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Comparative Genomic Hybridization of Human Malignant Gliomas Reveals Multiple Amplification Sites and Nonrandom Chromosomal Gains and Losses

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Nine human malignant gliomas (2 astrocytomas grade III and 7 glioblastomas) were analyzed using comparative genomic hybridization (CGH). In addition to the amplification of the EGFR gene at 7p12 in 4 of 9 cases, six new amplification sites were mapped to 1q32, 4q12, 7q21.1, 7q21.2-3, 12p, and 22q12. Nonrandom chromosomal gains and losses were identified with overrepresentation of chromosome 7 and underrepresentation of chromosome 10 as the most frequent events (1 of 2 astrocytomas, 7 of 7 glioblastomas). Gain of a part or the whole chromosome 19 and losses of chromosome bands 9pter-23 and 22q13 were detected each in five cases. Loss of chromosome band 17p13 and gain of chromosome 20 were revealed each in three cases. The validity of the CGH data was confirmed using interphase cytogenetics with YAC clones, chromosome painting in tumor metaphase spreads, and DNA fingerprinting. A comparison of CGH data with the results of chromosome banding analyses indicates that metaphase spreads accessible in primary tumor cell cultures may not represent the clones predominant in the tumor tissue. (Am J Pathol 1994, 144:1203–1218)

Human malignant gliomas represent the most common primary malignant brain tumors.[1,2] Therapeutic strategies that would considerably improve their poor prognosis could not be developed. Chromosome banding and molecular genetic analyses of these tumors have revealed a number of recurrent genetic changes. The most frequent findings were trisomy 7, monosomy 10 and 22, and partial deletions of 9p and 17p and gonosomal losses. In addition, changes in the ploidy range, chromosomal rearrangements, involving in particular chromosomes 1 and 9, and double-minute chromosomes (DMs) were observed.[3–12] Amplification, rearrangements, and overexpression of the epidermal growth factor receptor (EGFR) gene were reported to occur in approximately 40% of malignant gliomas and considered to play a pivotal role in tumorigenesis.[4,13–25] Despite this progress, our knowledge of specific genetic changes involved in the origin and development of both benign and malignant human gliomas is still insufficient.[26]

A new approach, termed comparative genomic hybridization (CGH), has recently been introduced.[27] For this procedure, two-color fluorescence *in situ* hybridization is applied to reference metaphase spreads with normal chromosome complements using a mixture of differentially labeled tumor DNA and normal genomic DNA. After detection of hybridized sequences with two fluorochromes, the respective fluorescence intensities were measured along the reference chromosomes. Fluorescence ratio changes reflect copy number changes of chromosomes or chromosomal segments within the tumor and thus provide a survey of genetic imbalances in tumor cells in a single *in situ* hybridization experi-

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ment. In addition to the identification of complete and partial chromosome gains and losses this method allows the mapping of DNA amplifications on reference metaphase chromosomes.[27-32]

Using CGH, we determined chromosome copy number changes and DNA amplifications in nine human malignant gliomas. These data were compared with the results of chromosome banding and DNA fingerprinting analyses. In one complex case the chromosomal origin of amplified DNA sequences harbored in DMs was confirmed using fluorescence *in situ* hybridization (FISH) on tumor metaphase spreads, whereas interphase cytogenetics of tumor nuclei using 16 chromosome band-specific yeast artificial chromosome (YAC) clones was applied to test the reliability of the CGH data. Our study demonstrates the potential of an integrated approach combining conventional and molecular cytogenetic methods to extend our knowledge of genetic changes in gliomas and other solid tumors.

Materials and Methods

Clinical and Pathological Data

Clinical and histopathological data of malignant gliomas from three female and six male unrelated patients are summarized in Table 1. Brain tumor material was obtained during surgery. Seven patients suffered from primary tumors and had not received chemotherapy or radiotherapy at the time of tumor resection. Two tumor samples (cases no. 2 and 9) were obtained from recurrent tumors. One patient (case no. 2) had not received additional treatment, whereas the second patient (case no. 9) underwent chemotherapy and radiotherapy after the first surgery. The histopathological diagnosis of paraffin-embedded tissue was performed according to the World Health Organization classification.[33]

Chromosome Preparations

Metaphase chromosome spreads from primary cell cultures of tumor samples were prepared as described.[12] Reference metaphase spreads for CGH experiments were prepared from blood of healthy donors (46,XX or 46,XY) using standard procedures.[34] Slides were stored in 70% ethanol at 4 C until use.

DNA Preparations

Tumor DNA was extracted as described from frozen tumor tissue samples.[15] Isolation of reference genomic DNA from peripheral blood lymphocytes of a healthy male donor (46,XY) was performed according to standard procedures.[35]

DNA Fingerprinting

DNA fingerprints from glioma DNA were generated using the oligonucleotide probes $(GT)_8$ and $(GTG)_5$ and compared with the constitutional band pattern obtained from the DNA of peripheral blood leukocytes of each patient. A full-length cDNA from the EGFR gene was used for Southern blot hybridization.[15]

Labeling of DNA Probes

Nick translation of DNA probes was performed following standard protocols.[36] Tumor DNA was labeled with biotin-1 × 6-dUTP (Boehringer Mannheim, Germany) and reference genomic DNA was labeled with digoxigenin-11-dUTP (Boehringer Mannheim). Alu polymerase chain reaction (PCR) products of YAC clones[37,38] and chromosome-specific DNA libraries were labeled with biotin-1 × 6-dUTP or digoxigenin-11-dUTP.

Table 1. *Clinical Data of Patients and Histopathological Data of Tumors*

Case No.	Lab No.	Age/Sex	Histopathological Diagnosis	Primary/ Recurrent Tumor	Localization
1	233	49/f	Anaplastic astrocytoma grade III	p	r. frontal
2	171	31/m	Anaplastic astrocytoma grade III	r	r. temporal
3	94	72/m	Glioblastoma	p	r. frontoparietal
4	256	59/m	Glioblastoma	p	thalamic
5	83	61/f	Glioblastoma	p	r. frontotemporal
6	143	59/f	Glioblastoma	p	r. parietal
7	178	60/m	Glioblastoma	p	l. parietooccipital
8	237	69/m	Glioblastoma	p	l. parietal
9	253	12/m	Glioblastoma	r	l. frontoparietal

f, female; m, male; p, primary tumor; r, recurrent tumor; l, left; r, right.

CGH

CGH was performed with tumor DNA as described[29] with the following modifications: slides with reference human metaphase spreads (46,XX or 46,XY) were denatured in 70% formamide, 2× standard saline citrate (SSC) at 72 C for 2 minutes and dehydrated through an ethanol series. To block highly repetitive sequences, an area of 18 × 18 mm^2 was prehybridized for 2 hours at 37 C with 50 μg of heat-denatured, unlabeled Cotl DNA fraction (BRL/Life technologies, Germany) in 50% formamide, 1× SSC, and 10% dextran sulfate. The coverslip was then removed and a mixture of 100 ng of biotinylated tumor DNA and 100 ng of digoxigenin-labeled reference genomic DNA added in 10 μl hybridization solution (50% formamide, 1× SSC, 10% dextran sulfate). Hybridization was performed for 3 days. Biotinylated DNA sequences were visualized by fluorescein isothiocyanate (FITC) conjugated avidin (Vector, Germany), whereas digoxigenin-labeled sequences were detected by indirect immunofluorescence using mouse anti-digoxin (Sigma, Germany) and goat anti-mouse Ig-tetramethylrhodamine isothiocyanate (TRITC) antibodies (Sigma). Chromosome preparations were counterstained with 4′-6-diamidino-2-phenylindole dihydrochloride (DAPI) (Serva, Germany).

