Journal of Neuropathology and Experimental Neurology

Vol. 55, No. 9
September, 1996
pp. 973–981

The Glial and Mesenchymal Elements of Gliosarcomas Share Similar Genetic Alterations

RUDOLF H. BOERMAN, MD, PHD, KARI ANDERL, JOHN HERATH, THOMAS BORELL, NICOLA JOHNSON, JANET SCHAEFFER-KLEIN, ALLEN KIRCHHOF, ANTON K. RAAP, PHD, BERND W. SCHEITHAUER, MD, AND ROBERT B. JENKINS, MD, PHD

Abstract. The cellular origin of the sarcomatous component of gliosarcomas is controversial. It is not clear if the sarcoma arises in transition from the glial cells that comprise the gliomatous component or independently arises from non-neoplastic mesenchymal cells of the tumor stroma. Using comparative genomic hybridization (CGH) along with cytogenetic analysis, fluorescence in situ hybridization (FISH) analysis, and polymerase chain reaction (PCR) analysis of microsatellite allelic imbalance, we have evaluated the genetic alterations in the gliomatous and sarcomatous components of five gliosarcomas. The glial element was grade 4 fibrillary astrocytoma (glioblastoma multiforme) in all five tumors. The sarcoma elements were fibroblastic without osseous, chondroid, or angiosarcomatous differentiation. Gain of chromosome 7, loss of chromosome 10, deletions of the chromosome 9 p-arm, and alterations of chromosome 3 were frequently observed, demonstrating that gliosarcomas can be genetically classified as belonging to the spectrum of glioblastomas. Furthermore, the sarcomatous and gliomatous portions of each gliosarcoma investigated were similar with respect to both the presence and absence of specific genetic alterations. This observation supports the hypothesis that the sarcomatous component of a gliosarcoma either arises from the same common precursor cell as the gliomatous portion, or it arises from the gliomatous portion itself.

Key Words: Comparative genomic hybridization (CGH); Genetic alterations; Gliosarcoma.

INTRODUCTION

The gliosarcoma, also termed "Feigin tumor," is a dimorphic neoplasm containing both gliomatous and sarcomatous elements (1–5). Its incidence has been variously estimated as 2 to 8% of malignant gliomas (6–8). The glial component is usually a grade 4 fibrillary astrocytoma or glioblastoma multiforme (GBM), although sarcomatous elements have been described in association with oligodendroglioma (9), subependymoma (10), neuroblastoma (11), and even metastatic carcinoma (12).

Despite numerous morphological and histochemical studies, the cellular origin of the sarcomatous elements is controversial (13–15). The sarcoma usually has histologic features of malignant fibrous histiocytoma (16). However, other types of sarcoma have also been described as having osteocartilagenous or even myxomatous features (10, 13, 17–19). Thus, the cell underlying the development of the sarcoma component appears to have the potential to differentiate toward a variety of mesenchymal cell types (13). Even though the sarcoma has been hypothesized to arise as an independent tumor (13, 14, 20) induced in the stromal mesenchyme by the glial tumor, the sarcoma could arise from the glioma itself or from a common precursor cell (15, 21). The existence of connective tissue within gliosarcomas would then constitute an example of fibroplastic metaplasia. Neoplastic astrocytes can form collagen, and some investigators have proposed to broaden the concept of gliosarcoma to include all collagen-producing astrocytomas (14, 15, 22). The existence of gliosarcomas as a separate entity has even been questioned, and some investigators have included them in the spectrum of GBM (15, 21).

The cellular origin of the sarcomatous component of gliosarcomas can be evaluated using genetic techniques. If the sarcoma and glioma arise from a common precursor, then both components should exhibit similar genetic alterations. However, if the sarcoma is induced by the GBM, then different genetic changes should be observed in the two elements because each component will have derived from a different precursor cell. The technique of comparative genomic hybridization (CGH) is a powerful new genetic tool, one especially suited for such experiments since it provides an overview of DNA sequence copy number changes without the necessity of cell culture (23). In this study, we have applied CGH and other complementary analyses to examine the genetic alterations in the glial and mesenchymal components of gliosarcomas.

From the Department of Laboratory Medicine and Pathology (JH, KA, TB, JS-K, AK, BWS, and RBJ), Mayo Clinic and Mayo Foundation, Rochester, Minnesota; the Department of Neurology (RHB), University of Nijmegen, The Netherlands; the Department of Cytochemistry and Cytometry (AKR), University of Leiden, The Netherlands; and Christ Church College (NJ), Oxford University, United Kingdom.

Correspondence to: Robert B. Jenkins, MD, PhD, Laboratory Genetics, Mayo Clinic, 200 First Street SW, Rochester, MN 55905.

Supported in part by a grant from the NIH (CA50905 to RBJ) and by the Dutch Cancer Society (RHB).

MATERIALS AND METHODS

Tumor Samples

The tumors for this study were selected from a series of 30 gliosarcomas present in the Mayo Clinic Tissue Registry and brain tumor bank, as well as from the consultation file of BWS. The primary criterion for case selection was the availability of frozen tumor, this being necessary to validate the CGH results obtained with DNA extracted from paraffin-embedded tissue.

TABLE 1
Clinical Findings on Five Patients with Gliosarcoma

Case no.	Age/Sex	Diagnosis	Location	Surgery	Radiotherapy	Chemotherapy	Survival (years from diagnosis/years from surgery)
1	60/M	Gliosarcoma	R-temporal	GTR	50 Gy	BCNU/Temozolamide	Dead (2.50/2.25)
2	67/M	Gliosarcoma	R-occipital	GTR	64.8 Gy	BCNU/MOP	Dead (1.70/1.50)
3	41/F	Oligodendroglioma	R-frontal	GTR	—	—	Dead (3.75/3.75)
		Recurrent gliosarcoma	R-frontal	STR	58 Gy	—	Dead (3.75/1.75)
		Recurrent gliosarcoma	R-frontal	STR	—	—	Dead (3.75/0.50)
4	67/M	Gliosarcoma	R-frontal	GTR	50 Gy	BCNU	Dead (0.60/0.60)
5	33/M	Gliosarcoma	L-frontal	GTR	60 Gy	—	Alive (2.00/2.00)

GTR = Gross Total Resection; STR = Stereotactic Resection; Gy = Gray.

