Imaging, Diagnosis, Prognosis

LOH in the HLA Class I Region at 6p21 Is Associated with Shorter Survival in Newly Diagnosed Adult Glioblastoma

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Abstract

Purpose: Glioblastoma (GBM) shows downregulated expression of human leukocyte antigen (HLA) class I, thereby escaping from cytotoxic T cells and limiting the efficacy of immunotherapy. Loss of heterozygosity (LOH) of HLA class I (6p21) and/or β-2 microglobulin (B2m) (15q21) regions represents irreversible downregulation. In this study, we examined the prevalence of these LOH events and their relations with overall survival in GBM.

Experimental Design: In a cross-sectional analysis on 60 adult patients with GBM, DNA from formalin-fixed, paraffin-embedded specimens were evaluated for 10 microsatellite regions of HLA class I, B2m, HLA class II, HLA class III, and 6q by PCR as well as immunohistochemical evaluation of HLA class I expression and CD8+ T-cell infiltration.

Results: LOH in HLA class I, B2m, HLA class II, HLA class III, and 6q regions was present in 41.4%, 18.2%, 9.4%, 77.8%, and 36.0% of informative cases, respectively. LOH of HLA class I was associated with shorter overall survival (HR = 4.89, P = 0.0078). HLA class I was downregulated in 22% to 43% of cases based on immunohistochemistry. Cases that displayed negative staining were significantly younger. HLA class I expression correlated with intratumoral CD8+ T-cell infiltration.

Conclusion: LOH in the HLA class I region is frequent in adult GBMs. The association of shorter survival with LOH in this region suggests a crucial role for these genes in immunosurveillance. Clin Cancer Res; 19(7); 1816–26. © 2013 AACR.

Introduction

Glioblastoma (GBM) is the most lethal adult primary brain tumor with a median life expectancy of 14.6 months (1). Previous studies have suggested that the immune system has a significant role in the pathogenesis of glioma. Reports of the decreased risk of developing glioma in individuals with a strong allergic history suggest that the immune system can be effective in opposing the development and progression of gliomas (2, 3). Indeed, effector T-cell infiltration has been found to be associated with longer survival of patients with GBM (4, 5).

The ability of the immune system to recognize and kill cancer cells relies on activated cytotoxic T cells (CTL) that recognize tumor antigens presented by the HLA class I peptide complex. A major mechanism of cancer immune escape seems to involve defects in antigen presentation by HLA class I and this mechanism negatively impacts the clinical course in some malignancies and the outcome of CTL-based immunotherapy (6).

The downregulation or loss of HLA class I or β-2 microglobulin (B2m), an essential component of the complete HLA class I molecule, can be divided into reversible or irreversible lesions, each representing entirely different mechanisms of downregulation (7). Provided that HLA class I genes, consisting of HLA-A, HLA-B, and HLA-C are intact, the expression of HLA class I can be restored in malignant glioma cells by the use of IFN-γ and -β (8). However, loss of heterozygosity (LOH) in 6p21 and 15q21, where HLA class I and B2m genes are located, respectively, represent irreversible lesions that are not amenable to epigenetic restoration of HLA class I expression. (9, 10) Although HLA class I expression is known to be downregulated in approximately 50% of GBMs (11), it is unclear whether LOH in the HLA class I and B2m regions are prevalent in GBMs, which is possible given the high chromosomal and microsatellite instability in these tumors (12). Furthermore, it is unknown whether LOH of these genes has any effect on patient survival, based on the rationale that these events could lead to decreased tumor antigen presentation and limited recognition by CTLs.
Translational Relevance

Glioblastoma (GBM) shows downregulated expression of human leukocyte antigen (HLA) class I to evade the recognition by CD8⁺ cytotoxic T cells. In this investigation, we found, for the first time, that loss of heterozygosity in the HLA class I region is associated with shorter overall survival in newly diagnosed patients with GBM. This sheds new light onto the allelic status of these well-characterized genes and its importance in GBM immunosurveillance. These findings provide greater understanding of how GBMs evade the immune system and allow further prognostication for patients with GBM.

In this study, we investigated the expression of HLA class I and the prevalence of LOH in HLA class I and B2m regions using a PCR-based microsatellite approach in patients who underwent standard treatment, including resection, radiation, and chemotherapy. We hypothesized that LOH in HLA class I and B2m regions, if prevalent in GBM, would be associated with lower survival. The results in this study support this hypothesis and provide important prognostic insights regarding the frequency and prognostic relevance of LOH in the HLA class I and B2m regions, as well as having implications for future immunotherapy trials.

