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Genetic Aberrations Defined by Comparative Genomic Hybridization Distinguish Long-Term from Typical Survivors of Glioblastoma[1]

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ABSTRACT

Glioblastoma (GBM) remains a highly lethal neoplasm, refractory to current therapies. The molecular genetic aberrations most closely related to clinical aggressiveness in GBM have been difficult to identify, perhaps due in part to the short survival range observed in cohorts of GBM patients. To address this, we characterized 39 tumors from rare patients (2–5% of all GBM cases) who experienced long-term survival (>3 years) using comparative genomic hybridization as a genome-wide screen. We then compared the frequency and type of aberrations with those in tumors from 24 typical or short-term survivors [STSs (<1.5 years)]. Losses of 9p and 10 and simple gains of chromosome 7 showed at least trends toward increased frequency in the STS group. Additional aberrations, including loss of 6q and gains of 19q and 20q, were significantly more frequent in the STS group. The presence of 19q loss was exclusive to the long-term survivor (LTS) group. Multivariate analyses indicated that 6q loss, 10q loss, and 19q gain were associated with short-term survival (all $P < 0.01$). The combination of any two of these three aberrations was seen in 16 of 24 STSs but only 1 of 39 LTSs. This comparison of rare LTSs with STSs (typical GBM survivors) identified 6q loss, 10q loss, and 19q gain, particularly when two or more of these were present, as most closely associated with aggressive clinical behavior in GBM. Loss of 19q may be a marker of long-term survival.

INTRODUCTION

GBM[3] is the most common primary brain tumor in adults. Despite current therapeutic modalities, the median survival for patients with newly diagnosed GBM has remained at approximately 12 months (1). At 2 years after initial surgical procedure, >90% of patients are deceased (2). Prognostic factors in these tumors include patient age and KPS (1). The search for robust molecular prognostic factors has been extensive, but robust markers of prognosis have not been described for GBM (3–6). The difficulty in identifying genetic aberrations associated with aggressive clinical behavior of these neoplasms may be due in part to the narrow range of survival experienced by these patients. However, identification of those aberrations most closely associated with aggressive clinical behavior remains an important goal if improved treatments are to be developed.

Aberrations that occur with high frequency include amplification of chromosome 7p (7) and losses of 9p and 10 (8, 9). However, other aberrations in these genetically unstable tumors have been noted, and a recent study identified 26 different CNAs that occurred in at least 20% of the cases (9). These data raise the challenge of identifying which aberrations in these tumors are related to our inability to adequately treat them. If novel therapies targeted against specific molecular aberrations are to be developed as adjuncts to chemotherapy and RT, aberrations most closely related to resistance need to be identified. LTSs (>3 years) represent only 2–5% of cases (10–13), and therefore little is known about the molecular genetic characteristics that define this potentially interesting group of tumors. Selecting and comparing these tumors with more typical STSs may therefore offer a means to gain insights into genetic changes important for the aggressive clinical behavior of GBM.

Prior clinical studies indicate that LTSs are younger than the median age, with high KPSs (a measure of patient function) at initial diagnosis (10, 12, 13). In addition, LTSs have often received aggressive surgical resections and multimodal therapy. Finally, a patient with GBM who lives at least 3 years has an increased likelihood of prolonged survival without tumor recurrence (14, 15), suggesting that these patients are fundamentally different from most GBM patients. Our prior studies suggest that LTS tumors, as a group, show differences in p53, mdm2, and proliferation rate compared with those of STSs (11–13, 16). Statistical differences were found, but there was significant overlap using these candidate markers. In this report, we use a genome-wide screen (CGH) on 39 LTS tumors and 24 STS tumors to identify additional differences to find patterns of genetic lesions that may better account for the disparate survival of LTSs *versus* STSs.