Essential clinicopathological data regarding the 5 patients whose tumors were studied are shown in Table 1. The frozen tumor samples were split into adjacent fragments, and one or more of the fragments were used for CGH analysis. The frozen tumor fragments adjacent to those used for CGH were sectioned and histologically evaluated. The adjacent fragments of cases 1, 4, and 5 contained both glioma and sarcoma, and those of case 3 contained only sarcoma. An adjacent fragment was not available for case 2. The samples from patient 3 were derived from the third operation.

Paraffin-embedded specimens were routinely fixed in 4% neutral buffered formalin for 12 hours to 3 days. In all cases the diagnosis of gliosarcoma was confirmed by a neuropathologist (BWS) using histochemical stains, e.g. hematoxylin/eosin (H&E), Gomori reticulin, phosphotungstic acid and hematoxylin (PTAH), and by immunocytochemical preparations (glial fibrillary acidic protein and S-100 protein). The tumors were classified in accordance with the WHO classification (5) and the criteria of Meis et al (16). The tumors were typical examples of gliosarcoma. The glial element was grade 4 fibrillary astrocytoma (glioblastoma multiforme) in all 5 tumors. The sarcomatous elements were fibroblastic in nature. No osseous, chondroid, or angiosarcomatous differentiation was noted. Paraffin blocks were selected which contained clearly distinct regions of glioma and sarcoma, each region measuring approximately 0.5 cm^2 unassociated with necrosis. Greater than 70% neoplastic tissue was present within these regions, the remainder being stromal or reactive tissue.

DNA Extraction

Reference genomic DNA from peripheral blood leukocytes of a healthy male donor as well as frozen tumor DNAs were isolated according to standard procedures (24).

DNA was derived from paraffin-embedded tissues as follows: Sixty-four 4μm sections were cut from the paraffin blocks. The first and last two sections were stained with H&E and reticulin for the purpose of identifying sarcoma, glioma, and normal tissue elements. Of the intervening consecutive sections, 25 were placed on glass slides for in situ hybridization and 35 on cellulose acetate sheets (PP2200 from 3M; cut to the size of glass slides and precleaned with 100% ethanol). The acetate slides were deparaffinized (2 × 10 minutes in Histoclear followed by 2 × 10 minutes in 100% ethanol). Specific areas of interest were cut out of the acetate slides with a scalpel and placed in microfuge tubes. Incubation with proteinase K (Sigma, St Louis, MO) was carried out for 3 days as described elsewhere (25, 26). DNA was extracted with the IsoQuick kit (ORCA Research, Bothell, WA). The DNA solution was added to a Microcon-100 microconcentrator (Amicon Inc, Beverly, MA) and eluted by centrifugation (27). DNA size was determined by gel electrophoresis (nondenaturing 1% agarose gels with ethidium bromide staining). DNA concentration was measured within the gel by fluorometry (MTI camera CCD72 and NIH Image 1.57 software) and compared to a standard of known concentration which was electrophoresed simultaneously.

Labeling of DNA

Nick translation was performed as described elsewhere (23, 26) using the Gibco kit (Gibco BRL, Gaithersburg, MD) and a modified 10× dNTP mix (0.4 mM dATP, dGTP, and dCTP; 0.8 mM dTTP; 500 mM TrisHCl [pH = 7.5]; 50 mM $MgCl_2$; 100 μg/mL BSA; and 100 mM 2-mercaptoethanol). The nick translation reaction time was 45 minutes; then the reactions were terminated by the addition of stop buffer from the Gibco kit. Direct (2 nmol FluoroGreen-dUTP and FluoroRed-dUTP [Amersham, Arlington Heights, IL]) and indirect labels (2 nmol biotin-16-dUTP and 2 nmol digoxigenin-11-dUTP [Boehringer Mannheim, Indianapolis, IN]) were compared for labeling efficiency, signal intensity, and variation on control specimens (see below). Indirect labels were used in all subsequent experiments; the tumor DNA was labeled with biotin and the reference DNA with digoxigenin. After nick translation, the sizes of the tester (tumor) and the reference DNA probes were determined by agarose gel electrophoresis. These sizes ranged between 200 to 3000 bp for all samples. Also, DNA concentration was measured by fluorometry and the amounts of DNA were adjusted so that the concentration of the tumor DNA probe equaled the concentration of the reference DNA probe.

CGH

To prepare the probe solution, 300 ng of both the labeled tester and reference DNA were precipitated together with 30 mg of Cot-1 DNA (Gibco) according to a standard procedure (24) and the pellet was redissolved in hybridization mixture (50% formamide, 2XSSC, 10% dextran sulfate, pH = 7.2). The

probe solution was denatured for 5 minutes at 80°C, quenched on ice, and allowed to preanneal at 37°C for 2 hours before application to the slides.

Metaphase chromosomes were cultured from PHA-stimulated peripheral blood lymphocytes of a normal healthy male donor according to standard cytogenetic procedures. Dropping fixed metaphase suspensions onto glass slides using routine cytogenetic techniques produced preparations with highly variable chromosome morphology and multiple chromosomal overlaps. These preparations gave inconsistent and incomplete CGH results. Dropping fixed metaphase suspensions onto slides in a controlled environment chamber (Thermatron Industries, Holland, MI) at a relative humidity of 50% and a temperature of 25°C resulted in preparations with very consistent chromosomal morphology and optimal chromosomal spreading (28). CGH results were consistent with these preparations. Slides containing metaphase spreads prepared by this method were incubated with RNase A (Boehringer; 100 mg/mL in 2XSSC) for 1 hour at 37°C, digested with pepsin (Sigma, 0.1 mg/mL in 0.01 M HCl) and postfixed with formaldehyde (Fisher Scientific, Pittsburgh, PA; 1% in PBS with 50 mM $MgCl_2$) (29). The slides were denatured in 70% formamide/2XSSC (pH = 7.2) at 80°C for 2 minutes in a hybridization oven, quenched in 70% ethanol at −20°C, and dehydrated in graded ethanols. Hybridization was carried out under sealed coverslips for 3 days at 37°C in a moist container.

