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## Clinical Stratification of Glioblastoma Based on Alterations in Retinoblastoma Tumor Suppressor Protein (RB1) and Association with the Proneural Subtype

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### Abstract

A recent study of CDK4/6-inhibitors in glioblastoma (GBM) xenografts identified retinoblastoma tumor suppressor protein RB1 status as a determinant of tumor therapeutic efficacy. Because of the need for clinically applicable RB1 testing, we assessed the utility of 2 complementary methods for determining RB1 status in GBM. Using fluorescence in situ hybridization (FISH) and immunohistochemistry (IHC), we analyzed 34 GBMs that had also undergone molecular characterization as part of *The Cancer Genome Atlas* (TCGA). By IHC, 4 tumors (11.8%) had complete loss of RB protein expression, including 2 with homozygous deletion of RB1 by FISH and 1 with hemizygous deletion of RB1 by FISH combined with a novel nonsense mutation in RB1. Consistent with these results, in an independent set of 51 GBMs tested by IHC we demonstrated loss of RB1 protein in 5 (9.8%). In GBM molecular subtype analysis of TCGA data, complete loss of RB1 transcript expression was seen in 18 of 170 tumors (10.6%) and these were highly enriched for, but not exclusive to, the proneural subtype ( $p < 0.01$ ). These data support the use of IHC for determining RB1 status in clinical GBM specimens and suggest that RB1 alterations may be more common in certain GBM subgroups.

### Keywords

Fluorescence in situ hybridization; Glioblastoma; Immunohistochemistry; Patient stratification; RB1; *The Cancer Genome Atlas* (TCGA)

## INTRODUCTION

Glioblastoma (GBM) is the most common primary malignant brain tumor of adults with a median survival of less than 2 years (1-3). Novel therapeutic options are desperately needed. GBMs are characteristically heterogeneous and contain different cell signaling pathway

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alterations (4, 5). Novel therapeutics are being designed to target some of these pathways but their success likely depends on patient stratification into molecular subgroups so that therapies are to specific subsets of patients with predicted favorable responses.

Aberrations in the CDKN2A/p16-CDK4/6-RB pathway are common in GBM (6-8) and they have been shown to be critical in gliomagenesis or tumor progression from lower-grade astrocytomas (9). CDK4 and CDK6 phosphorylate RB1, which induces the release of the transcription factor E2F, thus facilitating the transition of the cell cycle from the G1 to S phase. In normal cells this cell cycle transition is negatively regulated by the p16 protein, which binds and inhibits CDK4/6 function. Thus, alterations in expression of p16, CDK4, CDK6, or RB1 can result in dysregulation of the cell cycle (10). Indeed, *The Cancer Genome Atlas* (TCGA) project has shown that this pathway is altered in nearly 80% of primary GBMs with the most frequent genetic alterations being *CDKN2A* gene deletion or mutation, *CDK4* amplification, and *RB1* mutation or deletion (11). A recent preclinical study investigated the efficacy of a CDK4/6-specific inhibitor (PD-0332991) in intracranial GBM xenograft tumors and found it to be a potent inhibitor of GBM growth (12). As expected, however, this antitumor effect was not seen in RB1-deficient tumors because the latter cell signaling alteration is downstream of the drug target. Thus, stratification of patient tumors based upon RB1 status may be important.

Multiple mechanisms of bi-allelic RB1 gene inactivation have been identified in tumors, including combinations of deletions and point mutations (13). In astrocytomas, alterations in RB1 expression have been associated with increased tumor cell proliferation and decreased survival (14, 15). Assessment of RB1 status, however, is not currently performed clinically. Here, we used immunohistochemistry (IHC) for determining RB1 status in formalin fixed paraffin-embedded (FFPE) clinical GBM samples and validated this assay using profiling data from TCGA (11), including relative RB1 gene copy numbers and transcript expression levels. Copy number assessment was additionally assessed by FISH and concordance levels between the methodologies were determined.