MATERIALS AND METHODS

Case Selection. LTSs for this study were defined as patients who lived at least 3 years from the time of an initial surgical diagnosis of GBM. STSs were patients with a survival of <1.5 years from initial diagnosis. Because the LTSs as a group tended to be young, the STSs had an age constraint of <50 years, so that the two groups were comparable in age. All patients (LTSs and STSs) were diagnosed at first surgery as having GBM using WHO criteria. Cases were re-reviewed by a second pathologist (K. D. A.) to confirm the diagnosis. Several cases from the LTS group were reclassified as anaplastic oligodendroglioma on review and excluded. The finding of anaplastic oligodendroglioma in patients with a diagnosis of GBM who have survived long term has been emphasized previously (17). An additional case was reclassified as juvenile pilocytic astrocytoma and excluded. After a review of medical records, two cases who showed radiographic evidence of low-grade precursors before the first surgery were excluded. The LTS tissue samples were obtained from three institutions: (*a*) the UCSF; (*b*) the University of Calgary Tom Baker Cancer Center; and (*c*) the Mayo Clinic. All STSs were obtained from UCSF. Analysis was performed only on tissue obtained before therapy was initiated. All samples were tested according to an institutional review board-approved protocol at the UCSF.

CGH. DNA from frozen or formalin-fixed paraffin-embedded tissue was used for CGH. Tumor tissue was hand-dissected from normal tissue in cases where the block did not have >90% tumor, as determined by visual inspection of a H&E-stained slide. DNA extraction from paraffin blocks or slides was carried out using proteinase K digestion and phenol/chloroform extraction as described previously (4). DNA concentration was measured fluorometrically.

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[3] The abbreviations used are: GBM, glioblastoma; CGH, comparative genomic hybridization; STS, short-term survivor; LTS, long-term survivor; KPS, Karnofsky performance score; UCSF, University of California at San Francisco; DOP-PCR, degenerative oligonucleotide-primed PCR; CNA, copy number aberration; EGFR, epidermal growth factor receptor; RT, radiotherapy.

If there were small amounts of DNA (<200 ng) after extraction, DOP-PCR amplification of the material was done as described previously (18). DOP-PCR was performed on a total of 10 cases (4 from the STS group and 6 from the LTS group). We have previously shown that DOP-PCR CGH results in profiles equivalent to those of regular CGH (18). Otherwise, DNA was directly labeled using nick translation. Reference DNA was labeled with FITC. Tumor DNA was labeled with digoxigenin-11-dUTP (Roche).

CGH was performed according to the procedure described by Mohapatra *et al.* (9). Briefly, the labeled DNAs were hybridized to target metaphase slides (Vysis, Downers Grove, IL). After washing, the metaphases were incubated with rhodamine-conjugated antidigoxigenin antibody, washed, and counterstained with 4,6-diamino-2-phenylinodole in antifade solution. Red, green, and blue images were acquired with a quantitative image processing system, and the ratios of fluorescence intensity along the chromosomes were quantitated. A relative gain was scored when the mean test:reference ratio was >1.2, and relative loss was scored when the mean green:red ratio was <0.8. CNAs at or near centromeres were not scored. Amplifications were scored only when a bright intrachromosomal signal was observed with a red:green reference ratio of >3.

Statistical Analyses. Survival time was established as the interval from initial surgery to patient death or last official contact. A Mann-Whitney test was used to compare the median ages and KPS between the two cohorts. Fisher's exact test was used to compare the rate of individual CNAs between the two groups and to test for associations between aberrations. All tests were two-sided. A CNA had to be seen in at least 20% of the cases in either cohort to be considered for comparison between the LTS and STS groups. Stepwise multivariate logistic regression was used to determine the CNAs that best distinguished between the LTS and STS groups. Because gender was statistically significant on univariate, and age is generally considered a prognostic variable, they were included in all models. Because one aberration (loss of 19q) occurred only among the LTSs, it was not possible to include it in a multivariate logistic regression model where the test is based on maximum likelihood estimation. To verify that other variables were not statistically significant because of their association with this CNA, the variables that remained in the model using all cases were also tested considering only those for whom 19q was not lost. All three variables remained statistically significant, and the estimated odds ratios were similar. Cox proportional hazards regression was used to identify prognostic factors within the LTS group. Patients who were alive at last known contact were considered censored for the Cox analysis. Given the exploratory nature of this study, we report nominal *P*s without adjustment for multiple comparisons. This was done to decrease the chance of missing potentially important associations due to the reduction of power that accompanies standard methods of adjustment.