Materials and Methods

Patient specimens

Archival formalin-fixed, paraffin-embedded (FFPE) adult GBM samples (n = 64) belonging to a total of 60 patients obtained at the time of tumor biopsy or resection were obtained from the Brain Tumor Bank at the University of Pittsburgh (Pittsburgh, PA) and used for this cross-sectional study (see Supplementary Table S1). Deidentified patient information, including clinicopathologic data, was accessible only through designated honest brokers (Anatomic Pathology Broker System, Approval # IRB PRO12020252; Principal investigator: H. Okada). Nonstratified patient samples were selected by tissue availability from 2005 to 2011, inclusive by our honest broker from the database. Out of these 60 patients, 56 patients (48 newly diagnosed and 8 recurrent cases; one specimen per patient) were treated with resection, radiation, and chemotherapy.

Antibodies

The following primary monoclonal antibodies (mAb) were used at indicated dilutions (see Supplementary Table S2 for allele-specific recognition for each of the mAbs): EMR8-5 (1:3,000); HCA2 (1 μg/mL); and HC10 (0.25 μg/mL). The quality of HLA class I staining by each mAb was monitored by using surrounding normal cells, to establish any contamination from normal cells and ensure reliable LOH analyses (15). Lesions were scored as positive, heterogeneous, and negative, when the percentage of stained tumor cells in a specimen was more than 75%, between 75% and 25% inclusive, and less than 25%, respectively. For statistical comparisons, HLA class I immunoreactivity was dichotomized into negative and positive, which included heterogeneous and homogenous staining. CD8-positive cells were graded semiquantitatively by intratumoral and perivascular infiltration patterns on a 0 to 3 scale using a × 20 objective. Intratumoral infiltration was graded as 0 (no positive cells in any field), 1 (mild, 0–10 cells in some fields), 2 (moderate, individual positive cells in most fields), and 3 (strong, many individual positive cells in all fields or aggregation/clusters of positive cells in multiple foci). Perivascular infiltration was categorized by 0 (no positive cells around any vessel), 1 (mild, few positive cells around some vessels), 2 (moderate, 10–20 positive cells around some vessels), and 3 (strong, more than 10 positive cells around all vessels).

PCR-based microsatellite analysis

Each FFPE specimen was stained with hematoxylin and eosin to ensure that GBM was present. Areas with high density and purity (>90%) of tumor cells were marked for microdissection of adjacent sections to minimize contamination from normal cells and ensure reliable LOH analyses (16). Overlapping areas in up to 4 adjacent slides were used for microdissection. DNA was extracted from the microdissected tissue using QIAamp DNA Micro Kit (Qiagen) according to the manufacturer’s instructions.
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Table 1. Panel of microsatellite markers targeting chromosome 6 and 15

<table>
<thead>
<tr>
<th>Marker</th>
<th>Specific location</th>
<th>Size</th>
<th>Primer sequence</th>
<th>Annealing temperature (°C)</th>
</tr>
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<td>Tel.(1500–2500kb)/HLA-A</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>5’ GAAGGAGAATGGAATTTCCG</td>
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</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>5’ ACGGTCGTAACCTAACCT</td>
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</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>5’ ATCGAGGTAACAGCGAAAC</td>
<td></td>
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<td>58</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>5’ GAGCAAGGGAGGAGAGGAAT</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>5’ CAGTATAAGCCAGAAGTAC</td>
<td>57</td>
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<td></td>
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<td></td>
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<td></td>
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<td>5’ AGTCCCTGATGGGTG</td>
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</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>5’ QATTCAAGGCCAGGAGAT</td>
<td></td>
</tr>
</tbody>
</table>

