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Clonal expansion and epigenetic reprogramming following deletion or amplification of mutant IDH1

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IDH1 mutation is the earliest genetic alteration in low-grade gliomas (LGGs), but its role in tumor recurrence is unclear. Mutant IDH1 drives overproduction of the oncometabolite 2-OG and α-ketoglutarate (2HG), which competitively inhibits α-ketoglutarate-dependent enzymes and competitively inhibits others, including S-adenosylhomocysteine hydrolase (a class I HDAC inhibitor) and histone demethylases (5, 6). 2HG-mediated inhibition of these epigenetic modifiers in IDH mutant tumors is associated with increases in CpG island (CGI) DNA methylation (in gliomas, the glioma CpG island methylator phenotype, or G-CIMP), changes in histone modification patterns, and changes in chromosome topology (7–9). Among all adult gliomas, IDH mutant tumors are associated with the longest patient survival (10, 11). IDH mutations and high levels of 2HG are associated with a differentiation block, which can be released by treatment with mutant IDH inhibitors; however, the effects of IDH inhibition may depend on the disease, cell type, or tumor model (12–17). The impact of IDH mutations on glioma progression remains difficult to assess due to the difficulty of modeling these tumors. While cell lines overexpressing the mutant enzyme have provided key insights, they do not accurately represent the heterozygous mutant state of the disease. Considerable efforts have been invested to establish brain tumor initiating cell (BTIC) lines from IDH mutant gliomas and more closely recapitulate the biology of these tumors. However, patient-derived cells with endogenous expression of mutant IDH1 are largely refractory in culture, although xenographs from IDH mutant gliomas with tertiary mutations have been established (18, 19).

In paired LGG samples, IDH1 mutations were shown to be retained through tumor recurrence, in contrast to TP53, ATRX, BRCA, and SMARCA4 mutations that are occasionally lost (2, 3, 20). However, current reports suggest that the mutant or wild-type IDH1 allele can be deleted during tumor recurrence and may be selected against in vitro (21–25), raising the critical question of the role of IDH mutations beyond tumor initiation. Here we used longitudinally collected tumor samples to discover and characterize the dynamic molecular, cellular, metabolic, and clinical features of these tumors.

IDH1 | DNA methylation | 2HG | glioma | copy number

Heterozygous mutations in IDH1 or IDH2 (collectively, IDH) are the earliest and most common genetic alteration in low-grade gliomas (LGGs) and are frequently found in other cancers as well, including acute myelogenous leukemia (AML) (1–4). Mutant IDH produces high intracellular concentrations of the oncometabolite 2-OG, which can activate some α-ketoglutarate-dependent enzymes and competitively inhibit others, including S-adenosylhomocysteine hydrolase (a class I HDAC inhibitor) and histone demethylases (5–7). 2HG-mediated inhibition of these epigenetic modifiers in IDH mutant tumors is associated with increases in CpG island (CGI) DNA methylation (in gliomas, the glioma CpG island methylator phenotype, or G-CIMP), changes in histone modification patterns, and changes in chromosome topology (7–9). Among all adult gliomas, IDH mutant tumors are associated with the longest patient survival (10, 11). IDH mutations and high levels of 2HG are associated with a differentiation block, which can be released by treatment with mutant IDH inhibitors; however, the effects of IDH inhibition may depend on the disease, cell type, or tumor model (12–17).

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The authors declare no conflict of interest.

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Data deposition: New exome sequencing, RNA sequencing, and DNA methylation array data (accession no. EGAS00001001985) and whole genome sequencing (accession no. EGAS00001002618) have been deposited in the European Genome-Phenome Archive database. Code is available on GitHub (https://github.com/UCSF-Costello-Lab/IDH1_CNA).

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initially IDH1 mutant LGG with and without subsequent copy number alterations (CNAs) affecting IDH1. In addition, we developed cell culture and xenograft models that spontaneously and systematically lost a copy of IDH1, and compared their metabolic and epigenome changes with those seen in patient samples.

**Results**

**Tumors with Altered Copy Number at IDH1.** We performed exome sequencing on a cohort of 50 paired initial LGGs and their patient-matched recurrences (3, 26, 27) (SI Appendix, Table S1 and Dataset S1). All of the initial tumors had heterozygous mutations in IDH1 or IDH2; however, in two recurrences (in patients 14 and 169), the IDH1 mutant allele frequency (MAF) dropped below the 10% minimum detection threshold (Fig. 1A and SI Appendix, Table S2). Other mutations were retained (Fig. 1B and SI Appendix, Dataset S2), suggesting that recurrence in these cases involved deletion of the mutant IDH1 allele.

