A study of loss of heterozygosity at 70 loci in anaplastic astrocytoma and glioblastoma multiforme with implications for tumor evolution[1]

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Cancers that arise from astrocytes in the adult CNS present as either anaplastic astrocytomas (AAs) or as more aggressive glioblastomas multiforme (GBMs). GBMs either form de novo or progress from AAs. We proposed to examine the molecular genetic relationship between these CNS tumors by conducting a genome-wide allelic imbalance analysis that included 70 loci on examples of AA and GBM. We found significant loss of heterozygosity (LOH) at 13 discrete chromosomal loci in both AAs and GBMs. Loss was significant in both AAs and GBMs at 9 of these loci. AAs show the highest rates of LOH at chromosomes 1p, 4q, 6p, 9p, 11p, 11q, 13q, 14q, 15p, 17p, 17q, and 19q. GBMs showed the greatest losses at 1p, 6q, 8p, 9p, 10p, 10q, 11p, 13q, 17p, 17q, 18p, 18q, and 19q. GBMs also demonstrated significant amplification at the epidermal growth factor receptor locus (7p12). These data suggest that there are three classes of loci involved in glioma evolution. First are loci that are likely involved in early events in the evolution of both AAs and GBMs. The second class consists of AA-specific loci, typified by higher LOH frequency than observed in GBMs (4q, 6p, 17p, 17q, 19q). The third class consists of GBM-specific loci (6q, 8p, 10, 18q). Damage at these loci may either lead to de novo GBMs or permit existing AAs to progress to GBMs. Glioma-related LOH profiles may have prognostic implications that could lead to better diagnosis and treatment of brain cancer patients. *Neuro-Oncology 1, 169–176, 1999 (Posted to Neuro-Oncology [serial online], Doc. 98-28, June 16, 1999. URL <neuro-oncology.mc.duke.edu>)*

Cancers of the CNS are typically derived from astrocytes, with astrocytomas comprising 40–50% of adult onset CNS tumors (Ohgaki et al., 1995). Despite treatment with surgery, radiation, and chemotherapy, malignant astrocytomas frequently recur, and recurrent tumors are often more aggressive. Classification schemes based on histopathology generally recognize two stages (grades) of malignant astrocytomas: AAs[3] and GBMs (Burger et al., 1985; Kleihues et al., 1993). AAs are characterized by moderate hypercellularity, moderate pleomorphism, no necrosis, and possible vascular proliferation. AAs are generally more responsive to treatment but often progress to GBMs. Glioblastomas are the most malignant form of astrocytoma and are characterized by moderate to marked hypercellularity, moderate and marked pleomorphism, and necrosis (with or without pseudopalisading) (Burger et al., 1985). GBM is virtually incurable, with more than 50% of patients dying within the first year after diagnosis (Bruner, 1994; Furnari et al., 1996; Levin et al., 1997; Magnani et al., 1994).

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[3]Abbreviations used are as follows: AA, anaplastic astrocytoma; CALR, concordant allele loss rates; EGFR, epidermal growth factor receptor; GBM, glioblastoma multiforme; LOH, loss of heterozygosity.

Table 1. Primer, location, and loss of heterozygosity in AA and GBM"

