Loss of heterozygosity on chromosome 10q in glioblastomas, and its ass...

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Abstract

Background: Glioblastoma multiforme (GBM) is the most common malignant central nervous system neoplasm. Loss of heterozygosity (LOH) on chromosome 10q in these tumors has been found to show variable association with prognosis. Aim: To evaluate LOH 10q status in cases of GBM, and to correlate these results with patient characteristics, other genetic alterations, and survival. Material and Methods: Fresh tumor tissue and blood samples were obtained for 25 cases of GBM diagnosed over a 2-year period. LOH 10q assay was performed on blood and tumor DNA by a PCR-based method using four microsatellite markers. TP53 mutation analysis and fluorescence in situ hybridization for epidermal growth factor receptor (EGFR) were performed. Histopathology was reviewed and clinical data were analyzed. Results: LOH 10q was identified in 17 of 25 cases (68%). Losses were frequent with markers D10S1765 (12/20 informative cases; 60%) and D10S587 (12/17 informative cases; 70.5%) in the regions of 10q23.3 and 10q26.1, respectively. D10S540 for 10q25.1 showed LOH in 4/12 informative cases (33.3%) and D10S1770 for 10q26pter in none of the 25 cases. LOH with D10S1765 at the PTEN gene locus was found to correlate with overall LOH 10q status (P = 0.001). LOH 10q was more common in patients older than 40 years (16/19, 84.2%) than in those below (1/6, 16.7%) (P = 0.006). One of three pediatric patients included demonstrated LOH 10q. Survival rates for patients with LOH were lower than for patients with retained heterozygosity. Conclusion: LOH 10q is a frequent genetic abnormality in GBM in Indian patients, is seen more frequently in older adults, and its presence is associated with shorter survival. The single best marker to determine LOH 10q status is D10S1765 at the PTEN region.

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Introduction

Glioblastoma multiforme (GBM), World Health Organization (WHO) grade IV astrocytoma, is the most frequent
malignant central nervous system (CNS) tumor. [1] The incidence of this tumor ranges from 3 to 4 new cases per 100,000 population per year. [2] In India, data from the National Cancer Registry Program shows that GBM accounts for 19% of all brain tumors. [3] To date, the treatment of GBM remains a challenge to neuro-oncologists, as surgical cure is practically impossible due to the diffusely infiltrative nature of this tumor. In spite of recent advances in surgery, radiotherapy, and chemotherapy, the prognosis of GBM patients remains poor, with an overall survival rate of 15%-20% at the end of 1 year. [4] Thus far, histological evaluation has been the most reliable method for the diagnosis and characterization of these tumors. However, this is highly subjective and is not predictive of prognosis.

A number of genetic alterations are involved in the development of GBMs. [5],[6] Of these, loss of heterozygosity (LOH) on chromosome 10 is the most frequent, occurring in up to 85% of cases. [4],[7],[8],[9],[10],[11] A few case series have described LOH 10q as being associated with shorter survival and poor outcome, whereas some have found no such association. [4],[12],[13],[14],[15] In view of the reported association of LOH 10q with poor prognosis, this study was undertaken to assess the frequency of LOH 10q in GBMs in the Indian population, and to correlate LOH 10q status with histological parameters, other genetic alterations and with outcome. To the best of our knowledge, this is the first report on analysis of LOH 10q in GBM from India.

### Material and Methods

A total of 2888 brain tumors were received in the Neuropathology laboratory at our Institute over a period of 2 years between April 2008 and March 2010. Of these, 566 cases were diagnosed as gliomas (19.6%). GBMs comprised 35.3% of all gliomas received (200 cases). Of these 200 cases, 25 cases with adequate amount of frozen tumor tissue were randomly chosen for the study.

#### Tumor specimens

Tumor tissue was obtained at the time of surgery along with matched peripheral blood samples from 25 GBM patients. Portions of the resected tumors were snap frozen in liquid nitrogen and stored at −80°C until prior to DNA extraction. The remaining tissue was fixed in formalin and paraffin-embedded for histopathological examination. Hematoxylin and eosin (H and E)-stained sections were reviewed by three independent neuropathologists (CS, VS, and MCS), and consensus diagnoses were made according to the recent WHO Classification (2007). [1] Non-neoplastic cortical tissue from five epilepsy surgery specimens was used as control.

