Comparative Genomic Hybridization Analysis of Astrocytomas

Prognostic and Diagnostic Implications

Rodney N. Wiltshire,* James E. Herndon II,[†]
Annie Lloyd,* Henry S. Friedman,*[‡]
Darell D. Bigner,* Sandra H. Bigner,* and
Roger E. McLendon*

From the Departments of Pathology,* Pediatrics,[‡] and Cancer
Center Biostatistics,[†], Duke University Medical Center, Durham,
North Carolina

Astrocytoma is comprised of a group of common intracranial neoplasms that are classified into four grades based on the World Health Organization histological criteria and patient survival. To date, histological grade, patient age, and clinical performance, as reflected in the Karnofsky score, are the most reliable prognostic predictors. Recently, there has been a significant effort to identify additional prognostic markers using objective molecular genetic techniques. We believe that the identification of such markers will characterize new chromosomal loci important in astrocytoma progression and aid clinical diagnosis and prognosis. To this end, our laboratory used comparative genomic hybridization to identify DNA sequence copy number changes in 102 astrocytomas. Novel losses of 19p loci were detected in low-grade pilocytic astrocytomas and losses of loci on 9p, 10, and 22 along with gains on 7, 19, and 20 were detected in a significant proportion of high-grade astrocytomas. The Cox proportional hazards statistical modeling showed that the presence of +7q and -10q comparative genomic hybridization alterations significantly increased a patient's risk of dying, independent of histological grade. This investigation demonstrates the efficacy of comparative genomic hybridization for identifying tumor suppressor and oncogene loci in different astrocytic grades. The cumulative effect of these loci is an important consideration in their diagnostic and prognostic implications. (*J Mol Diagn* 2004, 6:166-179)

Astrocytic tumors are the most common primary central nervous system neoplasm that affects patients of all ages. They are diagnostically divided into four histologi-

cal grades according to the World Health Organization (World Health Organization) classification scheme, which correlates morphological features with prognostic significance.^{1,2} Currently, the best prognostic markers for patients with astrocytic tumors are the World Health Organization tumor grade, patient's age at the time of diagnosis, and clinical performance.³ The inherent subjectivity of histological grading has complicated tumor classification and has prompted a search for objective molecular protocols.^{4,5}

Cytogenetic analyses on World Health Organization Grades I, II, and III astrocytoma typically reveal normal karyotypes; however, a few specimens exhibiting polysomy for chromosome 7 and monosomy for chromosomes 10, 17p, and 22 have been reported.⁶ These earlier karyotypic investigations foreshadowed the molecular genetic analyses that detected limited genetic alterations in World Health Organization Grade I tumors. Mutations in the *TP53* (17p13) gene and over-expression of the platelet derived growth factor receptor, α (*PDGFRA*) (4q12) gene are reported in up to 50% of well-differentiated astrocytomas (A), World Health Organization Grade II. Additional alterations, including loss of heterozygosity (LOH) of 19q and mutations in the retinoblastoma (*RB*) (13q14) gene, are reported in anaplastic astrocytomas (AA), World Health Organization Grade III.^{2,7} Among glioblastoma multiforme (GBM), World Health Organization Grade IV, frequent cytogenetic abnormalities including polysomy of chromosome 7, monosomy of 9p and 10, and double minutes representing gene amplifications have been confirmed by molecular studies.^{6,8,9} *PTEN* (10q23) gene mutations, *CDKN2A/B* (9p21) gene deletions, and over-expression of the *EGFR* (7p12) gene have been clearly demonstrated in AA and GBM. Their involvement in the pathogenesis of astrocy-

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Address reprint requests to Dr. Roger E. McLendon, Duke University Medical Center, Department of Pathology, Box 3712, Durham, NC 27710. E-mail: mclen001@mc.duke.edu.

tomas and potential as prognostic and diagnostic markers are under investigation.⁹⁻¹⁶

Comparative genomic hybridization (CGH) is a powerful molecular cytogenetic technique that allows the examination of gross copy number changes in solid tumors. CGH analyses of gliomas have proven to be reliable and correspond to the data obtained from conventional studies.^{9,17,18} CGH has successfully detected recurrent changes in low-grade astrocytic tumors. Gains of 6q, 7, and 9q loci have recently been reported in pilocytic astrocytomas (PA), World Health Organization Grade I.^{19,20} Reproducible gains of 7q and 8q have been reported in A, while gains of 7 and 12p along with losses on 9p, 10, and 13 have been cited in AA.^{7,21-23}

To identify additional candidate loci associated with astrocytic tumors, this study used CGH to study 102 tumors ranging from World Health Organization Grades I to IV. The only significant numeric alteration seen in the low-grade astrocytoma was a loss of 19p loci in a subset of pilocytic astrocytomas. On the other hand, statistically significant losses of 9p and 10 loci and gains of chromosome 19 were detected in AA and GBM specimens, while additional gains of chromosomes 7 and 20 sequences were primarily observed in GBM samples. Multivariate and univariate analyses indicated that +7q and -10q were correlated with reduced patient survival, independent of tumor grade. The effect of 10q loss on patient survival was also independent of patient age. This investigation demonstrates the efficacy of CGH in identifying potential tumor suppressor and oncogenic loci in astrocytic grades. It also provided evidence that these loci can be used as independent diagnostic and prognostic markers for supplementing the World Health Organization tumor classifications and patient care.