polymorphic microsatellite markers spanning 6p21 and 15q21 were tagged with FAM at their 5’ ends (Integrated DNA Technologies, Inc.). The details of the microsatellite markers used in this study are given in Table 1. Relative positions of markers targeting chromosome 6 are displayed in Supplementary Fig. S1. An assumption in this study was that a microsatellite marker near a particular gene would serve as a surrogate marker for the allelic status of that gene, such that the LOH shown by a marker would indicate an allelic loss of the targeted gene. PCR was conducted on GeneAmp PCR System 2700 thermal cycler (Applied Biosystems) using KOD Xtreme Hot Start DNA Polymerase (EMD Millipore) and its recommended protocol with the optimized annealing temperature of each primer listed in Table 1. Products of amplification were first visualized by 2% agarose gel electrophoresis to ensure ample amplification and were subsequently subjected to capillary gel electrophoresis that was conducted at the Molecular Anatomic Pathology laboratory, University of Pittsburgh Medical Center on ABI3730 (Applied Biosystems) according to the protocol. PCR quality water was used as a negative control in all reactions. The signal waveforms from the microsatellite-based PCR were visually inspected for aberrant signals that may suggest inadequate amplification. The technician conducting the capillary electrophoresis analysis was blinded to the results. The relative fluorescence values (peak heights) were obtained for individual alleles and the ratio of peaks was calculated using GeneMapper software v.4.0 (Applied Biosystems). LOH for a specific marker was defined as having an allelic ratio fall outside a 99% confidence interval constructed from DNA obtained from peripheral blood of 10 or more normal individuals using the following equation proposed by Slebos and colleagues:

\[
\bar{X} \pm t_{n-1} \frac{s}{\sqrt{n}}
\]

where \(\bar{X}\) is the average log2(allelic ratio) from normal control samples, \(t_{n-1}\) is the t-score for the sample size available for a specific marker, s is the SD, and n is the number of normal samples available (17). Retention of heterozygosity (ROH) was defined as the lack of LOH findings with informative allele ratios. Samples with non-informative loci and no LOH for a particular marker are excluded from analysis.

**Statistical analysis**

Statistical analyses were conducted using STATA Version 12 and TIBCO Spotfire S+ 8.2 for Windows. The Mann-Whitney U test was used to compare continuous variables between 2 groups. Fisher exact test was used to analyze categorical data from 2 groups. Spearman rank correlation was used to analyze associations in immunoreactivity. Immunohistochemistry data and clinical correlations with LOH status were analyzed using both newly diagnosed and recurrent cases together. Exploratory analyses of LOH events with molecular data from the brain tumor bank, obtained by methods described previously (18–21), were conducted using univariate analyses. Samples with noninformative loci and no LOH for a particular marker are excluded from survival analyses. Analyses were conducted by excluding
noninformative loci due to the assumption that the loci could harbor LOH or balanced alleles. Including noninformative loci may then introduce bias to the results. Differences in overall survival (OS), defined as duration from initial biopsy/resection until death, between groups using only newly diagnosed cases were analyzed using standard proportional hazards regression modeling for censored time-to-event data; inference was based on the likelihood ratio test. Values unless otherwise specified are presented as mean ± SD; \( P \leq 0.05 \) was considered to be significant.

Results

Patient characteristics
The clinical features of patients (\( n = 56 \)) are shown in Supplementary Table S1. The mean age was 60.0 ± 12.7 years (range 25–88 years). There were 35 males and 21 females. Their median follow-up time was 9.5 months (range 0.7–78.5 months).

HLA class I expression in adult GBM
The results of immunohistochemical staining of 56 FFPE adult GBM with 3 different mAbs (HCA2, EMR8-5, and HC10) varied widely among specimens; representative images are depicted in Fig. 1 and Supplementary Fig. S2A. Using EMR8-5 mAb, 11 (22%), 13 (27%), and 25 (51%) cases displayed negative, heterogenous, and homogeneous expression, respectively. Using HCA2 mAb, 17 (33%), 15 (29%), and 20 (38%) cases displayed negative, heterogenous, and homogeneous expression, respectively. Using HC10 mAb, 23 (43%), 5 (9%), and 26 (48%) cases displayed negative, heterogenous, and homogeneous expression, respectively.

Figure 1. Immunohistochemical staining for HLA class I expression in adult GBM. Representative images of immunohistochemistry using HCA2 shown at ×10 magnification (A–D) and ×20 (F). Each scale bar represents 100 \( \mu \)m (A–D). Negative control was established using medulloblastoma, whereas endothelial cells serve as internal positive control (A). HCA2 staining showing negative (B), heterogeneous (C), and homogeneous (D) expression. Heterogeneous and homogeneous immunoreactivity are considered to be positive HLA I expression. A chart summary of HLA class I staining (E). Although some myeloid cells (CD11b\(^+\), dark blue) exhibit HLA class I (brown) immunoreactivity (white arrow), most infiltrating myeloid cells (CD11b\(^+\), dark blue) in GBM are almost negative for HLA class I (black arrow; F).
There was a positive correlation between age and HCA2 immunoreactivity ($P = 0.02$), between age and EMR8-5 immunoreactivity ($P = 0.054$); however, no correlation between age and HC10 immunoreactivity was found ($P = 0.53$). OS was not associated with immunoreactivity against EMR8-5, HCA2, or HC10 regardless of whether age was included in the analysis. Myeloid cells (CD11b+ cells) displayed heterogeneous immunoreactivity of HLA class I (Fig. 1F).