To test for the suspected CNA, we used loss of heterozygosity (LOH) analysis (28). For both recurrent tumors, the apparent LOH suggested an allelic imbalance, which was confirmed as single copy loss in total copy number (TCN) analysis (Fig. 1C and SI Appendix, Fig. S1A). We next searched all tumors for LOH at IDH1 or IDH2 and identified four additional cases (SI Appendix, Fig. S1 and Table S2). To conclusively determine the copy number at IDH1, we performed fluorescence in situ hybridization (FISH) at the IDH1 locus (Fig. 1D and SI Appendix, Table S3). (The exome data for one case was acquired from an outside institution and was not analyzed further.) In patient 27, IDH1 CNAs were present in both the initial and recurrent tumor, while the remaining cases presented with IDH1 CNAs at recurrence. From these analyses, we identified the genetic basis of potentially functional changes in IDH1 TCN during malignant progression: deletion of the mutant allele (patients 14 and 169), subclonal gain of the wild-type allele (patient 68), subclonal gain of the mutant allele (patient 17), and gain with additional subclonal amplification of the mutant allele (patient 21) (SI Appendix, Table S2).

To investigate how well these single samples represented the tumor overall, we analyzed new exome sequencing of spatially distinct tumor tissue from patients 14, 68, and 169, along with previously published data from patient 17 (3) (SI Appendix, Table S2 and Dataset S1). These intratumoral analyses suggest that IDH1 CNAs were present throughout the recurrent tumors, yet were undetectable in the initial tumor (Fig. 1A and SI Appendix, Fig. S1). Thus, the CNAs were followed by substantial clonal expansion.

**Copy Number Changes at IDH1 Impact mRNA and Protein Expression.** We next examined the impact of these genetic alterations on IDH1 mRNA and protein levels. In each case with transcriptome sequencing, the MAF from mRNA was very similar to the MAF in DNA (Fig. 1A and SI Appendix, Table S2), suggesting that all alleles were equally expressed. We then performed immunohistochemistry (IHC) with an IDH1 R132H-specific antibody on all available formalin-fixed, paraffin-embedded tumor blocks (SI Appendix, Table S3). We found that the ploidy and chromosome 2 (chr2) CNAs manifested at the protein level, ranging from absence of mutant protein following deletion of the mutant allele to dramatically increased staining intensity following amplification of the mutant allele (Fig. 1D and SI Appendix, Fig. S2).

**Decreased 2HG and Increased Proliferation Following Amplification or Deletion of Mutant IDH1.** We next addressed the functional consequences of IDH1 CNAs. IDH1 mutations are strictly heterozygous, maintaining a specific ratio of wild-type to mutant enzymes for maximal production of 2HG (22, 29–32). IDH1 CNAs may shift this balance and lead to a paradoxical decrease in the production of 2HG. We used NMR analysis of snap-frozen tumor tissue to calculate the level of 2HG and then exome-sequenced that tissue. In patient 14, 2HG was undetectable following loss of the mutant allele (SI Appendix, Fig. S3A and Table S2). Patient 21 had low levels of 2HG despite malignant progression to high-grade glioblastoma (GBM), which typically has elevated 2HG levels (33) (SI Appendix, Fig. S3 B and C and Table S2). This is consistent with biochemical evidence indicating that a relative abundance of mutant IDH1 decreases 2HG production (22, 31, 32). In contrast, in patient 17 2HG levels fell in a normal range for IDH1 mutant GBMs (34) (SI Appendix, Fig. S3 C...
and D), potentially due to the subclonal nature of the CNA. Interestingly, the initial tumor from patient 169 showed little detectable 2HG (SI Appendix, Fig. S3E), despite subsequent exome sequencing of the tissue showing intact chr2, although with decreased IDH1 MAF (Fig. L1 and SI Appendix, Fig. S1F and Table S2). This discrepancy may be due to a subclonal deletion, as IHC for mutant IDH1 was negative in several regions of the initial tumor. For all other tumors, exome-derived IDH1 MAFs on post-NMR tissue were consistent with other tissue samples from the same surgical resections (SI Appendix, Fig. S1 and Table S3). These data further support in vivo clonal expansion of the cell that sustained IDH1 CNA and had impaired 2HG production.