Materials and Methods

Tumor Samples

The specimens for this study were collected under Institutional Review Board approved guidelines and subjected to standard histological classification according to the World Health Organization brain tumor grading system.² The specifics of specimen collection and selection for study have been previously described by our laboratory.¹¹ The patients' clinical information is based on the available data obtained on the last date of contact, September 24, 2002 (see supplemental table 1 on <http://www.amjpathol.org>). A total of 102 astrocytomas were obtained from 68 males and 34 females with a median age of 35 years (ranging from 5 months to 80 years), and grouped according to histological grade. The tumors were comprised of 10 World Health Organization Grade I astrocytomas, 10 World Health Organization Grade II well-diffused astrocytomas (A), 26 World Health Organization Grade III anaplastic astrocytomas (AA), and 56 World Health Organization Grade IV glioblastoma multiforme(s?) (GBM). The GBM patients were grouped into two age groups, under the age of 45 (GBM-1 to -25) and 45 years and older (GBM-26 to -56). This grouping is

based on the observation that tumors from patients under the age of 45 tend to be progressive and those from older patients tend to be *de novo*. All of the tumor samples, except for A-1, A-2, AA-1, AA-6, GBM-2, and GBM-13 were previously studied for loss of heterozygosity, gene deletions, and mutations.¹¹

Comparative Genomic Hybridization

DNA extracted from frozen tumor specimens were examined for copy number alterations using a modified CGH protocol.^{9,18,24} Briefly, 1 μ g of the tumor DNA and normal reference DNA was labeled with SpectrumGreen and SpectrumOrange dUTPs (Vysis, Inc., Downers Grove, IL), respectively, by nick translation. Approximately 400 ng of each labeled DNA was combined with 15 μ g of Cot-1 blocking DNA and hybridized to denatured normal metaphase chromosomes for 3 days. The metaphase spreads were prepared from an Epstein-Barr virus-transformed lymphoblast cell line and stored for 5 days at -20°C before use. After hybridization and standard stringency washes, at least eight chromosome metaphases were imaged and analyzed with the Quips XL image analysis program (Applied Imaging, Santa Clara, CA). The CGH red:green ratio profiles of 0.85, 1.20, and 1.45 suggest a loss, simple gain, and high-level gain of DNA sequences, respectively.⁹ Heterochromatic, centromeric, and telomeric regions were excluded from the analysis because of inconsistent hybridization patterns.

Fluorescent in Situ Hybridization

Gains detected by CGH on chromosome 19 were verified with telomeric probes using a conventional FISH protocol. Briefly, frozen tissue sections (5 μ m) were denatured in 70% formamide/2X SSC for 4 minutes at 70°C. SpectrumGreen TelVysion 19p and SpectrumRed TelVysion 19q telomeric probes and the control SpectrumAqua CEP 18 (Vysis Inc., Downers Grove, IL) centromeric probe were combined, denatured, and hybridized to the tissue section according to the manufacturer's recommendations. Following routine stringency washes, the hybridization was viewed using a microscope equipped with the appropriate fluorescence filters. At least 100 cells were examined for all signals and the mean signal numbers were recorded. Frozen sections of normal brain tissue were analyzed to establish a reference FISH signal copy number using chromosome 19 TelVysion and chromosome 18 CEP probes. A mean signal number range of 1.50-2.30 was observed with the normal tissue, corresponding to a diploid chromosomal complement. To establish reference FISH signal copy number for simple gains and high-level gains, the SpectrumGreen CEP 7 was hybridized to selected frozen tumor sections. Prior CGH analysis of GBM-38 and AA-19 showed a ratio of 1.20 for chromosome 7 and tumor GBM-25 had a ratio of 1.45. The mean FISH signal copy number range of 2.40 to 2.5 corresponded to simple gains and a mean signal copy number greater than 2.5 corresponded to high-level gains. All of

Table 1. Summation of Clinical and CGH Data

	Grade I (n = 10)	Grade II (n = 10)	Grade III (n = 26)	Grade IV* (n = 25)	Grade IV† (n = 31)
Mean age (years)	15	25	43	30	61
Median survival (months)	nd‡	73.4	20	21	8
Altered chromosomes/tumor§	2	5	7	9	10
High-level gained regions¶	0	0	3	26	41
CGH alterations	-19p	none	-9p -10q +19p +19q	-9p -10p -10q -22 +7p +7q +19p	-9p -10p -10q +7p +7q +19p +19q +20p +20q

*Grade IV patients under the age of 45.

†Grade IV patients 45 years or older.

‡The median survival could not be estimated for WHO Grade I tumors.

