Loss of Heterozygosity on Chromosome 10 Is More Extensive in Primary (De Novo) Than in Secondary Glioblastomas

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SUMMARY: Glioblastomas develop de novo (primary glioblastomas) or through progression from low-grade or anaplastic astrocytoma (secondary glioblastomas). There is increasing evidence that these glioblastoma subtypes develop through different genetic pathways. Primary glioblastomas are characterized by *EGFR* and *MDM2* amplification/overexpression, *PTEN* mutations, and *p16* deletions, whereas secondary glioblastomas frequently contain *p53* mutations. Loss of heterozygosity (LOH) on chromosome 10 (LOH#10) is the most frequent genetic alteration in glioblastomas; the involvement of tumor suppressor genes, other than *PTEN*, has been suggested. We carried out deletion mappings on chromosome 10, using PCR-based microsatellite analysis. LOH#10 was detected at similar frequencies in primary (8/17; 47%) and secondary glioblastomas (7/13; 54%). The majority (88%) of primary glioblastomas with LOH#10 showed LOH at all informative markers, suggesting loss of the entire chromosome 10. In contrast, secondary glioblastomas with LOH#10 showed partial or complete loss of chromosome 10q but no loss of 10p. These results are in accordance with the view that LOH on 10q is a major factor in the evolution of glioblastoma multiforme as the common phenotypic end point of both genetic pathways, whereas LOH on 10p is largely restricted to the primary (de novo) glioblastoma. (*Lab Invest* 2000, 80:65–72).

Glioblastoma (World Health Organization [WHO] Grade IV) is the most frequent and malignant human brain tumor, occurring at a frequency of two to three new cases per 100,000 population and per year for most European and North American countries (Lantos et al, 1996). Despite progress in surgery and adjuvant therapy, patients with glioblastoma still have a dismal prognosis and they usually succumb to the disease within 1 year after diagnosis (Galanis et al, 1998; Leenstra et al, 1998). Glioblastomas may develop rapidly, with a short clinical history (primary or de novo glioblastoma), or more slowly through progression from low-grade (WHO Grade II) or anaplastic (WHO Grade III) astrocytoma (secondary glioblastoma) (Kleihues and Ohgaki, 1999). These glioblastoma subtypes, although largely indistinguishable histologically, constitute distinct disease entities that manifest in different age groups and develop through different genetic pathways. Primary glioblastomas occur in older patients and are characterized by *EGFR* amplification/overexpression and, less frequently, *MDM2* amplification, *PTEN* (*MMAC1*) mutations, and *p16* homozygous deletion, while secondary glioblastomas occur in younger patients and contain *p53* mutations as a genetic hallmark (Kleihues and Ohgaki, 1999).

Loss of heterozygosity (LOH) on chromosome 10 (LOH#10) is the most frequent genetic alteration in glioblastomas, reportedly occurring in up to 80% of cases (Albarosa et al, 1996; Fults et al, 1998; Ichimura et al, 1998; Karlbom et al, 1993; Kon et al, 1998; Maier et al, 1997; Rasheed et al, 1995; Sonoda et al, 1996; Voesten et al, 1997). LOH#10 is less frequent (~40%) in anaplastic astrocytomas (Albarosa et al, 1996; Bijleveld et al, 1997; Ichimura et al, 1998; Karlbom et al, 1993; Kon et al, 1998; Maier et al, 1997; Rasheed et al, 1995; Sonoda et al, 1996; Voesten et al, 1997), and rarely occurs in low-grade astrocytomas (Ichimura et al, 1998; Karlbom et al, 1993; Kon et al, 1998; Maier et al, 1997; Rasheed et al, 1995; Sonoda et al, 1996; Voesten et al, 1997). The majority of glioblastomas appear to have lost an entire copy of chromosome 10 (Albarosa, 1996; Fults et al, 1998; Ichimura et al, 1998; Kon et al, 1998; Maier et al, 1997; Rasheed et al, 1995; Voesten et al, 1997). In glioblastomas with partial LOH#10, at least three common deletions have been identified: (a) 10p14-pter (Ichimura et al, 1998; Karlbom et al, 1993; Kimmelman et al, 1996; Kon et al, 1998; Sonoda et al, 1996; Voesten et al, 1997); (b) 10q23–24 (Albarosa et al, 1996; Fults et al, 1998; Ichimura et al, 1998; Karlbom et al, 1993; Maier et al, 1997; Rasheed et al, 1995; Sonoda et al, 1996); and (c) 10q25-qter (Albarosa et al, 1996; Fults et al, 1998; Ichimura et al, 1998; Karlbom et al, 1993; Maier et al, 1997; Rasheed et al, 1995; Sonoda et al, 1996), suggesting the presence of multiple tumor suppressor

