Targetable Signaling Pathway Mutations Are Associated with Malignant Phenotype in IDH-Mutant Gliomas

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Abstract

**Purpose:** Isocitrate dehydrogenase (IDH) gene mutations occur in low-grade and high-grade gliomas. We sought to identify the genetic basis of malignant phenotype heterogeneity in IDH-mutant gliomas.

**Methods:** We prospectively implanted tumor specimens from 20 consecutive IDH1-mutant glioma resections into mouse brains and genotyped all resection specimens using a CLIA-certified molecular panel. Gliomas with cancer driver mutations were tested for sensitivity to targeted inhibitors in vitro. Associations between genomic alterations and outcomes were analyzed in patients.

**Results:** By 10 months, 8 of 20 IDH1-mutant gliomas developed intracerebral xenografts. All xenografts maintained mutant IDH1 and high levels of 2-hydroxyglutarate on serial transplantation. All xenograft-producing gliomas harbored “lineage-defining” mutations in CIC (oligodendroglioma) or TP53 (astrocytoma), and 6 of 8 additionally had activating mutations in PIK3CA or amplification of PDGFRA, MET, or N-MYC. Only IDH1 and CIC/TP53 mutations were detected in non-xenograft-forming gliomas (P = 0.0007). Targeted inhibition of the additional alterations decreased proliferation in vitro. Moreover, we detected alterations in known cancer driver genes in 13.4% of IDH1-mutant glioma patients, including PIK3CA, KRAS, AKT, or PTEN mutation or PDGFRA, MET, or N-MYC amplification. IDH/CIC mutant tumors were associated with PIK3CA/KRAS mutations whereas IDH1/TP53 tumors correlated with PDGFRA/MET amplification. Presence of driver alterations at progression was associated with shorter subsequent progression-free survival (median 9.0 vs. 36.1 months; P = 0.0011).

**Conclusion:** A subset of IDH1-mutant gliomas with mutations in driver oncogenes has a more malignant phenotype in patients. Identification of these alterations may provide an opportunity for use of targeted therapies in these patients. *Clin Cancer Res; 20(11); 1–12. ©2014 AACR.*

Introduction

Shortly after the discovery of recurrent mutations in the isocitrate dehydrogenase 1 (IDH1) and IDH2 genes, it was recognized that IDH-mutant diffuse gliomas have a clinical phenotype distinct from IDH1-wild-type gliomas (1–5). More recently, 2 major genetic subtypes of IDH1-mutant glioma have been identified: one genetic lineage is defined by TP53 and α thalassemia/mental retardation syndrome x-linked (ATRX) mutations and strong correlation with astrocytic histology (1, 2, 5, 6), and a second lineage is characterized by concurrent mutations in homolog of Drosophila capicua (CIC), Far Upstream Element Binding Protein (FUBP1), and the telomerase reverse transcriptase (TERT) promoter as well as tight association with 1p/19q codeletion and oligodendroglioma histopathology (2, 5, 7–12). Notably, the genomic alterations that frequently occur in the more common IDH1-wild-type primary glioblastoma (GBM; WHO grade IV), including EGFR gene amplification and rearrangement, PTEN mutation, and CDKN2A-CDKN2B deletion, are rare in IDH1-mutant gliomas (2, 5, 12, 13). Thus, IDH1-mutant gliomas are thought to arise via a molecular pathway that is distinct from primary GBM (5, 6).

Although IDH1-mutant diffuse gliomas carry a relatively better prognosis (1–4), most ultimately transform to a more...
Translational Relevance

Recurrent mutations in the isocitrate dehydrogenase 1 (IDH1) and IDH2 genes identify gliomas with a better prognosis. However, most IDH-mutant gliomas eventually transform to more aggressive tumors and become fatal. The genetic events that drive this later malignant behavior are largely unknown. Here, we find that a subset of IDH-mutant gliomas acquire mutations in cancer driver genes when they recur. Presence of these mutations is associated with more rapid subsequent progression. We also find that genetic subtypes of IDH-mutant gliomas utilize distinct oncogenic pathways during transformation to more malignant tumors. Moreover, IDH-mutant gliomas with driver mutations preferentially establish orthotopic xenograft tumors in mouse brain and are sensitive to targeted inhibition of the mutant gene product. Identification of targetable recurrent driver mutations provides novel therapeutic possibilities for the patients with IDH-mutant glioma who are most in need of new treatments.