Chromosome Painting

Chromosomal *in situ* suppression (CISS) hybridization of tumor metaphase spreads with whole chromosome paint probes was conducted as described.[36,39] Plasmid libraries from sorted chromosomes 4 and 7 were kindly provided by Dr. Joe Gray (University of California, San Francisco).[40]

Interphase Cytogenetics

Single cell suspensions were prepared from fresh tumor tissue as described,[12] then directly fixed with methanol/acetic acid and dropped on slides. These uncultured tumor nuclei were used for two-color FISH experiments with Alu PCR-amplified and -biotinylated sequences from YAC clones.[37,38] Sixteen YAC clones were selected for the present experiments (Table 4). For each clone the hybridization efficiency tested on nuclei from phytohemagglutinin-stimulated lymphocyte cultures was ≥95%, ie, nuclei with two distinct signals (data not shown). A minimum of 100 nuclei per YAC clone was evaluated following the criteria described by Hopman et al.[41]

Fluorescence Microscopy

A Zeiss Axiophot microscope equipped with a 100 W mercury lamp was used for epifluorescence microscopy. The filter sets for DAPI (LP 450-490, BP 365, FT 395), FITC (LP 515-565, BP 450-490, FT 510), and TRITC (LP 590, BP 546, FT 580) were specifically aligned to minimize image shifts. Microphotographs were taken using Agfachrome 1000 RS color slide films.

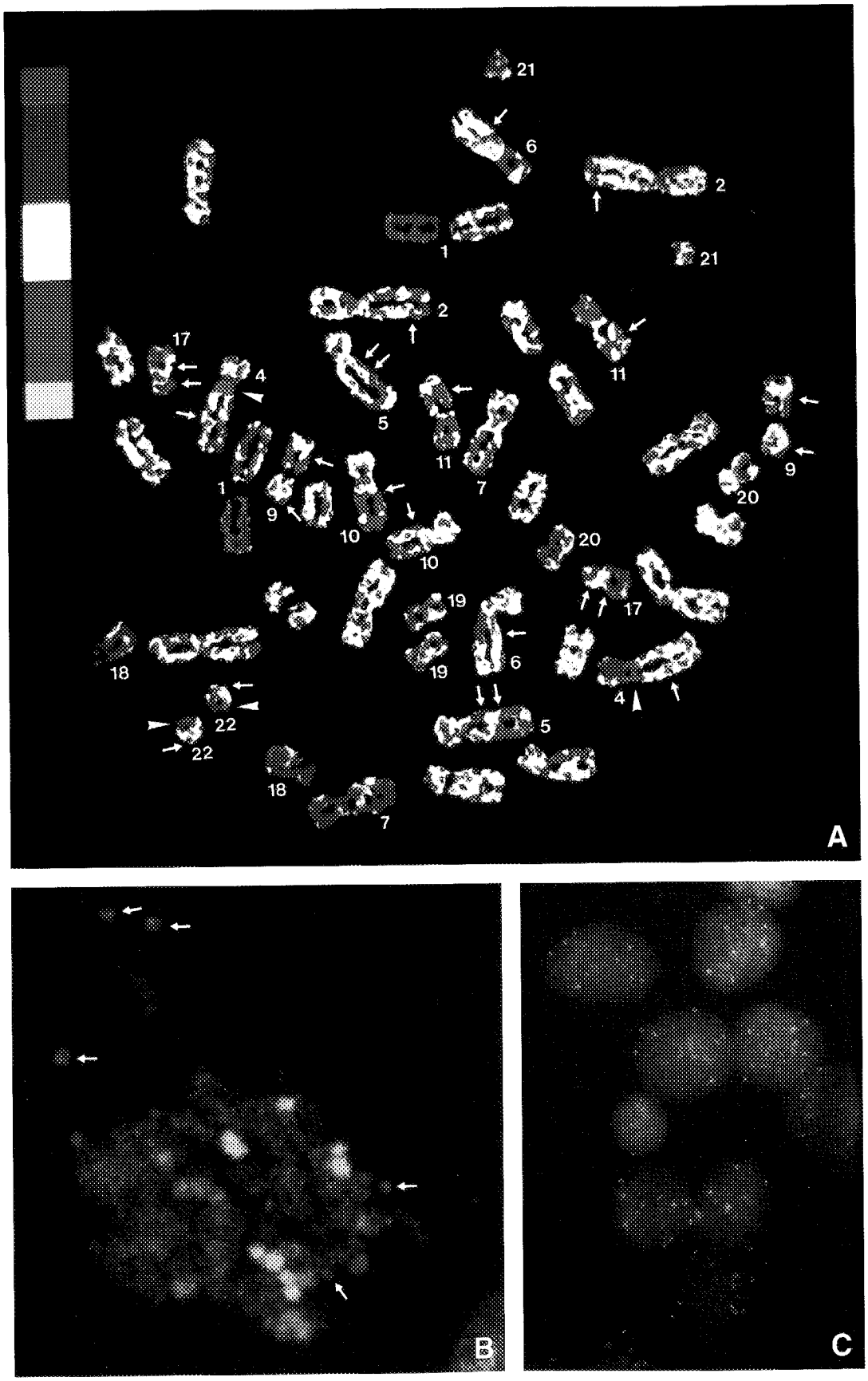
Digital Image Acquisition and Processing

Digital images from reference metaphase spreads subjected to CGH were recorded for each fluorochrome as described[29] using a cooled charge coupled device (CCD) camera (Photometrics, Tucson, AZ) connected to an epifluorescence microscope (Zeiss, Axiophot, Germany). For each case evaluation of chromosomal imbalances and amplification sites in gliomas was performed both by visual inspection and calculation of fluorescence ratio profiles. For visual inspection digitized FITC and TRITC images of 10 reference metaphase spreads and the corresponding ratio images were analyzed.[29] A five-color lookup table was established according to the results of CGH with test DNAs from cell populations with specific monosomies and trisomies (S. du Manoir et al, manuscript in preparation). Chromosomes were identified using DAPI banding patterns. Photographs were taken from the screen with Agfa RS 50 color slide film. For fluorescence ratio profiles computer programs were developed on the basis of TCL-Image (TNO Institute of Applied Physics, Delft, The Netherlands) running on a Macintosh Quadra 950. After determination of the chromosomal axis, individual FITC/TRITC profiles were calculated for each chromosome. Mean ratio profiles were determined from 10 metaphases. The central line in the profiles (Figures 2 and 5) represents the most frequently measured fluorescence ratio for each reference metaphase spread. The left and right vertical lines define threshold values for underrepresentation and overrepresentation of chromosome material (S. du Manoir et al, manuscript in preparation).

Results

Chromosome Gains and Losses Detected by CGH in Nine Malignant Gliomas

Clinical and histopathological data from the nine patients with malignant gliomas included in this study are presented in Table 1. DNA extracted from frozen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tumor samples was used for CGH analysis. For 10 reference metaphases ratio images were obtained (see Materials and Methods). Figure 1, A shows a typical example obtained from a recurrent glioblastoma (case no. 9). In a single ratio image, however, all genetic imbalances may not be detected simultaneously (see below). Analysis of a series of reference metaphase spreads is therefore indispensable for a complete evaluation. CGH analysis of case no. 9 revealed complex karyotypic changes including two amplification sites (4q12 and 22q12, for further details see below), full or partial gains of chromosomes 1, 4, 5, 6, 7, 8, 17, 18, 19, 20, and 21, and full or partial losses of chromosomes 2, 5, 9, 10, 11, 17, and 22. Cytogenetic analysis of these complex changes was not possible. Figure 2 demonstrates the average fluorescence ratio profiles for each reference chromosome from 10 metaphases. The central vertical line corresponds to the most frequently measured fluorescence ratio and the right and left lines represent the lower and upper limits of the normal range (see Materials and Methods). Accordingly, a central line ratio value for a given chromosome or chromosome segment reflects the presence of two copies in pseudodiploid tumor cells, three copies in pseudotriploid cells, four copies in pseudotetraploid cells, and so forth.