RESULTS

Clinical Characteristics. The median age of the 39 cases of the LTS group was 38 years (range, 0.25–75 years), which was similar to that of the 24 cases comprising the STS group (median age, 43 years; range, 9–50 years; $P = 0.5$, Mann-Whitney test). The median KPS for each group was 90. The median survival of the LTS group was 58 months, with 14 patients alive at the time of the last follow-up exam. The 14 LTSs alive at last contact had a median follow-up of 72.5 months (range, 38–179 months). The median survival of the STS group was 10 months (range, 1–17 months), and all STS patients were (according to the design of the study) deceased.

Histopathology. All cases with a diagnosis of GBM were rereviewed to ensure that the histopathological features met WHO criteria for GBM. As noted in "Materials and Methods," several cases from the LTS group were excluded from molecular analysis because they exhibited features of other gliomas. No differences in histopathological features, including the presence of necrosis or microvascular proliferation, could be discerned, nor could differences in GBM subtype (for example, giant cell, small cell, gemistocytic subtypes; data not shown).

Genetic Aberrations in GBMs from STSs and LTSs. A composite of CGH results for all STSs is shown by ideogram in Fig. 1*A*. The median number of aberrations was 6.5 (range, 2–14). Similar to previous studies on these tumors, the most frequent aberrations included simple gains on chromosome 7p and 7q, which occurred in 71% and 67% of cases, respectively, and losses of chromosome 10p (63%) and 10q (83%). Amplification of 7p was present in 25% (6 of 24 cases). Loss on chromosome 9p occurred in 58% of STSs. Additional gains occurring at relatively high frequency included 19p (50%), 19q (38%), 20p (25%), and 20q (33%). Losses included 6q (42%), 13q (21%), and 14q (29%).

The LTS cases are shown in Fig. 1*B*. The median number of aberrations was 4 (range, 0–9), which was significantly less than that in the STS group ($P < 0.01$, Mann-Whitney test). The most frequent aberrations were gains of chromosome 7 and loss of chromosome 10. Specifically, 7p gain and 7q gain were each seen in 18 of 39 (46%) LTSs. Chromosome 7p amplification was present in 6 of 39 (15%) cases. Chromosome 10q loss was seen in 16 of 39 (41%) LTSs, and 10p loss was seen in 13 of 39 (33%) cases. Loss of 19q was seen exclusively in LTSs and was present in 28% of cases (11 of 39 cases), frequently in association with 1p loss (6 of 11 cases).

Comparison of Aberration Frequency in STSs *versus* LTSs. Aberrations previously established as common in the majority of GBM, including gain of 7 and losses of 9p, 10p, and 10q, showed, at minimum, statistical trends ($P < 0.1$, Fisher's exact test) toward increased frequency in the STS group (Table 1). The exception to this was 7p amplification, which did not show a trend toward increased frequency ($P = 0.50$). Additional differences in CNA frequency between the two groups included 6q loss, 19p gain, 19q gain, and 20q gain, all of which were significantly more common in the STS group. Loss of 19q was observed only in the LTS group and was further divided by 1p status because combined 1p and 19q loss has been implicated as a marker of improved outcome in oligodendrogliomas (19, 20).

Multivariate Analyses. Aberrations that were significantly associated with survival on univariate analysis were included in logistic regression models. After stepwise elimination of variables with $P > 0.05$, loss of 6q and 10q and gain of 19q remained highly significant in multivariate analysis (Table 2). Loss of 19q could not be included in the initial logistic regression analyses because no occurrences of this aberration were found in STSs. With respect to the three aberrations mostly closely associated with short-term survival identified by logistic regression (−6q, −10q, and +19q), at least one of these three aberrations occurred in 22 of 24 STSs, as compared with 22 of 39 LTSs ($P < 0.01$ Fisher's exact test). A more striking distinction was observed by evaluating these lesions in combination: the combination of any two of these three aberrations occurred in 16 of 24 STSs but in only 1 of 39 LTSs ($P < 0.01$, Fisher's exact test).

