Am. J. Hum. Genet. 58:1260–1267, 1996

Deletion Mapping of Gliomas Suggests the Presence of Two Small Regions for Candidate Tumor-Suppressor Genes in a 17-cM Interval on Chromosome 10q

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Summary

The loss of genetic material on chromosome 10q is frequent in different tumors and particularly in malignant gliomas. We analyzed 90 of these tumors and found loss of heterozygosity (LOH) in >90% of the informative loci in glioblastoma multiforme (GBM). Initial studies restricted the common LOH region to 10q24-qter. Subsequently, the study of a pediatric GBM suggested D10S221 and D10S209, respectively, as centromeric and telomeric markers of a 4-cM LOH region. It is interesting to note that, in one subset of cells from this tumor, locus D10S209 seems involved in the allelic imbalance of a larger region, with D10S214 as telomeric marker. This 17-cM region contains the D10S587-D10S216 interval of common deletion recently defined on another set of gliomas.

Introduction

Glial neoplasms are the most common primary brain tumors and evolve through different grades of malignancy. Gliomas of astrocytic origin, in particular, can be classified as differentiated astrocytomas, anaplastic astrocytomas (AA), and glioblastomas multiforme (GBM). During the malignant progression to GBM, different sets of chromosomal alterations can take place, including trisomy of chromosome 7, structural abnormalities of chromosomes 17p, 9p, and 19q, and loss of chromosome 10 (Lang et al. 1994). Loss of heterozygosity (LOH) on chromosome 10 has been identified not only in gliomas (Rasheed et al. 1992; Karlbom et al. 1993; Fults and Pedone 1993) but also in renal cell carcinomas (Morita et al. 1991), non-Hodgkin lymphomas (Speaks et al. 1992), malignant meningiomas (Rempel et al. 1993), melanomas (Herbst et al. 1994), endometrial cancers (Peiffer et al. 1995), and prostate cancers (Gray et al. 1995), indicating that one or more putative tumor-suppressor genes (TSG) are located on this chromosome. Genetic losses affect more consistently the long arm of chromosome 10. This was also confirmed by experiments of "microcell fusion" of chromosome 10 in glioma cells causing the regression of the neoplastic phenotype both in vitro and in vivo (Pershouse et al. 1993). To define the minimal common region of LOH in gliomas, we have analyzed microsatellites on the long arm of chromosome 10 in tumor and constitutive DNA of 90 malignant gliomas.

Material and Methods

DNA Preparation and Amplification of Microsatellites

Tumor specimens were obtained from the surgery room. One part was used for histological analysis, and tumors were classified according to the World Health Organization revised criteria for classification (Kleihues et al. 1993). Other parts were frozen at −80°C for molecular analysis and, when enough material was available, used for the determination of the karyotype. Blood (usually 20 ml) was obtained from the patient a few days after the operation. DNA was isolated from surgical specimens and lymphocytes by cell lysis with guanidine thyocianate, using a nucleic acid extraction kit (IsoQuick, MicroProbe). When LOH was undetectable by microsatellite analysis, samples of neoplastic tissue were usually obtained under the guide of the pathologist (B.P.) by excising small fragments with a scalpel from paraffin-embedded material. These fragments were treated twice with octane to remove paraffin and washed twice with 100% ethanol to remove the solvent. After incubation with 200 μg/ml of Proteinase K in 100 μl of

Received September 27, 1995; accepted for publication February 26, 1996.

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0002-9297/96/5806-0020$02.00

50 mM TrisHCl pH 8.5, 1 mM EDTA, 0.5% Tween 20 (55°C for 3 h), the DNA was extracted by phenol-chloroform and precipitated by ethanol.

Microsatellite amplification was performed as described by Tenan et al. (1994). LOH was determined by visual inspection. When necessary, results were also analyzed by a computer-assisted system for quantification of electrophoresis gel imagery (Bio-Profil). Densitometric evaluations were performed defining a "window" for tumor or lymphocyte alleles. After subtracting the background, the software integrates the area and the intensity of each band and calculates the relative size.

FISH of Marker D10S108

Fifteen picomoles of the forward and reverse primer for the amplification of microsatellite marker D10S108 were each labeled with 15 pmol of [γ-^{32}P]ATP and used separately for the screening of replica filters of a λ-FIX human genomic DNA library (4×10^5 pfu). Prehybridizations and hybridizations were carried out at 42°C in the presence of 30% deionized formamide, 5 × sodium-phosphate-EDTA, 1% SDS, 1 × Denhardt, and 10% dextran sulphate. After overnight incubation and washing, one plaque-signal identified by both probes was isolated by further rounds of screening.