After hybridization, the slides were washed 3 × 5 minutes in 50% formamide/2XSSC (pH = 7.2) at 37°C, 3 × 5 minutes in 0.1XSSC (pH = 7.2) at 60°C and once in 4XSSC at room temperature. For immunocytochemical staining, the slides were preincubated with 0.5% dry milk powder in 4XSSC for 30 minutes at 37°C in a moist container. The DNA sequences were visualized by incubation with streptavidin-FITC (Vector Laboratories, Inc, Burlingame, CA) and anti-digoxigenin-Rhodamine (Boehringer) (both diluted 100-fold in 0.5% dry milk powder in 4XSSC) for 60 minutes at 37°C in a moist container and washed 3 × 5 minutes with 4XSSC/0.05% Tween. Chromosome preparations were dehydrated and counterstained with 4′-6-diamidino-2-phenylindoledihydrochloride (DAPI, Serva, Germany).

Image Analysis

The images were captured with a Zeiss Axiophot fluorescence microscope (Carl Zeiss Inc, Thornwood, New York) equipped with a 100 Neo-Fluar objective, a computer-controlled filter wheel, and a cooled CCD camera (Photometrics, Tucson, AZ). Analysis was carried out on a PowerMac computer loaded with Smartcapture IPLab Extensions software (VYSIS, Downers Grove, IL and Digital Scientific, Cambridge, United Kingdom). The image analysis procedure was performed using previously described methods (23, 30) with minor modifications. In order to obtain average CGH fluorescence ratio curves, 5 to 10 metaphase spreads were analyzed from each tumor.

Control Experiments and Abnormal Fluorescence Ratio Thresholds

We performed a number of control CGH experiments using DNAs derived from leukocytes of healthy male and female donors, from non-neoplastic brain tissue resected from patients with seizure disorders, and from short-term cell cultures derived from high grade astrocytomas. The average fluorescence ratio (FR) for CGH with normal DNA as tester was 1.0 (range 0.9 to 1.1) with a standard deviation of 0.1. The X-chromosome FR in CGH experiments using normal male against normal female DNA samples was 0.4 (SD = 0.1). Experiments with tester DNAs extracted from paraffin-embedded non-neoplastic brain tissue yielded similar results. Aberrations present in the cultured tumors were detected as FRs above 1.25 for numerical or regional chromosomal gain and below 0.75 for numerical or regional chromosomal loss. The ratios correlated to the known aberrant copy number obtained by routine cytogenetic analysis of the cultured tumors (data not shown). However, an exact linear relationship (31) between copy number and ratio could not be established because the short-term cultures were not monoclonal. Therefore, based on these experiments and the published requirements for quantitative analysis of CGH (32), FR thresholds of 0.75 and 1.25 were chosen to distinguish numerical and regional chromosomal loss and gain, respectively. A regional amplification was defined as an FR > 2.0. Finally, each gliosarcoma CGH experiment was performed simultaneously with a control hybridization using normal DNA as a tester to measure the normal autosome and X chromosome FR.

Validation by Reverse CGH, Fluorescence In Situ Hybridization (FISH) and Polymerase Chain Reaction (PCR) Analysis of Microsatellite Alleles

Reverse CGH (23) was carried out on all frozen specimens and a subset of the paraffin-embedded specimens to independently ascertain the alterations found in the initial experiments. This time the tester (tumor) DNA was detected with a red fluorophore, and the reference DNA was detected with a green fluorophore.

Fluorescence in situ hybridization (FISH) was performed on paraffin-embedded tissue sections using SpectrumOrange-labeled chromosome enumeration probes (CEP™, Vysis, Inc, Downers Grove, IL) for chromosomes 7, 10, and 17 as described elsewhere (33). Chromosomes 7 and 10 were chosen because gains of chromosome 7 and losses of chromosome 10 were the most common numerical abnormalities observed by CGH. They also constitute the most common alterations in GBM (34–38); furthermore, monosomy 10 has been described in gliosarcomas (39). Chromosome 17 was chosen because it represented a chromosome without numerical loss in the CGH experiments. However, chromosome 17 is of particular interest because its centromeres are usually paired in normal brain cells, and the loss of this pairing may have biological significance in gliomas (33, 40). The CEP signals were evaluated using a Zeiss Axioplan fluorescent microscope equipped for FITC, TRITC, and DAPI fluorescence. The number of signals within 300 nuclei were enumerated by two different observers using previously described criteria (41, 42).

Paired blood and tumor samples for three patients (cases 1, 2, and 3) were available for PCR analysis of microsatellite allele imbalance, which was performed as described previously (43). The markers evaluated were selected because either they mapped to regions showing loss or gain in the CGH experiments, or they mapped to regions known to be involved in

GBM. The films were evaluated by visual inspection; in cases of equivocal results, neighboring markers were evaluated. The markers used were: D1S199, D3S1284, D3S1309, D3S1268, D3S1306, D6S273, D6S263, D7S484, D7S486, D7S507, D7S522, D8S273, D9S126, D9S171, D9S157, D9S163, D10S226, D10S219, D11S987, D17S796, D17S786, D22S421, and D22S281 (all obtained from Research Genetics, Huntsville, AL).

RESULTS

CGH

CGH was performed using both DNA derived from frozen tumor tissue and DNA isolated from microdissected sarcomatous and gliomatous components present within paraffin-embedded tumor specimens. All results were rigorously controlled in repeated standard and reverse CGH experiments as described in Materials and Methods. Figure 1 illustrates typical CGH results from the frozen and paraffin-embedded tissue of case 1. The frozen tumor of case 1 showed gains of chromosome 7 and of the long arm of chromosome 3. Losses of the short arm of chromosome 9, the long arm of chromosome 11, and both arms of chromosome 10 were also detected. Evidence of loss of the short arm of chromosome 18 was consistently observed in several experiments but did not exceed the abnormal FR threshold criteria. The results from the paraffin-embedded regions showed gains of chromosome 7 and losses of chromosome 10 as well as the short arms of chromosomes 9 and 18 in both glioma and sarcoma. Loss of the long arm of chromosome 11 was detected only in the sarcomatous element.