DISCUSSION

LTSs of GBM are of investigational interest for several reasons. First, whereas >95% of GBM patients are no longer alive 3 years after initial diagnosis, those who do survive 3 years have a relatively good chance for extended survival. For example, among our 39 cases with >3-year survival, 18 patients (46%) lived 5 years or longer, and 9 patients (23%) lived 7 years or longer. Second, the narrow range of survival inherent in studies of unselected GBM populations makes it difficult to identify markers associated with outcome. Third, it is not clear which of the aberrations among the numerous changes in GBMs are most closely associated with poor outcome. This study is the first to screen a large cohort of LTSs for comparison with STSs to address these issues. The group we refer to as STSs in fact represent survival times typical of GBM. The comparison with the LTS group can be

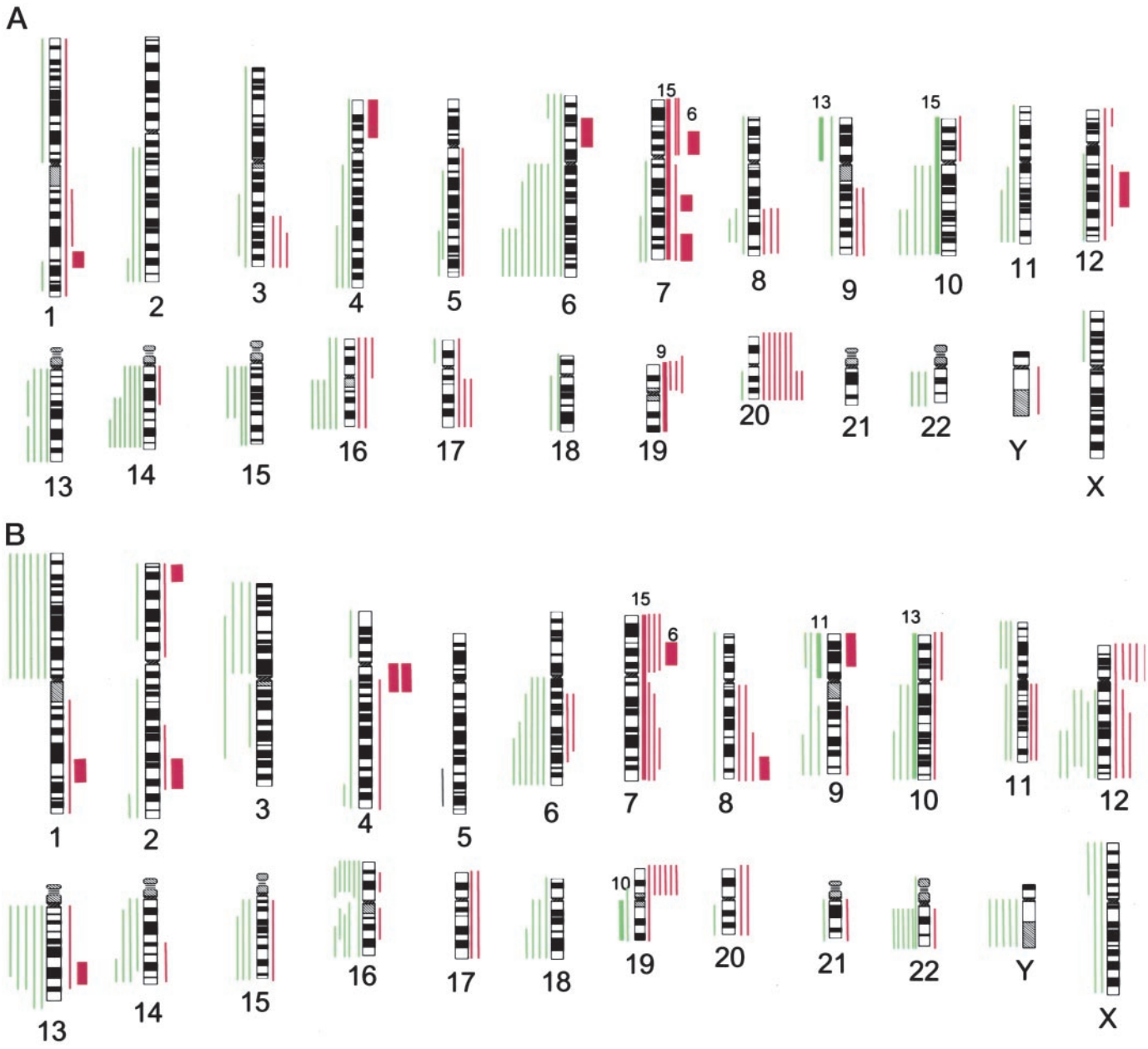

Fig. 1. Summary of CNAs in STSs and LTSs. *Lines* to the *left* of the chromosome ideogram show losses, and *lines* to the *right* of the chromosome ideogram show gains. Each *thin line* represents one CNA found in one tumor. Where a *thicker line* exists, the *number above* it represents the number of tumors showing that aberration. Amplifications are represented as *very thick lines* to the *right* of the ideogram. *A*, STSs; *B*, LTSs.

viewed as a means to identify those genetic changes most closely associated with the rapid demise typical of GBM patients.

In this report, we used a genome-wide screen to identify differences that may more completely account for the disparate survival. To accomplish this, we used CGH to metaphase spreads, (as compared with the recently described CGH to arrays; Ref. 21) because our samples were all archival paraffin-embedded tissue (some as many as 23 years old). Our results provide a starting