Identification of p18\textsuperscript{\text{INK4c}} as a Tumor Suppressor Gene in Glioblastoma Multiforme

David A. Solomon,\textsuperscript{1} Jung-Sik Kim,\textsuperscript{1} Sultan Jenkins,\textsuperscript{1} Habtom Ressom,\textsuperscript{1} Michael Huang,\textsuperscript{2} Nicholas Coppa,\textsuperscript{1} Lauren Mabanta,\textsuperscript{1} Darell Bigner,\textsuperscript{1} Hai Yan,\textsuperscript{3} Walter Jean,\textsuperscript{2} and Todd Waldman\textsuperscript{1}

\textsuperscript{1}Lombardi Comprehensive Cancer Center, Department of Neurosurgery, Georgetown University School of Medicine, Washington, District of Columbia, and \textsuperscript{2}Department of Pathology, Duke University School of Medicine, Durham, North Carolina

Abstract

Genomic alterations leading to aberrant activation of cyclin/cdk complexes drive the pathogenesis of many common human tumor types. In the case of glioblastoma multiforme (GBM), these alterations are most commonly due to homozygous deletion of p16\textsuperscript{INK4a} and less commonly due to genomic amplifications of individual genes encoding cyclins or cdks. Here, we describe deletion of the p18\textsuperscript{INK4c} cdk inhibitor as a novel genetic alteration driving the pathogenesis of GBM. Deletions of p18\textsuperscript{INK4c} often occurred in tumors also harboring homozygous deletions of p16\textsuperscript{INK4a}. Expression of p18\textsuperscript{INK4c} was completely absent in 43% of GBM tumors also harboring homozygous deletions of p16\textsuperscript{INK4a}. Expression of p18\textsuperscript{INK4c} was completely absent in 43% of GBM primary tumors studied by immunohistochemistry. Lentiviral infection of GBM cells with p18\textsuperscript{INK4c} was successfully achieved in 5/9 GBM xenografts, which lead to a senescence-like G\textsubscript{1} cell cycle arrest. These studies identify p18\textsuperscript{INK4c} as a GBM tumor suppressor gene, revealing an additional mechanism leading to aberrant activation of cyclin/cdk complexes in this terrible malignancy. [Cancer Res 2008;68(8):2564–9]...
Homozygous deletion of p18<sup>INK4c</sup> in GBM. In an effort to identify novel copy number alterations that drive the pathogenesis of GBM, we initially interrogated genomic DNA derived from 35 GBM cell lines and xenografts with Affymetrix SNP microarrays, as described in Materials and Methods. This analysis revealed focal deletions of chromosome 1p in 7 of 35 (20%) of samples (Fig. 1A). This ~200- to 300-kb region of chromosome 1 contains two annotated genes—p18<sup>INK4c</sup> and FAF1. p18<sup>INK4c</sup> is a cdk inhibitor that binds to and inhibits cdk4 and cdk6, and is a known tumor suppressor in mice (11, 12). FAF1 binds to the intracellular domain of Fas and is a proapoptotic signal transduction molecule (13). To determine whether either of these genes was the likely target of the deletion, we examined the copy number information at individual probesets to identify the consensus region of deletion (Supplementary Fig. S1). This analysis revealed that three probesets were deleted in all samples studied, narrowing the consensus region of deletion to a 56- to 133-kb interval containing only p18<sup>INK4c</sup>. These deletions of p18<sup>INK4c</sup> were confirmed by PCR and qPCR analysis using primer pairs specific to p18<sup>INK4c</sup> exon 1 (Supplementary Fig. S2; Fig. 1B). Finally, Western blot analysis showed the absence of p18<sup>INK4c</sup> expression in GBM cells with confirmed homozygous deletions (Supplementary Fig. S3).

