectively, were quantified as gray level images using a CCD camera (Photometrics). Fluorescence ratio images were calculated as described (du Manoir et al. 1993). The ratio profiles of individual reference chromosomes were determined by a program that was developed in the laboratory of T. Cremer and run on a Macintosh Quadra 950 (du Manoir et al. 1995). Briefly, after the determination of the chromosomal axis for each chromosome in every metaphase, individual FITC and TRITC profiles were calculated. These were used for the computation of the FITC/TRITC ratio profiles. The three vertical lines on the right side of the chromosome ideogram represent different values of the fluorescence between the tumor and the normal DNA (Fig. 2b). The values are 0.75, 1, and 1.25 from left to right. The curve shows the ratio profiles that were calculated as mean values of at least four metaphase spreads. Theoretically, a monosomy in the tumor would result in a ratio value of 0.5 and a trisomy in a value of 1.5. Values above 2 were regarded as amplified regions. Details of the software used are described elsewhere (du Manoir et al. 1995).

Results

Cytogenetic studies

Chromosome analysis of the G5 cell line has been performed at seven different passages of the culture. The modal numbers of chromosomes were between 62 and 81, most of them in the hypertriploid range (Table 1). Figure 1a shows a metaphase, of a G5 cell (passage 54) with 70 chromosomes and multiple dmin. The representative karyotype is shown in Fig. 1b (passage 20). Chromosomes 12, 14, 15, and 18 are present in three copies each, chromosomes 10, 11, and 13 in two copies, and chromosome 6 is present in one copy. Karyotyping of G5 cells revealed complex changes with the presence of 12 marker chromosomes. The chromosomal origin of these markers is described in Table 1. Markers M1, M2, M3,

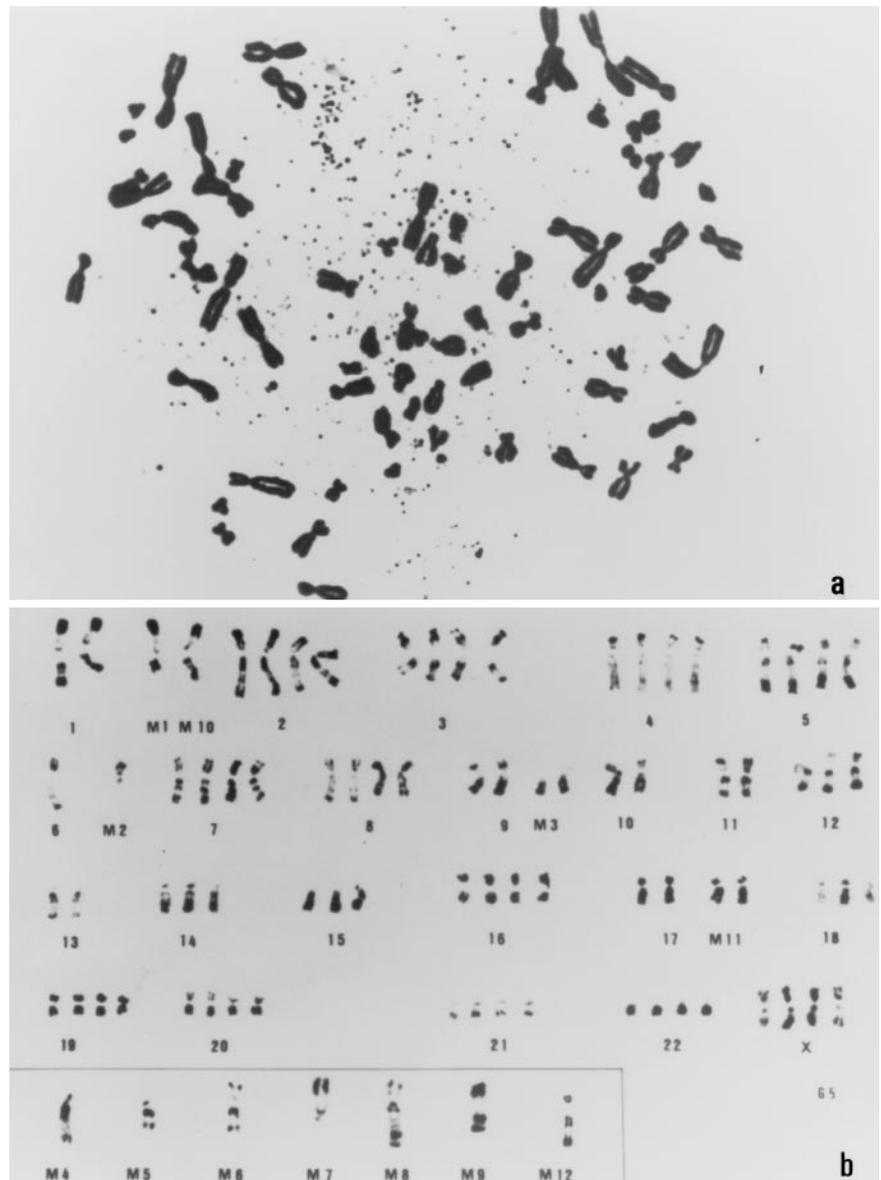
Table 1 Identification of marker chromosome origin