For FISH, λ.BMC10S108 DNA, after confirming by PCR the presence of the D10S108 CA repeat, was labeled by nick-translation with biotin-21 dUTP (Clontech Laboratories). A mixture of 100 ng of this probe and 20 ng of biotinylated DNA from an alpha-satellite probe of chromosome 10 was mixed with human COT-1 DNA (BRL) and salmon sperm DNA (Sigma) and resuspended in 25 µl of hybridization solution (50% formamide, 2 × SSC, 10% dextran sulfate). The probe was denatured at 75°C and allowed to preanneal at 43°C on glass slides carrying normal metaphases of human chromosomes pretreated with RNAse (Sigma) and 70% formamide. Hybridization was carried out at 37°C for 16 h. The biotinylated probe was detected by incubation with streptavidin-FITC at 5 mg/ml (Vector Laboratories). The signal was amplified by biotinylated anti-streptavidin (Vector Laboratories; 5 mg/ml), and slides were subsequently incubated in streptavidin-FITC, mounted with propidium iodide in antifading solution, and observed with a Zeiss fluorescence microscope containing a FITC filter (the detailed protocol for FISH can be obtained from I.M.).

Results

Initial Definition of a Common Region of LOH

During the last 3 years we have analyzed 90 malignant gliomas (53 GBM, 35 AA, and 2 anaplastic oligodendrogliomas), in an effort to define the minimal region of LOH on chromosome 10q. Karyotypes of 30 of these tumors have been reported (Magnani et al. 1994), and those of 22 other cases have also been determined (data available on request).

In an earlier phase of our work, we looked for LOH on 19 malignant gliomas (12 GBM, 5 AA, and 2 anaplastic olidendrogliomas) with microsatellites spanning a large portion of chromosome 10q, from locus D10S107 to D10S212 (Decker et al. 1992; Weissenbach et al. 1992). We found that allelic losses were always detectable in GBM but were quite uncommon in less malignant tumors (a partial analysis of 13 of these tumors was provided by Colombo et al. 1993). Among GBM, we detected LOH in 2/6 of the informative patients for D10S107, 6/9 for D10S109, 4/8 for D10S91, 6/8 for D10S110, 6/9 for D10S108, 8/8 for D10S187, 5/5 for D10S216, 8/8 for D10S169, and 6/6 for D10S212 (markers are ordered from centromere to telomere). These data showed that LOH was more evident on the telomeric region of chromosome 10 and that several GBM did not show loss LOH in the region centromeric to D10S108. This locus was therefore considered as a provisional centromeric flanking marker.

A human genomic library was screened with the primers for the corresponding microsatellite. The isolated clone (λ.BMC10S108) was used to investigate the subchromosomal localization of this locus by FISH. The combined probe, composed of alpha-satellite chromosome 10 and λ.BMC10S108, hybridized to metaphase spreads of chromosomes from normal lymphocytes, highlighting the centromere and the 10q23.3 region of each chromatid (fig. 1). This localization narrowed the position of locus D10S108 from the 10q22.3-q23.3 interval (Matise et al. 1994) down to a much smaller region on the boundary of 10q23.3-q24.1.

In agreement with this result, tumor GB29 (referred as MI-14 by Magnani et al. [1994]), which showed a 10q24-qter deletion on cytogenetic analysis, demonstrated conserved heterozygosity with microsatellites on loci D10S185, D10S108, and D10S198 and loss on loci D10S190 and D10S214 (fig 2). Other loci examined in this tumor are shown in figure 3.

We therefore concentrated our work on the 10q24-qter portion and extended our analysis to other 71 gliomas (30 AA and 41 GBM). A summary of the results for each locus analyzed is in table 1. The overall number of patients is 90, but none of them has been investigated for all loci: the number of tested loci reflects the different stages of the work, during which the minimal LOH region has been progressively defined, by us and others, and the number of available microsatellites increased. Thus, several loci that were initially of interest have been subsequently neglected.