Marker	Cyto. location	Approx. position	AA series (%)	GBM series (%)	Marker	Cyto. location	Approx. position	AA series (%)	GBM series (%)
D1S243	**1p**	**18/263**	**3/14; 21**	**3/23; 13**	**D10S196**	**10q11.2-21.2**	**81.1/144**	**0/17; 0**	**13/24; 54**
D1S214	1p36.3	18.8/263	2/19; 11	3/26; 12	**D10S1687**	**10q**	**105.6/144**	**1/18; 6**	**5/23; 22**
D1S255	**1p**	**49.8/263**	**4/14; 29**	**4/20; 20**	**D10S587**	**10q**	**134.7/144**	**2/18; 11**	**5/24; 21**
D1S211	1p	54/263	1/19; 5	0/26; 0	D10S1723	10q	134.9/144	0/15; 0	1/23; 4
D1S305	1q	190.5/263	1/9; 11	1/22; 5	D10S212	10qter	140.1/144	1/16; 6	3/26; 12
D1S213	1q	246/263	0/16; 0	0/21; 0	**D11S922**	**11pter**	**2.1/144**	**4/17; 24**	**7/25; 28**
TPO	2p24	8.3/255	1/13; 8	0/11; 0	**D11S988**	**11p**	**7.4/144**	**4/15; 27**	**4/22; 18**
D2S330	2p	18.2/255	0/14; 0	0/16; 0	D11S1328	11q	119.1/144	0/12; 0	1/17; 6
D2S391	**2p21-16**	**54.4/255**	**0/14; 0**	**0/20; 0**	D12S372	12pter-p13	11.3/143	0/19; 0	0/26; 0
D2S362	2q	241.5/255	2/14; 14	0/19; 0	D12S391	12p	25.5/143	2/15; 13	1/23; 4
D3S1768	3p24.22-22	32.9/214	2/18; 11	0/24; 0	D12S78	12q24	112.4/143	1/16; 6	2/17; 12
D3S1298	3p	36/214	2/18; 11	2/22; 9	D12S1075	12qter	115.6/143	1/10; 10	3/18; 17
D3S1300	3p21	56.1/214	3/16; 19	1/16; 6	**D13S137**	**13q14.3**	**49.6/114**	**3/12; 25**	**6/14; 43**
D3S1262	3q27	201.8/214	1/18; 6	0/23; 0	D13S285	13q34	112.1/114	0/13; 0	0/19; 0
D4S428	4p13	92.8/203	1/9; 11	1/18; 5	D14S261	14p	18.8/109	1/14; 7	0/18; 0
D4S407	**4q**	**150.2/203**	**5/12; 42**	**3/18; 17**	D14S302	14q24.3-32.1	95.6/109	1/10; 10	3/16; 19
D4S422	4q	172.1/203	1/19; 5	1/26; 4	**D14S62**	**14q**	**98.1/109**	**5/15; 33**	**3/18; 17**
D4S192	**4q25-34**	**171.6/203**	**3/14; 21**	**1/23; 4**	**D15S165**	**15pter**	**22/106**	**2/10; 20**	**0/19; 0**
D4S415	4q	194.1/203	1/18; 6	1/24; 4	D15S120	15qter	77.1/106	0/18; 0	0/20; 0
D5S392	5p	1.9/194	3/18; 17	2/23; 9	D16S521	16p13.3	10.8/98	2/15; 13	0/17; 0
D5S644	5q	111.5/194	0/13; 0	2/21; 10	D16S265	16q21	73.2/98	2/16; 13	3/20; 15
D6S344	**6p**	**6.9/183**	**4/14; 29**	**4/25; 16**	**D17S731**	**17p**	**11.2/92**	**6/15; 40**	**6/22; 27**
D6S292	**6q21-23**	**159.4/183**	**1/18; 5**	**5/22; 23**	**PS3I (P53)**	**17p13.1**	**31.3/92**	**5/8; 63**	**3/14; 21**
D6S471	6q	162.6/183	1/10; 10	5/15; 33	NF1	17q11.2	43.8/92	0/9; 0	3/17; 18
EGFR	**7p12**	**58.8/171**	**2/17; 12**	**10/26; 38**	**D18S63**	**18pter-11.22**	**8.8/85**	**1/17; 5**	**4/20; 20**
D7S466	7q31	131.6/171	0/17; 0	3/24; 12	**D18S46**	**18q21.1**	**61.2/85**	**1/14; 7**	**5/21; 24**
D8S264	**8p**	**4.8/155**	**1/15; 7**	**4/17; 24**	D18S474	18q	61.4/85	1/16; 6	3/23; 13
D8S277	8p23.1	7.5/155	0/17; 0	1/18; 6	DCC	18q	67.1/85	0/12; 0	2/22; 9
LPL	8p22	65.1/155	0/14; 0	1/18; 6	D19S221	19p13.2	12.7/67	0/19; 0	0/26; 0
D8S284	8q23	138.7/155	0/15; 0	0/23; 0	**D19S601**	**19q**	**60.2/67**	**9/12; 75**	**6/17; 35**
D9S157	**9p**	**17.5/145**	**5/15; 33**	**11/22; 50**	**D19S254**	**19q13.4**	**62/67**	**6/13; 46**	**6/19; 32**
D9S922	9q	88.8/145	0/15; 0	1/18; 5	D20S186	20p12-p11.2	21.2/72	1/18; 5	1/25; 4
D9S290	9q32-34.1	98.2/145	0/18; 0	1/22; 5	D21S1270	21q22	36.5/50	2/15; 13	3/22; 14
D10S249	**10p**	**5.3/144**	**3/16; 19**	**9/18; 50**	D22S929	22q12.1	25.7/56	2/15; 13	2/18; 11

AA, anaplastic astrocytoma; GBM, glioblastoma multiforme; *TPO*, thyroid peroxidase; NF, neurofibromatosis 1; *EGFR*, epidermal growth factor receptor; DCC, deleted in colorectal carcinoma; *LPL*, lipoprotein lipase. Position indicates approximate location in megabases per whole. Denominator in AA and GBM columns represents informative cases. Loci with at least one significant loss are denoted in boldface type.

Cytogenetic and LOH studies of malignant astrocytomas have shown that chromosome loss is more widespread in GBM than in AA (Fults 1996; von Deimling et al., 1995). These observations support the hypothesis that inactivation of multiple tumor suppressor genes located on different chromosomes is a driving force in astrocytoma progression. Allelic deletions on 9p, 11p, 13q, and 19q are equally prevalent in both AA and GBM, suggesting that inactivation of tumor suppressor genes located in these chromosomal regions precedes the formation of malignant astrocytoma. By contrast, loss on chromosome 10 occurs much more frequently in GBM than in AA, suggesting that loss of tumor suppressor genes on chromosome 10 mediates the transition from AA to end-stage GBM. GBMs have also been observed to develop rapidly, seemingly without clinical or histopathologic precursors, particularly in older patients (von Deimling et al., 1994). This observation, and the fact that individual GBM cases vary with regard to the chromosome sites showing deletions, suggests that not all GBMs arise by progressing through the AA stage. Some may arise by alternative pathways that should be recognizable by a different set of altered genes than are observed in AA.

Previous genetic studies of malignant astrocytomas have identified regions of oncogene activation (most commonly gene amplification of EGFR at 7p12) (Bello et al., 1994; Bijlsma et al., 1994; Fischer et al., 1994; Leenstra et al., 1994; Muleris et al., 1994; Schrock et al., 1996; von Deimling et al., 1994) as well as tumor suppressor gene inactivation as defined by LOH. LOH has been reported in gliomas at 9p (*MTS1/CDKN2A* and *MTS2/CDKN2B*), 13q (*RB*),

11p, 17p (*p53/TP53*), and 19q (Fults and Pedone, 1993; Collins, 1995; von Deimling et al., 1994; Schlegel et al., 1996; Debelenko et al., 1997; Kimura et al., 1996; Fults et al., 1992b; Thiel et al., 1992; Li et al., 1997). GBM (and other severe cancers) have been associated with the *MMAC/PTEN* gene on 10q (Steck et al., 1995; 1997).