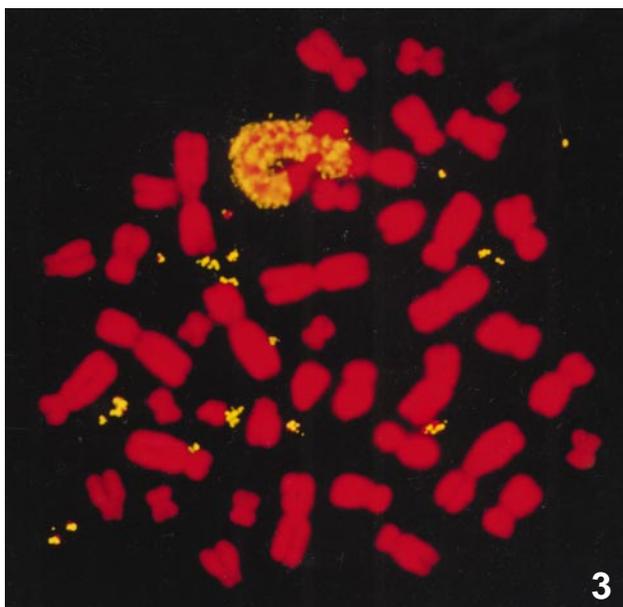
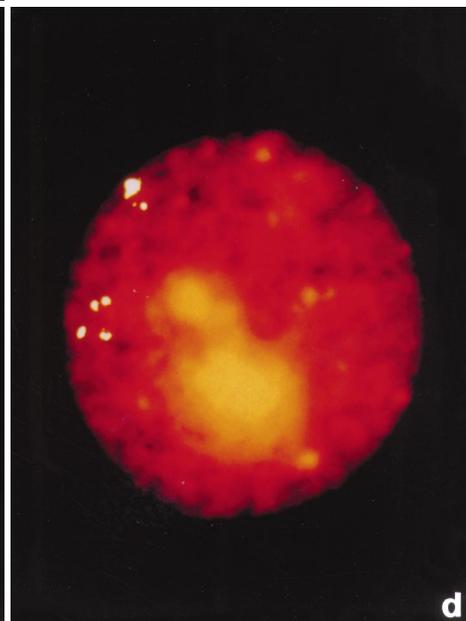
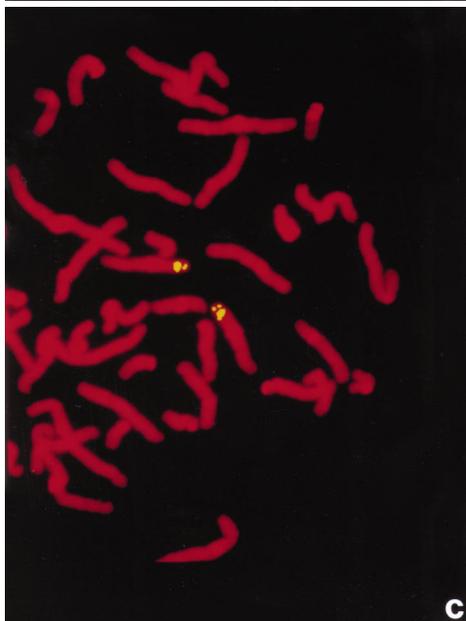
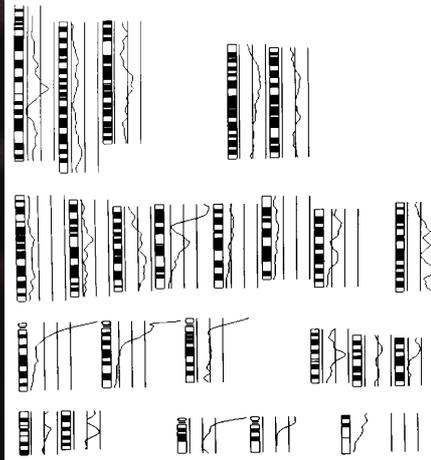
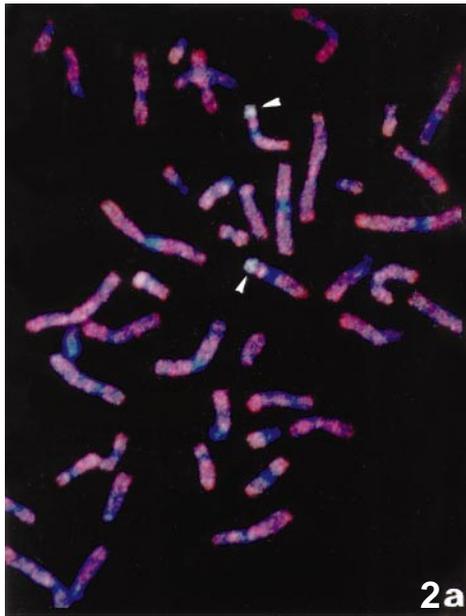
M1	del(1)(?pter→q31::q31→qter)
M2	del(6)(?pter→24::q24→qter)
M3	del(9)(?p21)
M4	add(13)(pter→q21::?)
M5	del(11)(?pter→p11::p15→qter)
M6	add(11)(?::p11→qter)
M7	add(1)(pter→q11::?)
M8	add(2)(pter→q32::?)
M9	del(3)(pter→q24::q26→qter)
M10	add(1)(pter→q31::?)
M11	del(7)(p12)
M12	add(?::q10→qter)