point for further characterization of genetic regions, which may be related to clinical aggressiveness in GBM. Differences identified between the STSs and LTSs in this study can be divided into those that are emphasized in the literature as critical in the molecular pathogenesis of GBM (7 gain, 9p loss, and 10 loss) and those that have been reported previously but are less well characterized (loss of 6q, gain of 19, and gain of 20).

Gain of 7 and Loss of 9p and 10. Our STS group showed frequencies of aberrations similar to those previously identified by others (22–26), including simple gains on 7 and losses on 9p, 10p, and 10q. These are the most common aberrations found by CGH, but they have not been shown to be prognostic in GBM in cohort studies (5, 9). In the current study, each of these CNAs showed at least trends toward increased frequency in the STS group. In this study, 7p gain was seen in 71% of STSs and 46% of LTSs ($P = 0.07$), and 7q gain was seen in 67% of STSs and 46% of LTSs ($P = 0.13$), and in both cohorts gains of chromosome 7 were typically gains of the whole chromosome. This finding supports the idea of multiple oncogenes on chromosome 7 being important for the clinical behavior of GBM.

Surprisingly, amplification of a region of 7p, which occurs in 30–40% of GBM cases and typically includes EGFR (7, 27), was not preferentially distributed in the STS cases (15% *versus* 25%, $P = 0.5$). Because EGFR amplification is felt to be specific for GBM (grade 4 astrocytoma) compared with anaplastic astrocytoma (grade 3), this supports the notion that the GBM histology of the LTS group was

Table 1 *Comparisons of copy number aberrations in LTSs and STSs*

All aberrations that showed a frequency of >20% in at least one of the groups are shown. The number and frequency of each aberration are shown and compared using Fisher's exact test.