In these average ratio profiles transitions between balanced, underrepresented, and overrepresented segments appeared less distinct than in the best resolved ratio images. This is due partly because these transitions were also not distinct in individual ratio profiles obtained in some reference metaphases, and because variations in the condensation of bands along individual chromosomes were not taken into account when the length of chromosomes was normalized for the calculation of the average ratio profiles.

In addition to the ratio profiles, the extent of partial chromosome gains or losses was therefore identified in optimally resolved ratio images of the respective chromosomes. Compared with the ratio image (Figure 1, A, case no. 9) an additional small aberration could be detected using the profile, ie, the exclusion of bands 11q12-21 from the loss of 11q. On the other hand, partial losses of 17p13 and 22q13 were clearly seen in the best resolved ratio images but were not apparent in the average ratio profiles of 10 chromosomes (see below). All other changes were detected concordantly.

A survey of the copy number changes revealed in all nine tumor samples is presented in Table 2 and Figure 3. Because CGH would not detect a gain or loss of a chromosome present in less than 50% of the cells (our unpublished data), each chromosomal imbalance reflects a genetic alteration present in most cells of a malignant glioma *in vivo*. Imbalances of chromosomes 7 and 10 were detected in all 7 glioblastomas and in 1 of 2 astrocytomas. Gain of the whole chromosome 7 was found in cases no. 1, 3, 4, 5, 6, 8, and 9, whereas case no. 7 showed a partial gain of 7pter-q31. Loss of the whole chromosome 10 was observed in cases no. 1, 3, 4, 5, 6, 7, and 8. Case no. 9 showed a partial loss of 10q22-qter. A gain of chromosome 19 was observed in four tumor samples (cases no. 5, 6, 8, and 9), whereas a partial gain of 19q13.2-qter was detected in one sample (case no. 2). Three samples revealed a gain of chromosome 20 (cases no. 4, 8, and 9). Partial losses of 9p and 22q were each detected in five samples (cases no. 1, 3, 6, 8, and 9 for 9p and cases no. 1, 3, 4, 7, and 9 for 22q). Losses restricted to the short arm of chromosome 17 were identified twice (cases no. 1 and 9), whereas in a third sample (case no. 4) a complete loss of chromosome 17 was noticed. Apparent consensus

Figure 1. A: *Fluorescence ratio image of a reference metaphase spread (46,XX) after CGH with glioblastoma DNA (case no. 9, FITC detection) and reference DNA (TRITC detection) prepared from normal male lymphocytes (46,XY). A five-color lookup table (left upper corner) was chosen for the visualization of pixel by pixel FITC/TRITC ratios. The white color suggests a balanced state of chromosomes or chromosome segments in the tumor. Green and blue colors represent moderately and highly increased ratio values indicative for the overrepresentation of the respective segments. Red and yellow colors point to the underrepresentation of chromosome segments. For chromosome identification DAPI banding was applied (data not shown). Blue coloring of chromosome bands 4q12 and 22q12 indicates two amplification sites* (arrow heads). Arrows *denote transition sites between an apparently balanced and underrepresented or overrepresented segments in several chromosomes. Identical transition sites are depicted on homologue chromosomes. The pericentromeric and paracentromeric heterochromatic regions and the short arms of the acrocentric chromosomes were excluded from the calculation of FITC/TRITC ratios and visualized as gray shaded regions in this image (see Materials and Methods). Note that colorization of specific chromosome segments was only considered significant if the same color was consistently obtained in a series of ratio images and confirmed by average ratio profiles (compare Figure 2). Original magnification ×630.* B: *Tumor metaphase spread (case no. 9) counterstained with DAPI (blue) after two-color chromosome painting with library DNA from flow sorted chromosomes 4 (visualized with TRITC, red) and 7 (visualized with FITC, green). Note the presence of four painted copies of chromosome 7 plus an additional chromosome fragment and the presence of chromosome 4 in approximately three copies. The red painting of numerous DMs (some of them indicated by arrows) demonstrates the presence of chromosome 4-derived sequences (compare Figure 6 for further demonstration) in accordance with the detection of an amplification on reference chromosomes 4 by CGH (compare Figures 1, A, 4, B, and 5). The microphotograph was taken by triple exposure of a color slide film. Original magnification ×630.* C: *Nuclei from an uncultivated single cell preparation of tumor tissue (case no. 9) after two-color CISS hybridization with two chromosome 1-specific YAC clones (compare Table 4). Clone HTY 3222 maps to 1p36 (TRITC detection, red signals). Clone HTY 3153 maps to 1q44 (FITC detection, green signals). Nuclei exhibit 3 to 5 red signals and 6 to 8 green signals. Note that focusing through the nuclei was required for accurate signal evaluation. Original magnification ×630.*

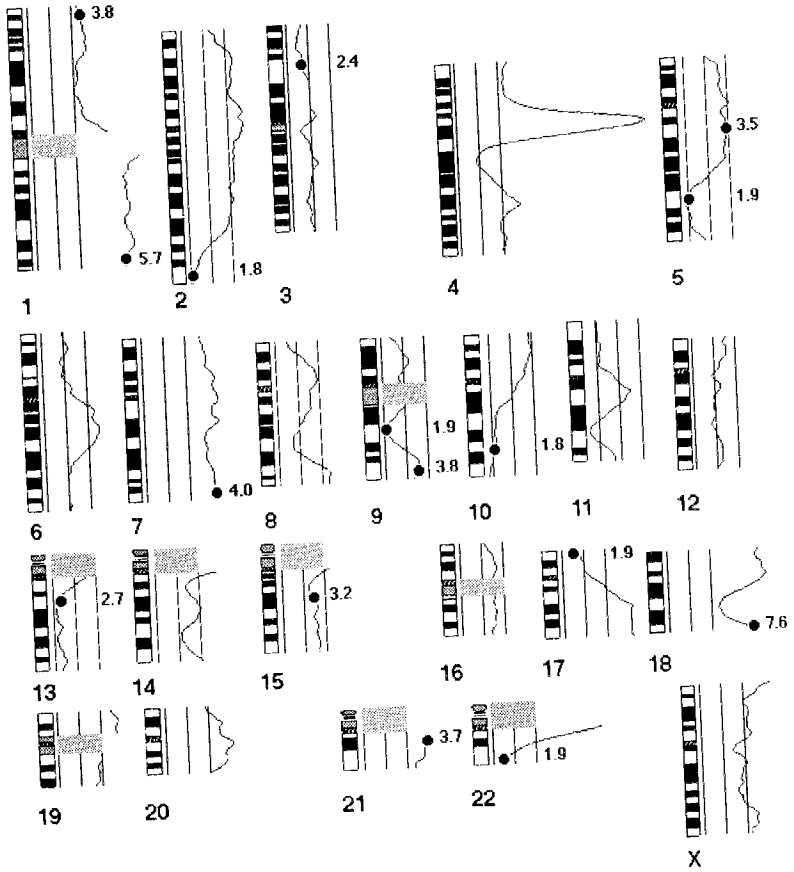

Figure 2. *Average fluorescence ratio profiles obtained from 10 reference metaphase spreads subjected to CGH with glioblastoma DNA (case no. 9; compare Figure 1, A). The central vertical line represents the ratio value typical for a balanced state of chromosome material, ie, two copies for pseudodiploid tumor cells and three copies for pseudotriploid cells. The right and left lines represent thresholds indicative for chromosomal gains (right line) and losses (left line). Numerous gains and losses are indicated in this chromosome copy number karyotype (compare Table 2). Note two amplification sites apparent at chromosome bands 4q12 and 22q12 (compare Figure 1, A). Due to the suppression with Cot1 DNA fraction, very low FITC and TRITC fluorescence intensities were recorded at heterochromatic pericentromeric and paracentromeric chromosome regions and on the short arms of the acrocentric chromosomes. These gray shaded regions were therefore excluded from evaluation. Black dots indicate the mapping positions of YAC clones used for confirmation of the CGH data by interphase cytogenetics. At the right of each dot the average signal number for 100 nuclei from an uncultivated cell sample of case no. 9 is given (compare Table 4).*

regions for these chromosomes included 9pter-23, 17p13, and 22q13. Gains or losses of several other chromosome regions were observed less frequently.