Herein, we hypothesized that additional “tertiary” genetic alterations, which have been noted to occur in IDH-mutant gliomas (7, 15, 26, 27), could be the drivers of the progressive malignant phenotype of IDH-mutant gliomas. We therefore tested 20 consecutive IDH-mutant glioma specimens from patients undergoing surgery at our institution for the ability to establish intracerebral xenografts in mice. We performed a comparative genetic analysis of all primary tumor specimens using a CLIA-certified molecular panel and discovered that the IDH-mutant gliomas that successfully established orthotopic xenografts (8 tumors) were enriched with additional tertiary oncogenic genetic alterations, including PIK3CA mutation and amplification of the PDGFRα, MET, and N-MYC genes. IDH-mutant glioma TICs generated from xenograft-forming tumors exhibit oncogenic addiction to tertiary mutations. In patients with IDH-mutant glioma, tertiary alterations seem to be acquired at the time of tumor progression and also associated with higher pathologic grade and shorter progression-free survival (PFS). These data indicate acquisition of tertiary alteration is associated with more aggressive tumor behavior and may predict the ability to establish intracerebral xenografts in mice.

Materials and Methods

Biologic samples and clinical data

From September 2011 to October 2012, we prospectively accrued 20 consecutive untreated (9 patients) and previously treated (11 patients) patients with IDH-mutant glioma undergoing resection at the Massachusetts General Hospital (MGH). Patients either had previously confirmed IDH1 mutation or were accrued if clinical suspicion of IDH-mutant glioma was high based on characteristic features such as young age, frontal location, and lesser degree of contrast enhancement and necrosis on neuroimaging (5). Progression of disease was confirmed by either tissue diagnosis or standard response criteria (28). All tumor samples and clinical information were collected under MGH institutional review board approved protocols, and informed consent was obtained from all patients. All mouse procedures were approved by the Subcommittee on Research Animal Care at MGH.

Glioma neurospheres and orthotopic xenografts

Fresh surgical specimens were enzymatically dissociated, and 2 to 5 × 10⁴ cells were stereotactically implanted into the right striatum of the brains of 7- to 10-week-old female severe combined immunodeficient mice as described (29). In some cases when an excess of tumor tissue was available, cells were briefly cultured in neurosphere medium as described (30) to enrich for TIC neurospheres, and a similar number of cells were implanted within 48 hours of in vitro culture. There was no notable difference in xenograft formation with either method. Mice were monitored for status twice per week and sacrificed when neurologic deficits became significant. A minimum 10-month observation period after implantation...
Driver Mutations in IDH1-Mutant Gliomas

was determined whether orthotopic xenografts developed, although all animals were sacrificed after 1 year to assess for tumor formation. Brains were removed for pathologic studies and tumors were excised to reestablish TIC neurosphere cultures. TICs were then either implanted into the brains of new mice or used for in vitro assays as described (29, 31).

**Histology and immunostaining**

Hematoxylin and eosin staining and immunohistochemistry (IHC) were performed on formalin-fixed paraffin-embedded (FFPE) sections as described (29, 31). Primary antibodies used for IHC were anti-IDH1 R132H (Dianova; 1:100), Ki-67 (Dako; 1:150), anti-CD31 (BD Pharmingen; 1:150), and anti-nestin (Santa Cruz; 1:400).

**Genotyping and FISH data**

Clinical molecular profiling was performed as described (32, 33). Briefly, the MGH SNaPshot assay is a multiplexed, PCR-based, single-base extension assay that interrogates 73 commonly mutated loci from 23 genes (AKT1, APC, BRAF, CTNNB1, EGFR, EML4-ALK, HER2, FGFR3, GNA11, GNAQ, GNAS, Hras, IDH1, IDH2, KIT, KRAS, MEK1, NOTCH1, NRAS, PIK3CA, PTEN, RET, TP53). Genomic PCR-based sequencing was used to sequence all coding exons of the IDH1, IDH2, PIK3CA, and PIK3R1 genes. PCR products were amplified from genomic DNA templates with Platinum Taq polymerase per manufacturer’s protocol using intron-based primers spanning the expressed coding sequences (Supplementary Table S2) then Sanger sequenced (Beckman Coulter Genomics). FISH assays for the EGFR, MET, and PDGFRα genes were performed using BAC probes CTD2113A18 (7p EGFR locus), CTB-13N12 (7q MET locus), CEP7 (centromere 7 control), RP11-58C6 (4q PDGFRα locus), and CEP4 (centromere 4 control) (Abbott) as described (32). BAC clone RP11-480N14 (chr2: 15991148-16158895) was used to make the N-MYC probe and ALK (2p23) Proximal Probe (Kreatech) was used for centromere 2 control. Gene/control probe copy number ratios of >2.0 were considered amplified. 1p and 19q status was determined using the Vysis 1p36/1q25 and 19q13/19p13 FISH Probe Kit (Abbott). 1p/1q and 19q/19p ratios of <0.75 were considered lost and >0.75 as maintained.