Of 37 AGs (including 35 AA and 2 anaplastic oligodendrogliomas), 5 showed complete LOH at all the informative loci tested, while in the remaining tumors the allelic pattern was unaltered. Among GBMs (53 tumors) allelic losses were detected at all loci in 42 cases and

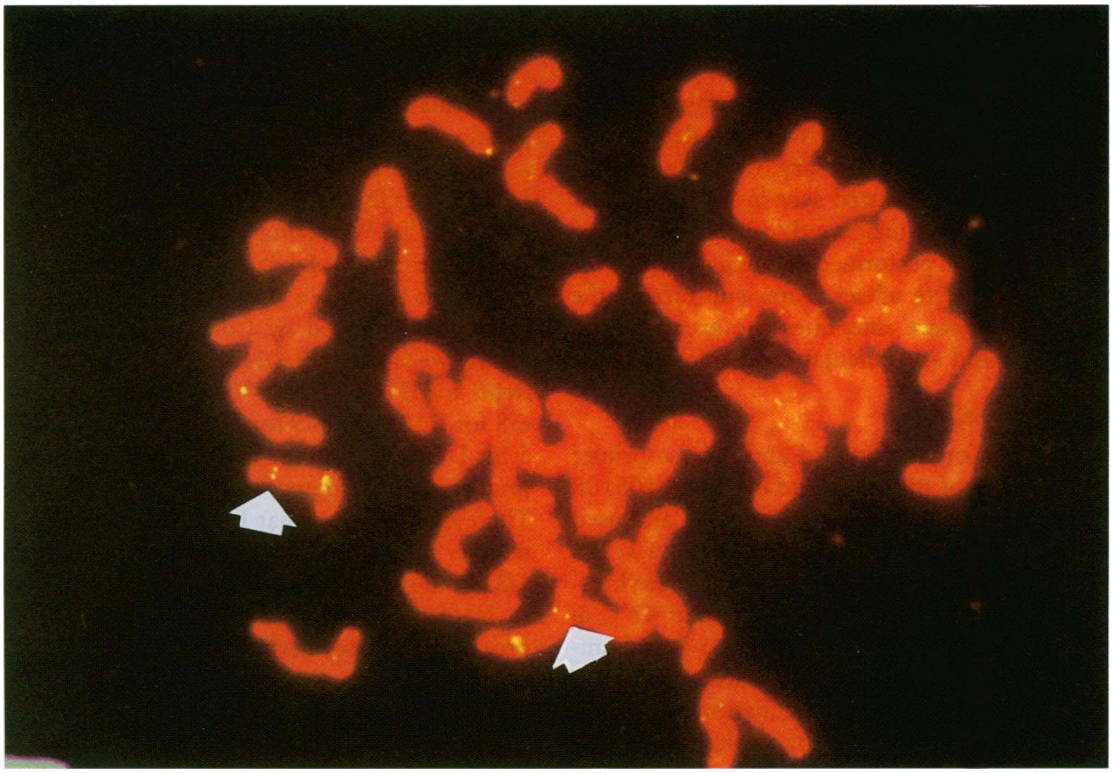

Figure 1 FISH of marker D10S108. Probes for the hybridization to metaphase spreads of normal chromosomes were clone λ-BMC10S108 and alpha-satellite DNA of chromosome 10. The hybridization signals of λ.BMC10S108 for each chromatid are indicated by *arrows*.

were absent in only 2. Partial losses were present in nine tumors, and the results in these informative cases are presented in figure 3. Overall, LOH was detected in 88.1% (252/286) of the informative loci and 19.2% (23/120) for GBM and AG, respectively. When the analysis was restricted to the 10q24-qter region (loci D10S192-D10S212) LOH was present in 94.5% (173/183) and 24.2% (22/91) of GBM and AG, respectively, suggesting that allelic losses on this region of chromosome 10 are a crucial step of the malignant evolution of gliomas. This is directly demonstrated by the analysis of patient AA3/GB11, who underwent surgery twice because of a recurrent AA. At the first histological analysis, the tumor showed only the features of an anaplastic astrocytoma, but in the recurring tumor islets in evolution to GBM were also detectable (fig. 4). We isolated DNA from paraffin-embedded fragments derived from AA and GBM cells and performed microsatellite analysis on these samples and on lymphocyte DNA. At all loci tested, the alleles in the AA retained heterozygosity, while LOH was always present in the part evolving to GBM (figs. 3 and 4).