An important question remains: Are there as yet uncharacterized LOH events that could serve as the signposts for additional tumor suppressor genes involved in the genesis or progression of malignant astrocytomas? Polymerase chain reaction–based microsatellite markers that detect polymorphisms within the abundant short tandem repeats throughout the human genome have made it possible to construct high-resolution genetic linkage maps of human chromosomes. The resolution of these maps can approach one centimorgan (Gyapay et al., 1994; Murray et al., 1994). The high polymorphism information content of microsatellite markers, as compared with earlier restriction fragment length polymorphism markers, results in a significantly greater sensitivity when attempting to detect allelic imbalance in patient tumor DNA samples. We carried out a comprehensive LOH analysis of 45 malignant astrocytoma patients using highly informative microsatellite markers for 70 distinct autosomal loci. We found several previously unreported allelic deletions. These suggest potential sites for novel tumor suppressor genes.

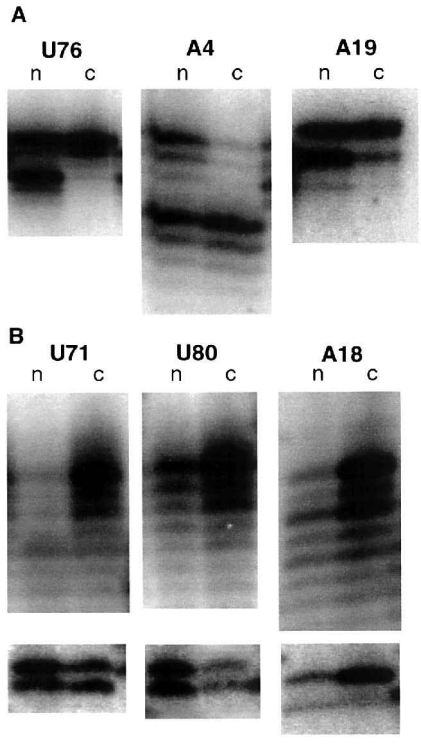

Fig. 1. Representative genetic data. A: Examples of allelic imbalance (LOH). Cases U76, A4, and A19 show LOH for markers D17S731, D14S261, and D19S601, respectively. B: Example of amplification at the EGFR locus in samples U71, U80, and A18. Lower panels show data for D9S922, which was coamplified to normalize for variation in template and thermocycler performance. N, normal; C, cancer.

Methods

DNA Samples

DNA was prepared either from lymphoblastoid cell lines or from resected brain tumors. Histopathologic grading was based on the classification scheme of Burger et al. (1985). Nineteen pairs of samples were from patients with AAs, and 26 pairs of samples came from patients with GBMs for a total of 45 sample pairs. Tumor resection was precise enough to ensure that 75% of the cellularity of each sample was derived from the targeted tissue. Cellular enrichment was about 90% in most samples. Tumor and lymphoblastoid cell line DNA samples were prepared as previously described (Fults et al., 1990).

Polymerase Chain Reaction, Gels, and Quantitation of LOH

Samples were independently evaluated for LOH at each of the polymorphic markers. The antisense primer was 5'-radiolabeled in a standard polynucleotide kinase reaction by 3,000 Ci/mmol [γ^{32}P]ATP at a molar ratio of 18:1 ([γ^{32}P]ATP:Primer) (Steck et al., 1997). DNA template (100 ng) was subjected to 30 cycles of polymerase chain reaction (denaturing at 94°C for 30 s, annealing at approximately 55°C for 1 min, and extension at 72°C for 1 min) carried out in a 15-µl reaction in a 96-well thermocycler. Amplified DNA was diluted 1:1 with stop solution (97% formamide, 1% EDTA, 0.1% bromophenol blue, and 0.1% xylene cyanol) and denatured at 85°C for 2 min. Denatured DNA (3 ml from each sample) was loaded onto a 7% denaturing polyacrylamide (19:1 acrylamide/bis-acrylamide) gel containing 32% formamide and 34% urea and was fractionated by gel electrophoresis for approximately 2.5 h at 60 W. Gels were transferred to filter paper, covered with plastic wrap, equilibrated with 10% methanol/10% acetic acid solution, and dried. Dried gels were exposed to X-ray film without an intensifying screen (usually 16 h).

The markers used and their approximate chromosomal locations are shown in Table 1. Details concerning marker polymorphisms and sequence can be obtained from the Genome Database (GDB^{TM}), formerly at The Johns Hopkins University, now at the Hospital for Sick Children in Toronto (Henson et al., 1994). The GDB World Wide Web URL is http://gdbwww.gdb.org/. Genetically informative samples showed two distinguishable alleles. The intensity ratio of the two allelic bands of DNA from normal tissue relative to that from gliomas in the same case was obtained from digitized data collected with a phosphorimager and analyzed with the Molecular Dynamics ImageQuant (Molecular Dynamics, Sunnyvale, Calif.) software package. A conservative ratio of 1.5 was used to define LOH in this study. Our 75% target cell enrichment was adequate to meet or exceed this ratio in cases with pervasive LOH. Experiments were repeated three times to validate the stability of the LOH phenotype. Microsatellite instability was not a major finding