§Mean number of chromosomes involved in detectable copy number changes.

¶Number of regions detected by CGH with a red:green ratio of at least 1.45.

||Statistically significant copy number aberrations.

the selected samples had CGH profiles of 1.0 for chromosome 18, which was used as an internal control.

Statistical Analysis

To assess whether the DNA sequence copy number changes, detected by CGH, occurred significantly above random chance, the oncotrees statistical software package (<http://www.ncbi.nlm.nih.gov/CBBresearch/Schaffer>) was implemented.^{25,26} The software uses a statistical program based on the method designed by Brodeur et al,²⁷ which takes into account the size of each chromosome arm and the established probabilities of each arm being altered in tumors. The statistical probability of the frequency of CGH alterations occurring non-randomly in each tumor grade was determined by the Fisher's exact test with a significant *P* value <0.05. The CGH alterations detected on the short (p) and long (q) arms of chromosomes are considered independent events.

Cox proportional hazards model was used to assess the relationship between each statistically significant CGH alteration and survival, as well as the joint effect of these alterations on survival. Statistical significance was established with a *P* value <0.05. Kaplan-Meier plots were graphed to display the relationship between the presence of CGH alterations and patient survival.

Results

CGH Analysis

Chromosomal copy number changes were detected in 95 of 102 (93%) astrocytomas with an increasing number of alterations accompanying higher tumor grades (Table 1). Among the 10 low-grade (PA and SEGA) specimens, 24 altered chromosomes were detected, while 51 were seen in 10 A, 188 in 26 AA, and 551 in 56 GBM. The oncotree statistical software was implemented to deter-

mine which copy number changes occurred significantly above random chance.^{25,26}

Within the World Health Organization Grade I sample population, recurrent numeric alterations were observed on chromosomes 7 and 19 (Figure 1). However, loss of 19p, noted in PA-3, -4, and -6, was the only alteration that occurred significantly above random (see supplemental table 1 on <http://www.amjpathol.org>). Comparatively, among A samples, gains and losses were detected on chromosomes 3, 7, 11, 13, 19, 22, X, and Y, but none were statistically significant (Figure 2). Genomic copy number changes were detected on all of the chromosomes in the AA tumors (Figure 3). Losses of 9p (8 of 26) and 10q (12 of 26) as well as gain of chromosome 19 (16 of 26) were evident in a significant proportion of the specimens. The smallest regions consistently lost were 9p21.3→p21.2 and 10q25.1 and the short arm of chromosome 19 was the smallest region consistently gained.

Similarly, alterations were identified involving all chromosomes in GBM tumors, but only losses of 9p and 10 along with gains of 7 and 19 occurred significantly above random chance (Figure 4, A and B). Under-representation of 9p loci was identified in 27 of 56 (48%) samples, with the entire short arm lost in 20 tumors. Reduced copy number of chromosome 10 was recorded in 44 of 56 (79%) cases and 27 of them lost the entire chromosome. Partial losses of chromosome 10 loci revealed that 10p14 and 10q21.2→q25 were consistently lost. Gain of chromosome 7 was detected in 43 of 56 (77%) GBM samples and a gain of the entire chromosome seen in 24 specimens. The CGH profiles indicated that the smallest gained regions were 7q11.2 and 7q34→qtr. Over-representation of chromosome 19 was observed in 21 of 56 (38%) cases. Five had a sole gain of 19p, which pinpoints it as the most refined region of gain.

The frequency of certain CGH alterations differed between the GBM age groups (Figure 4, A and B and Table

Figure 1. Composite of CGH alterations detected in 10 World Health Organization Grade I astrocytomas. Each **line** denotes the chromosome regions lost (**left**) or gain (**right**) in a single case.

1). Loss of chromosome 22 was evident in both age groups with the smallest commonly lost region, 22q13.3, observed in 15 of 56 (27%) specimens. However, its frequency reached significance among patients younger than 45 years of age and was just below the threshold among patients older than 45. Over-representation of the short arm of chromosome 19 was seen in a significant portion of both groups, but the gain of the long arm reached statistical significance only in the older GBM patients (12 of 31). Similarly, gain of chromosome 20 sequences was detected in a significant portion of the older age group (16 of 31), but not in the younger group (8 of 25). Among the older patients, 13 of 16 samples had a gain of 20q13.2→qtr loci and it was solely gained in GBM-35 and GBM-50, making it the smallest consistently gained region (Figure 4B). Forty-one high-level gained regions were detected in tumors from the older patients, compared to 26 distinguished in the younger age group (Table 1). The significance of the incidence of high-level gains could not be determined at this time.