Table 2 Proportion of cells with double minute chromosomes (*dmin*) and the number of *dmin* per cell in those containing them in different passages of the G5 cell line

Passage number	Number of cells scored	Percentage of cell with <i>dmin</i>	Number of <i>dmin</i> per cell (range)
p10	33	55	2.89 (1–15)
p11	27	30	17.13 (1–65)
p12	20	30	1.17 (1– 2)
p20	35	57	1.67 (1– 5)
p25	53	57.4	11.6 (1–95)
p33	14	64	12.78 (1–25)
p44	16	84	10.15 (1–35)
p54	36	83	27.26 (1–55)
p76	48	98	25.13 (1–65)

Fig. 1 a Standard coloration of a metaphase from the human glioblastoma cell line G5. Note the frequent occurrence of double minute chromosomes (*dmin*) throughout the metaphase (passage 54). **b** Representative R-banded karyotype of G5 cell line (passage 20). The hypertriploid cell line shows four copies of most of the chromosomes, whereas chromosomes 12, 14, 15, and 18 are present in three copies, and chromosomes 9, 10, 11, and 13 in two. Chromosome 6 occurs only once. Twelve marker chromosomes can be identified. The marker chromosomes that are included in the karyotype reflect recurrent findings. The marker chromosomes displayed in the inset were sporadic findings





◀ **Fig. 2 a** Comparative genomic hybridization with DNA extracted from the high grade astrocytoma cell line G5. Regions of balanced copy numbers appear *orange*, whereas an underrepresentation of genetic material is reflected by a shift towards the *red*. Note the strong *green* signals on the short arm of chromosome 9 (*arrows*), indicating an amplification of DNA sequences derived from this chromosomal region in the tumor genome. Sequences rich in heterochromatin were not labeled due to the suppression with *Cot1* DNA, and remain *blue* (DAPI stain). The image was acquired after triple exposure of a color slide film using a Leica DM RBE microscope equipped for epifluorescence and filter sets specific for DAPI, FITC, and rhodamine. **b** Average fluorescence ratio profile. Mean of the ratio profile calculation of five metaphases of the G5 cell line. 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 values are 0.75, 1, and 1.25 from left to right. The ratio profile (*curve*) was computed as a mean value of six metaphase spreads. Note that chromosomes 6, 10, 11, 13, and 14 are underrepresented in the tumor genome. The overrepresentation of genetic material on chromosome 9p is obvious. **c, d** High resolution mapping of the amplification on chromosome 9p. **c** Comparative genome hybridization on less condensed metaphase chromosomes. Chromosomes were counterstained with propidium iodide. The tumor DNA was detected with FITC. Note that two independent amplification signals can be discerned on chromosome bands 2p21–22 and 2p24. **d** The appearance of distinct hybridization signals observed in interphase nuclei

Fig. 3 Fluorescence in situ hybridization on a metaphase spread of a G5 cell with a chromosome 9-specific DNA library showing dmin (identified by propidium iodide staining) labeled with this probe. Note the labeling of a marker chromosome containing chromosome 9 material

M9, and M13 are consistently present in the cell line karyotypes.