#### Clinical data

Demographic profile of the 25 patients was noted. Patients older than 18 years of age were considered as adults, while those 18 years or younger were included in the pediatric age group. Patients with clinical and histopathological evidence of progression from a low-grade glioma were classified as having secondary GBMs; and the rest were regarded as being primary GBMs. [5]

#### DNA extraction

Genomic DNA was extracted from 25 mg of freshly frozen tumor tissue after bringing it to room temperature, and also from matched peripheral blood samples, using DNeasy Blood and Tissue Kit (M/s. Qiagen), according to the manufacturer’s protocol. H and E-stained sections of each specimen were reviewed to confirm the adequacy of the sample, that is, to assess cellularity and extent of necrosis, and to ensure minimal contamination by non-neoplastic stromal elements.

#### Analysis for loss of heterozygosity on chromosome 10q

Four polymorphic microsatellite markers spanning frequently deleted regions on chromosome 10q, namely, D10S1765 near the locus of PTEN (phosphatase and tensin homolog) gene, D10S540, D10S587 near the locus...
of DMBT1 (deleted in malignant brain tumors) gene, and D10S1770 (M/s. MWG, Ebrsberg, Germany) were selected after reviewing the available literature. [7],[9],[14],[16] The markers used and their chromosomal loci are shown in [Figure 1]a. In brief, 40 ng of DNA was amplified by polymerase chain reaction (PCR) (M/s. DNA Engine, Biorad, USA). The 25 µL reaction volume contained 2 µL of each primer (forward and reverse), 0.5 µL dNTPs (M/s. Fermentas, USA), 1.5 µL MgCl₂, and 0.2 µL of Taq DNA polymerase (M/s. Fermentas, USA). The reaction mix was subjected to initial denaturation at 95°C for 7 min, followed by 30 cycles of denaturation at 95°C for 30 s, annealing at 53-59°C (varying with each primer) for 30 s and extension at 72°C for 40 s, followed by a final extension step at 72°C for 5 min. All PCRs were run with a negative control in which the reaction mix contained water in place of DNA. After denaturing at 95°C for 10 min, PCR products were loaded onto 8% denaturing polyacrylamide gel containing 7 M urea and were fractionated by gel electrophoresis at 50 mA/300 V for 4-7 h. Ethidium bromide staining was used to visualize the fractionated bands on a UV transilluminator gel documentation system (Alpha Imager EP ver 1.2.0.1, M/s Alpha Innotech, Santa Clara, CA). A reduction of ≥ 50% in band intensity in tumor DNA as compared to corresponding blood DNA was considered as LOH [Figure 2].

Detection of other genetic alterations

Dual-probe fluorescence in situ hybridization (FISH) assay for epidermal growth factor receptor (EGFR) amplification was performed on paraffin-embedded sections of all cases, using a locus-specific probe for EGFR and a reference centromeric probe for chromosome 7 (CEP7) (Vysis, Downers Grove, IL, USA). Briefly, sections were deparaffinized and then dehydrated in 100% ethanol. This was followed by target retrieval in citrate buffer for 20 min and 0.04% pepsin digestion (P-7000; Sigma-Aldrich, St. Louis, MO, USA) for 30 min. Probe mixture (10 µL per slide) was applied on the sections, which were subjected to simultaneous probe/specimen denaturation at 73°C for 5 min and overnight incubation at 37°C in Thermobrite TM hybridization chamber (Vysis, Downers Grove, IL, USA). Following this, the sections were washed and counterstained with 4,6-diamidino-2-phenylindole (DAPI) (Vysis, Downers Grove, IL, USA) and visualized under a fluorescence microscope. Signals were counted in a minimum of 200 nonoverlapping, intact nuclei per slide. EGFR amplification was considered as present when more than 10% of tumor cells showed either EGFR:CEP7 ratio > 2 or innumerable tight clusters of EGFR probe signals. Polysomy was defined as >10% nuclei showing three or more signals for EGFR and CEP7.

Sequencing analysis for TP53 mutation was performed on 20 GBMs in which adequate DNA was available. TP53 coding regions from exons 5-8 were analyzed by direct sequencing as per the protocol described in the IARC TP53 database. [17]

MIB-1 labeling index

MIB-1 staining of paraffin-embedded sections was performed, using monoclonal antibody MIB-1 (Dako, Glostrup, Denmark) in a dilution of 1:200. Sections from lymph node were used as positive controls. The labeling index for MIB-1 was calculated as a percentage of positively stained nuclei per 1000 cells.

