Cell Surface Notch Ligand DLL3 is a Therapeutic Target in Isocitrate Dehydrogenase–mutant Glioma

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Abstract

Purpose: Isocitrate dehydrogenase (IDH)-mutant glioma is a distinct glioma molecular subtype for which no effective molecularly directed therapy exists. Low-grade gliomas, which are 80%-90% IDH-mutant, have high RNA levels of the cell surface Notch ligand DLL3. We sought to determine DLL3 expression by IHC in glioma molecular subtypes and the potential efficacy of an anti-DLL3 antibody–drug conjugate (ADC), rovalpituzumab tesirine (Rova-T), in IDH-mutant glioma.

Experimental Design: We evaluated DLL3 expression by RNA using TCGA data and by IHC in a discovery set of 63 gliomas and 20 nontumor brain tissues and a validation set of 62 known IDH wild-type and mutant gliomas using a monoclonal anti-DLL3 antibody. Genotype was determined using a DNA methylation array classifier or by sequencing. The effect of Rova-T on patient-derived endogenous IDH-mutant glioma tumorspheres was determined by cell viability assay.

Results: Compared to IDH wild-type glioblastoma, IDH-mutant gliomas have significantly higher DLL3 RNA (P < 1 × 10^-13) and protein by IHC (P = 0.0014 and P < 4.3 × 10^-6 in the discovery and validation set, respectively). DLL3 immunostaining was intense and homogeneous in IDH-mutant gliomas, retained in all recurrent tumors, and detected in only 1 of 20 nontumor brains. Patient-derived IDH-mutant glioma tumorspheres overexpressed DLL3 and were potently sensitive to Rova-T in an antigen-dependent manner.

Conclusions: DLL3 is selectively and homogeneously expressed in IDH-mutant gliomas and can be targeted with Rova-T in patient-derived IDH-mutant glioma tumorspheres. Our findings are potentially immediately translatable and have implications for therapeutic strategies that exploit cell surface tumor-associated antigens.

Introduction

Mutations in the isocitrate dehydrogenase (IDH) 1 and IDH2 genes identify a subtype of glioma with distinct biological, clinical, and radiographic features (1–5). These gliomas develop through early mutation of IDH (6), which results in accumulation of 2-hydroxylglutarate (7) and a genome-wide DNA hypermethylation phenotype (8), followed by acquisition of one of two sets of cooccurring genetic alterations: TP53 and ATRX mutations, or 1p/19q codeletion and mutations in TERT, CIC, and FUBP1 (9–12). More recent studies have identified a small subset of more aggressive IDH-mutant gliomas associated with lower global DNA methylation (13) and homozygous CDKN2A/B deletion (14), and alterations that are frequently acquired at recurrence, including temozolomide-induced hypermutation phenotype (15), Myc pathway alterations (16), and driver oncogenes and tumor suppressors (15–17).

The recent success in identifying the genetic alterations driving the development and progression of IDH-mutant glioma has yet to translate into successful novel therapies, however. Standard adjuvant treatment consisting of radiation and the procarbazine, CCNU, vincristine (PCV) chemotherapy regimen improves survival of patients with IDH-mutant glioma (18, 19); however, most
tumors eventually recur and are lethal. Because of the challenges experienced in directly targeting alterations driving IDH-mutant gliomagenesis, recent efforts have focused on immunotherapy and synthetic lethal strategies to selectively target IDH-mutant gliomas (20–23). However, these approaches await clinical validation.

The cell surface Notch ligand delta-like 3 (DLL3) has recently emerged as a therapeutic target in cancer, pioneered in pulmonary neuroendocrine tumors (24). A critical mediator of cellular development, DLL3 inhibits Notch pathway activation in cis and in trans by redirecting or retaining Notch and the Notch-activating ligand DLL1 to late endosomal/lysosomal compartments or the Golgi, respectively, and preventing their localization to the cell surface (25, 26). While DLL3 is predominantly localized to the Golgi apparatus (25, 27), Saunders and colleagues recently confirmed diffuse DLL3 expression on the cell surface membrane of pulmonary neuroendocrine tumor cells (24). Together with the absence of DLL3 expression in normal lung tissue, this study established DLL3 as a tumor-associated antigen and a tractable therapeutic target, leading to the development of rovalpituzumab tesirine (Rova-T), a DLL3-targeting antibody-drug conjugate (ADC) that is currently in clinical development, DLL3 inhibiting Notch pathway activation in cis and trans and preventing their localization to the cell surface (25, 26). While DLL3 is predominantly localized to the Golgi apparatus (25, 27), Saunders and colleagues recently confirmed diffuse DLL3 expression on the cell surface membrane of pulmonary neuroendocrine tumor cells (24). Together with the absence of DLL3 expression in normal lung tissue, this study established DLL3 as a tumor-associated antigen and a tractable therapeutic target, leading to the development of rovalpituzumab tesirine (Rova-T), a DLL3-targeting antibody-drug conjugate (ADC) that is currently in clinical trials (24, 28).