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genes. LOH#10 has been detected in 60% to 100% of glioblastomas with *EGFR* amplification (Lang et al, 1994; Leenstra et al, 1998; von Deimling et al, 1992) and in 40% to 80% of glioblastomas with a *p53* mutation (Lang et al, 1994; Leenstra et al, 1998), suggesting that LOH#10 is involved in the development of both primary and secondary glioblastomas.

The objective of the present study was to clarify whether the frequency and allelic patterns of LOH#10 differ between primary and secondary glioblastomas. We carried out deletion mapping on chromosome 10, using PCR-based microsatellite analysis in 17 primary glioblastomas (using normal DNA as a reference) and 13 secondary glioblastomas which progressed from low-grade astrocytomas (using normal or low-grade astrocytoma DNA as a reference). The presence and pattern of LOH#10 were correlated with other genetic alterations, including *EGFR* amplification and *p53* and *PTEN* mutations.

Results

LOH on Chromosome 10

Using 28 microsatellite markers, we examined a total of 840 polymorphic loci on chromosome 10 and obtained 621 (74%) informative results. Eight (47%) of 17 primary glioblastomas showed LOH#10. Of these, seven (88%) showed deletions at all informative loci;

this was interpreted as the loss of an entire copy of chromosome 10. Case 294 was exceptional, with deletions at all informative loci on 10p but with none on 10q (Figs. 1 and 2).

In secondary glioblastomas, LOH#10 was demonstrated in 7 of 13 (54%) cases, ie, at a frequency similar to that in primary glioblastomas ($p = 1.0$). However, in all cases, deletions were partial and typically located on 10q. One glioblastoma (case 25) showed LOH at all informative loci on 10q. In the remaining six cases, chromosomal deletions on 10q were partial. One tumor (case 70) showed an additional small deletion on 10p at D10S199. The most common deletion in all seven cases was on 10q25-qter distal to D10S1683, covering the *DMBT1* (Mollenhauer et al, 1997) and *FGFR2* (Moschonas et al, 1996) loci (Fig. 1).

For three secondary glioblastomas in which low-grade astrocytoma DNA was used as a reference, DNA from adjacent normal brain tissue (Cases 57 and 68) and peripheral blood leukocytes (Case 72) was also subjected to analysis. Allelic patterns of normal tissues and low-grade astrocytomas were concordant at all informative loci. In another case (Case 26), one of the two alleles showed significant decrease ($>50\%$) in signal intensity when compared with the remaining allele at markers D10S587, 1723, and 1700 (Fig. 1, marked as asterisk), which may suggest that LOH had already occurred in low-grade astrocytoma.

Figure 1.

Allelic patterns of chromosome 10 in 17 primary and 13 secondary glioblastomas. Case numbers are indicated at the top of each column. The overall frequency of loss of heterozygosity (LOH) on chromosome 10 is similar in both glioblastoma subtypes but the extent of chromosomal loss differs. Primary glioblastomas often show complete loss (10p, q) while in secondary glioblastomas, LOH is typically restricted to the long arm (10q).