Follow-up

Survival period was calculated from the date of surgery to the date of death, date of last contact if lost to follow-up, or the last day of the study period. Survival rates at 6 months and 1 year, as well as at the end of the study period were calculated.

Statistical analysis

Descriptive statistics, that is, mean and frequency distributions were calculated for all variables, using SPSS software. Chi-square test and Fisher’s exact test were applied to test association between categorized variables. t-Test and Mann-Whitney test were used to determine the difference between two means. Log-Rank test was used to compare survival distributions. P value of 0.05 was considered as the level of statistical significance.

Results
This is a prospective analysis of 25 cases of GBM (16 males, 9 females; age range 13-77 years; mean age 50.8 ± 18 years) for the presence of LOH on four different loci on chromosome 10q. Of these, 22 were adults [Table 1] and 3 were in the pediatric age group [Table 2]. The frontal lobe was the most common site involved (10 cases, 40%); rest of the tumors were located in the temporoparietal and occipital lobes, thalamus, basal ganglia, and paraventricular region. One of the 25 cases was classified as secondary GBM as the patient had previous history of a low-grade astrocytoma, and the remaining 24 cases were considered as primary GBM.

**Histopathological features**

All the cases examined showed the typical histomorphological features of GBM, with foci of palisading as well as confluent necrosis, and microvascular proliferation. Tumor cells showed nuclear hyperchromasia, atypia, and pleomorphism. Mitotic activity was noted, with mitotic count ranging from 5 to 38/10 hpf.

**LOH 10q assay**

Using 4 microsatellite markers, 100 loci were examined for LOH 10q in 25 cases of GBM. Seventeen cases demonstrated the presence of LOH 10q (68%) as depicted in [Figure 1]b. None of the five control samples from epilepsy surgery harbored LOH 10q. Of the 17 cases showing LOH, eight cases showed LOH with a single marker, seven cases with two markers and two cases with three markers. None of the cases showed LOH with all the four markers. LOH was demonstrated in 12 out of 20 cases (60%) informative for the marker D10S1765 for 10q23.3 near the PTEN locus, and in 12 of 17 cases (70.5%) informative for D10S587 for 10q26.1 near the DMBT1 locus. D10S540 for 10q25.1 showed LOH in four of 12 informative cases (33.3%), whereas none of the cases showed LOH with D10S1770, the marker for 10q26-pter. Combined LOH at the PTEN and DMBT1 loci was identified in nine of the 14 cases informative for both these markers (64.3%). On comparison of the LOH 10q status with occurrence of LOH with each of the four markers used, the presence of LOH with D10S1765 for PTEN was found to show significant correlation with LOH 10q status (P = 0.001).

**LOH 10q and age**

On classifying the patients into two groups, patients 40 years and older demonstrated LOH 10q more frequently (16 of 19 cases, 84.2%) than those younger than 40 years (1/6 cases, 16.7%) (P = 0.006). The mean age of patients showing LOH (55.65 years) was higher than the mean age of patients with retention of heterozygosity (40.63 years; (P = 0.115). Of the three pediatric patients included, 1 showed the presence of LOH 10q with the marker D10S540 [Table 2].

**EGFR amplification and TP53 mutation**

Among the 22 adult patients, EGFR amplification was identified in 10 cases (45.45%), and polysomy 7 in five cases (22.73%). LOH 10q was observed in 11 of 15 cases (73.3%), which showed increase in EGFR copy number (amplification or polysomy) and in 5 of 7 cases (71.4%) without increased EGFR copy number [Table 1]. None of the three pediatric GBMs demonstrated EGFR amplification, while two showed polysomy 7. One of these cases showing polysomy harbored LOH [Table 2].

TP53 mutations occurred in 2 of 20 cases examined (10%). The mutations were identified in exon 5 and exon 8 of the TP53 gene in either case, respectively. While the former showed LOH 10q with two markers, the latter did not demonstrate LOH. TP53 mutation and EGFR amplification were found to be mutually exclusive of each other.

**Secondary GBM**

The single case of secondary GBM demonstrated LOH with the markers D10S1765 and D10S587. It also showed the presence of a mutation in exon 5 of the TP53 gene. EGFR amplification/polysomy 7 were absent in this case.
MIB-1 labelling index

MIB-1 labeling index ranged from 10% to 80% (mean = 15.6%). The mean MIB-1 LI for cases with LOH 10q was 16.9%, while that in the no loss of heterozygosity (NLOH) group was 12.9% (P = 0.792).