Further Definition of the Minimal LOH Region

A particularly informative tumor (GB40) was obtained from a pediatric patient, M.P. Cytogenetic analysis of GB40 showed that the karyotype was apparently normal, but the molecular findings suggested that the LOH could be narrowed down to a region much smaller that the 40-cM interval approximately corresponding to 10q24-qter. This child started suffering with diplopia and vomiting at 6 years of age. Neuroradiological investigations demonstrated the presence of a malignant neoplasm in the brainstem (fig. 5*A*), and she subsequently underwent surgery to remove the exophytic portion of this tumor. The histological analysis was in agreement with the diagnosis of GBM, as shown in figure 5*B*, but regions of higher differentiation, resembling an anaplastic astrocytoma, were also detectable. After radiotherapy and chemotherapy the patient was asymptomatic for 1 year. She has been newly hospitalized for a recurrence and is under local chemotherapy with bleomycin.

The initial results of microsatellite analysis on DNA from the specimen obtained during surgery did not show a clear evidence of allelic imbalance. However, when we extracted genomic DNA from selected areas in the paraffin-embedded tumor that looked unequivocally malignant at the histological analysis (sample GB40-P.I), LOH was found at locus D10S190 (fig. 2). Heterozygosity was maintained in tumor DNA at all the other informative polymorphisms tested in sample GB40-P.I, so the minimal LOH region, spanning ~4 cM of genetic distance, was defined by centromeric and telomeric loci D10S221 and D10S209, respectively (fig. 2).