In their investigation of DLL3, Saunders and colleagues found DLL3 expression by RNA-Seq to be the highest in low-grade gliomas among more than 20 cancer types in The Cancer Genome Atlas (TCGA) dataset (24). Because 80%–90% of low-grade gliomas are IDH mutant (2, 3), we therefore hypothesized that DLL3 would be highly overexpressed in IDH-mutant glioma and that expression would be tightly associated with IDH-mutant gliomas compared with IDH wild-type glioma. Here, we analyzed a cohort of diffuse gliomas using IHC with an anti-DLL3 mAB and compared DLL3 expression between glioma molecular subtypes. We then tested the therapeutic potential of the anti-DLL3 ADC Rova-T using patient-derived glioma tumor cultures.

**Materials and Methods**

**Tumor samples**

For the discovery set, 20 nontumor brain tissue samples and 63 glioma tumor samples were obtained from Cooperative Human Tumor Network (CHTN), Conversant Bio, and RS Diagnostics. This set of tumors included the following diagnoses: glioblastoma, WHO grade IV (n = 30 including 1 recurrent tumor), oligodendroglioma, grade II (n = 11), anaplastic oligodendroglioma, grade III (n = 4, including 1 recurrent), astrocytoma, grade II (n = 3, including 1 recurrent), anaplastic astrocytoma, grade III (n = 4, including 1 recurrent), ’oligastrocytoma’, grade III (n = 5), and one each of pilocytic astrocytoma, pleomorphic xanthoastrocytoma grade II, ependymoma grade II, mixed ependymoma-subependymoma, ganglioglioma, and recurrent glioma NOS.

For the validation set, 62 gliomas with known IDH1/2 mutation status were obtained from the NYU pathology database to compare DLL3 expression in IDH-mutant glioma and IDH wild-type glioblastoma. This set included 26 IDH wild-type glioblastomas, including 1 recurrent tumor, and 36 IDH-mutant glioma tumors from 25 patients. Within IDH-mutant gliomas, there were 14 recurrent tumors, 11 of which had paired original tumors from the same patient. The pathologic diagnoses in this set included astrocytoma grade II (n = 17), anaplastic astrocytomas (n = 8), oligodendroglioma, grade II (n = 7), and anaplastic oligodendrogliomas, grade III (n = 4). All tumor samples, pathologic information, and molecular data were collected under NYU institutional review board–approved protocols.

**TCGA DLL3 mutation, copy number, and gene expression analyses**

Glioma tumor mutation, copy number, and normalized mRNA (RNA-Seq V2) TCGA datasets were downloaded from www.cbioportal.org (29, 30). For DLL3 expression analysis, tumors from the Glioblastoma TCGA, Cell 2013 database (31), and Brain Lower Grade Glioma (TCGA, Provisional) database (32) with both IDH1/2 mutation status and DLL3 RNA-Seq data available (a total of 433 tumors) were accessed on April 1, 2018. For DLL3 mutation and copy number analyses, all diffuse glioma, lower grade glioma (LGG), glioblastoma, medulloblastoma, and pilocytic astrocytoma datasets (total 4,634 sequenced cases) were accessed on June 23, 2018.