A special cytogenetical finding characterizing the G5 cell line is the presence of dmin. Of the metaphase cells, 30–98% were found containing various numbers of dmin. Table 2 shows an increase of both the percentage of cells contain dmin and the number of dmin per cell correlated with the culture passage number.

CGH analysis

CGH was performed with DNA extracted from the tumor cell line at passage 54. Figure 2a shows the result of a triple exposure of a color slide film using a filter set specific for DAPI, rhodamine, and FITC. Chromosomal regions rich in heterochromatin (e.g., centromeric repeats) remain free of hybridization signals (indicated by the blue DAPI staining) due to the suppression with *Cot1* DNA. Chromosomal subregions that are overrepresented in the tumor genome are labeled more intensely in green. The strongest green hybridization signal was observed on the short arm of chromosome 9, revealing a high level amplification of tumor DNA. In order to quantify the fluorescence intensities, we evaluated the hybridization using a CCD camera. Images were acquired for each fluorochrome with specific filter sets. Figure 2b shows the fluorescence ratio measured along the axis of each chromosome as a mean value of individual chromosomes from five metaphase spreads. In addition to the amplification of

sequences originating from chromosome 9p, the ratio data indicate an underrepresentation of chromosomes 6, 10, 11, 13, 14, and 18q.

When prometaphase chromosomes were used for CGH, it became obvious that not the entire short arm of chromosome 9 was affected by amplification; the amplified DNA sequences could be mapped to two distinct loci on bands 9p21–22 and 9p24 (Fig. 2c). In interphase nuclei, the amplification sites are clearly separated (Fig. 2d).

FISH analysis

The presence of amplification sites on the short arm of chromosome 9 suggested that dmin observed in this cell line could contain DNA sequences from chromosome 9p. We performed FISH with a chromosome 9-specific plasmid library on metaphase chromosomes of the G5 cell line. Most of dmin were found to be labeled with the chromosome 9-specific plasmid library (Fig. 3).

Discussion

Double minute chromosomes (dmin) are structures indicating gene amplifications which are frequently found in certain solid tumors types. In glioblastomas, dmin have been found in about 50% of cases investigated by classical cytogenetic methods. Conventional banding techniques, however, cannot identify the chromosomal origin of dmin. Recent developments in CGH technology have provided a tool to detect tumor DNA amplifications and to map their origin on normal reference chromosomes. The starting point in the characterization of amplified DNA sequences would be to determine their chromosomal map position by CGH. This allows choice of appropriate DNA probes for FISH analysis in order to localize the amplified DNA to dmin. In this study, we have applied a combined approach using classical chromosome analysis, CGH, and FISH to the human glioma G5 cell line in order to define the chromosomal origin of amplified sequences in dmin.

The karyotype of this cell line revealed a hypertriploid tumor, with a loss of chromosomes 6, 10, 11, 12, 13, 14, 15, 18, and several chromosome markers. Up to 95 dmin were counted per mitosis. CGH experiments showed an underrepresentation of chromosomes 6, 10, 11, 13, 15, and 18q. Thus, the CGH data on losses of chromosomal regions are in concordance with the results of conventional cytogenetic analysis except for chromosomes 12 and 14. This discrepancy may be attributable to chromosome 12 and 14 material being hidden in the marker chromosomes.

The most notable change detected by CGH, however, was an amplification of tumor DNA sequences originating from the short arm of chromosome 9. This overrepresentation of genetic material on chromosome 9 had no obvious correlation with the karyotype, suggesting that these amplified DNA sequences may be located on dmin. In or-

der to prove this assumption, we used a chromosome 9-specific DNA library for FISH analysis of G5 cell line metaphases. Most of the dmin were found to be labeled with this probe, confirming that the dmin contain DNA sequences originating from chromosome 9.

Upon inspection of the hybridization pattern after CGH on less condensed metaphase and prometaphase chromosomes as well as in interphase nuclei, it became apparent that the amplicon does not continuously comprise the entire chromosome 9p. We could discern two distinct loci on the short arm, the more telomeric one being consistently less intense than the second one. This is indicative of two distinct genetic loci amplified in the G5 cell line on 9p21–22 and 9p24.

Our results demonstrate the usefulness of an approach integrating classical cytogenetics, CGH, and FISH in studying the DNA content of dmin in an established cancer cell line. It does not provide direct information on the amplified genes but may focus the use of special molecular techniques on target chromosome regions. Further studies are needed to evaluate the relevance of this approach in fresh tumors, because the detection of DNA amplifications by CGH in clinical samples could be limited due to contamination by normal cells and the presence of various tumor cell subpopulations with different karyotypes (Kallioniemi et al. 1994b).

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