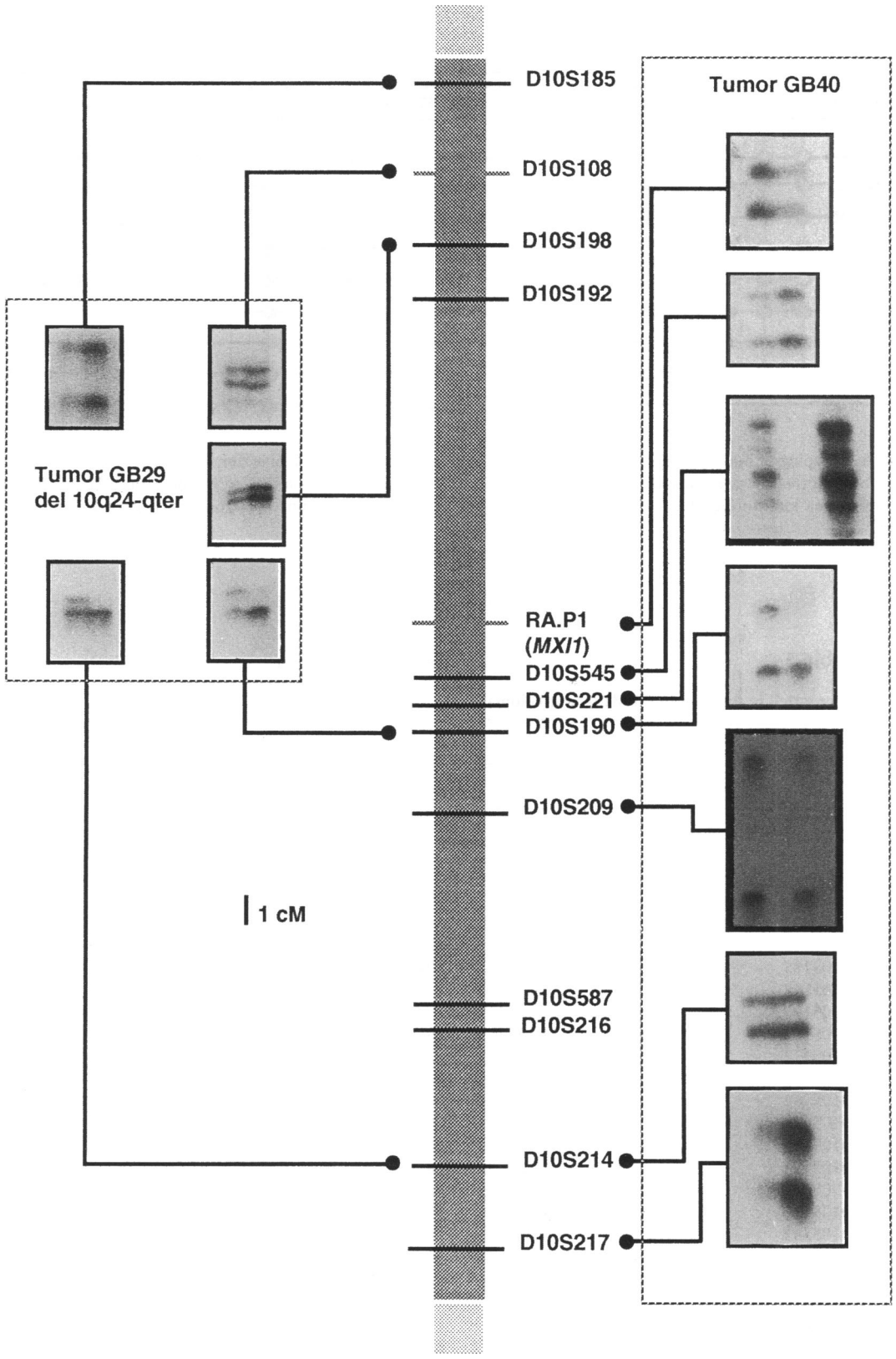

Figure 2 Microsatellite analysis of allelic imbalance of two key tumors, GB29 and GB40. In the *middle* is shown a schematic drawing of part of chromosome 10q. The genetic distance among loci (black bars) is according to Gyapay et al. (1994) and Chumakov et al. (1994). The positions of loci D10S108 and RA.P1-*MXI1* (gray bars) are presumptive and are based on FISH (this study and Edelhoff et al. 1994, respectively). *MXI1* has also been placed on YACs 936h5 and 966h9, which overlap with YACs bearing the microsatellite marker D10S597 (Gray et al. 1995). For each locus, amplification of lymphocyte and tumor DNA are shown, respectively, on the *left* and on the *right*. Tumor GB29 restricted the initial definition of the LOH region to 10q24-qter, from locus D10S190 to the telomere. Tumor GB40, subsequently, demonstrated LOH only on locus D10S190. However, the analysis of locus D10S209 in two of six samples extracted from paraffin showed that the densitometric allelic ratio of tumor DNA was ~65% that of lymphocyte DNA, suggesting the presence of LOH for this marker in a minority of tumor cells. In the sample in the figure, such ratio is 86%.

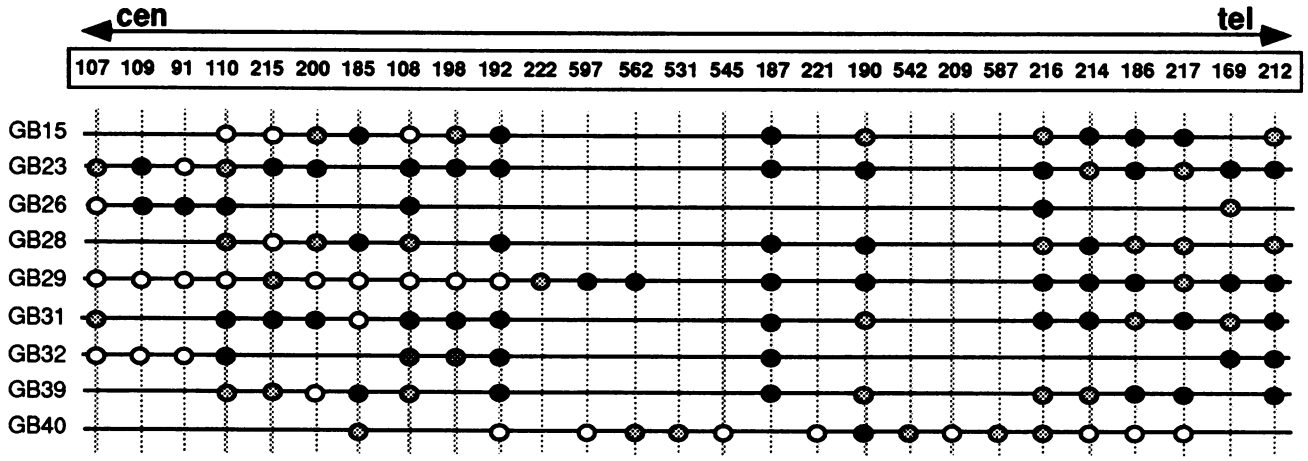

Figure 3 Allelotyping of human malignant gliomas. The figure reports the data of the nine tumors informative for mapping. Loci, shown in abbreviated form on the *top*, are ordered from centromere (*left*) to telomere (*right*), as indicated by the *arrow*. Tumor numbers are on the *left* (GB = glioblastoma multiforme). Black dots denote LOH; white dots denote maintained heterozygosity; gray dots indicate that the data are not informative.