Table 1. Loss of Heterozygosity on Chromosome 10 and Other Genetic Alterations in Primary and Secondary Glioblastomas

Patient No.	Age (year)/gender	Tumor location	Clinical history	LOH	#10	p53	misconducting mutation	PTEV missense mutation	EGFR/CF ratio
Primary Glioblastomas									
233	59/M	T, BG	2 months	-	-	-	-	-	0.90
234	43/M	F	1 month	+	+	-	-	61, CAT->CCT, His->Pro	0.72
256	56/F	T	10 days	-	-	253, ACC->GCC, Thr->Ala	-	-	0.93
257	50/M	F, BG	2 months	-	-	-	-	-	1.47
258	65/F	F	1 week	-	-	-	-	-	0.69
287	47/F	P	1 month	+	+	-	-	-	0.87
288	69/M	P	2 months	+	+	-	-	-	1.18
289	58/F	T	3 months	+	+	-	-	-	8.42
292	62/M	P	1 month	-	-	-	-	-	1.50
294	36/M	P	5 weeks	+	-	-	-	-	1.19
296	63/F	VE	4 months	-	-	-	-	-	0.98
300	71/F	TO	1 month	+	+	-	-	-	6.08
301	68/F	TP	1 week	+	+	-	-	-	4.54
302	64/M	TP	3 months	+	+	-	-	122, ATT->AGT, Ile->Ser	5.04
303	67/M	PO	2 weeks	-	-	-	-	-	2.07
314	40/M	F	3 weeks	-	-	126, TAC->TAA, Tyr->stop	-	-	14.1
344	47/M	P	2 weeks	-	-	-	-	-	0.92
Secondary Glioblastomas									
11	33/F	VE	Grade II	-	-	163, TAC->TGC, Tyr->Cys	-	-	1.96
25	44/F	T	Grade II	-	+	275, TGT->TTT, Cys->Phe	-	-	1.62
26	32/F	F	Grade II	-	+	163, TAC->TGC, Tyr->Cys	-	-	1.08
35	53/M	T	Grade II	-	-	273, CGT->TGT, Arg->Cys	-	-	1.17
57	26/M	F	Grade II	-	-	175, CGC->CAC, Arg->His	-	-	1.11
58	33/M	FT	Grade II	-	-	141, TGC->TAC, Cys->Tyr	-	-	1.05
59	40/M	FT	Grade II	-	-	175, CGC->CAC, Arg->His	-	-	1.36
60	31/F	T	Grade II	-	+	-	-	-	1.20
64	34/M	F	Grade II	-	+	300->89 bp del	-	-	1.93
68	52/F	TPO	Grade II	-	+	278, CCT->ACT, Pro->Thr	-	-	1.11
70	52/M	PO	Grade II	+	+	-	-	-	1.22
72	28/F	F	Grade II	-	-	301->2 bp del	-	-	2.02
295	47/M	FT	Grade II	-	+	245, GGC->AGC, Gly->Ser	-	-	0.96

F, frontal; T, temporal; P, parietal; O, occipital; VE, paraventricular; BG, basal ganglia.

Results of p53 mutations and PTEV mutations in secondary glioblastomas, except for cases 72 and 295, have been previously reported (Watanabe et al, 1998; Tohma et al, 1996). An EGFR/CF ratio of >2.5 was regarded as evidence of EGFR amplification (see "Materials and Methods").

Figure 2.

Representative results of loss of heterozygosity (LOH) on chromosome 10 in a primary (case 300) and a secondary (case 70) glioblastoma. Microsatellite markers are indicated on the left side of each panel. LOH is indicated by arrows. *N*, normal (blood); *II*, low-grade astrocytoma (World Health Organization Grade II); *IV*, glioblastoma (Grade IV).

Correlation of LOH#10 with Other Genetic Alterations

PCR-SSCP, followed by DNA sequencing, demonstrated *p53* missense mutations in 2 (12%) of 17 primary glioblastomas and in 11 (85%) of 13 secondary glioblastomas ($p = 0.0001$; Table 1). *PTEN* mutations were found in 2 (12%) of 17 primary glioblastomas but none in secondary glioblastomas (Table 1). *EGFR* amplification was detected by differential PCR in 5 (29%) of 17 primary glioblastomas but in none of the secondary glioblastomas (Table 1). *EGFR* amplification tended to be associated with complete LOH#10: 4 (80%) of 5 primary glioblastomas with

EGFR amplification showed an entire loss of chromosome 10, whereas only 3 (25%) of 12 glioblastomas without *EGFR* amplification showed complete LOH#10. However, this difference was not statistically significant ($p = 0.10$). There was no significant correlation between the presence of LOH#10 and other genetic alterations.