Chromosome 19 FISH Validation

The unexpectedly high frequency of chromosome 19 over-representation in AA and GBM specimens, in comparison with our earlier CGH analysis of GBM, prompted

a verification of the results by FISH analysis.⁹ The interpretation of the FISH study correlated with that of the CGH ratio profiles in selected samples. Two detailed examples are illustrated in Figure 5. GBM-39 had a CGH ratio greater than 1.45 for the entire chromosome 19 suggesting a high-level gain above diploidy (Figure 5A). The subsequent FISH analysis revealed a mean signal copy number of 2.67 and 2.58 for 19pter and 19qtr, respectively, corresponding to a high-level gain. A mean signal copy number of 1.82 was seen for the control chromosome 18. A simple gain (CGH ratio 1.20) for 19p and a normal DNA copy number (CGH ratio 1.0) for 19q was detected in GBM-25 (Figure 5B). The FISH analysis detected three signals (mean 2.43) for 19pter, and two signals for 19qtr (mean of 1.78 signals) and 2 for 18 centromere (mean of 1.60 signals), correlating with the CGH profiles.

The remaining samples included GBM-38 with a CGH ratio of 1.0 for chromosome 19 had a FISH mean signal copy number of 1.91 for 19p and 1.75 for 19q. GBM-6 had a CGH ratio of >1.45 and a mean signal copy number of 3.1 for both 19p and 19q. AA-3 showed a gain of chromosome 19 with a CGH ratio between 1.20 and 1.45 and a mean signal copy number of 3.73 for 19p and 3.79 for 19q. The CGH signal ratio which proved to be higher than the FISH copy number might be due to ge-

Figure 2. Composite of CGH alterations detected in 10 World Health Organization Grade II astrocytomas. Each **line** denotes the chromosome regions lost (**left**) or gain (**right**) in a single case.

netic heterogeneity, a finding further supported by the CGH profiles showing a wide variation in hybridization, compared to the other selected samples.

Patient Survival

A summation of the clinical information is depicted in Table 1 and it shows a positive correlation between increasing grade and patient age and an inverse relationship between tumor grade and patient survival. The lowest grade tumors (PA and SEGA) tend to associate with the youngest patients. The median survival could not be estimated for this group because 8 of 10 (80%) of patients remained alive after surgery and six remained alive without evidence of disease for 4 to 11 years. Comparatively, the grade II astrocytoma patients were primarily young adults with 50% of them surviving without evidence of disease for 4 to 8 years. Among AA patients, there was a continuing reduction in median survival and only 5 of 26 (19%) patients survived for 4 to 10 years without evidence of disease. Collectively, the GBM patients had a similar mean age (45.5 years) to AA patients, but the median survival (11.8 months) was less and only 2 of 56 (4%) were alive without disease after a 5-year follow-up. After age grouping of the GBM patients, the younger ones had a median survival more than two times that of the older age group (Table 1).

Relationship Between All Patients' Survival and CGH Alterations

The Cox proportional hazard model was implemented to examine the effects statistically significant CGH alterations and histological grade have on patient survival. The univariate analysis examined the effect of CGH alterations on survival without adjustment for histological grade (Table 2). Among all patients, the presence of +7p, +7q, and -9p was associated with at least a 1.8-fold increase risk of dying. Among the 26 patients with AA, the number of abnormalities among 7p, 7q, 9p, 9q, 10p, 10q, 19p, and 19q were determined. The Cox model was used to examine the relationship between survival and the total score, as well as each abnormality individually. The joint effect of all individual abnormalities on survival using the Cox model and a backwards-elimination modeling approach was used to determine an effect on survival of the abnormalities. However, none of the analyses showed a statistically significant relationship. In AA and GBM patients, the presence of -10p and -10q in their tumors was significantly associated with poorer survival and a 2.6-fold increase risk of death. Graphical representations of +7q and -10q relationships with survival are displayed on Kaplan-Meier survival plots in Figure 6A and B, respectively.

Figure 3. Composite of CGH alterations detected in 26 anaplastic astrocytomas (World Health Organization Grade III). Each line denotes the chromosome regions lost (left) or gain (right) in a single case. Thick lines illustrate regions with high-level gains. Brackets depict the smallest region of consistent overlap on chromosomes with statistically significant copy number changes.

Multivariate analyses were used to examine the joint effects of histological grade and the same CGH alterations on patient survival. Among the CGH alterations, only gain of chromosome 7 loci and loss of 10 loci remained significant prognostic predictors of poorer survival after adjusting for histological grade (Table 2). GBM patients had a 10-fold greater risk of dying ($P < 0.0001$) compared to patients with lower grade tumors (PA, SEGA, and A), regardless of the CGH alteration. GBM patients had a twofold higher risk of dying compared to AA patients when high-level gains, loss of 9p, and gain of 19 were considered ($P \leq 0.031$) and the difference approached statistical significance when gains of 7 and losses of 10 were present (Table 2).

The joint effect of the copy number changes of the individual chromosome arms (+7p, +7q, -10p, -10q), and histological grade on survival was analyzed among patients with AA and GBM. The presence of +7q ($P = 0.0288$) and -10q ($P = 0.0018$) alterations maintained a significant association with poorer survival and nearly a twofold increase risk of dying. Although each alteration involving the four chromosome arms may affect patient survival differently, the cumulative effect was associated

with a progressively worse survival for AA and GBM patients (Figure 7A). The accumulation of any two or more of these alterations was sufficient to be a significant predictor of poorer survival ($P = 0.0279$), independent of histological grade.