Table 1

Microsatellite Analysis in 90 Malignant Gliomas

	AG				GBM			
Locus	No.	MH	LOH	%LOH	No.	MH	LOH	%LOH
D10S107	7	4/4	0/4	.0	12	4/6	2/6	33.3
D10S109	7	6/6	0/6	.0	11	3/9	6/9	66.7
D10S91	7	5/5	0/5	.0	11	4/8	4/8	50.0
D10S110	7	5/5	0/5	.0	22	2/12	10/12	83.3
D10S215	1	1/1	0/1	.0	27	3/20	17/20	85.0
D10S200	0	0/0	0/0	...	17	2/8	6/8	75.0
D10S185	2	1/2	1/2	50.0	25	2/17	15/17	88.2
D10S108	7	5/5	0/5	.0	23	3/13	10/13	76.9
D10S198	1	1/1	0/1	.0	18	1/10	9/10	90.0
D10S192	20	11/15	4/15	26.7	38	2/29	27/29	93.1
D10S222	1	0/0	0/0	...	1	0/0	0/0	...
D10S597	2	0/2	2/2	100.0	11	1/6	5/6	83.3
D10S562	2	0/2	2/2	100.0	9	0/8	8/8	100.0
D10S531	1	0/1	1/1	100.0	1	0/0	0/0	...
D10S545	3	0/1	1/1	100.0	3	1/3	2/3	66.7
D10S187	14	11/12	1/12	8.3	28	0/25	25/25	100.0
D10S221	0	0/0	0/0	...	1	1/1	0/1	.0
D10S190	27	9/13	4/13	30.8	28	0/17	17/17	100.0
D10S542	0	0/0	0/0	...	2	0/2	1/2	50.0
D10S209	2	1/2	1/2	50.0	11	1/8	7/8	87.5
D10S587	0	0/0	0/0	...	5	0/4	4/4	100.0
D10S216	13	7/8	1/8	12.5	25	0/13	13/13	100.0
D10S214	16	6/7	1/7	14.3	28	1/17	16/17	94.1
D10S186	17	6/8	2/8	25.0	21	1/12	11/12	91.7
D10S217	14	7/8	1/8	12.5	28	1/16	15/16	93.7
D10S169	7	3/3	0/3	.0	12	0/8	8/8	100.0
D10S212	20	8/9	1/9	11.1	25	0/14	14/14	100.0

Note.—AG = anaplastic gliomas (anaplastic astrocytomas and anaplastic oligodendrogliomas); GBM = glioblastoma multiforme; No. = number of patients examined; MH and LOH = maintained heterozygosity and loss of heterozygosity, respectively, over the number of informative markers; %LOH = percentage of loss of heterozygosity.

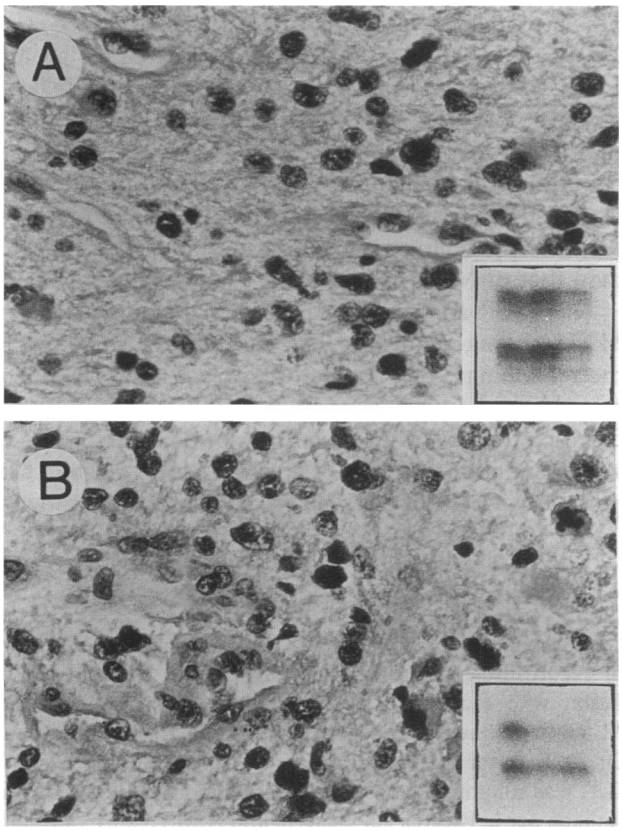

Figure 4 Histological and molecular analysis of tumor AA3/GB11. Panels *A* and *B* show the hematoxylin-eosin staining of AA and GBM regions, respectively, of recurrent glioma AA3/GB11. The insets show one of the analyzed microsatellites, D10S217. In both cases, the *left* lane shows amplification from lymphocyte DNA. In *inset A*, the *middle* and the *right* lanes show, respectively, maintained heterozygosity in DNA form the initial and the recurrent AA. In *inset B*, the *middle* and the *right* lanes show, respectively, LOH in DNA of the AA/GBM boundary region and of the GBM region.