Mutant IDH1 and 2HG reduce BTIC proliferation in vitro (25, 31, 35). Indeed, recurrent tumors with IDH1 CNAs underwent malignant progression to a higher grade (SI Appendix, Table S2). Therefore, we asked whether the observed IDH1 CNAs and decrease in 2HG were associated with more proliferative tumor clones. We counted Ki-67 positivity, a marker of actively cycling cells, in tumors with regionally heterogeneous IDH1 R132H IHC staining. In all three cases, the region with abnormal staining had a higher Ki-67 index than the region with typical staining (P = 0.008, paired t test) (Fig. 1D and SI Appendix, Table S4). Thus, clonal IDH1 CNA and reduction of 2HG are associated with a more proliferative state and outgrowth of a more aggressive tumor.

Systematic IDH1 Deletion in Vitro. A retrospective analysis showed successful establishment of BTICs from 8% (4 of 49) of IDH mutant gliomas, compared to 63% (62 of 99) from IDH wild-type gliomas (P = 1 × 10^-5, Fisher’s exact test) (SI Appendix, Table S5), illustrating the refractory nature of IDH mutant cells to in vitro culture. However, four BTIC lines were established from grade III or IV IDH mutant tumors, three of which harbored tertiary mutations that likely contributed to successful establishment (19, 25, 36, 37) (SI Appendix, Tables S6 and S7 and Dataset S3). However, these BTIC lines systematically lost either the mutant or wild-type IDH1 allele (SI Appendix, Fig. S44). Here we confirmed previous findings (23, 25), using a higher resolution copy number array, that each BTIC line derived from IDH1 mutant glioma has chr2 deletions encompassing IDH1 (Fig. 2A, and SI Appendix, Fig. S4B and Table S6) despite globally similar copy number profiles (SI Appendix, Fig. S4C). In contrast, chr2 alterations were rare in a panel of 38 IDH wild-type tumors and matched BTIC lines (Fig. 2B and C and SI Appendix, Fig. S5). Thus, selection of cells with chr2 deletions was exclusive to IDH1 mutant gliomas, suggesting that this deletion confers a selective growth advantage for IDH1 mutant gliomas in vitro (25).

We next asked whether IDH1 CNAs arose in vitro or if cells with the delon preexisted in the tumor. A low-frequency mosaic deletion in BT92 tumor tissue suggested the latter scenario (SI Appendix, Fig. S4B). CNA analysis of a separate piece of BT92 tumor tissue identified a subclonal chr2 deletion matching the deletion observed in the derived BTIC line (SI Appendix, Fig. S6). Together with the CNA identified in our cohort of paired primary gliomas, this suggests that cells with IDH1 deletions exist in the tumor and are selected for in vitro.

IDH1 Deletion in Vivo. We further set out to determine whether the selection for IDH1 CNA would be recapitulated in vivo. Primary cells isolated from a secondary GBM (BT257; Fig. 2D) formed spheres within the first week in culture (38) (Fig. 2E). Sequencing identified a rare IDH1 R132S mutation, which was maintained in vitro (Fig. 2F). Although these cells remained viable in culture for three months, they could not be successfully expanded. Thus, all remaining viable cells were used for orthotopic xenografts. Within a year, all animals developed large 2HG-producing IDH1 mutant tumors consistent with GBM (Fig. 2G and SI Appendix, Fig. S7 A-D). We maintained BT257 through serial xenografting, with survival ranging from three to six months (Fig. 2H and SI Appendix, Fig. S7D). While heterozygosity was maintained in the first xenograft, we observed a decrease in the height of the mutant peak in later xenografts (Fig. 2F). The BT257 tumor had no detectable alteration on chr2, with
the exception of a focal amplification of MYCN (Fig. 2J), yet we observed mosaic loss of chr2 being enriched from the primary BTICs to X6-BTICs, correlating with the loss of the IDH1 mutant allele (Fig. 2J and SI Appendix, Fig. S7E). IDH1 R132H and R132S produce different amounts of 2HG, which could result in subtle differences in selective pressure for IDH1 CNA events (35).

**IDH1 Copy Number-Altered Tumors Retain G-CIMP.** Given the widespread DNA methylation changes associated with IDH mutations, we investigated the DNA methylation dynamics of these tumors. All tumors retained G-CIMP based on an eight-site definition (8) and unsupervised hierarchical clustering with IDH mutant and wild-type gliomas from TCGA (11, 39) (SI Appendix, Fig. S8). Similarly, among 50 genes with G-CIMP-associated decreased expression (8, 25), none significantly changed expression following IDH1 CNA (SI Appendix, SI Materials and Methods). These analyses based on a subset of CpG sites, suggest a static methyleome during recurrence despite IDH1 CNA.