Relationship Between GBM Patient Survival and CGH Alterations

Among GBM patients, the effect that age and CGH alterations have on survival was examined (Table 3). Without adjustment for age, the univariate analysis showed at least a 1.89-fold greater risk for death when +7p, +7q, -10p, -10q, +19q, +20p, and +20q, were individually present. Furthermore, 35 of 56 (63%) GBM specimens had a combination of +7 and -10 alterations and 18 of 56 (32%) had a combination of +7, -9p, and -10. These combinations corresponded to a significant increase in the risk for poor survival, irrespective of age. In the multivariate analysis, the effects of -10p, -10q, and +20q remained statistically significant, when patient age was taken into account (Table 3). It was also discerned that

Figure 4. Composite of CGH alterations detected in (A) 25 progressive and (B) 31 *de novo* glioblastoma multiforme (World Health Organization Grade IV). Each **line** denotes the chromosome regions lost (**left**) or gain (**right**) in a single case. **Thick lines** illustrate regions with high-level gains. **Brackets** depict the smallest region of consistent overlap on chromosomes with statistically significant copy number changes.

Figure 5. Representative FISH analyses for the verification of chromosome 19 over-representation detected by CGH. **A:** An example of the FISH analysis on case GBM-39, whose accompanying CGH profile demonstrates a high-level gain (CGH ratio >1.45) on chromosome 19. **B:** An example of the FISH on case GBM-25 whose accompanying CGH profile shows a simple gain of 19p (CGH ratio = 1.20) and a normal diploid complement (CGH ratio = 1.0) of 19q. Hybridizations of the 19p (green signals) and 19q (red signals) telomeric probes, and the chromosome 18 centromeric probe (aqua blue signal) are shown in the left panels. The corresponding CGH profiles for chromosome 19 are shown in the right panels. The CGH profile ratio thresholds representing a normal diploid complement, loss, and gain of DNA sequences are indicated by the black line (ratio 1.0), red line (ratio 0.85), and the green line (ratio 1.20), respectively.

older GBM patients had a greater than twofold risk of dying compared to the younger patients, regardless of the CGH alterations ($P \leq 0.037$).

The joint effect of copy number changes on chromosome arms (+7p, +7q, -10p, -10q, +19p, +19q, +20p, and +20q) and patient age on survival was examined by multivariate analysis. The presence of -10q ($P = 0.0267$) had a 2.5-fold higher risk of dying, while the association with +20q ($P = 0.053$) was just below the statistical threshold, when adjusted for age. When the analysis was performed without adjustment for age, -10q ($P = 0.0144$) remained a significant predictor of poorer survival and +7q ($P = 0.038$) achieved significance. Although alterations involving each of the eight chromosome arms may have different effects on patient survival, the cumulative effect was associated with a progressively poorer survival of GBM patients (Figure 7B). Acquiring any three or more of these alterations was a significant predictor of poor survival for GBM patients ($P = 0.026$), independent of age.

Discussion

Histological tumor grading is a standard clinical methodology for assessing prognosis and determining therapy on patients with astrocytic gliomas.^{3,28} The subjective nature of histopathology has complicated this process; therefore, the identification of objective molecular markers that can help grade tumors and predict survival would be invaluable.^{4,5} Various genetic abnormalities associated with specific astrocytic grades provided valuable clues to the molecular pathogenesis of the disease, but none to date are routinely used as diagnostic and prognostic markers in clinical protocols.^{2,8,12,29} Our laboratory has focused on the use of molecular genetic and molecular cytogenetic techniques to identify those genetic alterations that can be used as diagnostic and prognostic markers. To this end, we subjected a group of astrocytomas to molecular genetic analyses and concurrently this CGH analysis.¹¹ The CGH results demon-

Table 2. Statistical Modeling Relating CGH Alterations to Astrocytoma Patient Survival