p18<sup>INK4c</sup> deletions in primary tumor samples from the Cancer Genome Atlas. The NIH has recently sponsored a large-scale project to perform genomic analysis on GBM and other tumor types. As part of this project, they have recently released raw Affymetrix SNP microarray data on 106 primary GBM tumors. We analyzed these data with dCHIP to determine the copy number status of p18<sup>INK4c</sup> in primary GBM tumors. As depicted in Fig. 1C, deletions of p18<sup>INK4c</sup> were present in five tumors (5%). These deletions were not present in constitutional DNA from these patients studied with the same Affymetrix SNP microarrays (data not shown), demonstrating that the deletions are somatic. The available clinical and pathologic details of these samples are presented in Supplementary Fig. S4. These data show that deletions of p18<sup>INK4c</sup> are identifiable in uncultured primary human GBM samples, albeit at a lower apparent frequency than in our cultured samples.

Admixed nonneoplastic cells complicate the identification of p18<sup>INK4c</sup> deletions in uncultured GBMs. We hypothesized that the lower apparent frequency of p18<sup>INK4c</sup> deletions in uncultured GBM samples was due, at least in part, to the presence of admixed nonneoplastic human cells that are eliminated during ex vivo growth. To directly test this, we performed SNP microarray analysis on a primary GBM tumor and a matched first-passage xenograft that we derived from the tumor. Deletion of p18<sup>INK4c</sup> was easily
detectable in the first passage xenograft but was much less apparent (and would have been missed) in the primary tumor from which the xenograft was derived (Fig. 2A). This experiment clearly shows that the presence of admixed normal human cells is one factor that confounds the identification of p18\textsuperscript{INK4c} deletions in primary tumors. Xenograft growth eliminates admixed nonneoplastic human cells, enabling more efficient detection of p18\textsuperscript{INK4c} deletions with available technologies.

**Heterogeneity of p18\textsuperscript{INK4c} deletion in GBM.** Despite eliminating the influence of admixed nonneoplastic human cells by \textit{ex vivo} growth, we did not observe \textit{complete} copy number reduction at the p18\textsuperscript{INK4c} locus in either GBM xenografts or uncultured primary tumors (Fig. 1A and C). To determine whether this was an issue with sample quality or instead reflected a more fundamental underlying biology, we examined the copy number at the p16\textsuperscript{INK4a} locus in the same GBM samples that harbored deletions of p18\textsuperscript{INK4c}. Deletions of p16\textsuperscript{INK4a} were present in 12 of these 14 samples (86%). As expected, in cell lines, the copy number for \textit{both} p16\textsuperscript{INK4a} and p18\textsuperscript{INK4c} was zero, reflecting the homogeneity of cultured cell lines (compare Fig. 2B to identical samples in Fig. 1A). In contrast, in more genetically heterogeneous xenografts and uncultured primary tumors, the copy number for p16\textsuperscript{INK4a} was near zero, whereas the copy number for p18\textsuperscript{INK4c} was 0.5 to 1.0 (compare Fig. 2B to identical samples in Fig. 1A and C). This analysis clearly shows that GBMs are homogeneous with regard to p16\textsuperscript{INK4a} deletions and more heterogeneous with regard to p18\textsuperscript{INK4c} deletions.

**Loss of p18\textsuperscript{INK4c} expression in GBM primary tumors.** We next used immunohistochemistry to measure p18\textsuperscript{INK4c} expression in individual cells in primary GBM tumors. In particular, we measured the expression of p18\textsuperscript{INK4c} protein in 35 primary GBM specimens as part of a GBM tissue microarray (Fig. 3A). Remarkably, expression of p18\textsuperscript{INK4c} was completely lost in 15 of the 35 (43%) samples studied by immunohistochemistry (examples in \textit{i} and \textit{ii}). In samples expressing p18\textsuperscript{INK4c}, staining was primarily nuclear, with some cytoplasmic staining observable as has been reported by Bartkova et al. (14). By comparison, expression of p16\textsuperscript{INK4a} (known to be commonly deleted in GBM) was lost in 21 of the 35 (60%) samples studied (examples in \textit{iii} and \textit{iv}).