DNA Amplification Sites Mapped by CGH

Eleven DNA amplifications could be determined by CGH and their origins mapped to seven different chromosomal locations (Table 3). In all cases the amplification sites were seen as distinct signals on both chromatids of the respective chromosomes in nearly all reference metaphase spreads. Figure 4, A presents a reference metaphase spread obtained in a CGH experiment with tumor DNA from case no. 5 indicating three different amplification sites. Figure 4, B shows examples of reference chromosomes 1, 4, 7, 12, and 22 with mapped amplification sites. The adjacent images of the DAPI banded chromosomes were used to determine the position of each site in a series of these reference chromosomes. For each case, average ratio profiles of chromosomes with mapped amplification sites were determined in 10 reference metaphase spreads (Figure 5). In three of the four cases with fluorescence peak values on chromosome 7 (cases no. 5, 6, and 8) these profiles showed an elevated FITC/TRITC ratio along the entire chromosome 7 indicating a gain of this chromosome in the respective tumor samples. In one case (no. 7) the ratio profile revealed a partial overrepresentation of 7pter-q31. The amplification of the whole p arm of chromosome 12 (case no. 2) is accompanied by loss of genetic material of the q arm as indicated by a shift of the high fluorescence ratio over 12p to values below the lower threshold along 12q. In contrast, the ratio profile for chromosome 1 (case no. 5) indicated a balanced representation of its genetic material with the exception of an amplification at 1q32. Similarly, a balanced state is indicated for chromosome 4 (case no. 3), which showed an amplification site mapped at 4q12. In case no. 9, which also showed an amplification at 4q12, an elevated FITC/TRITC ratio along the chromosome 4 indicated gains of chromosome regions 4p and 4q26-qter in most cells of this tumor. In four tumor samples (cases no. 5 to 8) a peak fluorescence detected over band 7p12 suggested the amplification of the EGFR gene. The EGFR gene amplification was further demonstrated by DNA fingerprinting (Table 3).

Table 2. *Results of CGH and Cytogenetic Banding Analysis*

Case No	Chromosomal Localization of Gains, Losses, and Amplification Sites (CGH results) / Karyotype
1	XX,−(5)(q33-qter),+7,−(9)(pter-23),+(9)(p11-p13),+(9)(q13-qter),−10,−15,−(16)(q22-qter),−(17)(p13),−(22)(q12-qter)
	43–44,XX,add(2)(q35),+7,−8,der(9)$_1$,+der(9)$_2$,−10,add(11)(p15),der(16)t(2;16)(q35;q34),i(17)(p10),der(17;19)(q10;q10),−19,−22[5]/86–88,idem×2,+der(7),+der(8),−15[5]
2	XY,−(Xp), −(2)(q34-qter),−(5p),−11,ampl.(12p),−(12q),−15,+(19)(q13.2-qter)
	46,XY[84]/44–47,XY,+7,−22[cp4]
3	XY,+(3)(q28),ampl.(4)(q12),+7,−(9)(pter-23),−10,−(22)(q13)
	46,XY,der(1)t(1;12)(p36;q15),+7,−10,del(12)(q15),−14,add(15)(p11),+21[22]/44–45,idem,−Y,dmin[8]
4	XY,−(4)(q27-qter),−6,+7,−10,−13,−14,−17,+20,−22
	46,XY[8]/45,X,−Y[23]
5	XX,ampl.(1)(q32.1),+7,ampl.(7)(p12),ampl.(7)(q21.2–3),−10,+19
	44–46,XX[6]/47,XX,+7,dmin[2]
6	XX,+7,ampl.(7)(p12),−(9)(pter-21),−10,+19
	49,XX,+3,+7,add(9)(p21),del(9)(p21),−10,+17,+19[6]/43–45,XX,+1,+3,+7,add(9)(p21),del(9)(p21),−10,+19[3]
7	XY,−(3)(q12–23),+7(pter-q31),ampl.(7)(p12),ampl.(7)(q21.1),−8,−10,−22
	45,X,−Y[11]/46,X,−Y,+7[6]
8	XY,+1p,+(1)(q21–41),+7,ampl.(7)(p12),−(9)(pter-23),−10,(14)(q12–13),+(19p)(high overrepr.),+19q,+20
	85–90,XXYY,der(5)t(5;7)(p10;q10);add(9)(p21)×2,−10×2,add(13)(p10)×2,−14×2,−15×2,ins(15;?)(q22;?)×2,der(16)t(7;16)(p13;q22)×2,+19×2,+20×2,−21,+mar1,+mar2[6]
9	XY,+(1p),+(1q)(high overrepr.),−(2)(q34-qter),+(4p),ampl.(4)(q12),+(4)(q26-qter),+(5)(q11–21),−(5)(q31-qter),+(6)(q12-q21),+7,+(8)(q24),−(9)(pter-23),−(9)(q21–22),−(10)(q22-qter),−(11p),−(11)(q22–23),−(17)(p13),+(17)(q21-qter),+(18p)(high overrepr.),+(18q),+19,+20,+21,ampl.(22)(q12),−(22)(q13)
	Polyploid with multiple structural aberrations and dmin

+, Gain of chromosomal material; −, loss of chromosomal material; ampl., amplification site, chromosomal localization of gene amplification; high overrepr., high overrepresentation of this chromosomal region.

The karyotypes of two cases (no. 3 and 5) were already published by Thiel et al in 1992. In critical cases the origin of marker chromosomes was confirmed by chromosomal *in situ* suppression (CISS) hybridization with appropriate whole chromosome paint probes. This approach, however, was restricted by the limited number of slides with metaphases from each case (G. Thiel and T. Lozanova, unpublished data).

Validity of CGH Data Tested by Chromosome Painting and Interphase Cytogenetics

To assess the validity of CGH to delineate multiple genetic imbalances in solid tumors, case no. 9 was paradigmatically chosen. The highest fluorescence value using both image ratios and intensity profiles was detected on 4q12. Therefore, this chromosomal band was considered to harbor DNA sequences presumably amplified in the DMs of this tumor. This expectation was proven by painting of the DMs in tumor metaphases using a painting probe for human chromosome 4 (Figures 1, B and 6). To test the validity of the copy number changes detected by CGH in this tumor, a series of FISH experiments was performed to interphase nuclei from uncultured tumor cells. Sixteen YAC clones (Table 4, Figure 1, C) showing a hybridization efficiency of ≥95% on normal lymphocyte nuclei (see Materials and Methods) were selected to test