Table 2 summarizes the alterations detected by CGH for both frozen and paraffin-embedded tumor specimens. As described above for case 1, a number of experiments resulted in either no detection of alterations in one of multiple CGH experiments or consistent detection of a number of alterations that did not exceed the abnormal FR threshold criteria. These regions have been included in Table 2 and placed in parenthesis because they may represent important alterations in gliosarcomas. In addition, although the p-arms of chromosomes 1 and 16 may give rise to false CGH interpretation of deletion (23), they are also included in Table 2 because these regions were apparently gained in some tumors.

The most frequent reproducible CGH alterations in the frozen specimens were: gain of some or all of chromosome 7 (4 cases), loss of chromosome 10 (2 cases), and loss of 9p (2 cases). These results were also present in the paraffin-embedded tumor tissue for two cases (cases 1 and 2). Gains of the chromosomes 1p-arm and 3q-arm were detected in two frozen tumors each. Alterations of 1q, 6p, 7p, 8q, 11p, 11q, and 16p were each detected only once in the frozen tumors studied.

The CGH experiments performed using the microdissected DNA from paraffin-embedded tumor specimens were generally successful. However, the yield of high-molecular DNA was low and in most instances no more than two CGH experiments could be performed on each component. An insufficient amount of glioma tissue from case 4 was available for analysis and the quality of DNA from case 3 was poor and did not give meaningful CGH measurements. The latter observation was surprising because the amount of DNA was adequate, the average DNA fragment length did not significantly differ from other DNA samples, and necrosis of the tissue was limited. The most likely explanation for this inconsistency would be that lengthy formalin fixation chemically damaged the DNA and/or the tissue underwent autolysis (see Dubeau et al, reference 25). This would be consistent with the dim FISH signals observed on sections taken from the same block (see below).

The tumor components of case 5 were apparently normal. Because normal genetic studies of GBM are rather unusual, standard and reverse CGH analysis of these tumors was repeated a total of 5 times without any FR variation ever exceeding an abnormal threshold criteria.

A comparison of CGH results shows that all numerical and most regional chromosomal abnormalities present in frozen tissue were also detected in paraffin-embedded tissue (see Table 2 and Fig. 1). Table 2 and Figure 1 also demonstrate that sarcomatous and gliomatous portions from all tumors were similar with respect to both the presence (case 1, 2, and 4) and absence (case 5) of CGH alterations. There were some minor differences between the components including loss of 11q in the sarcoma of case 1, loss of chromosome 3 in the sarcoma of case 2, and losses of 9p and 21p in the glioma of case 2.

Validation

CGH results were compared with routine cytogenetic data, PCR analyses of microsatellite allele imbalance and FISH studies. The results of these experiments are summarized in Table 2.

The cytogenetic analysis showed complex clonal karyotypes in cases 1 and 2 and a complex nonclonal karyotype in case 3. For case 1, the numerical chromosomal abnormalities and the loss of 9p detected by CGH match the cytogenetic results. For case 2, the numerical alterations and the deletions of the long arm of chromosome 18 detected by CGH also correlate with the cytogenetic observations. However, for case 3, the CGH alterations were not observed in the single abnormal cell that was available for karyotype analysis.

The results of PCR analysis of microsatellite allelic imbalance often matched the CGH data. For example, most regional or chromosomal losses detected by CGH in frozen tumor specimens were also detected as regional imbalances of microsatellite alleles. The sole exception for this was case 1 in which CGH detected the loss of 11q but microsatellite analysis did not. However, the regional