Aberration	LTSs (n = 39)	STSs (n = 24)	P
6q loss	15% (6)	42% (10)	0.04
7p gain	46% (18)	71% (17)	0.07
7q gain	46% (18)	67% (16)	0.13
7p amplification	15% (6)	25% (6)	0.50
9p loss	33% (13)	58% (14)	0.07
10p loss	33% (13)	63% (15)	0.04
10q loss	41% (16)	83% (20)	<0.01
13q loss	13% (5)	21% (5)	0.49
14q loss	10% (4)	29% (7)	0.09
19p gain	15% (6)	50% (12)	<0.01
19q gain	3% (1)	38% (9)	<0.01
19q loss	28% (11)	0% (0)	<0.01
19q loss/1p intact	13% (5)	0% (0)	0.15
19q loss/1p loss	15% (6)	0% (0)	0.07
20p gain	5% (2)	25% (6)	0.05
20q gain	5% (2)	33% (8)	<0.01

matched by one of the genetic signatures of primary GBM. This is also consistent with our previous data indicating that EGFR overexpression, by immunohistochemistry, is not significantly different between LTSs and STSs (16). That study also demonstrated that LTSs were more likely to overexpress p53 than were STSs, although there was no difference in the gene mutation rate to account for the higher rate of expression. In addition, the combination of proliferation index, mdm2 expression, and p53 expression best distinguished the two groups. Because the patient cohorts from this and our previous study of LTS are not identical, a direct comparison cannot be made, but taken together, both studies indicate molecular differences between GBM LTSs and typical GBM survivors, and these two groups are best distinguished by the examination of multiple markers.

Deletions of chromosome 9p are common in GBM. This region contains the tumor suppressor genes *CDKN2A/p16*, *CDKN2B/p15*, and *ARF/p14* that play important roles in cell cycle restriction via the pRb and p53 pathways and is seen in 40–70% of unselected GBMs (28). In this study, there was a statistical trend for 9p loss being present more frequently in STSs *versus* LTSs (58% *versus* 33%, respectively; $P = 0.07$). Chromosome 10 deletion is the most common chromosomal aberration found in GBM, occurring in 60–80% of unselected cases (9). In this study, loss of 10p and 10q was seen more commonly in the STSs than in the LTSs (63% *versus* 33% and 83% *versus* 41%, respectively). Loss of chromosome 10 and mutation of the *PTEN* tumor suppressor gene have previously been shown to be independently associated with poor survival outcomes in patients with anaplastic (grade 3) astrocytoma, although a prognostic association in GBM was not found (5, 29).

Overall, the results indicate that regions with high frequencies of aberrations reported previously in GBM, including gain of 7 and loss of 9p and 10, were, in general, more common in the STS group. Taken together, these data indicate that some of the aberrations known to be among the most common in GBM are associated with aggressive clinical behavior, although these clinical correlations may not be identifiable in cohort studies that, by design, do not include large numbers of LTSs.

Loss of 6q and Gain of 19 and 20q. Chromosome 6q loss was seen in a significantly higher proportion of typical survivors compared with LTSs (42% *versus* 15%). Loss of chromosome 6q is associated with a variety of human malignancies including melanoma and breast and cervical cancer, consistent with the presence of a putative tumor suppressor gene (30–35). Several studies have described a potential role of 6q loss in malignant gliomas (36–40). One of these studies, using loss of heterozygosity analysis, identified as many as five separate regions of deletion on 6q (37). With respect to clinical behavior, one report describes 20 GBM patients treated with surgery and RT. These patients were separated into two clinically distinct groups based on time to progression after RT. Using CGH, Weber *et al.* (26) found that 6q loss was more common in the group with the shorter progression-free interval, although this difference did not reach statistical significance.

Chromosome 19 is the second most commonly gained chromosome in GBM (after chromosome 7), as shown by both cytogenetic studies (41) and CGH (9), and our data indicate that chromosome 19 gain was strongly associated with short-term survival. Whereas isolated 19p gain was seen in several of our cases, no cases of isolated 19q gain were identified, which may suggest that 19p is the minimal region. However, the difference in frequency of 19q gain between the STS and LTS groups was larger, suggesting that selection for region(s) on 19q may be occurring. Although we refer to 19q gain as an important difference between STSs and LTSs, it is possible that 19p is also important because 19q gain did not occur in the absence of 19p gain. Gain of 19q has been reported as more frequent in GBM than in grade 3 anaplastic astrocytoma (42). It has not been previously implicated in survival in GBM, although one study (43) comparing radiosensitive and radioresistant GBMs suggested that combined gain of chromosome 7 and 19 was associated with radioresistance. Chromosome 20q gain was also significantly more frequent in the STS group. Gains of 20q have been implicated in glioma progression (44) and have been described in other solid tumors, including breast and ovarian cancer (45, 46). Consistent with this report, a previous investigation of GBM has found gains of 20q to be associated with more aggressive behavior (26).