Common CGH alterations*	No. of patients	No. of dead patients	Without adjustment for histology		With adjustment for histology		AA vs GBM		PA, SEGA, A vs GBM	
			Hazard ratio (95% CI)	p-Value†	Hazard ratio (95% CI)	p-Value	Hazard ratio (95% CI)	p-Value	Hazard ratio (95% CI)	p-Value
All tumor grades										
+7p	102	76	2.67 (1.66, 4.30)	<0.0001	1.75 (1.06, 2.89)	0.03	0.64 (0.37, 1.11)	0.111	0.10 (0.04, 0.30)	<0.0001
+7q	102	76	2.61 (1.61, 4.23)	<0.0001	1.91 (1.16, 3.15)	0.011	0.61 (0.36, 1.04)	0.071	0.10 (0.04, 0.29)	<0.0001
−9p	102	76	1.83 (1.16, 2.89)	0.009	1.25 (0.79, 1.99)	0.341	0.55 (0.33, 0.93)	0.026	0.09 (0.03, 0.26)	<0.0001
+19p	102	76	1.48 (0.94, 2.34)	0.09	1.43 (0.88, 2.34)	0.148	0.47 (0.27, 0.82)	0.008	0.09 (0.03, 0.24)	<0.0001
+19q	102	76	1.43 (0.88, 2.34)	0.156	1.42 (0.84, 2.40)	0.19	0.48 (0.28, 0.84)	0.01	0.09 (0.03, 0.24)	<0.0001
AA, GBM										
−10p	82	72	2.55 (1.55, 4.18)	0.0002	2.28 (1.36, 3.84)	0.0018	0.69 (0.40, 1.20)	0.189	nd	nd
−10q	82	72	2.65 (1.54, 4.56)	0.0004	2.53 (1.46, 4.39)	0.001	0.59 (0.35, 1.00)	0.051	nd	nd
High-level gains on 7	82	72	1.39 (0.84, 2.32)	0.204	1.24 (0.74, 2.08)	0.424	0.56 (0.33, 0.95)	0.031	nd	nd
Other high-level gains	82	72	1.16 (0.68, 1.98)	0.588	0.93 (0.53, 1.63)	0.807	0.53 (0.31, 0.91)	0.021	nd	nd
PA, SEGA, A										
−19p	20	4	1.34 (0.14, 12.89)	0.802	nd†	nd	nd	nd	nd	nd

*Statistically significant CGH alterations associated with specific tumor grades. PA and SEGA, WHO Grades I; A, WHO Grade II; AA, WHO Grade III; GBM, WHO Grade IV.

†Statistical significance, Bold type p-value < 0.05.

‡Analysis not applicable.

strated a trend of increasing genetic instability with advancing malignancy, and the prognostic significance of the alterations was estimated by their associations with patient survival (Table 1).

Significant CGH Losses

Under-representation of loci on chromosomes 9, 10, 19, and 22 were increasingly detected in advanced astrocytic grades and associated with patient survival. The loss of 9p loci was seen in all tumor grades, but its incidence reached statistical significance in AA and GBM samples (Table 1). As shown in published studies, loss of the entire short arm of chromosome 9 was typically involved.^{2,6,8,9,11,17} The few cases with partial deletions of 9p, showed that 9p21.3→p21.2 was consistently lost in AA, 9p22→p21 was lost in GBM under the age of 45, and

9p23 and 9p13→p10 loci were equally lost in the older GBM patients (Figures 3 and 4). This suggests multiple tumor suppressor genes on 9p are potentially important in the progression of AA and GBM, and possibly can be useful to further subdivide GBM patients. The Cox hazard proportional analysis showed that loss of 9p loci is associated with poorer survival, but failed to achieve significance when tumor grade and patients' age were taken into consideration (Tables 2 and 3). These results are consistent with published studies that indicate LOH 9p/*CDKN2* (9p21) deletions are primarily detected in high-grade astrocytomas and associated with poorer survival but not independent of patient age or histological grade.^{2,6,11,30-32} Evaluation of additional markers and genes on 9p, including 9p23 and 9p13→p10, would be beneficial in assessing their diagnostic potential for distinguishing high- and low-grade astrocytomas.

Figure 6. Kaplan-Meier plots depicting the effect of CGH alterations on the survival of astrocytoma patients determined by a univariate analysis using the Cox proportional hazards model. Patients with the CGH alterations (dotted lines), (A) gain of 7q ($P < 0.0001$) and (B) loss of 10q ($P = 0.004$) showed a significant reduction in survival compared to patients without these alterations (solid lines).

Figure 7. Kaplan-Meier plots illustrating the effect of the accumulation of CGH alterations on patient survival. **A:** AA and GBM patients with CGH alterations +7p, +7q, -10p, and -10q (**broken lines**) compared to those without these CGH alterations (**solid line**). **B:** GBM patients with CGH alterations +7p, +7q, -10p, -10q, +19p, +19q, +20p, and +20q (**broken lines**) compared to those without these CGH alterations (**solid line**).

Under-representation of chromosome 10 DNA sequences is another common CGH alteration detected in the high-grade astrocytomas of this study (Figures 3 and 4). Loss of both chromosome arms was frequently detected, corroborating previous studies.^{2,6,8,9,11,33} The oncotree statistical analysis showed that loss of 10q was significantly seen in both AA and GBM specimens (Table 1). Molecular genetic studies have reported loss of 10q loci in high-grade astrocytomas, with *PTEN* (10q23), and *DBMT1* (10q25.3→q26.1) being implicated in the pathogenesis of gliomas.^{2,9,11,13,17,30,34} Some studies have refined the potential tumor suppressor region to 10q25→qter.^{16,35–38} This was supported by the present CGH investigation that indicated 10q25.1 was the most common region lost in the high-grade tumors and by our previous LOH study of 117 gliomas.³⁹ Tumor suppressor genes, like *MX1* and *LIMB1*, have been mapped to this region, but their involvement in astrocytoma development have not been fully discerned.^{36,40,41} In contrast, the oncotree analysis showed that a significant loss of 10p

loci was restricted to GBM specimens, suggesting that the additional loss is necessary for the progression to higher-grade tumors. A consistent under-representation of 10p15 was detected in the GBM samples, agreeing with published LOH studies implicating this region in the progression of astrocytic tumors.^{33,42,43} Samples GBM-9 and 36 had a loss of 10p14 sequences without loss of 10p15 loci suggesting another potential tumor suppressor locus (Figure 4, A and B). These results indicate that loss of 10p14→p15 and 10q25.1 loci are important in the etiology of astrocytomas and may be individually useful as diagnostic markers for subdividing GBM tumors.