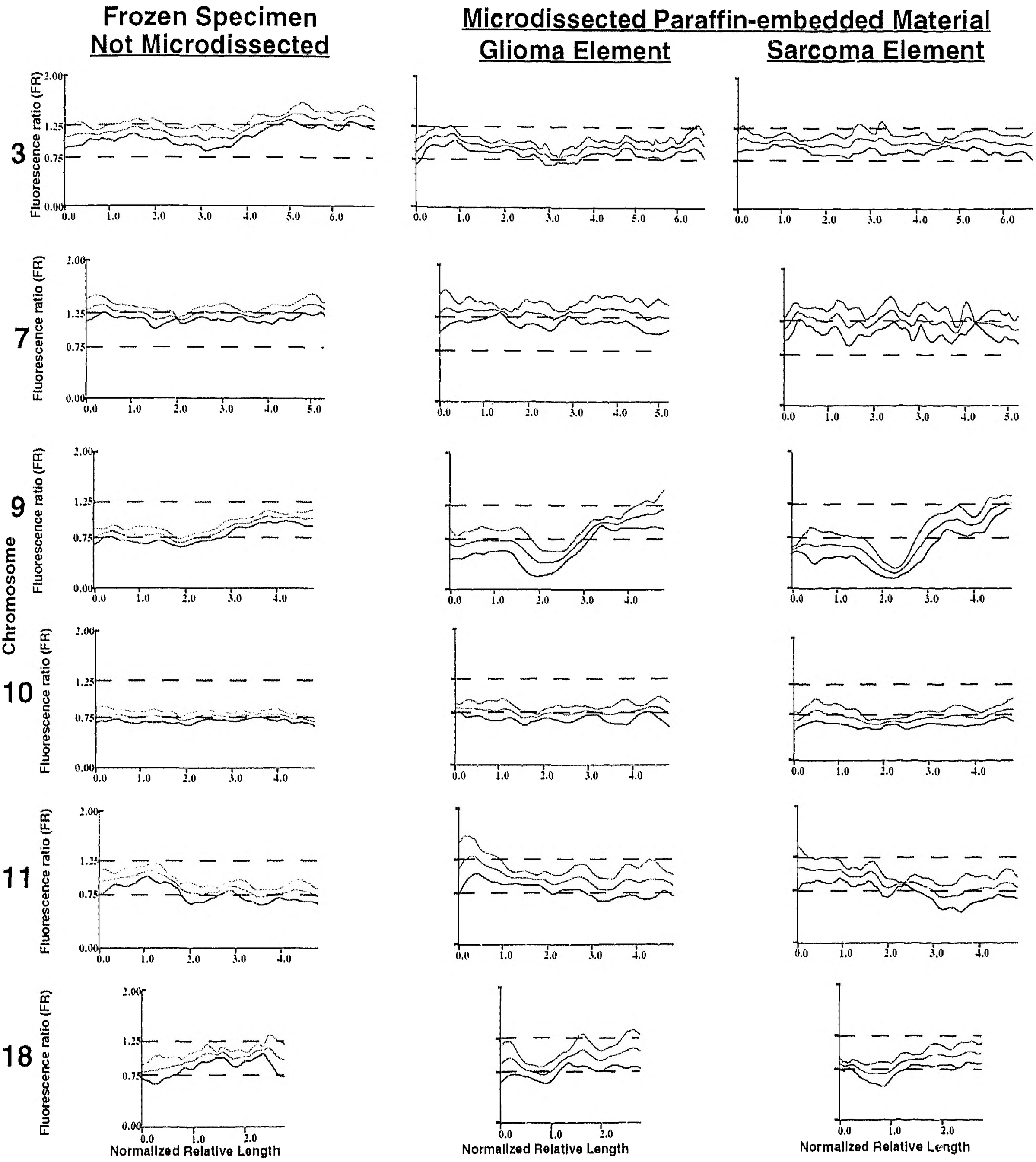

Fig. 1. An overview of the CGH alterations detected in frozen specimens and paraffin blocks of case 1. Column 1: CGH results on a frozen specimen. Columns 2 and 3: CGH results on microdissected gliomatous and sarcomatous components. The Y-axis for each graph displays fluorescence ratio (FR) for selected chromosomes. The mean FR, measured on 5 to 10 metaphases, is indicated by the red line. The standard deviation of the FR is indicated by the blue and green lines. The X-axis has been normalized to the relative length of the chromosome (as a percentage of the total haploid autosome length) with the p-arm to the left. The dashed horizontal lines indicate FR thresholds of 0.75 and 1.25 used to distinguish numerical and regional chromosomal loss and gain, respectively.

TABLE 2
Summary of Genetic Studies on Frozen Tumor Specimens and Microdissected Paraffin-embedded Gliomatous and Sarcomatous Components From Five Gliosarcomas

Case no./Tissue type	CGH Analysis		FISH analysis	Karyotype*	PCR analysis of microsatellite alleles	
	Gain	Loss			Imbalance	No imbalance
1) Frozen tumor (glioma and sarcoma)	3q, 7	9p, 10, 11q, (18p)**	ND***	44, XY, +7, −10, −11 × 2, add(1)(q42), add(9)(p13), +3–5mar[cp2]/45, X, −Y[4]/44, XX[4]	7p, 7q, 9p, 10p, 10q	1p, 3p, 3q, 6p, 8q, 11q, 17p, 22q
Paraffin-embedded regions:						
Glioma	7	9p, 10, 18p	+7, −10, split 17	NA	ND	ND
Sarcoma	7	9p, 10, 11q, 18p	+7, −10, split 17	NA	ND	ND
2) Frozen tumor (glioma and/or sarcoma)	1p, 1q, 7	9p, 10, (18q)	ND	47, XY, +7 × 2, −10, −11, t(1;20)(p22;p13), t(3;15)(q21;q22), der(18)t(1;18)(q11;q21), +mar[30]	9p, 10p, 10q	1p, 3q, 7p, 7q, 8q, 11q, 17p, 22q
Paraffin-embedded regions:						
Glioma	1q, 7	9p, 10, 18q, (21p)	+7, −10, split 17	NA	ND	ND
Sarcoma	7	3, 10, 18q	+7, −10, split 17	NA	ND	ND
3) Frozen tumor (sarcoma)	1p, 3q, 6p, 7p, 8q	(3p)	ND	46, XX, add(1)(q23), del(4)(p14), del(7)(q32), del(9)(p13), del(10)(p13), del(13)(q14), add(16)(q22)[1]	3p	1p, 3q, 7p, 7q, 8q, 10p, 10q, 11q, 17p
Paraffin-embedded regions:						
Glioma	not interpretable		+7, N10, split 17	NA	ND	ND
Sarcoma	not interpretable		+7, N10, split 17	NA	ND	ND
4) Frozen tumor (glioma and sarcoma)	7, (11p, 16p)	—	ND	ND	ND	ND
Paraffin-embedded regions:						
Glioma	ND	ND	+7, −10, split 17	NA	ND	ND
Sarcoma	7	—	+7, −10, split 17	NA	ND	ND
5) Frozen tumor (glioma and sarcoma)	—	—	ND	ND	ND	ND
Paraffin-embedded regions:						
Glioma	—	—	+7, −10, split 17	NA	ND	ND
Sarcoma	—	—	+7, −10, split 17	NA	ND	ND

* Cytogenetic studies were performed on a portion of the tissue prior to being frozen as previously described (Jenkins et al, 1989).

** CGH alterations in parentheses were either not detected in one of several repetitions, or were consistently detected but did not exceed the abnormal FR threshold criteria.

*** ND = Not Done; NA = Not applicable; — = No apparent anomalies; N10 = Apparently normal chromosome 10 centromere FISH counts.

or chromosomal gains detected by CGH were rarely detected by the microsatellite markers. This observation was expected, since a decrease in microsatellite allele intensity is more conspicuous than an increase. Nevertheless, the overall concordance of PCR and CGH analysis was 70% (with a concordance of 96% for losses only) providing further proof for the validity of the CGH procedure.

Similarly, FISH analysis of paraffin sections were generally concordant with the CGH results for all chromosomes evaluated. For example, chromosome 7 was apparently gained and chromosome 10 was apparently lost in cases 1 and 2 by both CGH and FISH. For case 3, neither FISH nor CGH analysis revealed any apparent chromosome 10 or 17 anomaly. In addition, FISH detected a gain of the chromosome 7 centromere in the paraffin sections of both components and CGH detected a gain of the chromosome 7 p-arm in the frozen sarcomatous tissue. In some instances, the FISH and CGH analyses were discordant. Loss of chromosome 10 and gain of chromosome 7 were detected by FISH in cases 4 and 5. However, only the gain of chromosome 7 was detected by CGH in case 4. Normal copy numbers for chromosome 17 were found in all samples; but in contrast to normal tissue, the homologues were no longer somatically paired in tumor cells. Overall, a concordance of 70% was found between FISH and CGH.