19q Loss. Chromosome 19q loss was observed only in the LTS group and can be divided by 1p status, based on previous studies of a related tumor, oligodendroglioma. Specifically, of the 11 LTS cases that showed 19q loss, 6 showed concomitant 1p loss, a marker of improved outcome in oligodendroglioma (19, 20). This pattern has also been reported in GBMs with a favorable outcome (47). Cases that showed combined 1p/19q loss were re-reviewed to ensure that they were not, in fact, diagnosable as oligodendroglioma or anaplastic oligodendroglioma, a finding that has been emphasized by others (17). Interestingly three of the GBMs with 1p/19q loss showed additional losses in 9p (two cases) and 10q (one case), both reported as mutually exclusive with 1p/19q loss in oligodendroglioma (19, 48). This suggests that at least some GBMs with 1p/19q loss share with anaplastic oligodendroglioma a favorable prognosis but are not necessarily "genetic" oligodendrogliomas with GBM histology. We point out that none of these cases were initially diagnosed as oligodendroglioma, and on re-review, those with oligodendroglial features were not included in this analysis. The remaining five cases that showed a 1p intact/19q loss pattern could represent the genetic equivalent of secondary GBM, although none of these cases showed 10q loss, which is also common in secondary GBM (49). On a practical level, 19q copy

Table 2 *Multivariate analysis (logistic regression) showing aberrations independently associated with survival*

All variables with associations $P \leq 0.05$ (except for isolated 19q loss, which was stratified by 1p status) were included in the initial model, and the variables were excluded in a stepwise fashion based on a lack of association until all variables were significant. Odds ratios (OR), 95% confidence intervals (95% CI), and *P*s are indicated. Results presented include adjustments for patient age and gender. KPS was not included in the model presented because of missing data on 10 cases. However, its inclusion left the model essentially unchanged.

Aberration	OR	95% CI	P
6q loss	11.0	2.0–61.2	<0.01
10q loss	12.3	1.9–79.8	<0.01
19q gain	124.7	5.4 to >999.0	<0.01

number may represent a useful clinical marker because loss was uniquely associated with the LTS group, and gain was (with one exception) was associated with the STS group.

Loss of 6q, 10q loss, and 19q gain were significantly different between the two groups of patients. When the three aberrations associated with short-term survival were examined for interrelationships, it is evident that the combination of these aberrations may better explain the differences between the two groups. The majority of the STS tumors (16 of 24) showed any two of these three aberrations, compared with only 1 of 39 of the LTS tumors. More specifically, whereas 10q loss was twice as common in the STS group (83% *versus* 41%; Table 1), 10q loss in the absence of either 6q loss or 19q gain was significantly less common in the STS group (4 of 20 cases, 20%) than in the LTS group (15 of 16 cases, 94%). These data are consistent with the idea that the aggressive behavior of GBM may be related to multiple key genetic lesions occurring concomitantly.

In sum, we genetically characterize a set of GBM tumors from a clinically interesting group of LTSs. Comparison with STSs reveals genetic regions that may be important in the biological behavior and therapeutic responsiveness of GBM. Tumors from LTSs exhibit fewer genetic aberrations, on average, than STS tumors. Aberrations previously implicated in the molecular pathogenesis of GBM (7 gain, 9p loss, and 10q loss) were, in general, less frequent in LTSs. Additional aberrations not previously emphasized as potentially prognostic (6q loss and 19q gain) were associated with the STS group. Conversely, loss on 19q was restricted to LTS patients and may represent a marker of improved outcome in GBM. These findings have implications for our understanding of the genetic factors related to the aggressive clinical behavior of GBM.

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