**Figure 2.** Heterogeneity of p18\textsuperscript{INK4c} deletions in GBM. \textbf{A}, copy number analysis of Affymetrix SNP microarray data shows deletion of p18\textsuperscript{INK4c} in a first-passage xenograft that is largely obscured by admixed normal cells in the primary tumor from which it was derived. \textbf{B}, copy number analysis of the p18\textsuperscript{INK4c} gene in cell lines (left), xenografts (middle), and primary tumors (right) harboring deletions of p18\textsuperscript{INK4c} reveals striking homogeneity with regard to p18\textsuperscript{INK4c} deletion in all sample types.
microscopy (Fig. 4C), and staining for senescence-associated β-galactosidase activity (Fig. 4D).

Infection with the p18\textsuperscript{INK4c} lentivirus led to a physiologic level of expression, comparable with the endogenous levels of expression found in M059J GBM cells harboring an intact p18\textsuperscript{INK4c} gene (Fig. 4A). Importantly, ectopic expression of p18\textsuperscript{INK4c} led to a rapid and complete senescence-like G\textsubscript{1} cell cycle arrest in cells with homozygous deletions of p18\textsuperscript{INK4c} but not in cells with an intact p18\textsuperscript{INK4c} gene (Supplementary Fig. S7; Fig. 4B–D; data not shown). Interestingly, expression of p18\textsuperscript{INK4c} in p18\textsuperscript{INK4c}-deficient SNB19 cells led to rapid and complete cell death (Fig. 4C).

Discussion

Here, we identify p18\textsuperscript{INK4c} as a new GBM tumor suppressor gene by describing homozygous deletions in GBM cell lines, xenografts, and primary tumors, and complete loss of expression in 43% of GBMs studied by immunohistochemistry. Furthermore, we show that re-expression of p18\textsuperscript{INK4c} at physiologic levels in GBM cells that lack it leads to immediate senescent-like arrest in the G\textsubscript{1} phase of the cell cycle.

p18\textsuperscript{INK4c} is a member of the INK4 family of cdk inhibitors, which includes p16\textsuperscript{INK4a}, p15\textsuperscript{INK4b}, p18\textsuperscript{INK4c}, and p19\textsuperscript{INK4d}. Members of this family bind to cdk4 and cdk6 and inhibit their ability to bind to D-type cyclins, thereby inhibiting the formation of an active cdk/cyclin complex and leading to cell cycle arrest. Deletions at the p18\textsuperscript{INK4c} locus have previously been implicated in the pathogenesis of other tumor types, including those of the brain (12, 15–18). Intriguingly, loss of both p18\textsuperscript{INK4c} and PTEN (both GBM tumor suppressors) has been shown to have synergistic effects on tumor formation in mice (19).

INK4 family members differ from each other in their patterns of expression and in the potency with which they bind individual cyclin/cdk complexes. p18\textsuperscript{INK4c} is thought to bind most potently to cdk6-specific complexes, although there is conflicting data on this point (11). The phenotypic consequences of binding to and inhibiting cdk4 and cdk6 with differing affinities are not well-understood.

We have shown that the presence of admixed nonneoplastic cells and intratumoral heterogeneity complicates the efficient identification of p18\textsuperscript{INK4c} deletions in uncultured primary tumors, using conventional technologies. However, it is also a formal possibility that greater frequency of p18\textsuperscript{INK4c} deletions in cell lines and xenografts are artifacts of \textit{ex vivo} culture.