Discussion

Entire or partial loss of chromosome 10 is a common genetic alteration in a variety of human cancers, including glioblastomas (Albarosa et al, 1996; Fufts et al, 1998; Ichimura et al, 1998; Karlbom et al, 1993; Kon et al, 1998; Maier et al, 1997; Rasheed et al, 1995; Sonoda et al, 1996; Voesten et al, 1997), malignant meningiomas (Rempel et al, 1993; Simon et al, 1995), endometrial carcinomas (Peiffer et al, 1995), prostate carcinomas (Gray et al, 1995), renal carcinomas (Morita et al, 1991), small cell lung carcinomas (Ried et al, 1994), non-Hodgkin's lymphomas (Speaks et al, 1992), and melanomas (Herbst et al, 1994; Isshiki et al, 1993). In glioblastomas, there appear to be at least three putative tumor suppressor loci, ie, 10p14-pter, 10q23-24, and 10q25-qter (Albarosa et al, 1996; Fufts et al, 1998; Ichimura et al, 1998; Karlbom et al, 1993; Kimmelman et al, 1996; Kon et al, 1998; Maier et al, 1997; Rasheed et al, 1995; Sonoda et al, 1996; Voesten et al, 1997). However, little is known whether the frequency and extent of LOH on chromosome 10 are different between primary and secondary glioblastomas. Ichimura et al (1998) carried out extensive LOH study in 198 astrocytic gliomas and showed that most glioblastomas lost one entire chromosome, while astrocytomas preferentially lost only 10p. However, the astrocytomas and glioblastomas that were analyzed were derived from different patients. Using comparative genome hybridization (CGH) analysis, Weber et al (1996) reported that 4 of 10 anaplastic astrocytomas and glioblastomas that had progressed from low-grade astrocytoma showed a reduced number of chromosome segments on 10q. Recently, we reported that LOH on 10q25-qter is often associated with abrupt morphologic transition from low-grade or anaplastic astrocytoma to a highly malignant, undifferentiated glioblastoma phenotype lacking glial fibrillary acidic protein (GFAP) expression (Fujisawa et al, 1999).

The present study is the first to demonstrate unequivocally that LOH#10 occurs at a similar frequency in primary and secondary glioblastomas but that the patterns of allelic loss are different. Entire loss of chromosome 10 was typical for primary glioblastomas, whereas partial LOH on 10q was characteristic for secondary glioblastomas. This pattern is consistent with the observation in this and previous studies (Lang et al, 1994; Leenstra et al, 1998; von Deimling et al, 1992) that glioblastomas with *EGFR* amplification, a genetic hallmark of primary glioblastomas, typically show complete loss of chromosome 10.

Several transformation-associated genes have been identified on 10q, including *PTEN* at 10q23.3 (Li et al,

1997; Steck et al, 1997), *LG1* at 10q24 (Chernova et al, 1998), *BUB3* at 10q24-q26 (Cahill et al, 1999), *MXI1* at 10q25.1 (Eagle et al, 1995), *hours-neu* at 10q25.1 (Nakamura et al, 1998), *abLIM* or *LIMAB1* at 10q25.1 (Kim et al, 1997; Roof et al, 1997) and *DMBT1* at 10q26.1 (Mollenhauer et al, 1997). The *PTEN* gene, which encodes a protein with homology to the catalytic domain of tyrosine phosphatase and to the cytoskeletal proteins tensin and auxilin (Li et al, 1997; Steck et al, 1997), is mutated frequently in glioblastomas (Boström et al, 1998; Chiariello et al, 1998; Duerr et al, 1998; Fults et al, 1998; Maier et al, 1998; Tohma et al, 1998; Wang et al, 1997), prostate carcinomas (Cairns et al, 1997; Suzuki et al, 1998), and endometrial carcinomas (Risinger et al, 1997; Tashiro et al, 1997). We previously reported that mutations in the *PTEN* gene, which is located on 10q23, are common (32%) in primary but rare (4%) in secondary glioblastomas (Tohma et al, 1998). *DMBT1* is deleted frequently in glioblastomas (Mollenhauer et al, 1997; Somerville et al, 1998) but *DMBT1* mutations have not yet been identified. Although growth suppression has been observed after microcell-mediated transfer of chromosome fragments from 10p14-15 into T98G glioblastoma cells (Kon et al, 1998), the putative tumor suppressor gene at this locus has not yet been identified.