## MATERIALS AND METHODS

### Tumor Material and Study Design

FFPE tumor tissue was obtained from the UCSF Brain Tumor SPORE Tissue Bank (CHR #10-01318) in accordance with ethical standards of the UCSF Institutional Review Board. GBM tissue microarrays containing central regions of tumor were constructed from 34 tumors previously profiled by TCGA (11). Each tumor was represented on the array with at least 2 tissue cores. FISH results were compared to corresponding TCGA array comparative genome hybridization (CGH) data (30 cases available). Immunohistochemistry (IHC) data for 33 cases were compared to corresponding TCGA transcript profiling data. A total of 33 tumors were analyzed by both FISH and IHC. A second independent set of 51 GBMs was also analyzed.

### FISH Analysis

FISH was performed on FFPE tissue microarray slides as described (16). Hybridization was achieved using Spectrum Orange-labeled RB1 (13q14) probe (Abbott Molecular, Abbott Park, IL). For each sample with adequate signal, a minimum of 100 non-overlapping nuclei was enumerated by 2 investigators (A.P., P.G.). A hemizygous RB1 deletion was defined as >50% of tumor nuclei showing only 1 signal (17). Homozygous deletion was defined by the lack of any RB1 signal in the majority of tumor cells despite the presence of signals within intratumoral non-neoplastic elements such as endothelial cells; this was required to rule out hybridization failure. FISH images were captured using an Olympus BX60 fluorescence

microscope with charge coupled device camera, Z-stack motor and a CytoVision basic workstation (Applied Imaging, Santa Clara, CA). FISH signals were scored as not deleted, hemizygous deletion, or homozygous deletion, and scoring was blinded to the results of IHC and profiling.

## IHC

IHC was performed for RB1 (BD Biosciences, San Jose, CA, G3-245) and Iba1 (Wako Chemicals USA, Inc., Richmond, VA, 019-19741) following antigen retrieval. The latter antibody identified intratumoral microglia and macrophages. An automated IHC staining process was used for all biopsies (Benchmark XT, Ventana Medical Systems, Inc., Tucson, AZ). Tumor RB1 protein status was determined by counting cells with positive nuclear staining in 3 20x fields. RB1-positive endothelial cells served as an internal positive control. All slides were reviewed by the same neuropathologist (J.J.P.) who was blinded to genomics and FISH data. IHC scores were scored as follows: 0 for expression in <20% of tumor cells; 1+ for expression in 20%-50% of tumor cells; and 2+ for positive expression in >50% of tumor cells. Digital images were captured using a microscope (Olympus, Center Valley, PA, Model BX41TF) and digital camera (Olympus, Model DP70).

## Genomic Data Analysis

Copy number data (HG-CGH-244) and gene expression data (AgilentG4502A)  $\log_2(\text{Tumor}/\text{Normal})$  were obtained from the TCGA Data Portal on December 20, 2010 and March 23, 2011. Gene deletion of RB1 was defined as a copy number loss resulting in a  $\log_2(\text{Tumor}/\text{Normal})$  less than or equal to  $-0.4$  (fold-change of copy number in tumor vs. normal less than 0.76), which included the RB1 gene (chr13:48,877,883-49,056,024). Loss of RB1 transcript expression was defined as a  $\log_2(\text{Tumor}/\text{Normal})$  of less than or equal to zero (fold change of gene expression in tumor vs. normal less than 1).

## Statistics

A two-tailed t-test was used to compare mean values. For Kaplan-Meier survival analysis, groups were compared using the Log rank test, and categories were compared using adjusted 2-sided Fisher exact test.

## RESULTS

### Concordance of RB1 FISH and Array CGH

We examined 34 GBMs by FISH and IHC and compared the results to corresponding array CGH and transcript profiling data (11). Eleven of 33 tumors (34%) exhibited deletion of at least 1 copy of RB1 and 2 tumors had homozygous deletion; 1 tumor did not have adequate tissue for evaluation (Fig. 1A, C, E). The FISH results were 100% concordant with array CGH data (11/11 tumors with homozygous or hemizygous loss of RB1 by FISH had decreased copy number of RB1 by array CGH; Table). Both cases with homozygous deletion of RB1 by FISH showed loss of transcript expression.