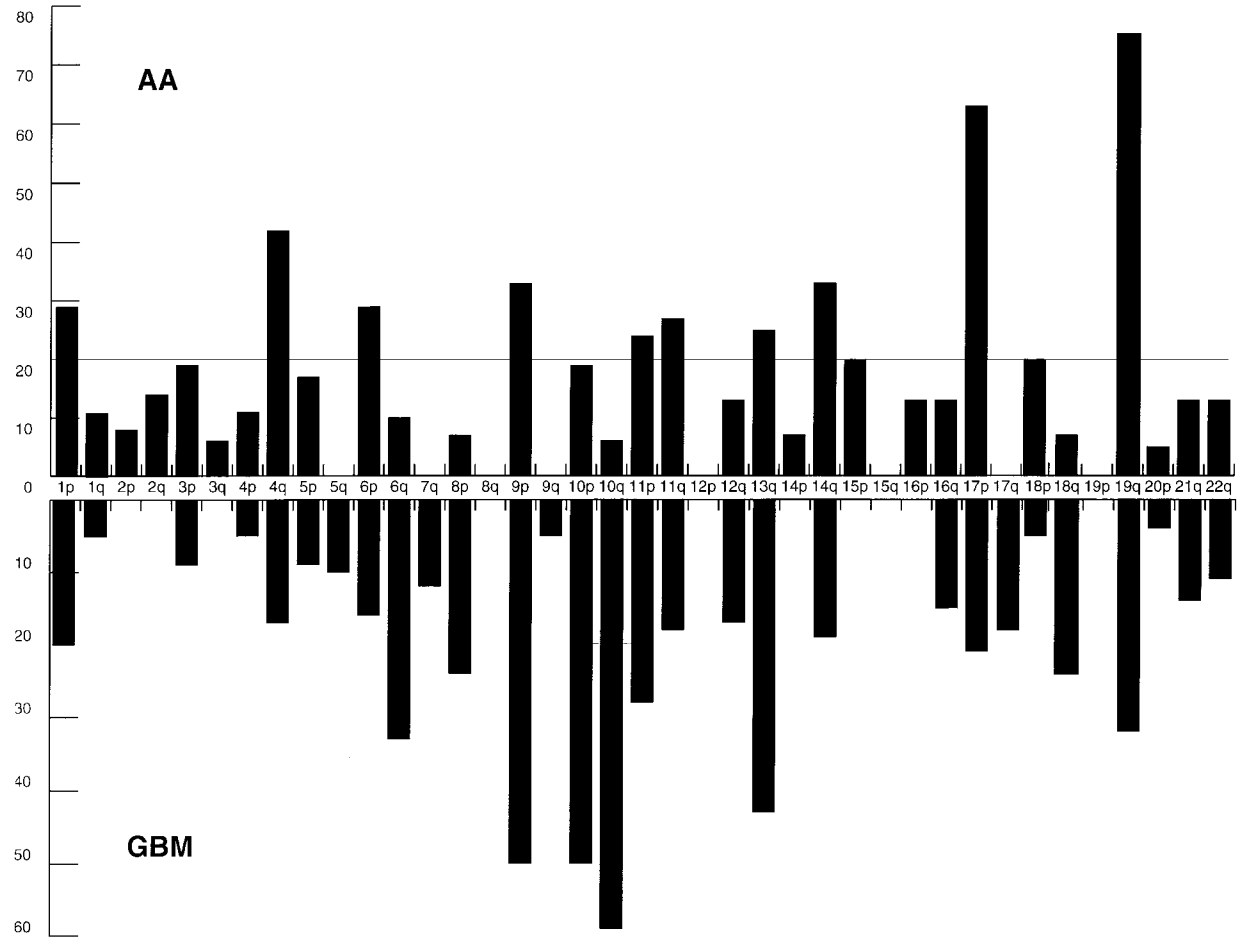

Fig. 2. Maximum observed LOH by chromosome arm. Upper bars represent anaplastic astrocytoma (AA), and lower bars represent glioblastoma multiforme (GBM). Units are percent loss. The line demarcates 20%, which was determined to be the threshold for statistical significance.

for these allelotyping studies. A total of two AA samples (11%, 2 events each) and only 1 GBM sample (4%, 3 events) showed multiple microsatellite instability events, and such cases were excluded from further analysis. Quantitation of *EGFR* amplification was accomplished by multiplexing *EGFR* primers with primers from chromosomal loci known to show little or no LOH and of a sufficiently different product size to allow for sufficient resolution of both products.

Statistical Analysis

Empirical observations lead us to suspect a 20% LOH rate at any given locus represented a significant, non-background loss. To determine if there was statistical significance to this observation, we used Fisher's exact test (Sokal and Rohlf, 1981) and compared loss rates between frequently and infrequently lost loci in both AA and GBM DNA sets. These analyses showed losses of 20% and above to be highly significant. Because multiple tests of associations across the genome are involved in each series analysis, the significance of a given test is determined by Bonferroni's experiment-wise protected alpha value (Wallenstein et al., 1980) in addition to the conventional alpha value of 0.05. The protected alpha value is obtained by simply dividing 0.05 by the number of tests involved in each series analysis. The relation of each pair of marker loci with respect to concordant allele loss rates (sharing of allele loss at two marker loci by the same individual) in each case was examined by Fisher's exact test (two-sided) using the statistical analysis package SAS (SAS Institute, Cary, NC). As noted earlier, corrections for multiple testing have been made.

Results

These studies investigated patterns of LOH in 19 patients with AA and 26 patients with GBM by using 70 autosomal genetic markers spaced at an average of 50 centimorgans across the 22 autosomes. Table 1 indicates the markers tested, approximate genetic locations, and observed LOH (expressed as a percentage of informative patients). Fig. 1 shows representative autoradiographic data. Statistical analysis between the loci showing maximum and minimum genetic changes by chromosome arm was performed for both AAs and GBMs. The significance of loss rates was

Table 2. Loss of heterozygosity by marker and cancer type at the most frequently lost loci

	AA series		GBM series	
Marker	% Loss	*P* value	% Loss	*P* value
D1S255 (1p)	29	0.0189	20	0.1027 (NS)
D4S407 (4q)	42	0.0014*	17	0.2202 (NS)
D6S344 (6p)	29	0.0189	16	0.2765 (NS)
D6S471 (6q)	10	0.5435 (NS)	33	0.0089
EGFR (7p12)	39	0.0003*	38	0.0001*
D8S264 (8q23)	7	1.0000 (NS)	24	0.0628 (NS)
D9S157 (9p)	33	0.0044	50	<0.0001*
D10S196 (10q)	0	0.6281 (NS)	54	<0.0001*
D11S922 (11pter)	24	0.0371	28	0.0061
D11S988 (11qter)	27	0.0242	18	0.1343 (NS)
D13S137 (13q21)	25	0.0586	43	0.0010*
D14S62 (14q)	33	0.0044	17	0.2202 (NS)
D15S165 (15pter)	20	0.1734 (NS)	0	0.4013 (NS)
PS31 (*P53*) (17p13.1)	63	0.0001*	21	0.1282 (NS)
D17S731 (17qter)	40	0.0006*	27	0.0123
D18S46 (18q21.1)	7	1.0000 (NS)	24	0.0376
D19S601 (19q)	75	< 0.0001*	35	0.0031

AA, anaplastic astrocytoma; GBM, glioblastoma multiforme; NS, not significant; *EGFR*, epidermal growth factor receptor.