**CD8**+ T-cell infiltration

As shown in Fig. 2 and Supplementary Fig. S2B, of the 41 cases with sufficient tissue for analysis, intratumoral CD8+ T-cell infiltration was not observed (grade 0) in 2 cases (4.9%), and was mild (grade 1) in 18 cases (43.9%), moderate (grade 2) in 15 cases (36.6%), and strong (grade 3) in 6 cases (14.6%). Perivascular CD8+ T-cell infiltration was not observed (grade 0) in 7 cases (17.0%), and was mild (grade 1) in 20 cases (48.8%), moderate (grade 2) in 12 cases (29.3%), and strong (grade 3) in 2 cases (4.9%). Overall, most cases had some degree of CD8+ T-cell infiltration, but rarely did the cases display strong, diffuse infiltration.

After dichotomizing each form of CD8+ T-cell infiltration into negative (grade 0 and 1) and positive infiltration (grade 2 and 3) categories, we found no associations between OS and intratumoral or perivascular CD8+ T-cell infiltration regardless of whether age was included in the analysis. However, intratumoral CD8+ T-cell infiltration significantly correlated with HLA class I immunoreactivity detected by EMR8-5 ($P < 0.01$) and HC10 ($P < 0.01$) but not by HCA2 ($P = 0.25$). There was not an association found in this study between perivascular CD8+ T-cell infiltration and HLA class I immunoreactivity, regardless of the mAb used.

**LOH analysis of 6p21 and 15q21**

LOH data are illustrated in Fig. 3A. Five markers that were used to assess the HLA class I region (D6S105, D6S265, D6S276, C.1.2.c, C.1.2.5) showed LOH in 12 of 29 informative cases (41.4%). A marker centromeric to HLA class II region (D6S291) showed LOH in 3 of 32 (9.4%) informative cases. Intriguingly, 35 of 45 (77.8%) informative cases for D6S273, which map to the HLA class III region, showed LOH. In addition, using D6S311 (which, if lost in addition to multiple 6p markers, may be inferred to be a marker to assess whole chromosome loss) showed LOH in 18 of 32 (56.0%) informative cases. Two markers mapping to B2m revealed LOH in 6 of 33 (18.2%) informative cases.

Overall, the LOH status in the HLA class I region was not associated with HLA class I immunoreactivity using EMR8-5, HCA2, or HC10. Furthermore, LOH in the HLA class I region was not associated with intratumoral or perivascular CD8+ T-cell infiltration. The associations between LOH events with available clinicomolecular data are summarized in Table 2. Of note, patients with LOH in the region of HLA class III were significantly younger (57.3 ± 12.8 vs. 67.2 ± 12.1 years, $P = 0.04$). Secondary GBMs are characterized by a high incidence of p53 mutation ($P < 0.01$). In our study, 28.6% of cases showed p53 aberration, as detected by immuno-

histrochemistry. In univariate analyses, we found no association between p53 with LOH in the HLA class I and B2m regions.

Kaplan–Meier survival curves are shown in Fig. 3B–F. Strikingly, survival analyses revealed that LOH in the HLA class I region was significantly associated with shorter survival in newly diagnosed GBM (median 13.1 vs. 8.6...
months; Fig. 3B), whereas LOH in the regions of HLA class II and class III were not (Fig. 3C and D). In univariate analysis using standard proportional hazards regression, the unadjusted HR for LOH in the HLA class I region was 4.89 ($P = 0.0078$). In univariate analysis using standard proportional hazards regression, LOH in the $B2m$ region showed a strong trend towards shorter survival (HR = 3.83, $P = 0.083$; Fig. 3E). Previous studies have found that young age is associated with longer survival (23, 24). In our study, when we dichotomized age about its median, we found a very weak association (P = 0.30) of old age with longer survival (HR = 0.7). We therefore decided not to include age as a covariate in the proportional hazards regressions. There was no association found between LOH of 6q and survival (Fig. 3F).