DISCUSSION

The nature of gliosarcoma has long been debated and has been the subject of numerous studies (1–22). Therefore, fundamental data regarding its molecular biology, particularly as compared to that of GBM, must be collected. We used CGH to evaluate the presence and absence of genetic alterations in gliosarcomas. We devoted considerable effort to optimize and validate our CGH experiments. The CGH procedure which we finally adopted for this study was reliable because the results were generally concordant with cytogenetic data, FISH analysis, and PCR analysis. Other groups have also confirmed the general validity of CGH (23, 44–46). In addition, we have demonstrated that CGH analysis can be successfully performed on DNA derived from paraffin-embedded specimens. These CGH analyses were generally concordant with CGH studies of matched frozen tissue specimens. However, three cases showed a small number of regional gains in the frozen specimens but not in the paraffin-embedded specimens (e.g. gains of 1p, 3q, 11p, and 16p). Given the overall concordance of the frozen and paraffin-embedded results, this likely reflects the difficulty of performing CGH experiments on a limited amount of paraffin-embedded tissue. Similar results have been described by Isola et al (26) for other tumors.

Two technical points of the CGH procedure must be discussed. First, accurate quantitation of the concentrations of the tumor and reference DNAs is critical for reliable CGH results. This is not always easy to attain, due to the inaccuracy of current standard DNA concentration measurement procedures (47, 48) and the large differences in sample quality, especially for tumor DNAs. Thus, after nick translation, we measured the DNA probe concentrations using fluorometry of gel-electrophoresed DNA. Then we adjusted the amounts of DNA to attain equal concentrations of tumor and reference DNA for reliable CGH results. Second, visualization and accurate measurement of CGH data is often impeded by the poor quality of the normal metaphase preparations used as the hybridization substrate. We observed that the use of an environmentally controlled chamber to prepare the slides ensures that the normal metaphases are optimally spread and of excellent morphology (28). This procedure greatly improves the selection of metaphases and simplifies CGH analysis.

The CGH method is a reliable and valid tool for the evaluation of genetic alterations in frozen and paraffin-embedded tumor specimens. The main advantage of CGH is that most major chromosomal abnormalities present within a tumor can be detected in a single experiment. This makes CGH attractive to perform compared to the laborious enumeration of a wide range of possible chromosomal anomalies by FISH. CGH can potentially select the chromosomal anomalies which would be appropriate to study by FISH. Moreover, CGH is almost always possible if frozen tissue is used for DNA isolation. However, CGH does have some disadvantages. First, CGH will only detect clonal anomalies if they are present in more than 50% of the cells from which the DNA was derived. This limit is defined by the 0.75 and 1.25 thresholds used in CGH (23, 31, 32). This may be the reason why CGH was unable to detect the loss of chromosome 10 in cases 4 and 5 and the gain of chromosome 7 in case 5 that were detected by FISH. Alternatively, this discrepancy may be attributed to the loss (or gain) of the centromeric region without alteration of the rest of the chromosome. This phenomenon is possible but is thought to be unusual. Another explanation might be sampling bias, but since adjacent tissue sections were used for FISH and CGH, this is also unlikely. Second, the abnormalities detected by CGH did not differ much from those detected by routine cytogenetic analysis. Therefore, because cytogenetics analysis can detect balanced translocations and often small deletions, it is still useful for detecting genetic anomalies. Third, although CGH is an elegant procedure, it is not easy to perform and requires rather sophisticated and expensive microscopic equipment and image analysis software. Finally, successful CGH requires meticulous attention to detail, whereby the procedure must be repeated in both standard and reverse conditions and the results should be checked using other methods.

This is the first study to provide an overview of the genetic alterations observed in gliosarcomas, specifically their separate glial and mesenchymal elements. Our CGH

observations are similar to those of prior CGH studies of malignant gliomas (49–51) and glioma cell lines (52) as well as other genetic studies of high grade gliomas (34–38). Gains of chromosome 7, losses of chromosome 10, and deletions of the chromosome 9 p-arm were common and firmly establish gliosarcomas within the spectrum of malignant astrocytic tumors (36, 39).

We did not detect loss of chromosome 17 (39) or an imbalance of 17p microsatellite alleles in gliosarcomas. The absence of these anomalies is interesting because they are common in high grade astrocytomas and often occur in combination with chromosome 10 and 9p losses (53, 54). The only abnormality detected was the loss of homologous pairing of the chromosome 17 centromeres in all sarcoma and glioma regions. Conversely, the normal areas from the same specimens retained homologous pairing (data not shown). This phenomenon may be of functional importance in normal brain cells (55), although its significance in tumors is still unclear (33, 40). Perhaps the most interesting observation is the involvement of chromosome 3 in three gliosarcomas. Although the number of cases is small, this may point to an important clonal alteration leading to the development of this complex tumor. Finally, the other gains (1p, 1q, 6p, 8q) and losses (11q, 18p, 18q) we observed by CGH have also been detected by prior CGH studies of gliomas (49–51) or glioma cell lines (52).

The most intriguing aspect of gliosarcoma biology is the emergence of the sarcomatous component. Our data presented in Figure 1 and in Table 2 indicate that the genetic makeup of both components is similar and often identical. These observations do not support the hypothesis of an independent sarcoma originating from an endogenous mesenchymal stem cell, residing within tumor stroma. Instead, our results are consistent with the hypothesis that the sarcomatous component arises by a process of "divergent differentiation" either from the same common precursor cell as does the gliomatous component or by metaplasia from the gliomatous component itself. It is remotely possible that the findings in this study simply reflect the contamination of the sarcomatous component with glioma tissue. However, the blocks were selected for the presence of clearly distinct glioma and sarcoma elements. Moreover, our results are supported by a prior FISH study (39) and by the recently published finding of identical mutations of the p53 gene within the sarcoma and glioma portions of 2 gliosarcomas (56).