The present study suggests that the involvement of different tumor suppressor gene(s) on chromosome 10 is between primary and secondary glioblastoma. Tumor suppressor gene(s) on chromosome 10p may play an important role in the evolution of primary, but not secondary, glioblastomas. Alternatively, the more extensive deletion on chromosome 10 in primary, rather than in secondary glioblastomas may result from greater chromosomal instability in primary glioblastomas. However, little is known about the mechanisms of chromosomal deletion, except for the general assumption that it is probably attributed to chromosomal instability caused by the disruption of mitotic checkpoints (Lengauer et al, 1998) or premature mitosis involving damaged DNA (Paulovich et al, 1997).

In conclusion, this study shows that LOH on chromosome 10 is more extensive in primary glioblastomas, rather than in secondary glioblastomas. Primary glioblastomas are characterized by an entire loss of chromosome 10, whereas in secondary glioblastomas, LOH is restricted to chromosome 10q, suggesting that different tumor suppressor genes on this chromosome are involved in the development of these glioblastoma subtypes.

Materials and Methods

Tumor and Blood Samples

Seventeen primary and 13 secondary glioblastomas were obtained from the patients operated on in the Department of Neurosurgery, University Hospital, Zürich, Switzerland. All patients with primary glioblastoma had a clinical history of <3 months and did not show any histologic or radiologic evidence of a pre-

cursor lesion, whereas all patients with secondary glioblastoma had surgeries for low-grade astrocytoma >6 months before the second operation for glioblastoma. Seventeen patients were men and 13 were women (Table 1). Tumors were fixed in formalin, embedded in paraffin for routine histopathologic analysis, and were classified according to the WHO grading system (Kleihues et al, 1993).

For all primary glioblastomas and two secondary glioblastomas (cases 72 and 295), aliquots of tumors were frozen immediately in liquid nitrogen and stored at -80° C until DNA extraction. Genomic DNA was extracted using TRIZOL Reagent (GIBCO BRL, Cergy Parroise, France) according to the manufacturer's instructions. Matched peripheral blood samples of these cases were obtained and DNA was extracted using QIAamp DNA Blood Kit (QIAGEN, Courtaboeuf, France).

For the cases in which frozen tissues were not available (low-grade astrocytoma and secondary glioblastomas, except for cases 72 and 295), DNA was extracted from paraffin sections (Brüstle et al, 1992; Fujisawa et al, 1999). In two secondary glioblastomas (cases 57 and 68), DNA was also extracted from the peritumoral brain tissue in paraffin sections. In case 72, DNA was extracted from paraffin-embedded, low-grade astrocytoma, frozen glioblastoma, and blood samples.

Analysis of LOH on Chromosome 10

LOH on chromosome 10 was studied by PCR-based microsatellite analysis (Fujisawa et al, 1999). Twenty-eight microsatellite loci were selected to cover chromosome 10, including reported common deletions on 10p14-pter (Ichimura et al, 1998; Karlbom et al, 1993; Kimmelman et al, 1996; Kon et al, 1998; Sonoda et al, 1996; Voesten et al, 1997), 10q23-24 (Albarosa et al, 1996; Fults et al, 1998; Ichimura et al, 1998; Karlbom et al, 1993; Maier et al, 1997; Rasheed et al, 1995; Sonoda et al, 1996), and 10q25-qter (Albarosa et al, 1996; Fults et al, 1998; Ichimura et al, 1998; Karlbom et al, 1993; Maier et al, 1997; Rasheed et al, 1995; Sonoda et al, 1996). All microsatellite markers were purchased from Research Genetics (Huntsville, AL); all were dinucleotide repeats, except for D10S1435 (tetranucleotide repeats). The size range and heterozygosity of each marker were obtained from the Genome Database (<http://gdbwww.gdb.org/>). The genetic map and distances of chromosome 10 were obtained from the enhanced location database at ftp://cedar.genetics.soton.ac.uk/pub/chrom_10/gmap (Collins et al, 1996).