LOH 10q and survival

The follow-up period ranged from 0.25 to 18 months (mean = 7.39 ± 5.9 months). At the end of the study period, of the 14 patients for whom follow-up was available, 11 were dead (one of who had developed a recurrence at 11 months and died subsequently), one was alive and two were lost to follow-up (at 0.5 and 5 months, respectively). The survival rate at 6 months was 50% and that at 1 year was 28.5%. Six-month survival rate for patients with LOH 10q was 37.5%, survival rate at 1 year was 12.5% and that at the end of 2 years was zero. On the other hand, the NLOH group had survival rates of 66.7% and 50% at 6 months and 1 year, respectively, and an overall survival rate of 16.7% at the end of 2 years, as shown in [Table 3]. [Figure 3] shows the Kaplan-Meier survival curves for the two groups of patients. The group showing LOH 10q had a shorter mean survival period of 6.03 months as compared to those with retention of heterozygosity (9.21 months). {Figure 3} {Table 3}

Discussion

While GBM may be diagnosed with relative ease by histopathological examination, further stratification of GBM patients into prognostic groups is not possible by light microscopy alone. Although a number of genetic alterations have been proposed to play a role in the pathogenesis of GBM, none of these alterations has definite prognostic connotations. Thus, the need arises for a definitive genetic marker that would help in determining the postsurgical management and prognostic stratification of GBM patients.

LOH 10q is described as the most common genetic abnormality in GBMs, present in 30%-88% of cases of GBM in various series. [4],[7],[9],[8],[10],[11] In our study, LOH 10q was seen in 17 of 25 cases (68%). Karlbom et al reported that the three regions of chromosome 10 that show LOH most frequently are 10p14-pter, 10q23-24, and 10q25-pter. [10] Subsequently, the PTEN and DMBT1 genes were identified at 10q23 and 10q26.1, respectively. [18],[19],[20],[21] In the present study, LOH was most commonly demonstrated at the loci of DMBT1 (70.5% of informative cases) and PTEN (60% of informative cases). PTEN is a regulator of cell-cycle progression and apoptosis via the PI3K-AKT pathway, and is frequently lost in GBMs. [22] LOH at the PTEN locus has been reported to occur in 36%-76% of GBMs in various series. [13],[14],[23],[24] Moreover, recently Mellinghof et al have shown that loss of PTEN in GBM leads to the development of resistance to therapy with EGFR kinase inhibitor agents, for example, geftinib. [25] With these developments, the importance of the PTEN gene assumes a new dimension as, in the near future, detection of PTEN alterations may be necessary to identify patient groups likely to benefit from these targeted therapeutic agents. Furthermore, in this study only LOH at the PTEN locus was found to be representative of the overall LOH status (P = 0.001), thus implying that if one marker was to be used to perform LOH 10q, a marker for this gene should be used, as it is more likely to detect LOH than the remaining markers. This is especially advantageous for smaller laboratories that do not have the resources to employ a large panel of markers for the detection of LOH 10q.

Although reported to be frequently lost in GBMs, the exact role of DMBT1 in the gliomagenesis pathway remains to be delineated. Similar to PTEN, LOH at the DMBT1 locus is also frequent in GBMs, ranging from 21% to 79%. [13],[14],[16],[26],[27] While we detected LOH at the DMBT1 locus in 70.5% of informative cases (12/17 cases), on comparison of DMBT1 LOH with overall LOH status, no significant correlation was noted.

Houillier and associates showed that LOH 10q occurs more frequently in older patients. [11] However, Tada et al and Gresner et al have refuted this association. [14],[26] In our study, a definite association was noted between LOH 10q and patient age. While 72.7% adults (16 of 22) showed LOH 10q, only one of three children (33.3%) showed this alteration. LOH 10q was observed more frequently in patients 40 years or older (84.2%) than those younger than 40 years (16.7%; P = 0.006). Similarly, LOH at the PTEN locus was found to occur at a
significantly higher frequency (12/16; 75%) in patients 40 years or older than in cases younger than 40 years (none out of 4) (P = 0.014). No such association was found between age and LOH at the DMBT1 locus, suggesting that the involvement of this gene may be an age-independent process. The mean age of patients showing LOH 10q (55.65 years) was found to be higher than that of those showing retention of heterozygosity (40.63 years), similar to the results of Lin et al. [13]

On histopathological examination, one case in our study showed the presence of oligodendroglioma-like areas. Although the WHO classification does not recognize GBM with oligodendrogliial component (GBM-O) as a distinct entity, some reports of genetic alterations in GBM-O are available. Our case showed LOH 10q, in contrast to the results of Kraus et al, who did not detect LOH 10q in 13 cases of GBM-O. [28] Vordermark and colleagues reported that GBM-O has a better prognosis than ordinary GBMs, with higher survival rates. [29] In agreement with this, our patient had a postop survival period of 10 months, which was longer than the mean survival of the pure GBM patients (7.2 months).