### Concordance of RB1 Immunohistochemistry and RB1 Expression Data

RB1 protein status was assessed in the same cohort of tumors by IHC (Figs. 1B, D, F, 2B, D, F). RB1-positive microglia/macrophages and endothelial cells served as internal positive controls. Due to tumor heterogeneity, including heterogeneity of tumor cell expression of RB1, known to be influenced by many factors including cell cycle (18, 19), and variation in density of the tumor-associated microglia/macrophages, tumors were determined to be RB1-intact if a minimum threshold of 20% of tumor nuclei were RB1-positive. In addition, immunostaining for microglia/macrophage marker Iba1 was used to highlight non-neoplastic

cells that retain RB1 protein (Fig. 2A, C, E, F). Loss of RB1 as determined by IHC (4 of 34 tumors; 11.8%) was very similar to RB1 loss based upon expression profiling with an overall concordance of 94% (31/33; Table). Although other non-neoplastic cells in the tumor microenvironment might express RB1, our threshold of 20% was sufficiently high to distinguish tumors with RB1 loss (mean RB1-positive nuclei 14.1% (range 11%-18%), n = 4) from tumors with intact but heterogeneous RB1-expression (mean RB1-positive nuclei 62% (range 36%-93%), n = 12, p < 0.001). Interestingly, one discrepant tumor lacking protein expression by IHC was hemizygotously deleted for RB1 by FISH and had a novel RB1 nonsense point mutation in the remaining allele (TCGA-08-0389). In that case, it seems likely that the TCGA expression data resulted from the detection of mutant RB1 transcript that does not encode detectable protein. In the case of TCGA-08-0512, the basis of undetectable protein expression is less clear. Nevertheless, these data suggest that RB1 IHC is highly effective for detecting alterations in RB1 protein expression.

### Concordance of RB1 FISH and Immunohistochemistry

Both tumors with homozygous deletions for RB1 were negative for RB1 by IHC (100%) (Fig. 3). Similarly, the 22/22 (100%) GBMs intact for RB1 by FISH were RB1-immunopositive. Of the 9 GBMs with hemizygous deletions of RB1 by FISH, 7 (78%) were positive by IHC. The remaining 2 hemizygotously deleted cases were negative by IHC, suggesting inactivation of the second allele with loss of RB1 protein expression. In fact, 1 of these tumors contained a novel nonsense mutation in RB1 further supporting that notion. The second tumor had no sequencing data available (Table). These data suggest that when there is homozygous deletion of RB1 by FISH, one can reasonably infer that the gene is inactivated whereas the gene is unlikely to be inactivated in cases with retained copy numbers. On the other hand, hemizygous deletion is unreliable for predicting tumor RB1 status because this assay alone cannot predict whether the retained allele is wild type or mutant.

### Associations of RB1 Status with GBM Subtype and Clinical Parameters

To determine the prevalence of loss of RB1 transcript expression in GBMs, we examined the available data from 170 tumors from TCGA (11). RB1 transcript expression was lost in 18 of 170 tumors (10.6%). To determine the frequency of RB1 loss in an independent set of GBMs, we immunostained an additional 51 FFPE GBMs. Similar to the frequency of RB1 loss noted for the TCGA analyzed tumors, we identified 5 GBMs with loss of RB1 protein expression (9.8%). GBMs have been stratified into different subtypes based upon patterns of genomic alteration and transcript expression level (20-22). To determine whether loss of RB1 expression was over-represented in any one GBM subgroup, we analyzed RB1 status in previously subtyped tumors (18). Loss of RB1 expression was a more frequent event in the proneural subtype (p < 0.01, adjusted 2-sided Fisher exact test; Fig. 4A). Furthermore, gene deletion of RB1 was more frequent in the proneural subtype, although this difference did not reach statistical significance (p = 0.08, adjusted 2-sided Fisher exact test). However, RB1 loss was not exclusive to the proneural subtype and occurred in all 4 subtypes. The clinical characteristics of patients with and without loss of RB1 expression were similar with respect to overall survival (Fig. 4B; n = 160), sex (female to male ratio of 64% vs. 58%, respectively; n = 170), and age (56 vs. 50 years, respectively; n = 170).