For all primary glioblastomas and two secondary glioblastomas, DNA from peripheral blood samples from the same patient was used as a reference, whereas for secondary glioblastomas except for two cases, DNA samples from low-grade astrocytoma from the respective patients were used as a reference. After PCR amplification, the allelic pattern for each marker was determined by comparing the electrophoretic pattern of DNA from glioblastoma with that of

reference DNA, ie, blood DNA or DNA samples extracted from low-grade astrocytoma. PCR was performed according to the instructions of Research Genetics with minor modifications. Briefly, 10 ng of DNA (1 μ l) samples of fresh-frozen tumors and blood samples or 1 μ l of DNA solution from paraffin sections were subjected to PCR with 2 μ l of 5 $\times$ PCR buffer, 200 μ M of each dNTP, 6 pmol of each sense and antisense primer, 1 μ Ci of [α - 32 P]-dCTP (ICN Biomedicals, specific activity 3000 Ci/mmol), 0.225 units of *Taq* polymerase (Sigma Chemical, St. Louis, Missouri), and 1.5 mM of MgCl₂ in a final volume of 10 μ l, with an initial denaturation of 95° C for 2 minutes, followed by 30 cycles (DNA from frozen tumors and blood sample) or 35 cycles (DNA from paraffin sections) of denaturation at 94° C for 45 seconds, annealing at 57° C for 45 seconds and polymerization at 72° C for 1 minute, and a final extension at 72° C for 7 minutes, using a Genius DNA Thermal Cycler (Techne, Cambridge, United Kingdom). PCR products were mixed with an equivalent volume of the denaturing solution containing 95% formamide, 20 mM EDTA, 0.05% xylene cyanol, and 0.05% bromophenol blue. Immediately after heating at 95° C for 5 minutes, 4 μ l of the mixture was loaded onto a 7% polyacrylamide/7 M of urea sequencing gel. Gels were run at 70 watts (W) for 3 to 6 hours, dried at 80° C, and autoradiographed for 24 to 96 hours. Gels were also exposed to Storage Phosphor screens (Molecular Dynamics, Sunnyvale, California). LOH was scored when signal intensity was reduced in glioblastoma by >50% of reference DNA, which was measured by densitometry (Bio-Rad model GS-670) or by a Phosphorimager (Molecular Dynamics).

Other Genetic Alterations

The genetic alterations in secondary glioblastomas, except for two cases, were reported previously (Tohma et al, 1998; Watanabe et al, 1996) (Table 1). For all primary glioblastomas and two cases of secondary glioblastomas (cases 72 and 295), the following genetic analyses were carried out.

PCR-SSCP Analysis and Direct DNA Sequencing for *p53* and *PTEN* Mutations. Prescreening for mutations by PCR-SSCP analysis was carried out, as previously described in exons 5–8 of the *p53* gene (Watanabe et al, 1996) and in exons 1–9 of the *PTEN* gene (Reis et al, 1999; Tohma et al, 1998; Watanabe et al, 1998). Samples which showed mobility shifts in the SSCP gels were further analyzed by direct DNA sequencing (Reis et al, 1999; Tohma et al, 1998; Watanabe et al, 1996).

Differential PCR for *EGFR* Amplification. To detect *EGFR* amplification, differential PCR was carried out, using the cystic fibrosis (*CF*) gene as a reference (Hunter et al, 1995), with some modifications (Tohma et al, 1998). The mean *EGFR/CF* ratio, using normal blood DNA sampling, was 1.11 with a standard variation of 0.11. An *EGFR/CF* ratio >2.6 was regarded as evidence of *EGFR* amplification (Rollbrocker et al, 1996). One primary glioblastoma, which showed

EGFR amplification in our previous study (Tohma et al, 1998), was used as a positive control.

Statistical Analyses

Fisher's exact test was carried out to analyze the contingency table for frequency of *EGFR* amplification, *p53* and *PTEN* mutations, and LOH#10 between primary and secondary glioblastomas.

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