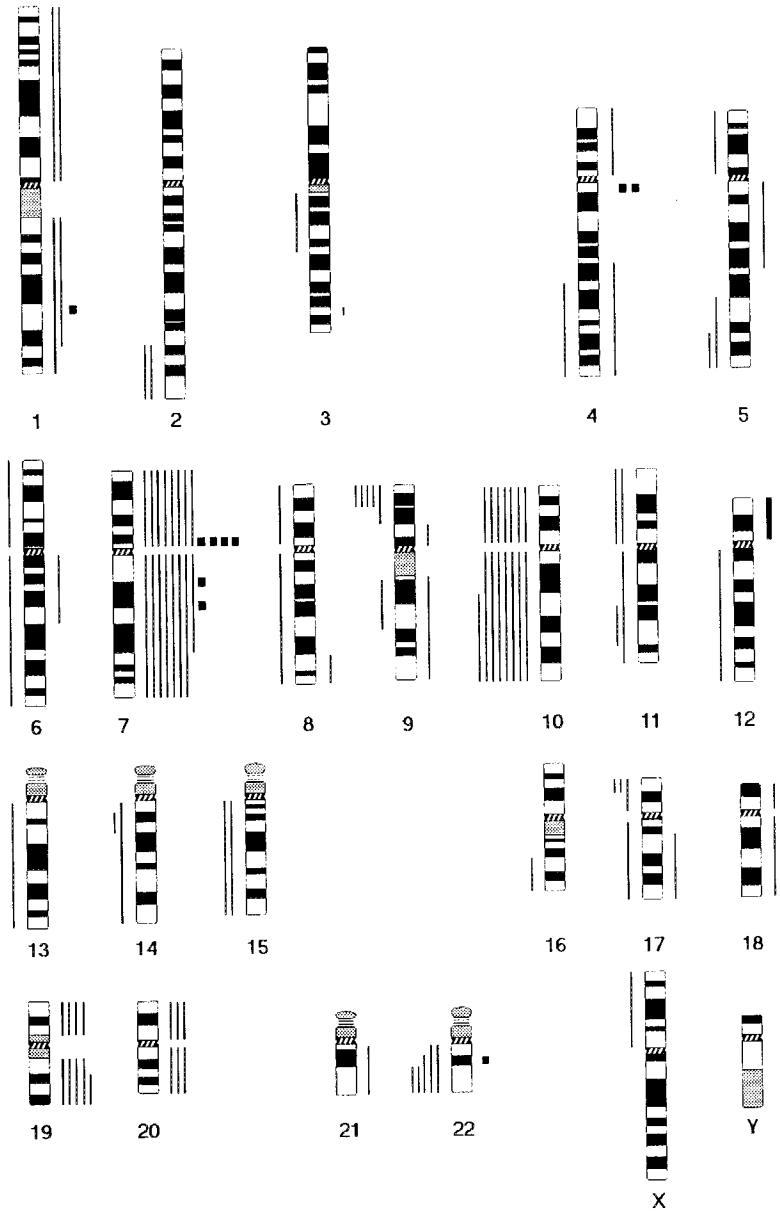

Figure 3. *Summary of genetic imbalances detected by CGH in nine human malignant gliomas. Losses are indicated by lines on the left of each chromosome scheme, whereas lines on the right represent gains. Chromosome bands to which amplification sites could be mapped are indicated by squares. The amplification of sequences present in an entire 12p is indicated by a thick line.*

the presence of chromosomal sites in the tumor cells for which average ratio values and/or visual inspection had indicated a balanced state, a loss, or a gain (Figure 2). For case no. 9 mean numbers of nuclear signals between 2.4 and 3.2 (with an average of 2.8 for all sites together) were counted after FISH with probes for three chromosomal sites noted in an apparently balanced state (Table 4, Figure 2). This result indicates that this tumor contained predominantly pseudotriploid cells. Probes for six chromosomal sites for which losses were observed by CGH yielded mean signal numbers between 1.8 and 2.0 (with an average of 1.9 for all sites together). For six chromosomal sites with increased ratio values mean signal numbers between 3.5 and 7.6 (with an average of 4.7) were determined.

Apparent discrepancies between average fluorescence ratios and mean signal numbers were noted for YAC clones HTY 3043 (9q34.3), HTY 3141 (17p13), and HTY 3149 (22q13). All three YACs were derived from a telomeric YAC library and define telomeric chromosome regions. The difficulties to obtain reliable average fluorescence ratios at the very tip of a chromosome will be discussed elsewhere (S. du Manoir et al, manuscript in preparation). Notably, the evaluation of FITC, TRITC, and ratio images from well

Table 3. *Chromosomal Localization of Amplification sites, Results of DNA Fingerprinting, and Cytogenetic Evaluation of dmins*

Case No.	Amplification Site	DNA Fingerprinting: EGFR Amplification	Cytogenetic Analysis: dmin
2	12p	–	–
3	4q12	–	dmin
5	1q32	+	
	7p12		dmin
	7q21.2–3	+	
6	7p12	+	–
7	7p12	+	–
	7q21.1		
8	7p12	+	–
9	4q12	–	dmin
	22q12		

The results of DNA fingerprinting of two cases (no. 3 and 5) were already published (15).

resolved individual chromosomes 9, 17, and 22 were in agreement with the results of the interphase cytogenetic analysis: enhanced FITC painting could be noted at 9q34, whereas 17p13 and 22q13 showed clearly less intense FITC painting.

Reference chromosomes for which the average ratio profiles indicate that different parts were present in different copy numbers in the tumor provide particularly interesting opportunities to compare the results of CGH and interphase cytogenetics. Ratio values for chromosome 1 indicated a gain for both the short and the long arm but the gain expected for the long arm was clearly more pronounced (Figure 2). In concordance with this ratio profile, two-color FISH with YAC clones specific for the short arm (1p36, red fluorescence) and the long arm (1q44, green fluorescence) of chromosome 1 yielded four signals for 1p36 and six signals for 1q44 in 75 and 70%, respectively, of the evaluated nuclei (Figures 1, C and 2, Table 4). CGH ratio values and mean signal numbers showed a linear correlation (Figure 7). For 9 of 16 YAC clones the signal numbers did not vary in 75% of the cells. This led us to conclude that this tumor contained one main clonal population. The more heterogeneous signal numbers of the other seven YAC clones may correspond to additive aberrations in this main clone or other less abundant individual clones.

Results of Chromosome Banding Analyses

In addition to the results of the CGH analyses the results of chromosome banding analyses of the nine malignant gliomas are listed in Table 2. Notably, chromosome banding and CGH analyses were performed in different laboratories and the results were exchanged for comparison only after both analyses had been completed independently. Numerous copy number changes of chromosomes were concordantly detected by both CGH and banding analyses. However, many striking discrepancies were also noticed (see Discussion). To test for the presence of the Y chromosome in tumors derived from males (Table 1, 6 cases) CGH was repeated, if necessary, on male reference metaphases. In all these cases the Y chromosome was specifically painted. In one case (no. 9) the presence of the Y chromosome was also confirmed by interphase cytogenetics with the Y-specific probe pYH 2.1[42] (data not shown).

Discussion

In this study numerous complete or partial chromosome gains and losses were identified by CGH in nine malignant gliomas. In the following, we will first compare the possibilities and limitations of CGH analysis to reveal genetic changes in these or other solid tumors with the possibilities and limitations of cytogenetic and other molecular genetic approaches. Second, we will discuss the implications of this study for the biology of malignant gliomas.

Comparison of CGH with other Approaches to Study Genetic Imbalances in Malignant Gliomas

Not all complete and partial chromosomal gains and losses detected by CGH could be identified by chromosome banding analysis. Conversely, some chromosomal aberrations found by banding analysis were not detected by CGH (Table 2). Several reasons may account for these discrepancies. CGH defines gains and losses that are present in the tumor sample *in vivo* but will only detect chromosome imbalances present in the majority of the cells (>50%, unpublished observations) from which DNA was prepared. In contrast, mitotic cells that are analyzed in a tumor cell culture may represent tumor subclones and normal cells selected by growth advantages *in vitro* and may contain additional chromosome aberrations that occurred during *in vitro* culture.