## DISCUSSION

GBM is a heterogeneous disease and stratification of patients based upon predicted response to specific agents may be critical for the success of targeted therapeutics. Because current diagnostic neuropathology relies on the analysis of FFPE material, broad applicability of a new assay currently requires it to be paraffin-based. Using FFPE material, we used FISH to

identify tumors with deletions of RB1 and IHC to identify tumors with loss of RB1 protein expression. In combination, the 2 methods provided information regarding potential mechanisms of RB1 alteration and RB1 status. Our data demonstrate loss of RB1 protein in roughly 10% of GBMs, consistent with previous estimates (11, 19).

Loss of heterozygosity of the RB1 gene on chromosome 13q is a relatively common event in astrocytoma (11, 23, 24). In a subset of tumors, alteration of the remaining RB1 allele and subsequent inactivation of RB1 disrupts the p16-CDK4/6-RB1 pathway (8, 19, 25, 26). The most common alteration of this pathway, homozygous CDKN2A deletion, results in suppression of RB1 protein function via elevated CDK4/6 activity. CDK4/6 is an attractive therapeutic target in tumors lacking CDKN2A/p16 function, but not in tumors lacking RB1 protein. Using IHC on clinical tumor samples we stratified patients into an RB1-intact group that was expected to benefit from CDK4/6 inhibitors and a group with loss of RB1 expression that would not be expected to benefit. IHC was a reliable method to detect RB1 status as predicted by our analysis of RB1 copy number by FISH and large-scale genomic data from TCGA, including expression array, array CGH, and sequencing (11). These data support the use of IHC to identify RB1 status for potential stratification of patients for clinical trials. Furthermore, they confirm the robust nature of the TCGA data and demonstrate its usefulness in the development and validation of clinical tests for patient stratification. In fact, expression array data available on the TCGA Data Portal (downloaded October 24, 2011) predict that 47 of 424 (11.1%) tumors analyzed have loss of RB1 expression. This estimate is consistent with our data showing loss of RB1 protein expression in 9 of 85 (10.6%) GBMs.

Two of 33 tumors were identified with hemizygous gene deletion by FISH but absent protein expression. Typical of tumor suppressor genes, multiple mechanisms may explain complete loss of RB1 protein in the setting of a hemizygous deletion, including point mutation, deletion, or alterations in transcriptional or translational regulation (27, 28). Consistent with this line of reasoning, sequencing of one of these cases demonstrated a novel nonsense point mutation in the remaining intact copy of RB1 predicted to result in a premature stop codon. Sequencing data was not available from the second case. We did not identify any tumors with loss of RB1 protein expression in the absence of gene deletion, but such occurrences have been described in other cancers (29, 30). Our data indicate that determination of RB1 protein status in tumor tissue reliably identifies the majority of cases with homozygous RB1 gene inactivation. It is possible, however, that in some cases epitope expression may persist in the context of a truncated, dysfunctional protein, yielding a false negative result. Such results are anticipated to be very infrequent.

We did not identify an association between RB1 status and survival in either our subset of tumors analyzed by IHC or in the 170 TCGA tumors analyzed by expression array. Although some reports have suggested such an association in GBM (31), RB1 may have a more direct correlation with prognosis in lower-grade astrocytomas including World Health Organization grade II and III tumors (14, 15, 32). Our finding that alterations in RB1 are more common in the proneural subtype of GBM may reflect similarities between genetic alterations in a subset of GBMs and lower-grade astrocytomas. Future studies will address this possibility and determine the utility of RB1 FISH and IHC for patient stratification in both low- and high-grade astrocytomas.