Loss of chromosome 10 loci has been cited to have prognostic value in astrocytomas.^{16,29,32} Analyses performed by our group and others showed that loss of 10q and mutations in the *PTEN* gene had a significant to marginal effect on patient survival without adjusting for histological grade or patients' age.^{10,11,13,31} In concert with these studies, the univariate and multivariate analyses performed in this investigation showed that a loss of

Table 3. Statistical Modeling Relating CGH Alterations to GBM Patient Survival

Common CGH alterations*	No. patients	No. dead patients	Without adjustment for age		With adjustment for age	
			Hazard ratio (95% CI)	p-Value†	Hazard ratio (95% CI)	p-Value
+7p	56	52	1.89 (1.01, 3.54)	0.046	1.40 (0.71, 2.77)	0.328
-7q	56	52	2.69 (1.33, 5.45)	0.006	1.70 (0.73, 3.97)	0.219
-9p	56	52	1.37 (0.79, 2.37)	0.261	0.99 (0.55, 1.78)	0.981
-10p	56	52	2.58 (1.36, 4.90)	0.0037	2.11 (1.07, 4.15)	0.031
-10q	56	52	3.14 (1.49, 6.63)	0.0027	2.47 (1.12, 5.44)	0.025
+19p	56	52	1.63 (0.92, 2.89)	0.094	1.78 (0.99, 3.21)	0.056
+19q	56	52	2.21 (1.17, 4.17)	0.017	1.86 (0.96, 3.59)	0.066
+20p	56	52	2.25 (1.17, 4.35)	0.015	1.89 (0.96, 3.71)	0.064
+20q	56	52	1.97 (1.10, 3.53)	0.022	1.82 (1.01, 3.28)	0.047
-22	56	52	1.24 (0.67, 2.28)	0.489	1.47 (0.79, 2.73)	0.224
High-level gain on 7	56	52	1.39 (0.84, 2.32)	0.204	0.80 (0.41, 1.55)	0.505
High-level gain on 19	56	52	1.48 (0.77, 2.81)	0.237	1.62 (0.84, 3.12)	0.149
Other high-level gains	56	52	1.05 (0.59, 1.86)	0.882	0.87 (0.48, 1.58)	0.656
+7 and -10	56	52	4.54 (1.38, 14.90)	0.013	3.10 (0.90, 10.70)	0.073
+7, -9p, and -10	56	52	5.54 (1.31, 23.33)	0.02	3.75 (0.86, 16.42)	0.079
Any high-level gains	56	52	1.05 (0.61, 1.82)	0.549	0.84 (0.48, 1.48)	0.551

*Statistically significant CGH alterations associated with GBM.

†Statistical significance: Bold type p-value < 0.05.

chromosome 10q had a significant effect on poorer patient survival. Unlike published reports, this analysis indicated that the prognostic association of -10q is independent of histological grade and patient age and implies that markers on 10q25.1 are potentially reliable prognostic indicators for high-grade astrocytomas.

Among World Health Organization Grade I tumors only loss of 19p was detected significantly above random chance (Table 1). A significant effect on survival was not observed because the majority of patients with low-grade tumors remained alive during the course of the study (Table 2). To our knowledge, loss of 19p loci has not been previously implicated in the progression of astrocytoma. However, published molecular genetic reports have suggested that the loss of 19q DNA sequences is necessary for malignant progression of astrocytic tumors.^{44,45} In the present study, under-representation of 19q loci was found in 11 of 102 (11%) tumors and independently by LOH in 20 of 102 (20%) (unpublished data). The frequency of the alteration was too low to appreciate any effect on the tumor biology. Partial and entire losses of 19q loci along with 1p losses have been reported in association with longer survival of oligodendroglioma patients and a subset of astrocytoma patients.^{9,10,17,18,32,46-49} This CGH study detected two astrocytomas, A-7 and GBM-14, with partial losses of both 1p and 19q, but neither of the patients survived longer than expected. Additional studies aimed at evaluating losses of 19 loci in low- and high-grade tumors would be beneficial in understanding the importance of this alteration in astrocytomas.