Recent analysis of G-CIMP–positive gliomas suggested that subclassification into G-CIMP high and G-CIMP low can better stratify by outcome (40). We wondered whether IDH1 CNA may drive a shift from G-CIMP high to G-CIMP low with its associated worse outcomes. We applied a random forest model built on TCGA data to classify each sample from the five patients with IDH1 CNAs and found that all samples from the three recurrences with clonal CNA were classified as G-CIMP low, while all other samples were G-CIMP high (Fig. 3A and SI Appendix, Table S8). This raises the possibility that G-CIMP–low tumors have altered methyleomes due to IDH1 CNA.

**IDH1 Copy Number-Altered Tumors Exhibit Methylation Reprogramming Outside of CGI.** We used principal component analysis (PCA) to provide a global view of DNA methylation patterns. Principal components (PCs) 1 and 2 defined using TCGA gliomas separated tumors by IDH mutation status; when we plotted the IDH1 CNA cases on the same PCs, several of the recurrences appeared more like the IDH wild-type TCGA tumors, while their initial IDH1 mutant counterparts resembled IDH1 mutant TCGA tumors (Fig. 3B). This dramatic shift within individual patients suggests that despite retention of G-CIMP, these malignantly progressed tumors have dynamically altered DNA methylation. Indeed, density plots, average methylation per tumor, and average change in methylation from the initial tumor to the IDH1 copy number–altered recurrence indicate a decrease in methylation of much larger magnitude than the decrease characteristic of malignant progression to GBM (26) (Fig. 3 C and D and SI Appendix, Fig. S9A).

Because the persistence of G-CIMP indicated retained hypermethylation within CGIs, we asked whether the hypomethylation occurred primarily outside of CGIs. Indeed, non-CGI CpG sites clearly drove the hypomethylation (Fig. 3E). While the pattern of hypomethylation was very strong in cases with clonal CNA (patients 14, 21, and 169), it was moderate or not detected in cases with subclonal CNA. Since the genomic DNA for methylation analysis was derived from a larger tumor area than the FISH analysis, subclonality might have muted the signal of any aberrant hypomethylation.

If the epigenetic changes in tumors with clonal CNA are mechanistically related to the decrease in 2HG as a consequence of IDH1 CNA, then there should be a common set of altered CpG sites. We calculated the change in methylation between initial and recurrent tumors at each assayed CpG site and found that patients 14, 21, and 169 grouped together in unsupervised hierarchical clustering and PCA (Fig. 4A and SI Appendix, Fig. S8B). Similarly, the three highest pairwise correlations were among patients 14, 21, and 169 (SI Appendix, Fig. S9C). Taken together, these analyses demonstrate that similar CpG sites are altered following IDH1 CNA, suggesting a common effector.

To identify the CpGs that lose methylation following IDH1 CNA, we compared the change in methylation between initial and recurrent tumors with and without IDH1 CNA and identified 29,018 CpG sites with significant hypomethylation specific to recurrence with IDH1 CNA (Fig. 4B). These CpG sites were underenriched in CGI and overenriched in shores/shelves and non-CGI genomic spaces ($P < 0.0001$, permutation test) (Fig. 4C). While globally promoter CpG sites were underenriched, there was clear enrichment of CpG sites in CGI were excluded ($P < 0.0001$, permutation test) (Fig. 4C). We performed gene ontology enrichment analysis on genes with significant CpG sites in their promoters while excluding CpG sites in CGI and found modest enrichments (Dataset S4).

**Methylation Reprogramming Following IDH1 CNA in Vitro and in Vivo.** To further confirm that the identified DNA methylation changes are related to IDH1 CNA, we profiled the BTICs and xenografts, along with a second sample from each patient lacking the CNA: tumor tissue, an earlier passage of BT142 before CNA, and a primary culture derived from an earlier surgery that expanded...
but did not establish (BT61, matching recurrent sample BT92). All samples were G-CIMP–positive (SI Appendix, Fig. S8). Unsupervised hierarchical clustering with the initial-recurrent tumor pairs showed that samples with CNA, whether cell line, xenograft, or primary tumor tissue, clustered together (Fig. 4D).

We then examined the 29,018 CpG sites that were associated with CNA in the initial-recurrent tumor pairs. If the methylation changes identified in the initial-recurrent tumor pairs are a signature of IDH1 CNA, then those same CpG sites should also lose methylation in these models. We calculated the change in methylation at each CpG site for each patient (SI Appendix, Table S6). Examination of the methylation changes in the 29,018 CpG sites revealed decreased methylation in those CpG sites, with a median methylation change of −0.07, compared with −0.0001 in the background CpG sites (Fig. 4E). Thus, a common pattern of methylation change is evident across in vitro BTICs, an in vivo xenograft, and paired initial-recurrent patient tumors, suggesting that IDH1 CNA truly leads to reprogramming of the DNA methylation.