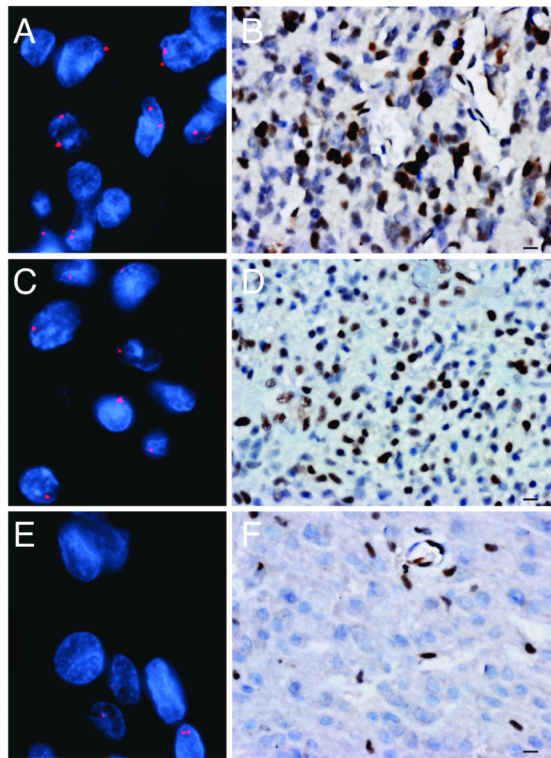
## Acknowledgments

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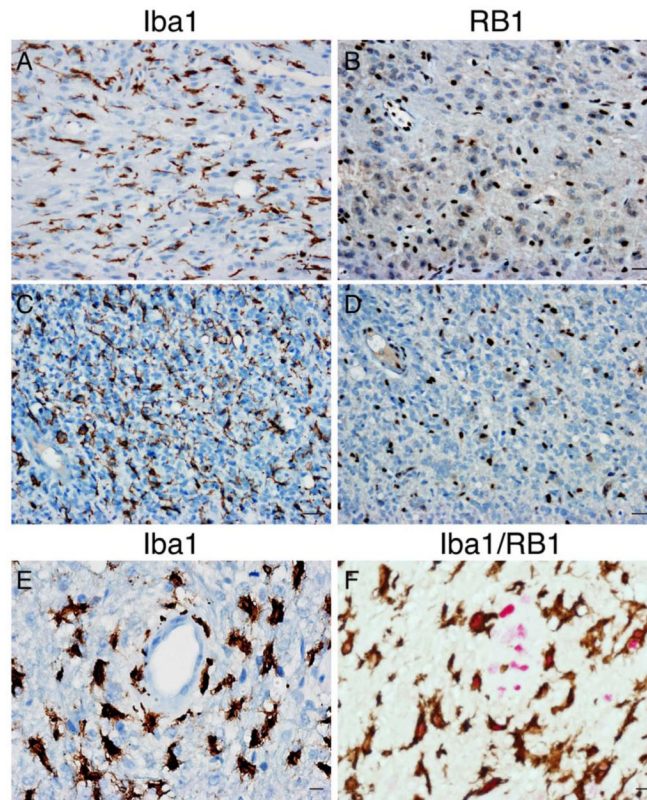
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**Figure 1.**