**Gas chromatography-mass spectroscopy data**

Ten to 20 mg of frozen tumor tissue was homogenized and extracted with methanol/chloroform. Metabolites were derivatized with N-(tert-butyldimethylsilyl)-N-methyl-trifluoroacetamide, with 1% tert-butyldimethylchlorosilane (Sigma) and analyzed on a 6890N GC system (Agilent Technologies) combined with a 5975B Inert XL MS system (Agilent Technologies). 2-Hydroxyglutarate (2HG) fragment 433 m/z and the glutamate fragment 432 m/z were used for further analysis (34). 2HG and glutamate concentrations were normalized to the internal standard norvaline and tissue weight as described (35).

**In vitro drug and shRNA inhibition studies**

Dissociated TICs were seeded to 96-well plates at 7,000 to 8,000 cells per well. Serially diluted inhibitor was added to wells, and cells were further cultured for 5 days. Viability of cells was measured by CellTiter-Glo assay (Promega) and the EC50 values were determined. All in vitro inhibitor assays were performed on TICs within 5 passages from dissociation from tumor. JQ1 and BYL719 were gifts from J.E. Bradner and J.A. Engelman, respectively. Crizotinib and sunitinib were obtained from Selleck Chemicals.

To knockdown N-myc expression, lentivirus vectors carrying shRNA for N-MYC (Sigma, pLKO.1) were packaged using ScreenFect (Wako)-mediated cotransfection of vector and packaging plasmid DNAs to 293T cells. MGG152 cells were infected with lentivirus in the presence of polybrene (6 μg/mL), selected with puromycin (0.5 μg/mL) for 3 days, and silencing confirmed with Western blot analysis. Cell viability and sphere formation were assessed 7 days later.

**Statistical analyses**

Statistical analysis was performed with JMP software. For parametric analyses, 2-tailed t tests were used and for analysis of frequencies of nominal data, 2-tailed Fisher exact test was used. Data are expressed as mean ± SD. Survival was analyzed by the Kaplan–Meier method with 2-sided log-rank statistics.

**Results**

**Establishment of intracerebral xenografts with endogenous IDH mutation**

We studied the potential of 20 serially collected patient IDH1-mutant glioma specimens to form xenograft after stereotactic implantation in immunocompromised mouse brains. Two tumors were low-grade (WHO grade II), 10 were anaplastic (WHO grade III), and 8 were GBMs, of which 3 were “secondary” GBMs that had transformed from a known lower-grade glioma (Table 1). All 20 gliomas in our study had IDH1 mutations, which occur far more frequently than IDH2 mutations in gliomas (2, 36). In parallel, we cultured TIC-enriched cells using the neurosphere system. Notably, 8 of 20 IDH1-mutant glioma tumors generated intracerebral xenografts, with the time to lethal xenograft ranging between 1 and 7 months (Fig. 1, Table 1 and Supplementary Fig. S1). All xenografts closely recapitulated parent human tumor phenotypes histologically (Fig. 1; Supplementary Figs. S1–S3, and Table 1). In vitro proliferation of the different lines varied, with doubling times ranging from 2.5 days (MGG152) to 10 days (MGG79, MGG108, MGG132). All lines could be passaged for at least 5 passages, however 2 lines (MGG119, MGG152) could be stably cultured for at least 4 months. All xenografts harbored endogenous IDH1 R132H mutation and retained the mutant allele upon serial passage in vitro or serial transplantation in orthotopic xenograft (Supplementary Figs. S4 and S5). Orthotopic xenografts were confirmed by GC-MS to have high-level production of the mutant-specific metabolite, 2HG (Supplementary Table S1).
Table 1. Orthotopically implanted patient IDH-mutant gliomas