Chromosome painting and interphase cytogenetics confirmed the results of the CGH analyses. Interphase cytogenetics[39,41,43] was particularly useful to evaluate samples of uncultured tumor cells for the presence of chromosomal gains and losses indicated by CGH. It should be noted that CGH does not provide information on the ploidy level of a given tumor. Such information can be obtained by interphase cytogenetics with DNA probes that map to chromosome sites showing fluorescence ratio values at the central

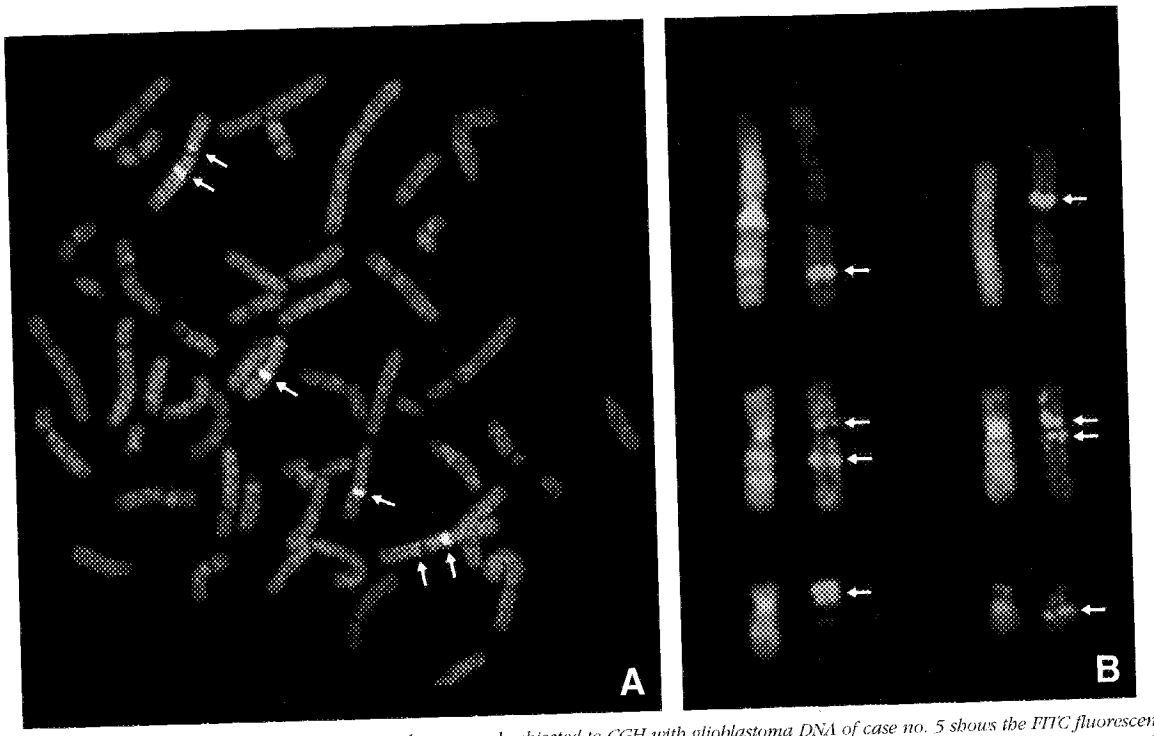

Figure 4. A: *Microphotograph of a reference metaphase spread subjected to CGH with glioblastoma DNA of case no. 5 shows the FITC fluorescence of hybridized tumor DNA sequences. Three amplification sites at chromosome bands 1q32, 7p12, and 7q21.2-3 are indicated by* arrows. *Mapping positions were identified by comparison with DAPI banded chromosomes (compare* B). B: *Mapping of seven different amplification sites to reference metaphase chromosomes by CGH. Gray scale pictures of the chromosomes were taken with a CCD camera and pseudocolored using image processing (blue, DAPI counterstaining; green, hybridization signals of FITC-detected tumor DNA). Intensely fluorescence sites were recorded on chromosome bands 1q32 (case no. 5), 4q12 (case no. 9), 7p12 and 7q21.2-3 (case no. 5), 7p12 and 7q21.1 (case no. 7), 12p (case no. 2), and 22q12 (case no. 9). TRITC fluorescence images of these chromosomes reflecting the hybridization of reference DNA sequences showed a homogenous staining except for the weakly stained heterochromatic regions (data not shown). Average fluorescence ratio profiles indicating complete or partial gains and losses of these chromosomes are demonstrated in Figure 5. Original magnification ×630.*

line (see Results) or by measurements of the DNA content of individual tumor nuclei using flow cytometry or image analysis microscopy (not performed in this study).

Molecular genetic approaches, for example the search for a loss of heterozygosity (LOH) in certain regions of a tumor genome, are mostly performed in a highly focused manner. If screening of a whole tumor genome is attempted in this way, large numbers of hybridization or PCR experiments have to be conducted requiring a set of informative probes or oligonucleotide primers spanning the entire genome. In contrast to CGH, however, LOH studies provide the advantage to distinguish between alleles. A loss of a particular chromosome region followed by reduplication of the remaining chromosome can be identified by LOH but not by CGH. Both CGH and studies of LOH have in common that they provide information for genetic changes present in the large majority of cells but do not allow studies of clonal heterogeneity. This limitation may be overcome in the near future. CGH analysis after degenerate oligonucleotide primed (DOP)-PCR[31] allows to study samples comprising only a few tumor cells. Recent improvements of universal DNA amplification suggest even the possibility that CGH may become possible on a single cell basis.[44] We anticipate that the isolation of a few nuclei or even a single nucleus from appropriately frozen, fixed, and paraffin-embedded tissue sections will make it possible to study the genetic basis of the histopathologically characterized cellular heterogeneity of malignant gliomas and other solid tumors.

In our study DMs were observed in 3 of 9 malignant gliomas by cytogenetic analysis. This result is in agreement with other investigations where DMs were observed in approximately 30% of malignant gliomas.[6,12] However, using CGH 11 DNA amplifications on 7 different chromosomal locations were detected in 7 of 9 cases. These findings support the assumption that karyotyping may underestimate the frequency of gene amplification.[28]

In conclusion, each of the cytogenetic and molecular cytogenetic approaches used in this study, ie, chromosome banding, CGH, chromosome painting, and interphase cytogenetics, revealed both information confirming the results of the other approaches and unique information. Although CGH is a superior approach to establish copy number karyotypes