It is also notable that the same GBM samples harboring heterogeneous deletions of p18\textsuperscript{INK4c} also often harbor remarkably homogeneous deletions of p16\textsuperscript{INK4a}. This finding was important as it enabled us to rule out issues of sample quality as a trivial explanation for our finding of heterogeneity. Furthermore, it suggests that homozygous deletion of p16\textsuperscript{INK4a} is an early event in the pathogenesis of GBM, whereas inactivation of p18\textsuperscript{INK4c} seems to occur later in the neoplastic process.
p18INK4c seems to be inactivated in GBM predominantly by homozygous deletion. This is similar to the situation for p16INK4a, in which homozygous deletion is the major mechanism of inactivation in GBM (although point mutations in p16INK4a also occur, albeit at a lower frequency; ref. 20). In the case of p16INK4a, this has been rationalized by suggesting that there is selection pressure for loss of p14ARF as well. It is possible that there is similar selection pressure for simultaneous codeletion of p18INK4c and FAF1 (or an adjacent as yet uncharacterized gene or noncoding RNA) during the pathogenesis of GBM.

In summary, here we have identified p18INK4c as a tumor suppressor gene that is genetically inactivated by homozygous deletion during the pathogenesis of GBM. Additional detailed studies are warranted to identify the phenotypic consequences of p18INK4c deletion during the pathogenesis of GBM.

Figure 4. Reconstitution of p18INK4c leads to senescence in GBM cells. A, Western blot for p18INK4c 24 h postinfection shows lentiviral reconstitution of p18INK4c expression in p18INK4c-null LN229, U87MG, and T98G GBM cells (SNB19 not shown). The level of reconstituted expression is only slightly higher than the endogenous levels present in p18INK4c-proficient M059J cells. B, cell cycle distributions 60 h postinfection with control or p18INK4c-expressing lentiviruses. Infection with p18INK4c lentivirus causes G1 cell cycle arrest in p18INK4c-deleted LN229 and T98G cells but not in p18INK4c-proficient M059J cells. C, phase contrast microscopy of cells 7 d postinfection shows that reconstitution of p18INK4c expression in p18INK4c-deficient LN229 and T98G cells leads to morphologic changes resembling senescence, whereas expression of p18INK4c in SNB19 cells leads to frank cell death. D, reconstitution of p18INK4c expression in LN229 cells leads to induction of senescence-associated 1-galactosidase activity. Similar results were observed in T98G cells but not in p18INK4c-proficient M059J cells (data not shown).

Acknowledgments

Received 11/28/2007; revised 1/24/2008; accepted 2/11/2008.

Grant support: Georgetown University School of Medicine (T. Waldman and W. Jean), a training grant T32-CA009686 from the National Cancer Institute, NIH (S. Jenkins), and the Lombardi Comprehensive Cancer Center is funded by P30-CA051008 from the National Cancer Institute, NIH.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

We thank Michelle Lombard and Karen Creswell for assistance with flow cytometry, Aaron Foxworth and Syid Abdullah for assistance with animal husbandry, Marcela White of the Brain Tumor Tissue Bank at the London Health Sciences Centre in Ontario, Canada for her assistance with the procurement of high quality GBM specimens, and Yardena Samuels for her comments on the manuscript.

The results published here are, in part, based on data generated by The Cancer Genome Atlas pilot project established by the National Cancer Institute and National Human Genome Research Institute. Information about The Cancer Genome Atlas and the investigators and institutions who constitute The Cancer Genome Atlas research network can be found at http://cancergenome.nih.gov.
References


Identification of p18\textsuperscript{INK4c} as a Tumor Suppressor Gene in Glioblastoma Multiforme

David A. Solomon, Jung-Sik Kim, Sultan Jenkins, et al.


Updated version
Access the most recent version of this article at:
doi:10.1158/0008-5472.CAN-07-6388

Supplementary Material
Access the most recent supplemental material at:
http://cancerres.aacrjournals.org/content/suppl/2008/04/15/0008-5472.CAN-07-6388.DC1

Cited articles
This article cites 20 articles, 14 of which you can access for free at:
http://cancerres.aacrjournals.org/content/68/8/2564.full.html#ref-list-1

Citing articles
This article has been cited by 12 HighWire-hosted articles. Access the articles at:
/content/68/8/2564.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.