<table>
<thead>
<tr>
<th>MGG</th>
<th>Histology</th>
<th>Sex</th>
<th>Age at Dx</th>
<th>Prior treatment</th>
<th>Time to xeno(^*) (Mos.)</th>
<th>IDH1 mutation</th>
<th>Confirmed secondary (lineage) alteration</th>
<th>MGMT</th>
<th>Tertiary alteration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Generated xenograft</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>60</td>
<td>GBM-O</td>
<td>F</td>
<td>45</td>
<td>None</td>
<td>4</td>
<td>R132H</td>
<td>CIC mutant</td>
<td>M</td>
<td>PK3CA H1047L</td>
</tr>
<tr>
<td>79</td>
<td>Recurrent AOA</td>
<td>M</td>
<td>33</td>
<td>RT, SRS</td>
<td>3</td>
<td>R132H</td>
<td>TP53 mutant</td>
<td>n.t.</td>
<td>PDGFRA amp</td>
</tr>
<tr>
<td>88</td>
<td>Secondary GBM</td>
<td>F</td>
<td>41</td>
<td>RT, TMZ x 10 cycles, LBH589+BEV, CPT-11+BEV</td>
<td>6</td>
<td>R132H</td>
<td>TP53 mutant</td>
<td>M</td>
<td>None detected</td>
</tr>
<tr>
<td>108</td>
<td>GBM-O</td>
<td>M</td>
<td>30</td>
<td>None</td>
<td>5</td>
<td>R132H</td>
<td>TP53 mutant</td>
<td>M</td>
<td>PK3CA R93Q</td>
</tr>
<tr>
<td>117</td>
<td>Secondary GBM</td>
<td>F</td>
<td>32</td>
<td>RT</td>
<td>3</td>
<td>R132H</td>
<td>TP53 mutant</td>
<td>U</td>
<td>PDGFRA amp</td>
</tr>
<tr>
<td>119</td>
<td>Secondary GBM</td>
<td>M</td>
<td>56</td>
<td>RT</td>
<td>7</td>
<td>R132H</td>
<td>TP53 mutant</td>
<td>M</td>
<td>None detected</td>
</tr>
<tr>
<td>132</td>
<td>Recurrent AOA</td>
<td>M</td>
<td>32</td>
<td>TMZ chemoradiation + 11 TMZ cycles</td>
<td>4</td>
<td>R132H</td>
<td>TP53 mutant</td>
<td>U</td>
<td>MET focal amp</td>
</tr>
<tr>
<td>152</td>
<td>GBM with PNET component</td>
<td>M</td>
<td>34</td>
<td>Gliadel, TMZ chemoradiation</td>
<td>1</td>
<td>R132H</td>
<td>TP53 mutant</td>
<td>M</td>
<td>N-MYC amp</td>
</tr>
<tr>
<td>No xenograft</td>
<td></td>
<td></td>
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<td></td>
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<td></td>
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<tr>
<td>71</td>
<td>AOA</td>
<td>M</td>
<td>51</td>
<td>None</td>
<td>—</td>
<td>R132H</td>
<td>1p/19q non-codel</td>
<td>M</td>
<td>None detected</td>
</tr>
<tr>
<td>78</td>
<td>LGO</td>
<td>M</td>
<td>41</td>
<td>None</td>
<td>—</td>
<td>R132H</td>
<td>1p/19q codel</td>
<td>M</td>
<td>None detected</td>
</tr>
<tr>
<td>81</td>
<td>GBM</td>
<td>M</td>
<td>27</td>
<td>None</td>
<td>—</td>
<td>R132H</td>
<td>TP53 mutant</td>
<td>M</td>
<td>None detected</td>
</tr>
<tr>
<td>82</td>
<td>AOA</td>
<td>M</td>
<td>34</td>
<td>TMZ x 12 cycles</td>
<td>—</td>
<td>R132H</td>
<td>1p/19q codel</td>
<td>U</td>
<td>None detected</td>
</tr>
<tr>
<td>83</td>
<td>Recurrent GBM</td>
<td>M</td>
<td>43</td>
<td>RT + gefitinib followed by gefitinib x 8 years</td>
<td>—</td>
<td>R132H</td>
<td>TP53 mutant</td>
<td>n.t.</td>
<td>None detected</td>
</tr>
<tr>
<td>96</td>
<td>Recurrent AOA</td>
<td>M</td>
<td>24</td>
<td>TMZ chemoradiation + 6 TMZ cycles, isotretinoin x 10 cycles</td>
<td>—</td>
<td>R132H</td>
<td>TP53 mutant</td>
<td>U</td>
<td>None detected</td>
</tr>
<tr>
<td>103</td>
<td>Recurrent AOA</td>
<td>M</td>
<td>26</td>
<td>PCV x 6 cycles then RT, TMZ x 7 cycles, BEV+TMZ x 12 cycles</td>
<td>—</td>
<td>R132H</td>
<td>1p/19q non-codel</td>
<td>M</td>
<td>None detected</td>
</tr>
<tr>
<td>109</td>
<td>Recurrent AO</td>
<td>F</td>
<td>35</td>
<td>TMZ x 12 cycles, RT</td>
<td>—</td>
<td>R132H</td>
<td>1p/19q codel</td>
<td>n.t.</td>
<td>None detected</td>
</tr>
<tr>
<td>116</td>
<td>AA</td>
<td>M</td>
<td>24</td>
<td>None</td>
<td>—</td>
<td>R132C</td>
<td>TP53 mutant</td>
<td>U</td>
<td>None detected</td>
</tr>
<tr>
<td>124</td>
<td>LGOA</td>
<td>F</td>
<td>38</td>
<td>None</td>
<td>—</td>
<td>R132C</td>
<td>1p/19q non-codel</td>
<td>U</td>
<td>None detected</td>
</tr>
<tr>
<td>126</td>
<td>AO</td>
<td>F</td>
<td>56</td>
<td>None</td>
<td>—</td>
<td>R132H</td>
<td>1p/19q codel</td>
<td>n.t.</td>
<td>None detected</td>
</tr>
<tr>
<td>130</td>
<td>AOA</td>
<td>F</td>
<td>52</td>
<td>None</td>
<td>—</td>
<td>R132H</td>
<td>1p/19q codel</td>
<td>n.t.</td>
<td>None detected</td>
</tr>
</tbody>
</table>