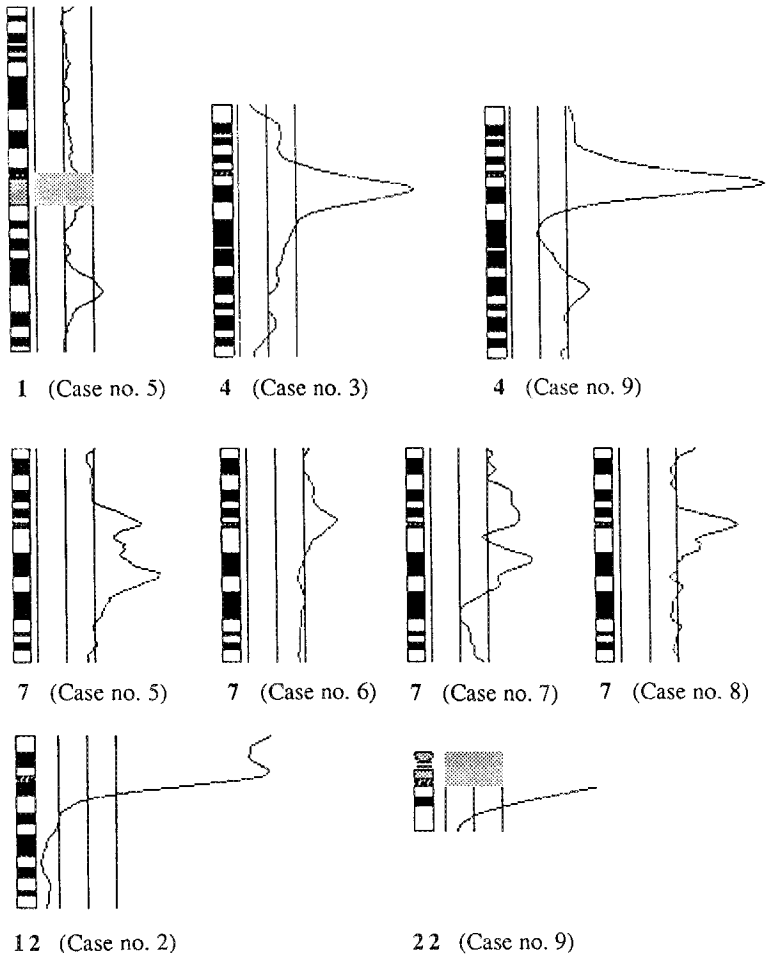

Figure 5. *Peak FITC/TRITC values of average fluorescence ratio profiles from chromosomes 1, 4, 7, 12, and 22 indicate amplification sites (from left to right) at 1q32 (case no. 5), 4q12 (cases no. 3 and 9), 7p12 and 7q21.2-3 (case no. 5), 7p12 (case no. 6), 7p12 and 7q21.1 (case no. 7), 7p12 (case no. 8), 12p (case no. 2), and 22q12 (case no. 9). For explanation of the three vertical lines see legend to Figure 2. Note that in addition to the amplification sites a partial gain of 4p and 4q26-qter is indicated in case no. 9. Overrepresentation of chromosome 7 is indicated in cases no. 5, 6, and 8, whereas a partial gain of 7pter-q31 is noted in case no. 7. In case no. 2 the gain of 12p is accompanied by a loss of 12q. The gray shaded paracentromeric heterochromatin of chromosome 1 and the short arm of chromosome 22 were excluded from the calculation of average ratios (for explanation compare legend to Figure 2).*

avoiding the artifacts of tumor cell cultures, chromosome banding, chromosome painting, and FISH with band-specific DNA clones provide possibilities to study the actual genetic composition of marker chromosomes and the distribution of amplified sequences in DMs and/or marker chromosomes of individual tumor cells. Reciprocal rearrangements can be identified by these other approaches but not by CGH. A comprehensive evaluation of cytogenetic changes in solid tumors should therefore be based on an integrated approach. The application of CGH as a routine diagnostic tool should be strongly faciliated in the future by the development of automated CGH tests.[29]

Implications of CGH Data for the Study of Genetic Events in the Development of Malignant Gliomas

Six new amplification sites were detected by CGH in 5 of 9 malignant gliomas and localized to chromosome bands 1q32, 4q12 (2×), 7q21.1, 7q21.2-3, 12p, and 22q12. The following potential candidate genes were reported to map to the amplified genomic regions[45] and might play a role in the regulation of growth control in glial tissue: CD45 (a leukocyte common antigen), polymeric immunoglobulin receptor, CD46 complement membrane cofactor protein, and transforming growth factor beta 2 map to 1q31-41. The involvement of cell surface factors like CD45 or CD46 in glioma development may be considered because CD44, the human lymphocyte homing receptor, was found to be strongly expressed in high grade gliomas but weakly in normal brain cells.[46] The amplification of 4q12 was detected in two unrelated cases. Among others the genes for alpha polypeptide of the platelet-derived growth factor receptor (PDGFRA) and alpha fetoprotein were mapped to this chromosomal region. Overexpression and an aberrant transcript of PDGFRA was detected in glioblastomas.[21,47] On 12p potential genes that might be involved in tumorigenesis of gliomas include the genes for CD4 and CD9, fibroblast growth factor 6, tumor necrosis factor receptor 1, and the v-Ki-*ras*2 oncogene homologue. The platelet-derived growth factor

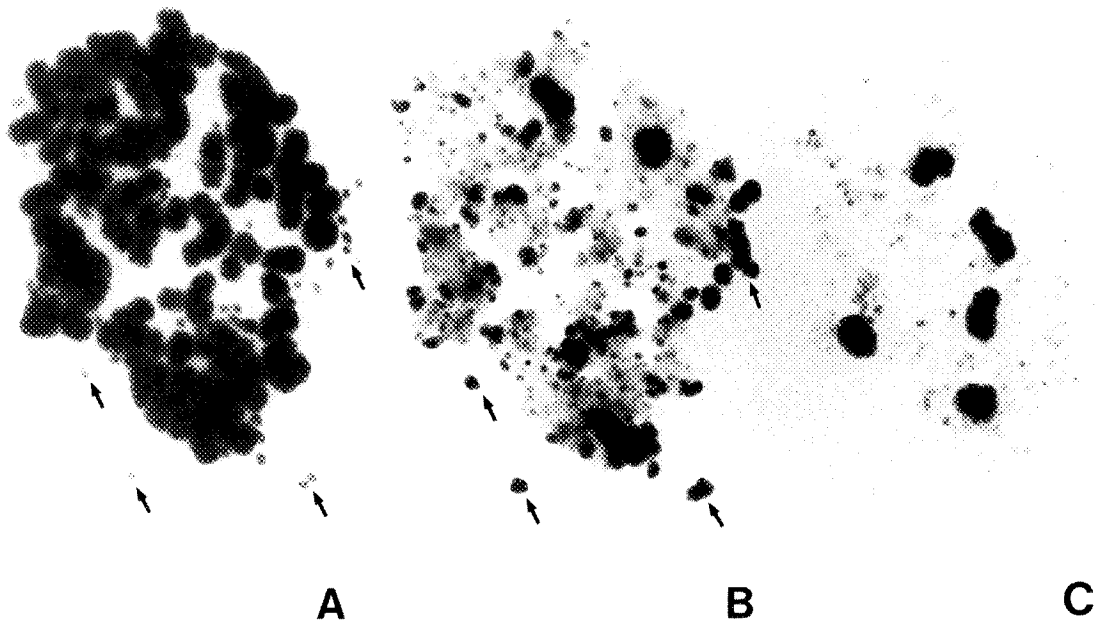

Figure 6. A: *DAPI stained tumor metaphase spread (case no. 9) shows numerous DMs (some of them indicated by arrows).* B: *The same metaphase spread after CISS hybridization with library DNA from flow sorted chromosomes 4 detected with TRITC. In addition to three painted chromosomes the specific painting of DMs is noted indicating the presence of chromosome 4-derived sequences.* C: *Simultaneous painting of the same metaphase spread with library DNA from flow sorted chromosome 7 demonstrates the painting of five chromosomes but none of the DMs (compare Figure 1, B). Original magnification ×630.*

Table 4. *Comparison of the Results of CGH Analysis of a Pseudotriploid Glioblastoma with Interphase Cytogenetics Using Band-Specific YAC Clones (case no. 9)*