Under-representation of chromosome 22 was detected primarily in the GBM samples, corroborating previous CGH and cytogenetic studies (Table 1).^{8,9,17} The CGH profiles of this study, pinpointed 22q13.3 as the smallest region consistently deleted, which also agrees with LOH studies suggesting that this region has a tumor suppressor gene involved in astrocytoma progression.^{17,50,51} Although tumor suppressor genes, like *ST13* and *SCUB31* have been mapped to this region, more work needs to be done to access their involvement in glioblastoma genesis.^{52,53} Despite the association of the loss of 22 with GBM, a significant effect on the survival of the GBM patients was not observed with this sample population (Table 3). Designing probes for 22q13.3 might be useful for evaluating the loss of this region as a potential diagnostic marker for GBM.

Significant CGH Gains

Increased copy numbers of chromosomes 7, 19, and 20 were detected in a significant portion of astrocytomas and associated with patient survival. Gain of chromosome 7 was seen in all tumor grades and the frequency reached statistical significance in GBM (Figures 1 to 4 and Table 1). The CGH profiles of this study refined the consistent regions of over-representation to 7q11.2 and 7q34→qter (Figure 4, A and B). Although many genes and expressed sequence tags are known in these regions, none to date have been implicated in the patho-

genesis of astrocytomas. Other studies have implicated gain of chromosome 7 as a predictor of shorter survival for patients with specific astrocytic tumors.^{14,29,54} To our knowledge, the univariate and multivariate analyses of this study is the first to illustrate a significant association between gains of 7q loci and poorer patient survival regardless of histological grade (Tables 2 and 3). However, we did not find that +7q had a significant effect on patient survival between GBM age groups. This suggests that potential oncogenes in 7q11.2 and 7q34→qter can be used as independent diagnostic and prognostic markers for subclassifying high-grade astrocytomas, but not for subdividing GBM patients.

Within the GBM samples, high-level gains were seen on chromosome 7, including the *EGFR* locus. This is consistent with other genetic studies suggesting oncogenic activity associated with chromosome 7 and *EGFR* are important in astrocytoma progression.^{2,6,17,55,56} The univariate and multivariate analyses indicated that these high-level gains did not have a significant effect on survival (Tables 2 and 3). This is supported by studies suggesting that gene amplifications alone are not significant predictors of poorer survival, independent of patient age or grade.^{30,45,57-61} However, other studies have demonstrated that amplifications and over-expressions of the *EGFR* gene have prognostic value in the context of all astrocytic grades, thus, re-enforcing its role as an important oncogene for astrocytic progression.^{13,62-64}

Over-representation of chromosome 19 loci was recognized in a significant number of AA and GBM specimens, which contrasts with our previous CGH analysis of GBM biopsies (Table 1).⁹ Therefore, a FISH analysis was beneficial for corroborating the results of this study (Figure 5). Review of the CGH literature revealed that gains of chromosome 19 have been detected in astrocytic tumors and gain of 19p was commonly seen, which is supported by this investigation.^{10,14,21,22,37,65-68} Unlike the published reports, high-level gains on chromosome 19 were detected in GBM samples and 19p13.3 was discerned as the smallest consistent high-level gained loci (Figure 4A). To date, none of the oncogenes mapped to 19p have been implicated in the etiology of astrocytoma. Our data also corroborate another study that illustrated +19q as a significant predictor of short-term survival for GBM patients (Table 3).¹⁰ These results suggest that gains of 19p loci can be useful in distinguishing between high- and low-grade astrocytoma, but the prognostic value of 19q markers must be further evaluated.

The third most common over-represented loci identified in this study involved chromosome 20. This present CGH analysis showed gain of 20 loci in the high-grade tumors with its incidence reaching statistical significance among GBM patients over the age of 45 (Table 1). In line with this data, a collective CGH study of 337 astrocytomas illustrated a high incidence of +20q.¹⁷ In contrast, one published report implicated 20p11.2→p12 as harboring oncogenes relevant for glioma progression.⁶⁶ Further scrutiny of the CGH profiles in this study indicated that 20q13.2→qter was consistently gained in *de novo* GBM (Figure 4B). Univariate and multivariate analyses illustrated that the presence of +20q either reached or

approached statistical significance as a marker for poor survival of GBM patients (Table 3). This is supported by another study that indicated gain of chromosome 20q was associated significantly with short-term GBM survivors.¹⁰

Our analysis demonstrates the power of CGH to identify recurrent genetic changes in high-grade astrocytomas that can localize potential tumor suppressor and oncogenic loci. This study corroborates published data by identifying common chromosome alterations in astrocytoma. Moreover, additional candidate loci are localized and for the first time evidence for prognostic application, independent of patient age and grade, is provided. Further evaluation of 7q11.2, 7q34→qter, 10p15→p14, 10q25.1, 19p13.3, 20q13.2→qter, and 22q13.3 loci is needed for a better understanding of their importance in astrocytoma progression, supplementing histological grading, and prognostic predictions. Designing molecular probes corresponding to these loci to screen clinical cohorts of low- and high-grade astrocytomas would be beneficial in further localizing these loci. The continued investigation of the individual as well as cumulative effects of these loci on disease progression and patient survival would be essential to future genetic studies of astrocytic tumors.

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