The loci listed were those with >20% allele loss rates either in AA or GBM. The significance of allele loss rates was determined by Fisher's exact test (two-sided). Each test examined whether the observed allele loss rate at a given locus differed from the empirical background allele loss rate <20%. In fact, the background rate was computed to be the average of the allele loss rates <20% at all loci examined for this study, excluding the above 17 markers.

*Significant after correction for multiple testing (P = 0.05/17 = 0.0029).

determined by Fisher's exact test (two-sided). The background rate of loss was determined to be <20% at all loci examined for this study (Table 2). Therefore, all losses of 20% and above are highly significant ($P = 0.0029$).

The chromosome arms exhibiting nonrandom LOH at significant frequencies (≥ 20%) are listed in Table 1 in boldface type. Approximate megabase location is included for every locus, and chromosomal band location is also given (where possible). Locus band and megabase location is available from the Genome Database (GDB) (Henson et al., 1994) at the World Wide Web URL http://gdbwww.gdb.org/. Approximate chromosomal sizes are used as defined by Newton E. Morton (1991). Fig. 2 summarizes the peak observed LOH by chromosome arm as AA- and GBM-specific histograms. Amplification of the EGFR region at 7p12 was observed in 12% of AA samples and 38% of GBMs.

We compared LOH test results in the patient groups with AA and GBM for loci showing an LOH frequency ≥20% (Table 2). When compared in this way, three distinct groups of chromosomal loci became apparent. In the first group—D9S157 (9p), D11S922 (11p), D13S137 (13q), D17S731 (17q), and D19S601 (19q)—the frequency of LOH was significantly above background in patients with AA and patients with GBM. In the second group—D6S471 (6q), D8S264 (8p), D10S249 (10p), D10S196 (10q), and D18S46 (18q)—LOH was significant only in patients with GBM. In the third group—D1S243 (1p), D1S255 (1q), D4S407 (4q), D6S344 (6p), D11S988 (11q), D14S62 (14q), TP53 (17p), and D19S601 (19q)—the frequency of LOH was significantly higher in patients with AA.

To identify any pairs of concomitantly lost markers, we examined CALR at pairs of markers by cancer type (Table 3). Three primer pairs—D4S407-D17S731, D6S344-D13S137 (in AAs), and D8S264-D11S922 (in GBMs)—showed evidence of nonrandom CALR, but became nonsignificant (although borderline) when corrected for multiple testing. These observations, although nonsignificant, are strengthened by the fact that intrachromosomal pairs D11S922-D11S988 and PS31(P53)-D17S731 showed CALR of a similar magnitude.

Discussion

Data from our comprehensive LOH analysis of the AA and GBM panels support previous findings in the field and identify several potential chromosomal locations for novel tumor suppressor genes. Overall, the present study revealed higher LOH frequencies than reported previously in an allelotype analysis of malignant astrocytoma that used restriction fragment length polymorphism markers (Fults et al., 1990).

We report amplification at the EGFR locus. Our findings are in agreement with previous reports of high levels of amplification (38%) in GBMs coupled with lower amplification levels (12%) in AAs. Several groups (Leenstra et al., 1994; Reifenberger et al., 1996; von Deimling et al., 1992a) have attempted to classify gliomas based on EGFR amplification, LOH status at 17p (p53), and LOH at chromosome 10. We report increasing EGFR amplification with increased LOH at chromosome 10 in GBMs, which correlates well with previous studies. AAs show

Table 3. Concordant allele loss rates at pairs of marker loci by cancer type significant at 5% level

Marker pair[a]	AA-series % CALR	AA-series *P* value[b]	GBM-series % CALR	GBM-series *P* value[b]
D4S407-D17S731	44.4	0.0079	—	—
D6S344-D13S137	33.3	0.0476	—	—
D11S922-D11S988	21.4	0.0028	13.6	0.0458
D8S264-D11S922	—	—	17.6	0.0223
PS31(*P53*)-D17S731	—	—	23.1	0.0035

CALR, concordant allele loss rates; AA, anaplastic astrocytoma; GBM, glioblastoma multiforme.

[a]Marker loci with >20% allele loss rates in either series. The relationship between each pair of marker loci with respect to concordant allele loss rates in each case series was examined by Fisher's exact test (two-sided).

[b]The above reported significant results become nonsignificant when correction for multiple testing per marker is taken into consideration ($P = 0.05/16 = 0.0,031$) in each case series, except the marker pair D11S922-D11S988 for the AA-series and PS31(*P53*)-D17S731 for the GBM-series.

lower losses on chromosome 10 and low amplification of EGFR, coupled with high losses at 17p, again in agreement with previous studies.

As expected, we found high frequency LOH on chromosome arms containing tumor suppressor genes with known involvement in astrocytomas. These genes include *MTS1/CDKN2A* and *MTS2/CDKN2B* (9p), *PTEN/MMAC1* (10q), *DMBT1* (10q), *RB* (13q), and *TP53* (17p) (Frankel et al., 1992, Fults et al., 1992a, Henson et al., 1994, Kamb et al., 1994, Jen et al., 1994, Li et al., 1997, Steck et al., 1997).