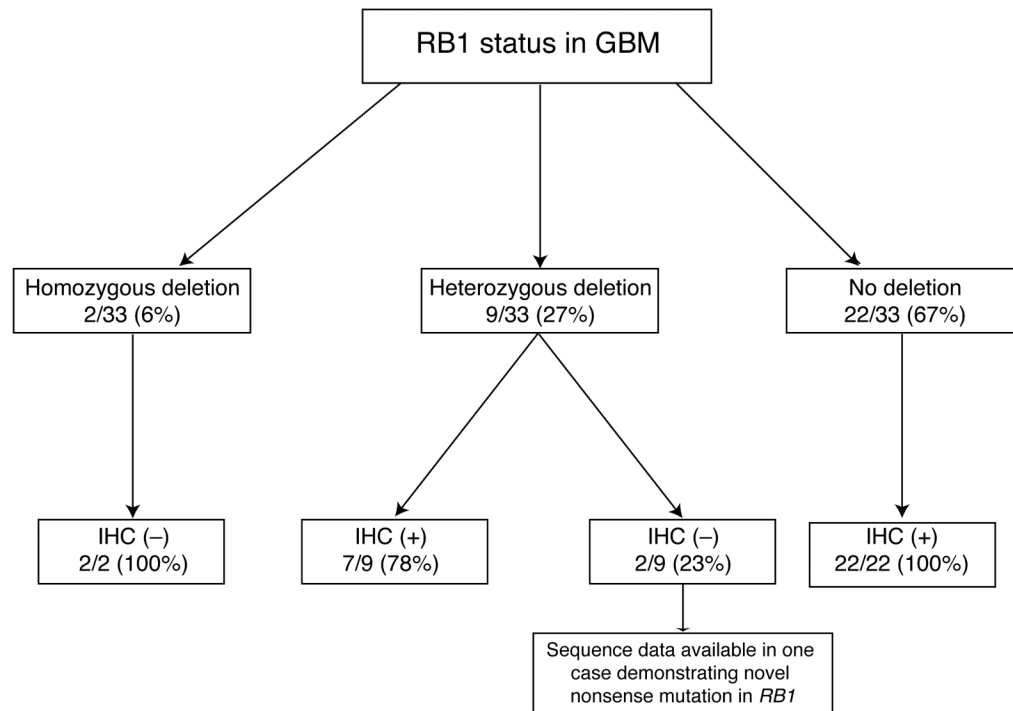
Determination of RB1 status in glioblastoma (GBM) by fluorescence in situ hybridization (FISH) and immunohistochemistry (IHC). Representative images of tumors analyzed for RB1 copy number by FISH (red) and for RB1 protein expression by IHC (brown). **(A-D)** Cells of tumors with intact RB1 primarily have 2 signals per nucleus by FISH **(A)** and are positive for RB1 by IHC **(B)**. A hemizygotously deleted tumor primarily has 1 RB1 signal per nucleus by FISH **(C)** and is positive for RB1 by IHC **(D)**. A homozygotously deleted tumor primarily has no signal for RB1 by FISH **(E)**. The presence of 2 signals in nucleus at bottom right likely represents a non-neoplastic cell and no positivity for RB1 protein by IHC **(F)**. The positive nuclei of endothelial and inflammatory cells provide a positive internal control. Bar: **A, C, E** = 10  $\mu$ m; **B, D, F** = 40x.