**DLL3 IHC**

For the discovery set, DLL3 IHC was performed on 5-μm-thick formalin-fixed, paraffin-embedded (FFPE) tissue sections using a proprietary monoclonal unconjugated, mouse anti-human DLL3 antibody, clone SC16.65 (AbbVie-Stemcentrx) optimized on 5-μm sections of human DLL3–overexpressing HEK-293T (HEK-293T-hDLL3, positive control) and naïve HEK-293T cell lines (negative control) sectioned onto plus slides (Thermo Fisher Scientific, catalog no. 22-042-924), or murine IgG2A isotype control on the Ventana platform (Ventana Medical Systems) as described previously (28). For the validation set, chromogenic IHC was performed using SC16.65 (provided by Abbvie-Stemcentrx), optimized on the same positive and negative control slides (HEK-293T-hDLL3 and naïve HEK-293T cell lines) on the Ventana platform (Discovery XT instrument). Sections were incubated for 1 hour at 60°C. Sections were deparaffinized and antigen retrieved using Low pH, Envision Flex Retrieval solution in a Dako PT Link with preheat temperature of 65°C, retrieval temperature...
of 97°C for 20 minutes, and cool-down end temperature of 65°C. Slides were rinsed with instrument reaction buffer and endogenous peroxidase activity was blocked. SC16.65 was diluted 1:4,000 in Ventana diluent (catalog no. 251-018) and incubated for 1 hour at 37°C. Primary antibody was detected with goat anti-mouse horseradish peroxidase–conjugated multimer incubated for 16 minutes. The complex was visualized with 3,3 diaminobenzidine and enhanced with copper sulfate. Slides were washed in distilled water, counterstained with hematoxylin, dehydrated, and mounted with permanent media. Negative controls consisted of isotype (IgG2a, matched concentration) and primary antibody diluent substituted for primary antibody on sections of HEK-293T, HDDR cell line. A patient-derived xenograft expressing endogenous DLL3 (24) and a naïve HEK-293T cell line were used as positive and negative controls, respectively, and included with the study sections. IHC scoring for the discovery set was performed independently by two teams; two board-certified neuropathologists (D. Zagzag and M. Snuderl) at NYU and a pathology researcher with more than 15 years of IHC experience at Abbvie Stemcentrx (K. Isse). Scoring was performed blinded to the pathology report and diagnosis. At the time of scoring, the IDH mutation/molecular status of the tumor samples were undetermined. Surface DLL3 expression was quantified by counting the percentage of cells with expression and by converting the staining intensity (range, 0, no staining; 1, weak; 2, moderate; 3, strong) and the percentage of cells with expression to an H-score (range, 0–300). For the validation set, the DLL3-immunostained tumor samples were coded and randomized to blind the neuropathologists (M. Snuderl and D. Zagzag), and surface DLL3 expression was scored following the same criteria. Molecular analysis and determination of IDH mutation and 1p/19q codeletion status

In all tumors, DNA was extracted from FFPE. Areas with the highest available tumor content were selected. Extraction was carried out using the automated Maxwell system (Promega). DNA methylation was analyzed by the Illumina EPIC Human Methylation array assessing 850,000 CpG sites (MethylationEPIC BeadChip 850k microarray, Illumina), according to the manufacturer’s instructions at the NYU Molecular Pathology laboratory as described previously (33).

For tumors in the discovery set, the glioma molecular subtype was determined using a recently developed random forest classifier tool (34) which analyzes whole-genome DNA methylation data generated from Illumina MethylationEPIC 850k arrays and classifies tumors into one of 82 designed CNS tumor classes. This DNA methylation classifier tool predicts IDH-mutant gliomas with high specificity and sensitivity, and also determines 1p/19q deletion status based on analysis of the copy number profile generated from 850k/EPIc data. Specifically, the methylation class family “Glioma, IDH1 mutant” comprises the methylation classes “astrocytoma, IDH1 mutant,” “astrocytoma, IDH1 mutant, subtype high grade” and “oligodendroglioma, IDH1 mutant and 1p/19q codeleted.” The methylation class family “Glioblastoma, IDH1 wild-type” comprises the subclasses RTK I to III, mesenchymal, MYCN, and midline. Furthermore, this DNA methylation classifier tool determines MGMT promoter methylation status using the MGMT-TP27 model (35).

For tumors in the validation set, we included a set of known glioblastomas confirmed to be IDH1/2 wild-type either by the DNA methylation classifier tool (“Glioblastoma, IDH1 wild-type”) or by a clinical targeted next-generation sequencing (NGS) Ion Torrent hotspot panel that included all mutation hotspots of IDH1 and IDH2 (Thermo Fisher Scientific) with a minimal 500x coverage of target areas. All FFPE slides were reviewed and the slide/block with the highest tumor cell content, at minimum 80%, was utilized for molecular studies. For the IDH1-mutant glioma cohort in the validation set, IDH1 mutation status was determined by IHC for IDH1 R132H or by the clinical targeted NGS panel when IHC was negative. Four-micron sections were cut from FFPE blocks from tumor specimens and stained with hematoxylin/eosin and examined by IHC with an antibody against IDH1 R132H (clone H09, Dianova).