Abbreviations: MGG, mass general glioma; Dx, diagnosis; Xeno, xenograft; Mos., months; GBM-O, glioblastoma with oligodendroglioma component; AOA, anaplastic oligoastrocytoma; AO, anaplastic oligodendroglioma; AA, anaplastic astrocytoma; LGO, low-grade oligodendroglioma, LGOA, low-grade oligoastrocytoma; RT, radiation therapy; SRS, stereotactic radiosurgery; TMZ, temozolomide; BEV, bevacizumab; PCV, procarbazine, CCNU, Vinristine regimen; codel, codeleted; amp, amplified; M, methylated; U, unmethylated; n.t., not tested.

\(^*\)Months elapsed until first intracranial xenograft developed and became lethal.
We sought to identify the mechanisms accounting for the ability to generate orthotopic xenograft in our set of IDH-mutant gliomas. There was no clear distinction in host factors such as patient age, sex, or prior treatment (radiation or various combinations of radiation and different chemotherapies; Table 1) between gliomas that formed xenograft versus those that did not. Generally, tumors that were able to form xenografts had GBM (WHO grade IV) histology (6/8 xenografts vs. 2/12 in the non–xenograft-forming subset) and lacked 1p/19q codeletion (7/8 xenografts).

We conducted a comparative genetic analysis on the primary tumor specimens to assess whether additional oncogenic alterations might account for differences in the ability to generate xenograft. All 8 xenograft-forming tumors and 10 of 12 non–xenograft-forming tumors harbored the IDH1 R132H variant, which accounts for ~90% of the IDH1 mutations in gliomas (2, 12, 36), and the remaining 2 non–xenograft-forming gliomas harbored IDH1 R132C. Interestingly, one glioma xenograft (MGG79) was homozygous for IDH1 R132H mutation and the corresponding orthotopic xenograft produced high levels of 2HG (Supplementary Fig. S4 and Table S1). This finding is in contrast with a previous report suggesting a wild-type IDH1 allele is necessary for 2HG production (37).

Focused genotyping analysis of all implanted primary tumors was conducted with our CLIA-certified clinical molecular profiling platform (32), which surveys 25 commonly mutated cancer-associated genes. We specifically assessed for “tertiary” genetic alterations, which we defined as nonlineage mutations, because lineage-associated mutations (astrocytic ATRX/TP53 mutations or oligodendroglial...
CIC/TERT mutations and 1p/19q codeletion) occur in virtually all low-grade IDH-mutant gliomas. Strikingly, 6 of 8 xenograft-forming tumors harbored tertiary mutations whereas none were detected in non–xenograft-forming patient gliomas (P = .0007; Table 1).

The detected tertiary alterations included hotspot-activating mutations in PIK3CA (2/6) and high-level focal amplifications in the PDGFRA (2/6) and MET (1/6) genes (Table 1). In addition, we sequenced the entire coding regions of PIK3CA, which encodes p110α, and PIK3R1, which encodes the phosphoinositide 3-kinase (PI3K) regulatory subunit p85α, in all xenograft-forming tumors and detected no other mutations. Notably, one GBM had focal primitive neuroectodermal tumor (PNET) histopathology (MGG152; Fig. 1A) and was unusually malignant in both the patient (5-month overall survival) and in orthotopic xenograft (lethal within 1 month; Table 1). A previous study detected C-MYC or N-MYC amplification in a significant proportion of malignant gliomas-harboring PNET-like components (38); therefore, we interrogated these genes in MGG152 and identified high-level N-MYC amplification (Fig. 2A). We confirmed that all the tertiary genetic alterations detected in the primary tumors were maintained in the respective orthotopic xenografts (Fig. 2A), suggesting these mutations play a role in the tumor initiating capability of IDH1-mutant gliomas.