**Discussion**

Although it is known that decreased HLA class I expression is frequent in GBM (11), it was unclear whether LOH in the region of HLA class I, an irreversible mechanism of downregulation, is common in GBM and has any impact on the clinical course. Our results provide evidence of the frequency of this genetic aberration in GBM and show a significant association between LOH of these regions and shorter survival.

LOH of HLA class I genes, consisting of HLA-A, HLA-B, and HLA-C, has been suggested to be the most common irreversible mechanism underlying HLA class I downregulation (10). Our reported prevalence of 41.4% in the current study is comparable with other studies in colorectal carcinoma (40%), melanoma (23%), bladder carcinoma (35%), laryngeal carcinoma (53%), and head and neck squamous cell carcinoma (49%; refs. 10, 25–28). No previous investigations reported any association of LOH of the HLA class I and $B2m$ regions with survival. Strikingly, our data showed a significant association of LOH in these 2 regions with poorer survival in patients with GBM. Surprisingly, we did not find any association between LOH and HLA class I expression, even with the use of 3 different mAbs that target different allotypic components of the molecule. Similar results have been reported in head and neck squamous cell carcinomas in which, with all 6p21–23 loci considered together, only LOH of D6S291 was found to be associated with HLA class I expression (28).

Multiple targets involving the other intact HLA class I allele and components of the antigen-presenting machinery, such as transporter associated with antigen processing and low molecular weight polypeptides proteins (29), may act in concert to result in detectable downregulation of HLA class I, analogous to Knudson multiple hit hypothesis (30). The latter hypothesis would help to consolidate our findings of (i) the lack of association between LOH of HLA class I region and protein expression, (ii) lack of association between LOH of HLA class I region and CD8$^+$ T-cell infiltration seen in this group, and (iii) the significant survival difference between LOH and ROH of the HLA class I region. These temporal events may not be captured by analyzing tissue samples obtained at the time of diagnosis. Although haploinsufficiency of HLA class I protein due to LOH is an obvious mechanistic explanation for shorter OS, mechanisms unrelated to immune escape may also be involved. It was postulated that loss of HLA class I surface expression on human melanoma cells grown in immunodeficient mice with no autologous immune response could generate more oncogenic tumor cells in vivo (31). Furthermore, HLA class I may have effects on cell-cycle gene expression, invasion, and intrinsic tumorigenicity that are not amenable to detection by immunohistochemistry and accounted for by extrinsic immunologic interactions (32). Precise mechanisms for these observations, in which no interaction with the immune system is needed, remain to be elucidated.

Survival analyses aimed at HLA class I immunoreactivity and linking it with LOH events are complicated due to the various antibodies available, such that contradicting results have been shown even in the same cancer type (33). Facoetti and colleagues were not able to show any survival difference by HLA class I expression, which the authors attributed to insufficient sample size (11). That would be particularly true in this study as our sample size is slightly more than half of theirs. Furthermore, mAbs used for the current study are not allotype-specific. Although we included the use of EMR8-5, which has been purported as a true pan-HLA class I antibody based on its reactivity against recombinant alleles (34), the high number of serotypes for each HLA-A/B/C precludes the existence of a true pan-HLA class I mAb. In light of that, the strong association of LOH of HLA class I with OS presents a potential biomarker for GBM without the variability inherent in immunohistochemistry.

Intriguingly, CD8$^+$ T-cell infiltration has been repeatedly shown to correlate with survival in patients with GBM, where the rationale involves proper antigen presentation by tumor cells (4, 5). Although we were not able to reproduce these findings possibly due to limited amounts of available tissues, our results have shown for the first time, to our knowledge, a positive correlation between HLA class I expression and the degree of intratumoral, but not perivascular, CD8$^+$ T-cell infiltration in adult GBM. These 2 patterns of CD8$^+$ T-cell accumulation in melanoma specimens have been shown to have a significant association with patient survival (35). Surprisingly, not many CD11b$^+$ cells are convincingly positive for HLA class I, suggesting that these CD11b$^+$ cells may be immature myeloid cells. Overall, CD8$^+$ T-cell infiltration was observed to be related to the HLA class I expression of tumor cells, especially, as areas with high density of tumor cells were selected for this study.