1p/19q codeletion status for IDH1-mutant gliomas in the validation set was previously determined utilizing a loss of heterozygosity (LOH) PCR CLIA laboratory developed assay. Normal (peripheral blood mononuclear cell) and tumor DNA were extracted. The polymorphic chromosomal markers of 1p (7 loci, D1S1612, D1S430, D1S199, D1S224, D1S162, D1S171, and D1S1161) and 19q (4 loci, D19S601, D19S412, D19S112, and D19S559) were used to evaluate 1p/19q LOH in the tumor by PCR. The amplified PCR products were fluorescently labeled and were analyzed by capillary gel electrophoresis. Normal and tumor DNA were compared to determine whether there was allelic loss of chromosome 1p and/or 19q. To detect 1p/19q LOH, tumor tissue was no less than 75% of the total sample tissue.

Western blot analysis

Western blots were performed as described previously (21) with modification to visualize using a LI-COR Odyssey Detection System (LI-COR Biosciences); membranes were blocked using LI-COR blocking buffer and IRDye (LI-COR) secondary antibodies were used. Primary antibodies used were: SP347 (anti-DLL3, Ventana; provided by Abbvie Stemcentrx), anti-IDH1 R132H (clone H09, Dianova), GPDH (Thermo Fisher Scientific). Extracts of SHP77 (high DLL3 expression), NCI-H69 (low to moderate DLL3 expression), and NCI-H211 (no DLL3 expression) cells were provided by Abbvie Stemcentrx.

Cell culture

U87 cells constitutively expressing GFP (U87-GFP) or IDH1 R132H (U87-IDH) were engineered previously in our laboratory (21). U87-GFP, U87-IDH, and HEK-293T cells were cultured in DMEM + 10% FBS. The patient-derived glioma tumorspheres MGG18 (IDH1 wild-type), MGG119 (IDH1 R132H mutant), MGG152 (IDH1 R132H mutant), and MGG18-IDH, an IDH wild-type GBM line engineered with a tetracycline-inducible IDH1 R132H gene, were all previously derived in our laboratory and were cultured in serum-free neural stem cell medium as described previously (17, 21). To induce mutant IDH1 expression, MGG18-IDH1 cells were cultured with doxycycline (Sigma-Aldrich, 1 μg/mL) for 72 hours.

Cell viability and apoptosis assays

Compounds used for cell viability assays included two antibody–drug conjugates (ADC): SC16LD6.5 (rovalpituzumab tesirine, Rova-T), which consists of SC16, an anti-DLL3 humanized mAb, conjugated to LD6.5, a DNA-damaging pyrrolobenzodiazepine dimer toxin and IgG1LD6.5, which consists of a nontargeting human IgG conjugated to LD6.5 (24). SC16LD6.5 and IgG1LD6.5 were provided by Abbvie Stemcentrx.
Cells (500 for U87-GFP and HEK-293T; 4,000 for MGG18 and MGG152; and 8,000 for MGG119) were dissociated into single cells and seeded into 96-well plates at 50 μL per well on day 1. On day 2, SC16LD6.5 or IgGLD6.5 were serially diluted in culture media and added to wells and cells were cultured for 8 days. Each sample concentration was tested in triplicate. Cell viability was measured by CellTiter-Glo assay (Promega) according to the manufacturer’s instructions, using a Synergy HTX multimode plate reader (BioTek). The luminescence values for each sample were normalized to untreated well values, and percent cell viability was plotted as a function of sample concentration. Data were analyzed with GraphPad Prism software using a four-parameter logistic nonlinear regression model.

Apoptosis assays were performed by staining with Annexin V according to the manufacturer’s instructions (BD Pharmingen) in combination with DAPI. Cells were washed with PBS, stained with PE-Annexin V and DAPI, and then measured using a FACS can flow cytometer (BD Biosciences). The relative cell distribution was analyzed using FlowJo software (FlowJo, LLC).

Statistical analysis
Nonparametric tests were used to compare groups in data generated by RNA-Seq and IHC. The Wilcoxon–Mann–Whitney U test was used to compare two groups and the Kruskal–Wallis test was used when comparing more than two groups, with follow-up multiple comparison testing using Dunn method with false discovery rate control using the two-stage linear step-up procedure of Benjamini, Krieger, and Yekutieli. All tests were performed in GraphPad Prism 7.