**Tertiary genetic alterations are drivers in IDH-mutant glioma xenografts**

We then tested the effect of small molecule inhibitors targeted at the identified tertiary mutations on in vitro proliferation. We examined the impact of BYL719, a
highly selective inhibitor of p110α (39), on the viability of MGG108 (PIK3CA R93Q) and MGG152 (PIK3CA/PIK3R1 wild type) TICs. We observed more potent inhibition of MGG108 than MGG152 cells (IC50 wild type) TICs. We observed more potent inhibition of MGG108 (with significantly shorter subsequent PFS (median 9.0 months; 95% CI, 35.7–73.0, without, 3A and B). Notably, there was no difference in PFS from the time of initial diagnosis (median 55.6 months; 95% CI, 24.6–93.1, with tertiary alteration vs. 54.1 months; 95% CI, 35.7–73.0, without, P = 0.76; Fig. 3A), implicating the tertiary alteration detected at progression as the driver of malignant degeneration in these patients. There was no detectable difference in overall survival; however, there were few deaths in either group (13/56 total patients), therefore longer follow-up time is needed to assess this endpoint.

Several tertiary genetic events were recurrently identified (Table 2), including activating mutations in PIK3CA (9 tumors) and KRAS (4 tumors) and PDGFRA amplification (4 tumors). PIK3CA and KRAS mutations were most often detected in tumors with at least a component of oligodendroglioma histology (7/9 PIK3CA mutant and 3/4 KRAS mutant tumors). Interestingly, all 4 PDGFRA-amplified tumors were purely astrocytic (P = 0.026). When specific oncogenes were compared by IDH genetic lineage rather than histopathology (within the set of tumors with tertiary mutations and confirmed lineage mutations), we identified significant associations between 1p/19q codeleted tumors and intracellular signaling pathway gene mutations (KRAS or PIK3CA, P = 0.002) and between receptor tyrosine kinase amplification (PDGFRA or MET) and non–1p/19q codeleted (TP53 mutant) tumors (P = 0.029).

To assess whether tertiary alterations are acquired at tumor progression, we examined paired tumor specimens from newly diagnosed and progressive tumors from 6 patients in whom we detected tertiary mutations. For 2 patients with PDGFRA amplification and 1 patient with focal MET amplification, we confirmed these alterations were present in the progressive but not in the initial tumor specimens (Fig. 3C). In 2 of 3 patients with PIK3CA mutant, the PIK3CA mutations were present only in the progressive tumor (Fig. 3C). The third patient (Patient 6 in Table 2) had PIK3CA E545G mutation in both the diagnostic and progressive tumor specimens. This patient was treated with 12 cycles of temozolomide and progressed just 24.6 months after diagnosis, a relatively rapid time to progression for low-grade oligodendrogliomas with 1p/19q codeletion.

Discussion

IDH-mutant diffuse gliomas nearly always progress after radiation therapy and chemotherapy and eventually transform to more malignant tumors. Effective therapeutic options at that stage are lacking. Herein we demonstrate that the subset of IDH-mutant gliomas that acquire an aggressive phenotype late in the disease are driven by specific tertiary oncogenic alterations. These tertiary alterations are associated with more malignant tumors in patients and increased tumor-forming ability in mice. Importantly, these tertiary alterations represent potential therapeutic targets for patients with IDH-mutant gliomas who are most in need of treatment.

Our data suggest that after acquisition of lineage-defining mutations, IDH-mutant gliomas may follow multiple pathways to transformation (Fig. 4). 1p/19q codeleted tumors tend to activate the PI3K/mTOR or Ras intracellular signaling pathways and TP53-mutant tumors tend to amplify growth factor receptor tyrosine kinases. Our findings are consistent with prior reports; recurrent PIK3CA mutations have been detected in a subset of IDH-mutant, 1p/19q codeleted anaplastic oligodendrogliomas (7, 26) and 2 recent reports observed enrichment of PDGFRA amplification in IDH-mutant versus IDH-wild-type primary GBM (27, 41). In addition, we find that activation of N-myc may
Table 2. Genetic alterations in *IDH*-mutant glioma patients at MGH