Our results showing a statistically significant younger age for specimens displaying negative HLA class I immunoreactivity present a paradoxical scenario. It is established that patients with GBM, who are younger at diagnosis, have a better prognosis than those who are older (36). In younger patients, it is possible that immune cells, capable of effectively targeting and killing adequately presented tumor cells, negatively select for tumor cells that lack proper antigen presentation. We did not find an association between age and OS, possibly due to our sample size.
A surprisingly high number of GBM specimens in our study showed LOH in the region of HLA class III. HLA class III region encodes many proteins with immune functions, including components of the complement system, cytokines (TNF-α, lymphotoxin A, and lymphotoxin B), HSPs, and many genes with no obvious immune-related functions (37). Of immunologic interest, TNF-α has been shown to evoke robust antitumor functions by increasing cytotoxic T-cell activity (38), enhancing monocyte, granulocyte, and natural killer cell cytotoxicity (39), and activating downstream proinflammatory cytokines (40). Our data showing a high prevalence of LOH in the HLA
Class III region are consistent with the above aspects of immunosuppression when the antitumor effects are lost. Our survival analysis was limited by the small number of patients with ROH.

Frequent allelic losses were detected using D6S311. Our finding of LOH of 6q in 36% of cases is in close agreement with 25% (tumor grade not defined) previously reported by Miyakawa and colleagues (41). The region of 6q has been implicated to harbor tumor suppressor genes and represent more aggressive tumors and lower survival in other cancers (42–44). In our study, however, we did not find an association between LOH of D6S311 with OS.

An obvious limitation in the present study is the cross-sectional design, which does not allow us to discern the
temporal relationships and causality of LOH in the region of *HLA class I* and survival. However, there is strong biologic plausibility concerning the role of HLA class I in tumor recognition by the immune system. Another concern is the lack of normal, autologous tissue from each patient to conduct traditional comparisons in our LOH analyses. However, we have chosen to use an artificially constructed confidence interval derived from healthy donor DNA to define a stringent criteria for LOH and avoid false-positive findings. Any false positive would inherently bias our results towards the null hypothesis, thereby making our current findings more convincing. The gain of alleles might produce a false-positive LOH finding or “pseudo-LOH” but any false positive would inherently bias our results towards the null hypothesis and add confidence to our findings. Finally, the lack of association between age and OS in this selected population was likely due to sampling variance, and the effects of age as a function of LOH of the HLA class I region on OS should be further studied with a larger population.

In conclusion, our findings could have potential prognostic value for adult patients with GBM and provide new insight into the GBM immunogenetics.

### Disclosure of Potential Conflicts of Interest

H. Okada has ownership interest (including patents) in and is a consultant/advisory board member of Stemline Therapeutics, Inc. No potential conflicts of interest were disclosed by the other authors.

### Table 2. Exploratory analyses: association of LOH events with available clinicomolecular data

<table>
<thead>
<tr>
<th>LOH in HLA class I</th>
<th>LOH in HLA class II</th>
<th>LOH in HLA class III</th>
<th>LOH in B2m</th>
<th>LOH in 6q</th>
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<td>Age*</td>
<td>0.82</td>
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<td>p53 (immunohistochemistry)*</td>
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<td>% cells with hyperdiploidy (Chromosome 7)*</td>
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<td>1.00</td>
<td>1.00</td>
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**NOTE:** MIB1, p53 expression, were assessed using immunohistochemistry. EGFR amplification, chromosome 7 hyperploidy, and % cells with p16 deletion were assessed using FISH. LOH of 1p and 19q were determined using both FISH and PCR. LOH of 9p, 10q, and 17p were determined using PCR only. Overall, loss of any 1 marker was considered as LOH for this study. MGMT promoter methylation was assessed using methylation-specific PCR. The above analyses were performed and quantified as described previously (18–21).

*Fisher exact test.

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**Acknowledgments**

The authors thank Justin Murphy (Molecular Anatomic Pathology Lab at the University of Pittsburgh) for conducting the capillary electrophoresis and Stephanie Bortoluzzi (Division of Neuropathology at the University of Pittsburgh) for compiling patient data.

**Grant Support**

This study was supported by grants from Doris Duke Clinical Research Fellowship (to J.T. Yeung), Musella Foundation for Brain Tumor Research and Information (to H. Okada), and the NIH (2R01 NS055140 and 1P01 CA132714; to H. Okada). This project used UPCI shared resources (Biostatistics Facility) that are supported in part by NIH P30CA047904.

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Received September 10, 2012; revised January 16, 2013; accepted February 4, 2013; published OnlineFirst February 11, 2013.
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LOH in the HLA Class I Region at 6p21 Is Associated with Shorter Survival in Newly Diagnosed Adult Glioblastoma


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