CGH Results, Gains, and Losses	Interphase FISH YAC Clone	Localization	Mean of 100 cells	Numbers of Signals Per Cell (%)									
				0	1	2	3	4	5	6	7	8	9
Balanced state	HTY 3113	3p21	2,4	2	5	45	42	6	0	0	0	0	0
Balanced state	YAC7	13q14	2,7	2	7	36	49	5	1	0	0	0	0
Balanced state	HTY 3010	15q21	3,2	3	2	10	51	30	4	0	0	0	0
Balanced state	HTY 3043	9q34.3	3,8	1	1	8	8	77	4	1	0	0	0
−(2)(q34-qter)	HTY 3118	2q36–37	1,8	3	18	74	4	1	0	0	0	0	0
−(10)(q22-qter)	HTY 3179	10q24–25	1,8	1	15	81	3	0	0	0	0	0	0
−(5)(q31-qter)	HTY 3213	5q31	1,9	2	16	74	8	0	0	0	0	0	0
−(9)(q21–22)	HTY 3143	9q21	1,9	2	7	90	1	0	0	0	0	0	0
−(22)(q13)	HTY 3149	22q13	1,9	3	11	77	8	1	0	0	0	0	0
−(17)(p13)	HTY 3141	17p13	2,0	1	8	86	4	1	0	0	0	0	0
+(5)(q11–21)	HTY 3026	5q13–14	3,5	1	1	16	17	62	3	0	0	0	0
+21	HY129	21q21	3,7	2	2	4	14	73	5	1	0	0	0
+(1p)	HTY 3222	1p36	3,8	2	0	8	8	75	7	0	0	0	0
+7	HTY 3172	7q36	4,0	3	2	7	10	41	32	3	2	0	0
+(1q)	HTY 3153	1q44	5,7	0	0	2	2	7	11	70	8	0	0
+18	HTY 3045	18q23	7,6	0	0	2	2	0	0	7	8	70	11

YAC clone YAC7 was kindly provided by Dr. Eric Green and YAC clone HY 129 was a gift from Drs M.-C. Poitier and M. Goedert. The other 14 YAC clones were mapped in our laboratory (A. Jauch, H. Donis-Keller, T. Cremer, unpublished data).

beta polypeptide (PDGFB) gene provides a candidate gene on 22q. The expression of PDGFB is higher in gliomas than in peritumoral and normal nervous tissue[48] and both PDGFA and PDGFB are higher expressed in glioblastomas than astrocytomas.[49] Further cytogenetic, molecular genetic, and biochemical studies are necessary to identify the amplified DNA sequences and to understand their influence on tumor biology.

Trisomy of chromosome 7 is the most frequent chromosomal aberration in astrocytomas ranging from 15% in benign low grade astrocytomas to

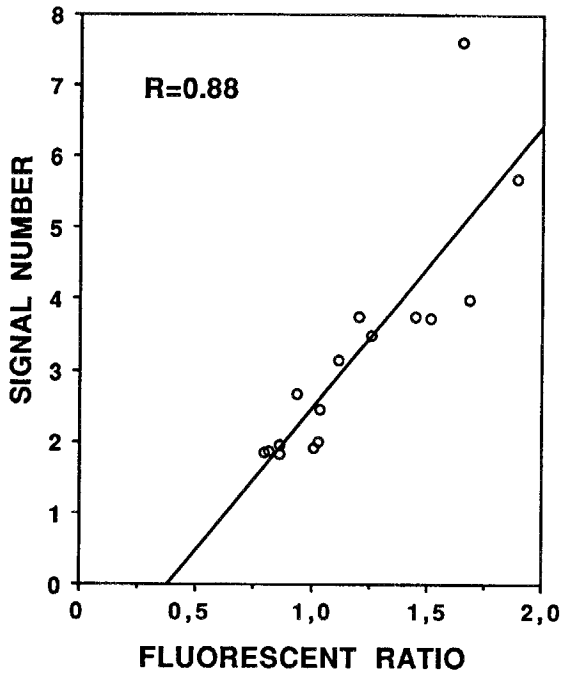

Figure 7. *Abscissa: average fluorescence ratios calculated for 16 chromosome sites in 10 reference metaphase spreads subjected to CGH with glioblastoma DNA (case no. 9). Ordinate: average signal numbers of the same chromosome sites evaluated by interphase cytogenetics in uncultured cell samples of this case with 16 human YAC clones (compare Figure 2, Table 4). A linear correlation with a regression coefficient of 0.88 is observed between the average signal numbers and the average fluorescence ratio values.*

up to 80% in malignant high grade astrocytomas.[2,7,8,11,12,50] CGH analysis observed an overrepresentation of chromosome 7 in 1 of 2 anaplastic astrocytomas (grade III) and in all glioblastomas (7 cases). These results demonstrate that overrepresentation of chromosome 7 is present in the majority of tumor cells *in vivo*. The independently performed G banding analysis revealed a clonal trisomy 7 in 5 of 8 karyotyped cases. Still a causative role of chromosome 7 overrepresentation in the pathogenesis of gliomas has been disputed, because trisomy 7 has been observed in normal epithelial tissues.[51] However, the distribution of amplification sites detected by CGH shows a preferential involvement of this chromosome supporting the pivotal role of chromosome 7 sequences in the biology of human gliomas. These amplifications include the EGFR gene, which was overrepresented in 4 of 9 cases in our study (see above). In two cases two additional amplifications sites were mapped to this chromosome at 7q21.1 and 7q21.2-3. Further investigations of normal brain tissue and of a larger number of low grade astrocytomas using CGH and interphase cytogenetics will help to study the role of this chromosome in the origin and progression of malignant gliomas.

The loss of chromosome 10 is widely accepted as a marker of malignancy in human gliomas. The search for possible tumor suppressor genes on this chromosome has been actively pursued with studies for loss of heterozygosity.[2,11,52–54] Cytogenetic results indicated a loss of a whole chromosome 10 in approximately 30 to 60% of malignant gliomas.[7,8,12] In our collection karyotyping revealed monosomy 10 for 4 of 8 analyzable cases. Using CGH, however, this specific aberration was noted in 8 of 9 cases. All seven glioblastomas revealed losses of chromosome 10 with a restriction to 10q21-qter in one case.

Chromosomes 9, 17, 19, and 22 are preferred targets for chromosomal aberrations in gliomas but less frequently involved than chromosomes 7 and 10. Cytogenetic and molecular analyses revealed structural aberrations and LOH of chromosomes 9, 17, and 22.[55–59] Using CGH, partial losses of 9p and 22q have been observed in 5 of 9 cases each with consensus regions mapped to 9pter-23 and 22q13. The latter region is also frequently involved in meningiomas.[60,61] The partial loss on chromosome 17p was obtained in 3 of 9 cases with the consensus region of 17p13. Mutations and deletions of the p53 gene were revealed in LOH studies of several types of tumors including gliomas.[53,55] However, restriction fragment length polymorphism analyses of 40 gliomas suggested that a tumor suppressor gene other than p53 is located on 17p13 and involved in progression to malignancy.[56] This is supported by studies of malignant astrocytomas[62] and pediatric primitive neuroectodermal tumors.[63] Contradictory findings have been reported on the involvement of chromosome 19. Trisomy 19 was observed by cytogenetic analysis, whereas loss of constitutional heterozygosity and partial deletions were reported in LOH studies.[11,64] This CGH analysis revealed a gain of chromosome 19 in 4 of 9 cases and a partial gain of 19q13.2-qter in 1 case. However, we do not know the allelic status of the cases with the extra copies of chromosome 19, and partial deletions detected by LOH may reflect submicroscopic deletions. Combined CGH and LOH studies are necessary to shed light on this problem.

The loss of the Y chromosome is a common observation of cytogenetic analyses in gliomas.[7,8,12] However, it was discussed also as a culture artifact, because interphase cytogenetics revealed the loss of the Y chromosome less frequently.[65,66] In six malignant gliomas from male patients banding analysis indicated a loss of the Y in two cases. Using CGH analysis the Y chromosome was detected in all six male cases.

We have begun to apply CGH analyses to low grade astrocytomas to improve the knowledge of primary changes in glial cells. Furthermore, the study of recurrent tumors will help to define an order of genetic events involved in the progression of gliomas.

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