Additionally, we found significant LOH frequencies at loci (6p, 10p, 11p, 14q, 17q, and 19q) where allelic deletions were reported previously in human gliomas, but where the putative tumor suppressor gene has not yet been identified (Fults et al., 1990, 1992b; Liang et al., 1994; von Deimling et al., 1992b). Several markers yielded novel observations. Marker D1S255 showed approximately 24% LOH in both AAs and GBMs. LOH on 1p has not been reported in gliomas at these levels.

Two markers showed high losses in GBMs but not in AAs. Marker D6S471 on 6q showed 33% LOH and marker D18S46 showed 24% LOH in GBMs. This data suggests that losses of tumor suppressor genes on 6q and 18q may be involved in the formation of GBMs. The fact that LOH at these two loci was 10% or less in the AA series suggests that these putative genes, like the previously described genes on chromosome 10, are terminal genetic events associated with end-stage GBM.

In several instances we found high rates of LOH in AAs coupled with lower LOH rates in GBMs, suggesting a specific involvement of these regions in the development of astrocytomas. Markers D4S407 (4q) and D14S62 (14q) showed higher LOH rates in AAs than in GBMs. This pattern of LOH may have important ramifications with regard to the controversy surrounding the genesis of AAs. Similar observations have been previously reported for chromosomes 17p (p53) and 19q (Campomenosi et al., 1996; von Deimling et al., 1992b).

Clinically, gliomas present in two ways. GBMs often arise de novo, without apparent progression from less malignant precursors, particularly in elderly patients. In younger patients, the more frequent observation is that of GBMs progressing from less malignant tumor phenotypes by stepwise progression through increasingly malignant tumor stages, often in spite of treatment. These observations have led to two models for the progression of malignant gliomas. One model holds that a series of discrete losses to specific tumor suppressors or gains at oncogenic loci leads through a pathway beginning with a normal cell and progressing through a serial evolution of increasingly malignant phenotypes, which ultimately results in GBM (von Deimling et al., 1995). The rapid growth of GBMs as compared with astrocytomas could explain the observations of seemingly spontaneous GBMs. One could imagine a subclinical astrocytoma that progresses to a rapid GBM tumor, causing the histopathologic evidence of the preceding astrocytoma to be unclear or otherwise obfuscated. The second model maintains that AAs and GBMs arise from a lesion-specific pattern of genetic damage. The observation of what appears to be tumor progression in a patient would actually be the result of the two forms arising from independent clones of transformed cells, each tumor foci having followed its own, presumably unique, pathway.

Analysis of our data suggests that these two clinical models are not mutually exclusive and that there are three types of loci involved in glioma evolution. First are loci that are probably involved in early events of the evolution of AAs or GBM, typified by significant rates of LOH in both types of lesions (7p amplification, 9p, 13q, 11p, and 17q). Suppressor genes at these sites are probably involved in both AAs and GBMs, and their inactivation during the progression of the tumor most likely precedes the AA stage. The second class consists of loci that appear to be AA-specific, typified by patterns where LOH frequency is significantly higher in AAs than in GBMs (1p, 1q, 4q, 6p, 11q, 14q, 17p, and 19q). The low levels of loss at these loci in GBMs may indicate that our GBM cases comprised a mixed population of tumors. Some may have progressed from less malignant astrocytoma stages, while others may have arisen by the de novo pathway. Alternatively, loss of these AA-specific loci may define a subset of AAs that do not progress to the GBM stage, or do so rarely. The third class of loci are those that appear to be GBM-specific. These are typified by significant rates of LOH in GBMs but not in AAs (6q, 8p, 10, and 18q). Genetic damage at these loci could either lead to de novo GBMs or permit existing AAs from the first two categories to progress into GBMs. Some investigators have tried to use GBM-specific loci to subclassify these two types of GBMs (von Deimling et al., 1993).

Using analysis of CALR, we hoped to discern any inter-relationships between pairs of loci and apparent predisposition to AAs, GBMs, or both. While several loci were found to be concordantly lost, these results become nonsignificant when corrected for multiple testing (Table 3). These findings gain importance when taken together with the CALR rates between loci on the same chromosome (chromosomes 11 and 17). Although these rates are not biologically significant, they serve as evidence that our

methodology is sound and raise the possibility that the interchromosomal pairs (D4S407-D17S731 and D6S344-D13S137) that are lost at similar rates could prove significant with a larger pool of samples from which to draw. Likewise, other primer pairs could emerge as significant targets for CALR with a larger pool of tested loci.

Our observations are consistent with the notion of an interrelated but potentially independent genetic evolution of gliomas. The widespread, nonrandom chromosome loss we observed in malignant astrocytomas indicates that the number of tumor suppressor genes involved in human gliomas exceeds that currently known. The novel chromosome deletion sites we report here may serve as targets for higher resolution deletion mapping studies and future positional cloning projects to identify novel tumor suppressor genes. These genes may serve as targets for future treatments for patients with this lethal form of cancer.

References

Bello, M.J., de Campos, J.M., Kusak, M.E., Vaquero, J., Sarasa, J.L., Pestana, A., and Rey, J.A. (1994) Molecular analysis of genomic abnormalities in human gliomas. *Cancer Genet. Cytogenet.* **73**, 122–129.

Bijlsma, E.K., Leenstra, S., Westerveld, A., Bosch, D.A., and Hulsebos, T.J. (1994) Amplification of the anonymous marker D17S67 in malignant astrocytomas. *Genes Chromosomes Cancer* **9**, 148–152.

Bruner, J.M. (1994) Neuropathology of malignant gliomas. *Semin. Oncol.* **21**, 126–138.

Burger, P. C., Vogel, F. S., Green, S.B., and Strike, T.A. (1985) Glioblastoma multiforme and anaplastic astrocytoma. Pathologic criteria and prognostic implications. *Cancer* **56**, 1106–1111.