Results

DLL3 is overexpressed in IDH-mutant glioma by RNA-Seq
Using TCGA datasets from 21 different cancer types, Saunders and colleagues found that low-grade glioma (LGG) had the highest DLL3 mRNA by RNA-Seq, while glioblastoma also had high expression relative to other cancers (24). On the basis of these data, we hypothesized that IDH-mutant gliomas would have significantly elevated DLL3 expression, and within gliomas, DLL3 expression would be mainly associated with IDH-mutant gliomas versus IDH wild-type glioma. To test this, we analyzed tumors in the TCGA LGG and glioblastoma datasets that had both IDH1/2 mutation status annotated and DLL3 RNA-seq data available (Supplementary Table S1).

We initially compared DLL3 expression by glioma grade. There was a significant difference between lower grade (WHO grade II and III) glioma (n = 284) and glioblastoma (WHO grade IV; n = 147), with lower grade gliomas having 3.2-fold higher expression (median read count 3,726 vs. 1,180; P = 2.7 × 10−8, Fig. 1A). There was also a significant difference in DLL3 expression when separating grade II and III tumors [median read counts of 4,467, 2,935, and 1,180 for grade II (n = 139), III (n = 145) and IV (n = 147), respectively, P = 3.1 × 10−11] and when each grade was compared with another (grade II vs. grade III, P = 0.0037, grade II vs. grade IV, P = 4.2 × 10−11, grade III vs. grade IV, P = 0.0002), with a 3.8-fold difference between grade II and IV (Fig. 1B).

Given that 70%–90% of grade II and III gliomas are IDH-mutant (2, 3), we compared the difference between IDH-mutant (n = 241) versus IDH wild-type (n = 192) glioma and found a striking difference in DLL3 expression, with IDH-mutant glioma harboring 5.2-fold greater DLL3 expression (median read count 4,733 vs. 916, P < 1 × 10−15, Fig. 1C). DLL3 overexpression in gliomas was not a consequence of DLL3 mutations or gene amplification, as these events were exceedingly rare. Among all glial and neuronal tumors in the TCGA dataset with available sequence data, including diffuse gliomas, pilocytic astrocytomas, and medulloblastomas, only 4 tumors had DLL3 gene amplification (all glioblastomas), 3 DLL3 missense mutations were detected (two diffuse gliomas, one medulloblastoma), and 1 DLL3 fusion was detected in one oligodendroglioma (Supplementary Table S2).

Figure 1.
Elevated expression of DLL3 mRNA in IDH-mutant glioma. Shown are mapped reads to DLL3 transcripts as determined by whole transcriptome RNA-sequencing in TCGA tumor samples. A and B, DLL3 mRNA reads by glioma grade. C, DLL3 mRNA reads by IDH mutation status. The Wilcoxon–Mann–Whitney U test was used to compare two groups (A and C) and the Kruskal–Wallis test was used to compare three groups (B), with follow-up multiple comparison testing using Dunn method with false discovery rate control using the two-stage linear step-up procedure of Benjamini, Krieger, and Yekutieli. All P values were two-tailed.
DLL3 expression is intense and homogeneous in IDH-mutant glioma by IHC

To confirm DLL3 protein expression in gliomas and assess the relationship of protein expression in different subtypes of glioma, we performed IHC using a highly specific mAb for DLL3 (SC16.65) on a discovery set of 63 gliomas and 20 nontumor brain tissue samples. DLL3 staining was scored independently by two different teams (Abbvie Stemcentrx and NYU) and scoring was blinded to the IDH mutation status and molecular subtype of the tumors. Representative images of DLL3 IHC are shown in Fig. 2.

In addition to scoring DLL3 staining, DNA was extracted from available tumor FFPE scrolls in the discovery set (n = 56) and analyzed using a DNA methylation array classifier tool to classify glioma molecular subtype (34). In total, 46 tumors could be classified using the classifier tool; 10 tumors had no match due to insufficient or poor-quality tumor DNA (Fig. 3A; Table 1; Supplementary Table S3). There were 17 classified as IDH wild-type glioblastoma, 19 as IDH-mutant gliomas (including 9 IDH mutant, 1p/19q codeleted and 10 IDH mutant, noncodeleted tumors), and 10 tumors of other major designated classes. These 10 'other' tumors consisted of pediatric and young adult glioma variants including pilocytic astrocytoma, subependymoma, ganglioglioma.