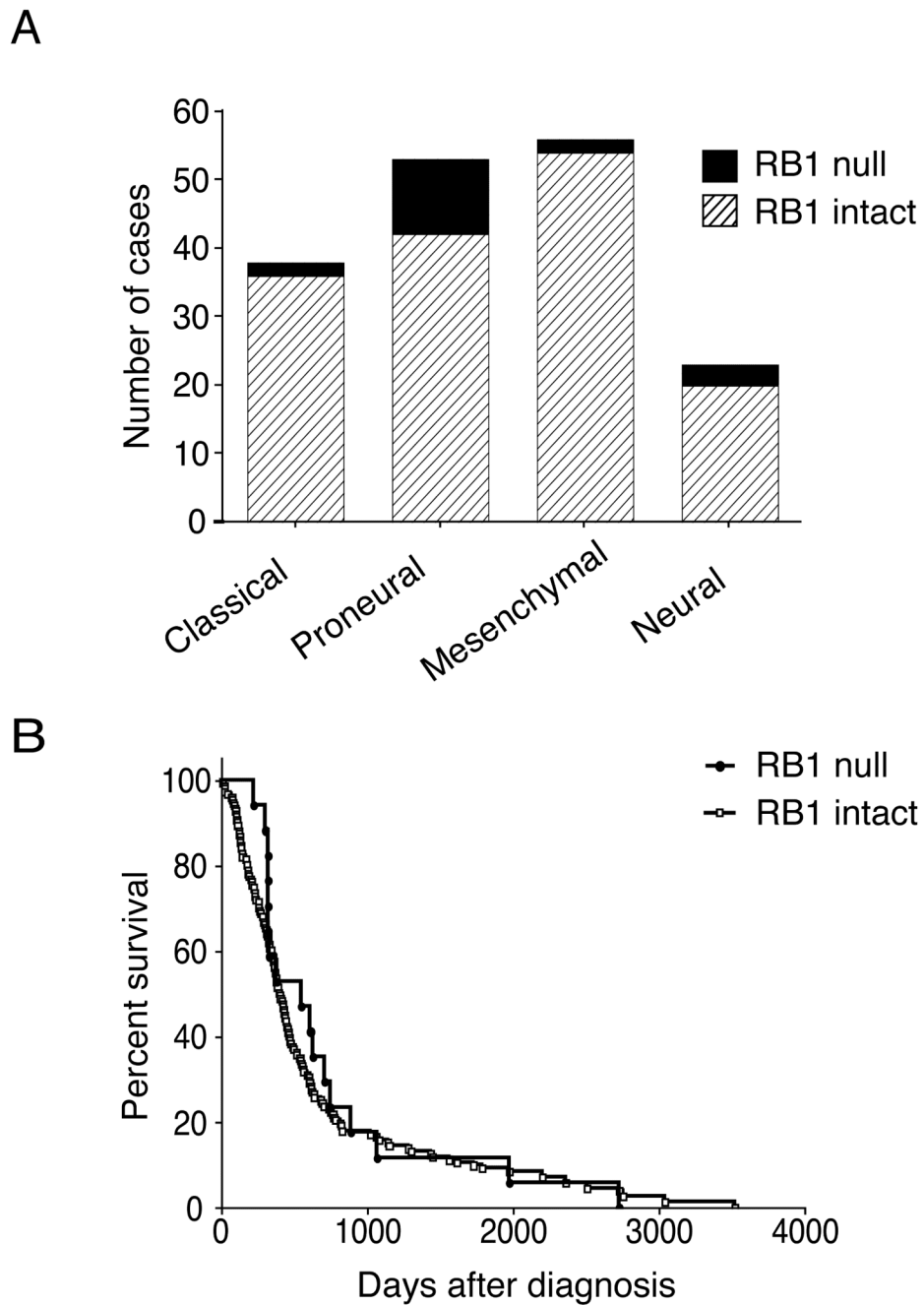
**Figure 2.**

Assessment of RB1 protein loss in tumor cells. (A-E) Two RB1-immunonegative tumors were either homozygous (A, B) or hemizygous (C, D) deleted for RB1 by fluorescence in situ hybridization. A cut-off value for RB1-positivity of 20% tumor nuclei was used to distinguish RB1-negative tumor cells (B, D) from RB1-positive non-neoplastic elements including endothelial cells and microglia/macrophages (A, C, E; Iba1-positive). Tumor cell nuclei are generally larger than those of non-neoplastic cells. (F) Double immunostaining for Iba1 (brown) and RB1 (red) highlights RB1-positive microglia/macrophages. Bars: A-D = 30  $\mu$ m; E, F = 10  $\mu$ m.



**Figure 3.**

Agreement between fluorescence in situ hybridization (FISH) and immunohistochemistry (IHC) for detecting RB1 loss in 33 glioblastomas (GBM). IHC and FISH showed high concordance when FISH showed either retained copy numbers or homozygous deletions. The majority of tumors with hemizygous RB1 deletion by FISH retained RB1 expression by IHC.



**Figure 4.** Loss of RB1 transcript expression is associated with the proneural glioblastoma (GBM) subtype, but is not associated with altered survival. **(A)** Loss of RB1 transcript expression was identified in 18 of 168 (10.5%) of The Cancer Genome Atlas (TCGA) tumors; these were over-represented in the proneural subtype as defined by Verhaak et al (18) ( $p < 0.01$ , adjusted two-sided Fisher exact test). **(B)** Loss of RB1 transcript expression was not associated with a difference in overall survival on univariate analysis (11).