<table>
<thead>
<tr>
<th>Pt</th>
<th>Age at Dx</th>
<th>Sex</th>
<th>Initial histology</th>
<th>Initial WHO grade</th>
<th>OS (mos)</th>
<th>PFS (mos)</th>
<th><em>IDH1</em> mutation</th>
<th>Confirmed secondary (lineage) alteration</th>
<th>Treatment before detection of tertiary alteration</th>
<th>Tertiary alteration (amino acid substitution or copy number ratio)<em>a</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>60</td>
<td>M</td>
<td>Oligoastrocytoma</td>
<td>II</td>
<td>78.3b</td>
<td>21.9</td>
<td>R132H</td>
<td>codel</td>
<td>RT; TMZ (18 cycles)</td>
<td>AKT1 (E17K), KRAS (G12R), PIK3CA (H1047L)</td>
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<tr>
<td>2</td>
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<td>165.1b</td>
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<td>codel</td>
<td>TMZ (12 cycles); RT</td>
<td>KRAS (G13D)</td>
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<td>159.8b</td>
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<td>codel</td>
<td>PCV (6 cycles); RT</td>
<td>KRAS G12R</td>
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<td>codel</td>
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<td>KRAS (G12A)</td>
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<td>codel</td>
<td>TMZ (12 cycles)</td>
<td>PIK3CA (E545G)</td>
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<td>M</td>
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<td>62.4b</td>
<td>55.7</td>
<td>R132H</td>
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<td>PIK3CA (E542K)</td>
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<td>38</td>
<td>M</td>
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<td>74.4</td>
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<td>codel</td>
<td>Concurrent RT/TMZ + adjuvant TMZ (12 cycles); TMZ (10 cycles)</td>
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<td>no codel</td>
<td>PCV (6 cycles); RT</td>
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<td>F</td>
<td>GBM</td>
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<td>12.2b</td>
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<td>MET amplification (&gt;25:1 in 10–15% cells)</td>
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<tr>
<td>14</td>
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<td>GBM</td>
<td>IV</td>
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<td>93.2</td>
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<td>N-MYC amplification (&lt;25:1 in 1/50 cells)</td>
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<td>TP53 R273H</td>
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<td>PDGFRA amplification (&lt;25:1 in 1/200 cells)</td>
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<td>16</td>
<td>35</td>
<td>M</td>
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<td>III</td>
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<td>24.4b</td>
<td>R132H</td>
<td>TP53 R273C</td>
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<td>PDGFRA amplification (&gt;25:1)</td>
</tr>
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<td>M</td>
<td>Anaplastic astrocytoma</td>
<td>III</td>
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<td>57.5</td>
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<td>no codel</td>
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<td>M</td>
<td>GBM</td>
<td>IV</td>
<td>25.6b</td>
<td>25.6b</td>
<td>R132H</td>
<td>TP53 R273C</td>
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<td>PDGFRA amplification (&gt;25:1)</td>
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<td>II</td>
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<td>19.1</td>
<td>R132H</td>
<td>TP53 R273C</td>
<td>RT</td>
<td>PDGFRA amplification (&gt;25:1)</td>
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<td>61</td>
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<td>GBM-O</td>
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<td>9.4b</td>
<td>R132H</td>
<td>TP53 R273C</td>
<td>None</td>
<td>PDGFRA amplification (&gt;25:1)</td>
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Abbreviations: Pt, patient; Dx, diagnosis; OS, overall survival; mos, months; GBM-O, glioblastoma with oligodendroglioma component; codel, 1p/19q codeleted; TMZ, temozolomide; ddTMZ, dose dense temozolomide; RT, radiation therapy; NA, not available.

*a* Ratio of specific gene probe to centromere control.

*b* Event has not yet occurred.
be a particularly malignant transformation pathway in IDH-mutant glioma. A recent study similarly observed a correlation between c-Myc expression and shorter time to transformation within IDH-mutant gliomas (42). Together with these reports, our data suggests IDH-mutant gliomas may undergo a sequenced genetic evolution analogous to IDH2-mutant acute myeloid leukemia, where stepwise acquisition of distinct classes of mutations results in more aggressive disease (43).

The mechanisms by which IDH-mutant gliomas acquire tertiary mutations are largely unknown. A recent study observed that a subset of IDH-mutant low-grade gliomas developed a hypermutation phenotype (15, 44) after treatment with temozolomide. All of the hypermutated gliomas in their dataset harbored driver mutations, many in the PI3K/mTOR signaling pathway (15). In our dataset, 10 of 20 patients with tertiary mutation were previously treated with alkylating chemotherapy (Table 2). Five of our 10 chemotherapy-treated patients had PI3K/mTOR pathway mutations in their tumors, and interestingly one tumor had activating mutations in 3 oncogenes (PIK3CA, AKT, and KRAS), suggesting it may be hypermutated. However, 9 patients as well as 2 xenograft-forming tumors from our implantation study harbored tertiary alterations and had no prior treatment, indicating that genomic instability resulting from DNA-damaging therapy is not the sole evolutionary path to malignant progression in IDH-mutant gliomas. The drivers of tertiary mutation in untreated IDH-mutant tumors remain to be determined.