In conclusion, we believe that the term gliosarcoma describes a morphological entity and should continue to be used as such. The genetic alterations of both the glioma and the sarcoma elements of gliosarcoma are similar to one another and to the pattern of genetic aberrations observed in GBM. These observations support a common origin of both elements. No single genetic alteration has been linked to the peculiar morphology of this tumor, although alterations of chromosome 3 were common in this group of gliosarcomas and have been thought to be less common in GBM (34–36, 49–50). Importantly, gliosarcoma remains an interesting biological entity for further investigation. The tumor appears to represent an example of either a precursor cell differentiating toward two histologically different components, or of mesenchymal metaplasia of neoplastic glial cells. Further studies will be required to determine which of these pathways underlies the "divergent differentiation" of gliosarcomas.

ACKNOWLEDGMENTS

We thank Karen Erwin and Gladys Hebl for their assistance in preparing this manuscript.

REFERENCES

1. Stroebe H. Uber entstehung and bau der hirngliome. Beitr Pathol Anat Allg Pathol 1895;18:405–86
2. Feigin IH, Gross SW. Sarcoma arising in glioblastoma of the brain. Am J Pathol 1955;31:633–53
3. Feigin I, Allen LB, Lipkin L, Gross SW. The endothelial hyperplasia of the cerebral blood vessels with brain tumors and its sarcomatous transformation. Cancer 1958;11:264–77
4. Rubinstein LJ. The development of contiguous sarcomatous and gliomatous tissue in intracranial tumours. J Pathol 1956;71:441–59
5. Kleihues P, Burger PC, Scheithauer BW. Histological typing of tumours of the central nervous system. World Health Organization. International histological classification of tumours. Berlin: Springer-Verlag, 1993
6. Bannerjee AK, Sharma BS, Kab VK, Ghatak NR. Gliosarcoma with cartilage formation. Cancer 1989;63:518–23
7. Morantz RA, Feigin I, Ransohoff J. Clinical and pathologic study of 24 cases of gliosarcoma. J Neurosurg 1976;16:662–72
8. Burger PC, Scheithauer BW. Tumors of the central nervous system. Atlas of tumor pathology. Third series. Fascicle 10. Armed Forces Institute of Pathology, Washington DC: 1994
9. Pasquier B, Coudere P, Pasquier D, Hong Panh M, N'Golet A. Sarcoma arising in oligodendroglioma of the brain. Cancer 1978;42:2753–58
10. Tomlinson FH, Scheithauer BW, Kelly PJ, Forbes GS. Subependymoma with rhabdomyosarcomatous differentiation: Report of a case and literature review. Neurosurgery 1991;28:761–68
11. Horten BC, Rubinstein LJ. Primary cerebral neuroblastoma. A clinicopathological study of 35 cases. Brain 1976;99:735–56
12. Schwarz I, Feigin I. Sarcoma arising in a metastatic carcinoma of the brain. Acta Neuropathol 1963;3:74–78
13. Ho K-L. Histogenesis of sarcomatous component of the gliosarcoma: An ultrastructural study. Acta Neuropathol 1990;81:178–88
14. Cerda-Nicolas M, Kepes JJ. Gliofibromas (including malignant forms), and gliosarcomas: A comparative study and review of the literature. Acta Neuropathol 1993;85:349–61
15. Jones H, Steart PV, Weller RO. Spindle-cell glioblastoma or gliosarcoma? Neuropathol Appl Neurobiol 1991;17:177–87
16. Meis JM, Martz, KL, Nelson JS. Mixed glioblastoma multiforme and sarcoma: A clinicopathologic study of radiation therapy oncology group cases. Cancer 1991;67:2342–49
17. Paulus W, Jellinger K. Mixed glioblastoma and malignant mesenchymoma, a variety of gliosarcoma. Histopathol 1993;22:277–79
18. Hayashi K, Ohara N, Jong Jeon H, Akagi S, Takahashi K, Akagi T, Namba S. Gliosarcoma with features of chondroblastic osteosarcoma. Cancer 1993;72:850–55