Concordance of RB1 Status as Determined by Fluorescence In Situ Hybridization and Immunohistochemistry Compared to *The Cancer Genome Atlas*

Table

Tumor ID	RB1 deletion by FISH	Copy number (log <sub>2</sub> ratio) <sup>a</sup>	RB1 protein by IHC <sup>b, c</sup>	RB1 expression (log <sub>2</sub> ratio) <sup>d</sup>	RB1 somatic mutation <sup>e</sup>	GBM Subtype <sup>f</sup>
08-0520	Homozygous	-2.406	0	-0.255	NA	N
08-0350	Homozygous	-2.146	0	-0.145	NA	P
08-0386	Hemizygous	-0.399	0	-0.33	NA	N
08-0389	Hemizygous	-0.682	0	0.093	Novel nonsense mutation	NA
08-0349	Hemizygous	-0.638	1	0.036	NA	N
08-0359	Hemizygous	-0.549	1	0.584	No	P
08-0347	Hemizygous	-0.473	1	0.544	No	P
08-0511	Hemizygous	-0.704	2	0.474	NA	C
08-0517	Hemizygous	-0.828	2	0.515	NA	P
08-0516	Hemizygous	-0.606	2	0.402	NA	NA
08-0357	Hemizygous	-0.571	2	0.735	NA	C
08-0246	Not Del	-0.322	1	0.835	No	C
08-0512	Not Del	-0.302	1	-0.7509	NA	M
08-0360	Not Del	-0.270	1	0.648	No	M
08-0375	Not Del	-0.050	1	0.691	No	C
08-0380	Not Del	-0.041	1	0.959	No	
08-0518	Not Del	-0.036	1	1.006	NA	C
08-0356	Not Del	-0.032	1	1.061	NA	NA
08-0355	Not Del	-0.016	1	1.016	NA	C
08-0385	Not Del	NA	1	0.532	No	P
08-0345	NA	-0.080	2	1.333	NA	NA
08-0510	Not Del	-0.132	2	1.108	NA	M
08-0514	Not Del	-0.088	2	1.063	NA	C
08-0353	Not Del	-0.083	2	1.158	No	NA
08-0390	Not Del	-0.003	2	1.148	No	M
08-0522	Not Del	0.048	2	0.986	NA	M
08-0392	Not Del	-0.083	2	0.625	NA	M

Tumor ID	RB1 deletion by FISH	Copy number (log2 ratio) <sup>a</sup>	RB1 protein by IHC <sup>b, c</sup>	RB1 expression (log2 ratio) <sup>d</sup>	RB1 somatic mutation <sup>e</sup>	GBM Subtype <sup>f</sup>
08-0531	Not Del	-0.063	2	0.777	NA	C
08-0244	Not Del	-0.056	2	0.711	NA	NA
08-0521	Not Del	-0.004	2	0.591	NA	NA
08-0358	Not Del	-0.003	2	0.85	NA	C
08-0344	Not Del	0.020	2	0.615	No	P
08-0525	Not Del	0.041	2	1.399	NA	NA
08-0381	Not Del	NA	2	NA	NA	NA

CGH, comparative genomic hybridization; FISH, fluorescence in situ hybridization; GBM, glioblastoma; IHC, immunohistochemistry; NA, data not available; No Del, no deletion; RB1, Retinoblastoma Tumor Suppressor Protein; TCGA, The Cancer Genome Atlas.

<sup>a</sup>Copy number [Tumor/Normal] data from TCGA, HMS.harvard HG-CGH-244A; gene deletion defined as  $[\log_2(\text{Tumor/Normal})]$  less than or equal to  $-0.4$  (fold change of copy number in tumor vs. normal less than or equal to 0.758) (11).

<sup>b</sup>Mean IHC score for tumors with decreased RB1 copy number by array CGH was significantly different than the mean IHC score for tumors with intact RB1 (0.95 vs. 1.48,  $p < 0.05$ ;  $n = 32$ ).

<sup>c</sup>The mean IHC score for tumors with loss of RB1 transcript expression was significantly different than the mean IHC score for tumors with intact RB1 expression (0 vs. 1.42,  $p < 0.00005$ ;  $n = 33$ ).

<sup>d</sup>Expression [Tumor/Normal] data from TCGA, AgilentG4502A\_07; loss of RB1 expression defined as  $[\log_2(\text{Tumor/Normal})]$  less than 0 (fold-change of expression in tumor vs. normal less than 1).

<sup>e</sup>Sequencing data from TCGA, hgsc.bcm.edu (11).

<sup>f</sup>GBM subtype as determined by Verhaak et al (18). P, proneural; N, M, Mesenchymal; C, Classical