Campomenosi, P., Ottaggio, L., Moro, F., Urbini, S., Bogliolo, M., Zunino, A., Camoriano, A., Inga, A., Gentile, S.L., Pellegata, N.S., Bonassi, S., Bruzzone, E., Iannone, R., Pisani, R., Menichini, P., Ranzani, G.N., Bonatti, S., Abbondandolo, A., and Fronza, G. (1996) Study on aneuploidy and p53 mutations in astrocytomas. *Cancer Genet. Cytogenet.* **88**, 95–102.

Collins, V. P. (1995) Genetic alterations in gliomas. J. Neurooncol. 24, 37–38.

Debelenko, L.V., Zhuang, Z., Emmert-Buck, M.R., Chandrasekharappa, S.C., Manickam, P., Guru, S.C., Marx, S.J., Skarulis, M.C., Spiegel, A.M., Collins, F.S., Jensen, R.T., Liotta, L.A., and Lubensky, I.A. (1997) Allelic deletions on chromosome 11q13 in multiple endrocrine neoplasia type 1-associated and sporadic gastrinomas and pancreatic endocrine tumors. *Cancer Res.* **57**, 2238–2243.

Fischer, U., Wullich, B., Sattler, H.P., Gottert, E., Zang, K.D., and Meese, E. (1994) DNA amplifications on chromosomes 7, 9 and 12 in glioblastoma detected by reverse chromosome painting. *Eur. J. Cancer* **30A**, 1124–1127.

Frankel, R. H., Bayona, W., Koslow, M., and Newcomb, E.W. (1992) *p53* Mutations in human malignant gliomas: Comparison of loss of heterozygosity with mutation frequency. *Cancer Res.* **52**, 1427–1433.

Fults, D. (1996) *Molecular Themes in Glial Tumor.* Baltimore, Md.: Williams and Wilkins.

Fults, D., and Pedone, C. (1993) Deletion mapping of the long arm of chromosome 10 in glioblastoma multiforme. *Genes Chromosomes Cancer* **7**, 173–177.

Fults, D., Pedone, C.A., Thomas, G.A., and White, R. (1990) Allelotype of human malignant astrocytoma. *Cancer Res.* **50**, 5784–5789.

Fults, D., Brockenmeyer, D., Tullous, M.W., Pedone, C.A., and Cawthon, R.M. (1992a) *p53* Mutation and loss of heterozygosity on chromosomes 17 and 10 during human astrocytoma progression. *Cancer Res.* **52**, 674–679.

Fults, D., Petronio, J, Noblett, B.D., and Pedone, C.A. (1992b) Chromosome 11p15 deletions in human malignant astrocytomas and primitive neuroectodermal tumors. *Genomics* **14**, 799–801.

Furnari, F. B., Huang, H.-J.S., and Cavenee, W.K. (1996) Molecular biology of malignant degeneration of astrocytoma. *Pediatr. Neurosurg.* **24**, 41–49.

Gyapay, G., Morissette, J., Vignal, A., Dib, C., Fizames, C., Millasseau, P., Marc, S., Bernardi, G., Lathrop, M., and Weissenbach, J. (1994) The 1993–94 genethon human genetic linkage map. *Nature Genet.* **7**, 246–339.

Henson, J.W., Schnitker, B.L., Correa, K.M., von Deimling, A., Fassbender, F., Xu, H.J., Benedict, W.F., Yandell, D.W., and Louis, D.N. (1994) The retinoblastoma gene is involved in malignant progression of astrocytomas. *Ann. Neurol.* **36**, 714–721.

Jen, J., Harper, J.W., Bigner, S.H., Bigner, D.D., Papadopoulos, N., Harkowitz, S., Willson, J.K., Kinzler, K.W., and Vogelstein, B. (1994) Deletion of p16 and p15 genes in brain tumors. *Cancer Res.* **54**, 6353–6358.

Kamb, A., Gruis, N. A., Weaver-Feldhaus, J., Liu, Q., Harshman, K., Tavtigian, S.V., Stockert, E., Day, R.S., III, Johnson, B.E., and Skolnick, M.H. (1994) A cell cycle regulator potentially involved in genesis of many tumor types. *Science* **264**, 436–440.

Kimura, M., Abe, T., Sunamura, M., Matsuno, S., and Horii, A. (1996) Detailed deletion mapping on chromosome arm 12q in human pancreatic adenocarcinoma: Identification of a 1-cM region of common allelic loss. *Genes Chromosomes Cancer* **17**, 88–93.

Kleihues, P., Burger, P.C., and Scheithauer, B.W. (1993) *Histological Typing of Tumours of the Central Nervous System.* Second edition. Berlin: Springer-Verlag.

Leenstra, S., Bijlsma, E. K., Troost, D., Oosting, J., Westerveld, A., Bosch, D.A., and Hulsebos, T.J. (1994) Allele loss on chromosomes 10 and 17p and epidermal growth factor receptor gene amplification in human malignant astrocytoma related to prognosis. *Br. J. Cancer* **70**, 684–689.

Levin, V., Leibel, S., and Gutin, P.H. (1997) Neoplasms of the central nervous system. In: DeVita, V.T., Rosenberg, S.A., and Hellman, S. (Eds.), *Cancer: Principles and Practice of Oncology.* Fifth edition. Philadelphia: Lippincott-Raven.

Li, J., Yen, C., Liaw, D., Podsypanina, K., Bose, S., Wang, S.I., Puc, J., Miliaresis, C., Rodgers, L., McCombie, R., Bigner, S.H., Giovanella, B.C., Ittmann, M., Tycko, B., Hilshoosh, H., Wigler, M.H., and Parsons, R. (1997) PTEN, a putative protein tyrosine phosphatase gene mutated in human brain, breast, and prostate cancer. *Science* **275**, 1943–1947.