To further investigate this tumor, five other samples (GB40-P.II and GB40-P.1, -P.2, -P.3, and -P.4) were excised from paraffin-embedded material, and PCR-amplified alleles were evaluated densitometrically. The average ratio between the integrated area of the larger and the smaller allele was 99% of the ratio of control lymphocyte DNA for locus D10S221, 59% for locus D10S190 (45% for sample GB40-P.I, shown in 2), and 76% for locus D10S209. This latter value reflects the situation of two samples (GB40-P.II and -P.2) in which the tumor-allele ratio was 65% and 66% of control DNA, respectively, suggesting that groups of neoplastic cells in the corresponding areas of the tumor present larger genetic losses involving D10S209 and extending toward the telomere. The tumor/control allele ratio of other loci shown in figure 3 is 100% for D10S545, 95% for D10S214 and D10S217, and 104% for RA.P1. (This is the name of the *MXI1* polymorphism described by Albarosa et al. 1995.) Markers D10S587 and D10S216 were not informative.

Discussion

Our results suggest that virtually all malignant gliomas undergo some genetic loss on 10q24-qter in order to evolve as GBM. We consider this large telomeric region to be a candidate to contain a putative TSG, on the basis of the initial study of 19 malignant gliomas, a definition also supported by other reports (Rasheed et al. 1992; Fults and Pedone 1993; Karlbom et al. 1993). It is interesting to note that several lines of evidence suggest the presence of a latent intercalary centromere at 10q25 (Vouillaire et al. 1993), a finding that could provide some hint on the mechanisms underlying the genetic damage of this region in gliomas and other tumors. The activation of such a latent centromere in gliomas could in fact give rise to an unstable dicentric chromosome that could subsequently undergo a breakage with loss of genetic material.

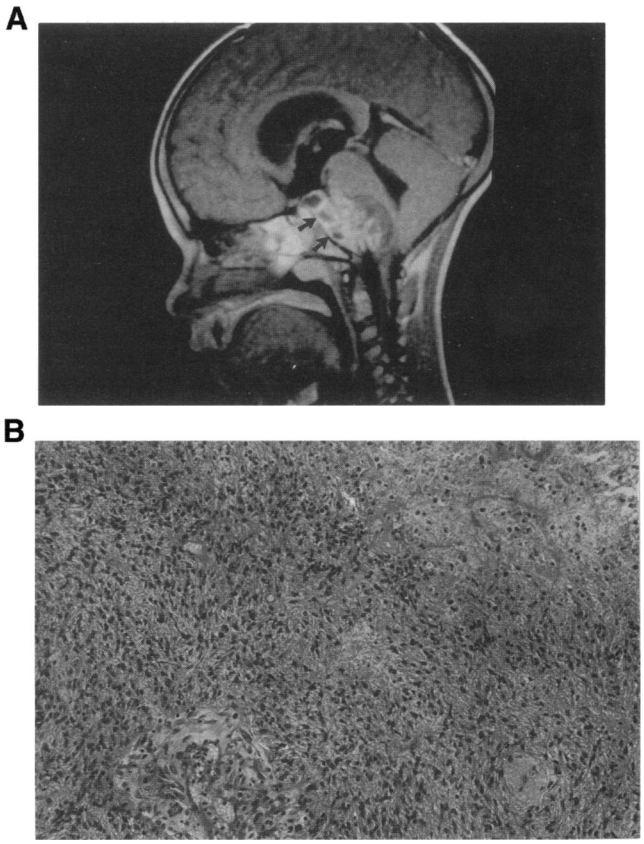

Figure 5 Radiological and histological features of tumor GB40. *A*, Magnetic resonance 0.5T. T1W sagittal section (after contrast administration), showing an enlargement of the brainstem due to a tumor with irregular, mainly ringlike enhancement and an exophytic expansion (indicated by *arrows*) in the prepontine cistern. *B*, Hematoxylin-eosin staining of tumor GB40 (200×). The picture shows a region with high cellular density, nuclear polymorphisms, circumscribed necroses, and vascular proliferation, all consistent with the diagnosis of GBM. Positive immunostaining for the glial fibrillary acidic protein was also present (not shown in this figure). Allelic deletions (see fig. 3) were present in DNA from paraffin-embedded areas of the tumor showing a high grade of malignancy, such as the one depicted here.