Discussion
IDH mutations are typically retained upon recurrence, leading to the initial hypothesis that these mutations play a role in tumor recurrence. Our paired initial-recurrent gliomas show that glioma cells can delete or amplify the mutant or wild-type allele, altering the balanced ratio of wild-type and mutant IDH1 and leading to disrupted or abolished production of 2HG. Cells with IDH1 CNAs then clonally expand to occupy a substantial portion of the recurrent tumor, suggesting a growth advantage. This is confirmed by the in vitro and in vivo models described here, in which deletion of either the mutant or wild-type IDH1 allele occurs systematically in IDH mutant-derived BTICs, but not in IDH wild-type–derived BTICs. This selection for CNA in vitro is reminiscent of tumor-suppressor genes for which the frequency of homozygous deletion or biallelic loss in primary cultures is elevated relative to tumor tissue (41, 42). In the case of IDH1 CNA, however, the unifying feature is loss of the heterozygous mutant state. In almost all of these cases, patients received temozolomide (TMZ) before the recurrence with IDH1 CNA (SI Appendix, Table S1), raising the possibility that TMZ treatment may contribute to outgrowth of the CNA cells, perhaps by preferentially killing the tumor cells that retain heterozygous IDH1 mutations (43).

Although derived from a relatively small number of cases, the molecular, cellular, and metabolic data presented here, along with genetics-focused case reports, suggest that mutant IDH1 is not strictly required for recurrence (21–24). Model systems expressing mutant IDH1 also suggest an abbreviated period during which inhibition of mutant IDH1 affects cell proliferation (44). Recurrent tumors with IDH1 CNA have some resemblance to the G-CIMP–low subtype (40). Taken together, these data suggest that loss or inhibition of mutant IDH may be associated with a more aggressive phenotype.

Small-molecule inhibition of mutant IDH1 in glioma cell lines results in few DNA methylation changes (15, 17), yet our samples demonstrated massive hypomethylation and decreased 2HG in cells with clonal IDH1 CNA. This may reflect the differences of in vitro drug therapy relative to tumor evolution in patients or differences among IDH1 inhibitors (45). A central question is whether mutant IDH is a reasonable target for therapy. Early clinical trial results suggest some efficacy in patients with IDH1 mutant AML (4), but not yet in IDH mutant gliomas. IDH1 mutant AML and gliomas have several key differences. While IDH1 mutations in gliomas are prognostic of better outcomes, studies in AML report conflicting impacts on survival (46). AML has a higher frequency of IDH2 mutations and a more even distribution of different IDH1 mutations. This, combined with the different subcellular localization of IDH1 and IDH2, affects the concentration of αKG, the NADP/NADPH ratio, and production...
of 2HG, and may account for different prognostic effects (32, 46). However, IDH mutations in AML, as in gliomas, are early events that drive an altered epigenome and block cellular differentiation (7). The efficacy of IDH inhibitors in AML may be related to successful reversal of the differentiation block, a key feature of other AML therapies (16, 47).

It is unlikely that an inhibitor of mutant IDH1 would have an impact on tumor cell populations with IDH1 deletion, since the target enzyme will not be present. Thus, we propose that longitudinal monitoring of the IDH1 locus during treatment with IDH1 inhibitors be incorporated into clinical trials to better understand the role that such deletions may play in drug response. In addition, mutations in IDH1 are associated with a wide range of epigenetic, gene expression, and metabolic changes with potential as therapeutic targets, possibly independent of whether the tumor retains or loses mutant IDH1 (17, 48, 49).

Materials and Methods

Sample use was approved by the University of California San Francisco’s Committee on Human Research, and this research was approved by the University of California, San Francisco’s Institutional Review Board. Additional samples were collected following protocols approved by the University of Calgary’s Human Research Ethics Board. All patients provided informed written consent. Animal studies were performed following institutional ethical guidelines and protocols approved by the University of Calgary’s Animal Care Committee.

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New exome sequencing, RNA sequencing, and DNA methylation array data (accession no. EGAS00001001854) and whole genome sequencing (accession no. EGAS00001002618) have been deposited in the European Genome-Phenome Archive database.EGA accession numbers for previously published data are provided in SI Appendix, Dataset S1. Code is available on GitHub (https://github.com/UICSF-Costello-Lab/IDH1_CNA).

Full details of all the protocols used in this study are provided in SI Appendix, Materials and Methods.