The overall frequency of tertiary mutations in our dataset was 13.4%, which may be reflective of the focused nature of our genotyping panel. Our data suggests resampling of tumor specimens at the time of progression may increase...
IDH-mutant gliomas develop and progress through an ordered sequence of oncogenic alterations. IDH-mutant gliomas are initiated by the occurrence of IDH1 or IDH2 mutation and widespread hypermethylation of CpG islands (CpG island methylator phenotype, CIMP) in a glial progenitor cell population. The subsequent acquisition of TP53 and ATRX mutations results in development along an astrocytoma pathway, whereas the codeletion of 1p and 19q (t(1;19)) along with the island methylator phenotype, CIMP) in a glial progenitor cell population. The subsequent acquisition of these "lineage-defining" mutations, IDH-mutant astrocytomas and oligodendrogliomas may follow several "tertiary" genetic pathways during the transformation to malignant or high-grade tumors. Astrocytomas tend to amplify growth factor receptor genes such as PDGFRA and MET, whereas oligodendrogliomas tend to develop activating mutations in intracellular signaling genes such as PIK3CA and KRAS. Alternative tertiary pathways include Myc activation and other undescribed mutations. Acquisition of tertiary genetic alterations may result in more malignant behavior. RTK, receptor tyrosine kinase; MAPK, mitogen-activated protein kinase.

By identifying recurrent, functionally significant tertiary genetic alterations, we set the stage for targeted therapy in IDH-mutant gliomas. Pharmacologic inhibitors for all of the tertiary alterations identified in our work are in development. Notably, our data suggests PDGFRA amplification warrants investigation in TP53-mutant, IDH-mutant gliomas. Outcomes for this subgroup are poor relative to the 1p/19q codeleted subtype (6) possibly because of the uncertain efficacy of chemotherapy in these tumors given their lack of 1p/19q codeletion (46, 47). We detected recurrent mutations in KRAS, which have been reported to be rare in gliomas (45, 48, 49). Recent clinical data suggest that cancers driven by KRAS mutation may be sensitive to MEK inhibitors (50). In addition, our data suggests investigation of Myc pathway inhibitors (40) may be justified for particularly aggressive IDH-mutant gliomas, because these may harbor C-MYC or N-MYC amplification or inappropriate c-Myc expression. Analogously, a recent study reported that Myc pathway inhibition potently reduces viability of acute myeloid leukemia cells driven by mutant IDH2 (43).

Finally, development of anticancer agents in IDH-mutant gliomas has been limited by the scarcity of biologically accurate preclinical models that are serviceable for testing therapeutics. We have established a panel of endogenous IDH-mutant intracerebral glioma xenografts that represent a powerful platform for studying IDH-mutant tumor biology and for answering fundamental questions about treatment of IDH-mutant gliomas. Future work utilizing these orthotopic xenografts may help determine the optimal therapeutic strategy for IDH-mutant gliomas, which may involve inhibition of mutant IDH1, inhibition of tertiary genetic alterations, alteration of metabolic or epigenetic pathways, or a combination thereof.

Figure 4. Molecular taxonomy of IDH-mutant glioma progression. We propose that IDH-mutant gliomas develop and progress through an ordered sequence of oncogenic alterations. IDH-mutant gliomas are initiated by the occurrence of IDH1 or IDH2 mutation and widespread hypermethylation of CpG islands (CpG island methylator phenotype, CIMP) in a glial progenitor cell population. The subsequent acquisition of TP53 and ATRX mutations results in development along an astrocytoma pathway, whereas the codeletion of 1p and 19q (t(1;19)) along with the island methylator phenotype, CIMP) in a glial progenitor cell population. The subsequent acquisition of these "lineage-defining" mutations, IDH-mutant astrocytomas and oligodendrogliomas may follow several "tertiary" genetic pathways during the transformation to malignant or high-grade tumors. Astrocytomas tend to amplify growth factor receptor genes such as PDGFRA and MET, whereas oligodendrogliomas tend to develop activating mutations in intracellular signaling genes such as PIK3CA and KRAS. Alternative tertiary pathways include Myc activation and other undescribed mutations. Acquisition of tertiary genetic alterations may result in more malignant behavior. RTK, receptor tyrosine kinase; MAPK, mitogen-activated protein kinase.

Disclosure of Potential Conflicts of Interest

D. Dias-Santagata has ownership interest (including patents) in a patent application (12/799,415). D. Dias-Santagata is a consultant/advisory board member for BioReference Laboratories Inc. L.W. Ellison is a consultant/advisory board member for BioReference Laboratories. D.R. Borger is a consultant/advisory board member for BioReference Laboratories. M. Vander Heiden has ownership interest (including patents) in Agios Pharmaceuticals. M. Vander Heiden is a consultant/advisory board member for Agios Pharmaceuticals. T.T. Batchelor has commercial research grants from AstraZeneca, Pfizer, Millennium, Roche, Merck, Novartis, Kirin, Spectrum, and Argenus. No potential conflicts of interest were disclosed by the other authors.
Authors' Contributions

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