The identification of a pediatric GBM (GB40) with a limited loss of genetic material on 10q25 allowed to narrow down considerably the region candidate to contain one or more tumor-suppressor genes involved in GBM formation. The analysis of a first sample from paraffin-embedded material pointed to a 4-cM LOH region flanked by markers D10S221 and D10S209 (Gyapay et al. 1994). The further investigation of contiguous samples suggested the presence of a cellular population that harbors a larger deletion, extending to the more-telomeric marker D10S214.

The deletion mapping of another series of gliomas has recently led to the definition of a minimal overlapping deletion region between markers D10S587 and D10S216 (Rasheed et al. 1995). These two markers, uninformative in our patient, are contained in the 17-cM interval flanked by loci D10S221 and D10S214. Thus, two possibilities arise: (i) the larger region of deletion, present in a subpopulation of neoplastic cells of GB40, might contain two (or more) TSG located, respectively, in the D10S221-D10S209 and in the D10S587-D10S216 interval; or (ii) the small, centromeric LOH region of tumor GB40 does not contain a TSG, and its loss is only prodromic to that of the real target, which is in the D10S587-D10S216 region. Whatever the case, we trust that our results, and their combination with those of Rasheed et al. (1995), will pave the way to a new phase of work, making feasible the assembling of YAC contigs and the subsequent isolation of candidate TSG.

While expanding our LOH analysis to a larger series of gliomas, we tested by SSCP different fragments of the coding sequence of two putative TSG that map on 10q24-qter, *MGMT* (Gardner et al. 1991), and *MXI1* (Edelhoff et al. 1994). The product of the *MGMT* gene, highly conserved during the evolution, repairs the DNA damage caused by mutagenic alkylating agents reacting with DNA at the O^6-position of guanine so that the antineoplastic action of alkylating, chemotherapeutic drugs, such as carmustin (BCNU), can depend on the deficient function of this enzyme (Pegg 1990). A decreased *MGMT* activity was actually found in several gliomas (Mineura et al. 1994), but our SSCP analysis of 90% of the *MGMT* coding sequence in 10 GBMs did not reveal mutations (data not shown). Although these data should be regarded as preliminary, they are in agreement with the possibility that an altered expression of this gene affects only a minority of malignant gliomas (as also suggested by the observation that chemotherapy is only effective in a small percentage of these tumors) and is more likely caused by an abnormal methylation of its promoter (Costello et al. 1994).

The product of the other gene tested, *MXI1,* is negatively regulating proteins of the Myc family, thanks to its interaction with the Myc activator, Max (Zervos et al. 1993). Furthermore, *MXI1* mutations were found in prostate cancers with 10q24-q25 deletions (Eagle et al. 1995). Thus, a deficient function of the Mxi1 protein could contribute to glioma tumorigenesis by making available large amounts of Max either for c-Myc activation (in glioma cell lines the half-life of c-Myc is four-to-sixfold longer than normal [Shindo et al. 1993]) or for some other unkown interaction (in the nervous system the apparent concentration of Max is much greater than that of Myc proteins [Zervos et al. 1993]).

The SSCP analysis we performed involved 65% of *MXI1* coding sequence. The exon-intron structure of the remaining 35%, on the 5′ end, could not be determined by either inverse or regular PCR analysis, and the corresponding λ-clone(s) was not found by screening of a genomic library (Eagle et al. 1995). Also in this case, mutations were not detected by SSCP (data not shown).

Acknowledgments

We thank the surgeons and the personnel of the Department of Neurosurgery of the Istituto Nazionale Neurologico C. Besta, who made this study possible. We also thank Dr. M. G. Bruzzone for help with neuroradiological analysis and Dr. D. Riva for sharing informations on patient M.P. (GB40). G.F. was supported by a research grant from the Associazione Italiana per la Ricerca sul Cancro, and A.M.F.C. by Consiglio Nazionale delle Ricerche grant 94.02680.CT 04.

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