Liang, B.C., Ross, D.A., Greenberg, H.S., Meltzer, P.S., and Trent, J.M. (1994) Evidence of allelic imbalance of chromosome 6 in human astrocytomas. *Neurology* **44**, 533–536.

Magnani, I., Guerini, S., Pollo, B., Cirenei, N., Colombo, B.M., Broggi, G., Galli, C., Bugiani, O., DiDonato, S., Finocchiaro, G., and Conti, A.M.F. (1994) Increasing complexity of the karyotype in 50 human gliomas. *Cancer Genet. Cytogenet.* **75**, 77–89.

Morton, N. E. (1991) Parameters of the human genome. *Proc. Natl. Acad. Sci. U. S. A.* **88**, 7474–7476.

Muleris, M., Almeida, A., Dutrillaux, A.M., Pruchon, E., Vega, F., Delattre, J.Y., Poisson, M., Malfoy, B., and Dutrillaux, B. (1994) Oncogene amplification in human gliomas: A molecular cytogenetic analysis. *Oncogene* **9**, 2717–2722.

Murray, J.C., Buetow, K.H., Weber, J.L., Ludwigsen, S., Scherpbier-Heddema, T., Manion, F., Quillen, J., Sheffield, V.C., Sunden, S., Duyk, G.M., Weissenbach, J., Gyapay, G., Mels, R., Albertsen, H., Plactice, R., Odelberg, S.,

Ward, D., Dausset, J., Cohen, D., and Cann, H. (1994) A comprehensive human linkage map with centimorgan density, Cooperative Human Linkage Center (CHLC). *Science* **265**, 2049–2054.

Ohgaki, H., Schauble, B., zur Hausen, A., von Ammon, K., and Kleihues, P. (1995) Genetic alterations associated with the evolution and progression of astrocytic brain tumours. *Virchows Arch.* **427**, 113–118.

Reifenberger, J., Ring, G.U., Gies, U., Cobbers, L., Oberstrass, J., An, H.X., Niederacher, D., Wechsler, W., and Reifenberger, G. (1996) Analysisof p53 mutation and epidermal growth factor receptor amplification in recurrent gliomas with malignant progression. *J. Neuropathol. Exp. Neurol.*, **55**, 822–831.

Schlegel, J., Scherthan, H., Arens, N., Stumm, G., and Kiessling, M. (1996) Detection of complex genetic alterations in human glioblastoma multiforme using comparative genomic hybridization. *J. Neuropathol. Exp. Neurol.* **55**, 81–87.

Schrock, E., Blume, C., Meffert, M.C., du Manoir, S., Bersch, W., Kiessling, M., Lozanowa, T., Thiel, G., Witkowski, R., Ried, T., and Cremer, T. (1996) Recurrent gain of chromosome arm 7q in low-grade astrocytic tumors studied by comparative genomic hybridization. *Genes Chromosomes Cancer* **15**, 199–205.

Sokal, R.R., and Rohlf, F.J. (1981) *Biometry: The Principles and Practice of Statistics in Biological Research.* Second edition. New York: W.H. Freeman and Company.

Steck, P.A., Ligon, A.H., Cheong, P., Yung, W.K., and Pershouse, M.A. (1995) Two tumor suppressive loci on chromosome 10 involved in human glioblastomas. *Genes Chromosomes Cancer* **12**, 255–261.

Steck, P.A., Pershouse, M.A., Jasser, S.A., Yung, W.K., Lin, H., Ligon, A.H., Langford, L.A., Baumgard, M.L., Hattier, T., Davis, T., Frye, C., Hu, R., Swedlund, B., Teng, D.H., and Tavtigian, S.V. (1997) Identification of a candidate tumour suppressor gene, MMAC1, at chromosome 10q23.3 that is mutated in multiple advanced cancers. *Nature Genet.* **15**, 356–362.

Thiel, G., Losanowa, T., Kintzel, D., Nisch, G., Martin, H., Vorpahl, K., and Witkowski, R. (1992) Karyotypes in 90 human gliomas. *Cancer Genet. Cyogenet.* **58**, 109–120.

von Deimling, A., Louis, D.N., von Ammon, K., Petersen, I., Hoell, T., Chung, R.Y., Martuza, R.L., Schoenfeld, D.A., Yasargil, M.G., and Wiestler, O.D. (1992a) Association of epidermal growth factor receptor gene amplification with loss of chromosome 10 in human glioblastoma multiforme. *Neurosurg*. **77**, 295–301.

von Deimling, A., Louis, D.N., von Ammon, K., Petersen, I., Wiestler, O.D., and Seizinger, B.R. (1992b) Evidence for a tumor suppressor gene on chromosome 19q associated with human astrocytomas, oligodedrogliomas, and mixed gliomas. *Cancer Res.* **52**, 4277–4279.

von Deimling, A., von Ammon, K., Schoenfeld, D., Wiestler, O.D., Seizinger, B.R., and Louis, D.N. (1993) Subsets of glioblastoma multiforme defined by molecular genetic analysis. *Brain Pathol.* **3**, 19–26.

von Deimling, A., Bender, B., Jahnke, R., Waha, A., Kraus, J., Albrecht, S., Wellenreuther, R., Fassbender, F., Nagel, J., Menon, A.G., Louis, D.N., Lenartz, D., Schramm, J., and Wiestler, O.D. (1994) Loci associated with malignant progression in astrocytomas: A candidate on chromosome 19q. *Cancer Res.* **54**, 1397–1401.

von Deimling, A., Louis, D.N., and Wiestler, O.D. (1995) Moleclar pathways in the formation of gliomas. *Glia* **15**, 328–338.

Wallenstein, S., Zucker, C.L., and Fleiss, J.L. (1980) Some statistical methods useful in circulation research. *Circ. Res.* **47**, 1–9.