Amplification of the EGFR gene is known to occur in around 35%-50% of primary GBM, [4],[11],[30] and only rarely in secondary GBM. [31] Previous authors have described an association between presence of EGFR amplification and LOH 10q. [11],[16],[22] Ohgaki et al stated that LOH 10q and EGFR amplification was the most frequent combination of genetic alterations identified in their series. [4] In our study, LOH 10q was demonstrated in 73.3% of adult patients with EGFR amplification/polysomy 7. On the other hand, 71.4% of adult cases without EGFR alterations also showed LOH, thus indicating that LOH 10q status is independent of EGFR amplification status. TP53 mutation, which occurs in 25%-30% of GBMs is considered the genetic hallmark of secondary GBM, is seen in two thirds of these tumors and only infrequently in primary GBM (<30%). [4],[31],[32] While Ohgaki et al and Kato et al noted an association between LOH10q and TP53 mutation in a significant number of cases, Houillier et al found no such association. [4],[11],[24] Two of our cases harboured TP53 mutation, one of which was a secondary GBM and demonstrated LOH. Both cases with mutation did not show EGFR amplification. Thus, in our study, LOH 10q was demonstrated in cases showing EGFR amplification as well as in those with TP53 mutation, indicating LOH10q is involved in genetic pathways of both primary and secondary GBM.

Pediatric GBM is a rare entity and not much data is available on the frequency of LOH 10q in this age group. Kraus et al did not detect LOH 10q in 6 pediatric GBMs studied by them. [33] A study by Tada et al included three children, two of which demonstrated LOH 10q (66.7%). [14] Rickert et al detected losses on chromosome 10q in four of 13 childhood GBMs (30.8%). [34] The present study included three pediatric GBMs, one of which demonstrated LOH with D10S540, while the other three loci showed retention of heterozygosity. Interestingly, a similar observation has been made by Albarosa et al, where LOH was identified in the same region of chromosome 10, that is, 10q25 in a case of pediatric GBM. [9] This tumor also showed retention of heterozygosity for all other informative loci on 10q. Thus, the possibility arises that this region on chromosome 10q harbors a tumor suppressor gene different from those involved in the pathogenesis of adult GBM. Further studies to identify tumor suppressor gene(s) located in this region and to elucidate their biological role in pediatric GBM will contribute significantly to our understanding of the oncogenesis of these rare tumors.

LOH 10q has been variably associated with poor patient outcome. Leenstra et al found that patients showing LOH 10 had a shorter median survival period. [35] Lin et al reported that the median survival time in patients with LOH at the PTEN locus (42 weeks) was significantly shorter than that of those without LOH (87 weeks). [13] Similarly, Tada et al demonstrated that LOH at loci near PTEN is associated with a significantly unfavorable prognosis. [14] However, other series have not corroborated these findings. Houillier et al found no correlation between LOH 10q and survival. [11] More recently, Wager et al noted no association with overall survival for either PTEN or DMBT1. [36] In the present study, the mean overall survival period of patients with LOH 10q was 6.03 months while that of patients not showing LOH was 9.21 months. Survival rates were lower for patients with LOH 10q compared to those with retained heterozygosity [Table 3], [Figure 3].

To summarize, LOH on chromosome 10q is a frequent genetic alteration in GBM and was demonstrated in 68% of our cases. In a majority of cases, the loci of tumor suppressor genes PTEN and DMBT1 demonstrated LOH. LOH 10q occurred in relatively older patients, predominantly older than 40 years. The occurrence of LOH 10q was independent of the presence of EGFR amplification or TP53 mutation. Patients with LOH 10q had a shorter survival period, suggesting its potential role as a marker for prognostication in GBM patients. However, further
studies with larger patient population are necessary to validate this observation. As a significant correlation of PTEN LOH with overall LOH 10q status was observed, it is suggested that PTEN be used as the marker of choice in the detection of LOH 10q in settings where it is not feasible to use more than one microsatellite marker due to economic constraints.

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