19. Barnard RO, Bradford R, Scott T, Thoma DG. Gliomyosarcoma: Report of a case of rhabdomyosarcoma arising in a malignant glioma. Acta Neuropathol (Berl) 1986;69:23–27
20. Lalitha VS, Rubinstein LJ. Reactive glioma in intracranial sarcoma: A form of mixed sarcoma and glioma ("sarcoglioma"). Cancer 1979;43:246–57
21. Paulus W, Jellinger K. Desmoplastic spindle-cell glioblastoma or gliosarcoma? Neuropathol Appl Neurobiol 1992;18:207–8
22. Bozoky B, Kemeny E, Krenacs T, Razga T, Barzo P. Gliosarcomas as a result of sarcomatous differentiation of glial tumor cells. Brain Tumor Pathol 1991;8:45–49
23. Kallioniemi O-P, Kallioniemi A, Piper J, Isola J, Waldman FM, Gray JW, Pinkel D. Optimizing comparative genomic hybridization for analysis of DNA sequence copy number changes in solid tumors. Genes Chrom Cancer 1994;10:231–43
24. Sambrook J, Fritsch EF, Maniatis T. Molecular cloning: A laboratory manual. Cold Spring Harbor, New York Cold Spring Harbor Laboratory, 1989
25. Dubeau L, Chandler L, Gralow JR, Nichols PW, Jones PA. Southern blot analysis of DNA extracted from formalin-fixed pathology specimens. Cancer Res 1986;46:2964–69
26. Isola J, DeVries S, Chu L, Ghazvini S, Waldman F. Analysis of changes in DNA sequence copy number by comparative genomic hybridization in archival paraffin-embedded tumor samples. Am J Pathol 1994;145:1301–8
27. Hunter SB, Abbott K, Varma VA, Olson JJ, Barnett DW, James CD. Reliability of differential PCR for the detection of EGFR and MDM2 gene amplification in DNA extracted from FFPE glioma specimens. J Neuropathol Exp Neurol 1995;54:57–64
28. Spurbeck JL, Zinsmeister AR, Meyer KJ, Jalal SM. The dynamics of chromosome spreading. Am J Med Genet 1996;61:387–93
29. Nederlof PM, ven der Flier S, Wiegant J, Raap AK, Tanke HJ, Ploem JS, van der Ploeg M. Multiple fluorescence in situ hybridization. Cytometry 1990;11:126–31
30. Kallioniemi A, Kallioniemi O-P, Sudar D, Rudovitz D, Gray JW, Waldman F, Pinkel D. Comparative genomic hybridization for molecular cytogenetic analysis of solid tumors. Science 1992;258:818–21
31. Piper J, Rutowitz D, Sudar D, et al. Computer image analysis of comparative genomic hybridization. Cytometry 1995;19:10–26
32. du Manoir S, Kallioniemi O-P, Lichter P, et al. Hardware and software requirements for quantitative analysis of comparative genomic hybridization. Cytometry 1995;19:4–9
33. Dalrymple SJ, Herath J, Borell TJ, Moertel CA, Jenkins RB. Correlation of cytogenetic and fluorescence in situ hybridization (FISH) studies in normal and gliotic brain. J Neuropathol Exp Neurol 1994;53:448–56
34. Rey JA, Bello MJ, de Campos JM, Kusak ME, Ramos C, Benitez J. Chromosomal patterns in human malignant astrocytomas. Cancer Genet Cytogenet 1987;29:201–21
35. Bigner SH, Mark J, Burger PC, Mahaley MS Jr, Bullard DE, Muhlbaier LH, Bigner DD. Specific chromosomal abnormalities in malignant human gliomas. Cancer Res 1988;48:405–11
36. Jenkins RB, Kimmel DW, Moertel CA, Schultz CG, Scheithauer BW, Kelly PJ, Dewald GW. A cytogenetic study of 53 human gliomas. Cancer Genet Cytogenet 1989;39:253–79
37. Arnoldus EPJ, Noordermeer IA, Peters ACB, Voormolen JHC, Bots GTAB, Raap AK, van der Ploeg M. Interphase cytogenetics of brain tumours. Genes Chrom Cancer 1991;3:101–7
38. Ransom DT, Ritland SR, Moertel CA, et al. Correlation of cytogenetic analysis and loss of heterozygosity studies in human diffuse astrocytomas and mixed oligoastrocytomas. Genes Chrom Cancer 1992;5:357–74
39. Paulus W, Bayas A, Ott G, Roggendorf W. Interphase cytogenetics of glioblastoma and gliosarcoma. Acta Neuropathol 1994;88:420–25
40. Arnoldus EPJ, Noordermeer IA, Peters ACB, Raap AK, van der Ploeg M. Interphase cytogenetics reveals somatic pairing of chromosome 17 centromeres in normal human brain tissue, but no trisomy 7 or sex chromosome loss. Cytogenet Cell Genet 1991;56:214–16
41. Hopman AHN, van Hooren E, van der Kaa CA, Vooijs GP, Ramaekers FCS. Detection of numerical chromosome aberrations using in situ hybridization in paraffin sections of routinely processed bladder cancers. Mod Pathol 1991;4:503–13
42. Poddighe PJ, Moesker O, Smeets D, Awwad BH, Ramaekers FCS, Hopman AHN. Interphase cytogenetics of hematological cancer: Comparison of classical karyotyping and in situ hybridization using a panel of eleven chromosome specific DNA probes. Cancer Res 1991;51:1959–67
43. Ritland SR, Ganju V, Jenkins RB. Region-specific loss of heterozygosity on chromosome 19 is related to the morphologic type of human glioma. Genes Chrom Cancer 1995;12:277–82
44. Kallioniemi A, Kallioniemi O-P, Citro G, et al. Identification of gains and losses of DNA sequences in primary bladder cancer by comparative genomic hybridization. Genes Chrom Cancer 1995;12:213–19
45. Matsumura K, Kallioniemi A, Kallioniemi O-P, et al. Deletion of chromosome 17p loci in breast cancer detected by fluorescence in situ hybridization. Cancer Res 1992;52:820–23
46. Sakamoto M, Sakunaga H, Yang-Feng TL, Gray JW. Comparative genomic hybridization and loss of heterozygosity studies in ovarian cancer. Am J Hum Genet 1993;53:356
47. Glasel JA. Validity of nucleic acid purities monitored by 260 nm/280 nm absorbance ratios. BioTechniques 1995;18:62–63
48. Huberman JA. Importance of measuring nucleic acid absorbance at 240 nm as well as at 260 and 280 nm. BioTechniques 1995;18:636
49. Schrock E, Thiel G, Lozanova T, et al. Comparative genomic hybridization of human malignant gliomas reveals multiple amplification sites and nonrandom chromosomal gains and losses. Am J Pathol 1994;144:1203–18
50. Schlegel J, Scherthan H, Arens N, Stumm G, Kiessling M. Detection of complex genetic alterations in human glioblastoma multiforme using comparative genomic hybridization. J Neuropath Exp Neurol 1996;55:81–87
51. Kim DH, Mohapatra G, Bollen A, Waldman FM, Feuerstein BG. Chromosomal abnormalities in glioblastoma multiforme tumors and glial cell lines detected by comparative genomic hybridization. Int J Cancer 1995;60:812–19
52. Mohapatra G, Kim DH, Feuerstein BG. Detection of multiple gains and losses of genetic material in ten glioma cell lines by comparative genomic hybridization. Genes Chrom Cancer 1995;13:86–93
53. Leon SP, Zhu J, Black PMcL. Genetic aberrations in brain tumors. Neurosurg 1994;34:708–22
54. Dalrymple SJ, Jenkins RB. Molecular genetics of astrocytomas and meningiomas. Curr Opin Neurol 1994;7:477–83
55. Borden J, Manuelidis L. Movement of the X chromosome in epilepsy. Science 1988;242:1687–91
56. Biernat W, Aguzzi A, Sure U, Grant JW, Kleihues P, Hegi M. Identical mutations of the p53 suppressor gene in the gliomatous and the sarcomatous components of gliosarcomas suggest a common origin from glial cells. J Neuropath Exp Neurol 1995;54:651–56

Received March 12, 1996
Revision received June 4, 1996
Accepted June 6, 1996