On the basis of the differences in gene expression, we compared the DLL3 IHC scores by H-score (scored at Abbvie Stemcentrx) and percent of positive tumor cells (scored independently at NYU) between IDH-mutant glioma and IDH wild-type glioblastoma and found a marked difference in DLL3 expression (P = 0.0014 for H-score, P = 0.003 for percent of DLL3-positive tumor cells; Table 1; Fig. 3B and C). Notably, the majority of IDH-mutant gliomas had intense and homogeneous membranous DLL3 expression, whereas expression in IDH wild-type glioblastoma was largely absent, and when present was patchy or scattered (Fig. 2). This distribution was reflected in the IHC scoring; the median H-scores and the median proportion of DLL3-positive tumor cells was 250 and 80%, respectively, for IDH-mutant glioma, and 15% and 15%, respectively, for IDH wild-type glioblastoma. Of note, 19 of 20 nontumor brain tissue samples were negative for DLL3 while scattered neurons were positive for DLL3 in one sample, and DLL3 expression was only observed in a few of the glioma variants (Table 1; Supplementary Table S3).

Among IDH wild-type glioblastomas, approximately half (8/17, 47%) had either no expression or only scattered cells expressing DLL3. When expressed, scattered and patchy staining was characteristic of this subtype, and only 4 (24%) tumors expressed DLL3 in ≥50% of tumor cells, with no tumor (0/17) ≥80% positive (Table 1). In contrast, the majority of IDH-mutant gliomas (11/19, 58%) had ≥80% DLL3-positive tumor cells. This homogeneity of DLL3 positivity in IDH-mutant glioma compares

Figure 2.
favorably with pulmonary neuroendocrine tumors, where the mean DLL3 H-scores range from 125 to 200 (24).

We then collected a validation set of known IDH wild-type glioblastomas and known IDH-mutant gliomas archived at NYU to test the association of DLL3 expression by IHC and IDH-mutant glioma. We performed IHC using the same anti-DLL3 antibody (Fig. 3D and E; Supplementary Tables S4 and S6). In the validation set, we confirmed that DLL3 expression in IDH-mutant gliomas was intense, homogeneous, and significantly higher than in IDH wild-type glioblastoma, with median H-scores of 130 versus 10 (P = 4.3 × 10⁻⁶) for IDH-mutant glioma and IDH wild-type glioblastoma, respectively, and median proportion of positive tumor cells of 60% versus 5% (P = 1.7 × 10⁻⁷), respectively (Table 1).

In addition, we evaluated the expression in recurrent IDH-mutant gliomas to assess whether DLL3 expression was retained at progression. We evaluated 14 recurrent tumors, of which 11 had paired original tumors, and found intense, homogeneous DLL3 expression in most cases (Figs. 2 and 3D and E). Interestingly, DLL3 expression was high in the recurrent cases, and for most paired cases the recurrent tumor had higher expression, although these differences were not statistically significant (Fig. 4G). One tumor was recurrent after radiotherapy (Fig. 2K and L; Supplementary Table S4), however, and DLL3 expression was retained in the progressive tumor.

DLL3 expression in glioma molecular subtypes
Because both IDH-mutant glioma and IDH wild-type glioblastoma can each be further subdivided into molecularly distinct subtypes, we explored whether DLL3 expression differed between these subtypes. In the discovery set, we found that high DLL3 expression was nearly universal in the 1p/19q codeleted subtype of IDH-mutant glioma (Supplementary Tables S3 and S5). All IDH-mutant, 1p/19q codeleted gliomas expressed DLL3, with the
lowest H-score being 50, while in 8 of 9 (89%) tumors DLL3 was positive in \( \geq 50\% \) of tumor cells and in 6 of 9 (67%) DLL3 was positive in at least 90% of tumor cells, including two high-grade (grade III) tumors. Among \( IDH \)-mutant, noncodelated gliomas (astrocytoma subclass), DLL3 expression was also high although not to the same extent in the discovery set, there was a significant difference in DLL3 expression between the two subclasses (\( P = 0.012 \) for H-score, \( P = 0.01 \) for percent of positive tumor cells), with median H-scores and DLL3 positive tumor cell proportions of 27% and 90%, respectively, for the \( 1p/19q \) codeleted subclass and 85% and 55%, respectively, for the astrocytoma subclass. In the validation set, however, there was no statistically significant difference between the astrocytoma and \( 1p/19q \) codeleted subset of \( IDH \)-mutant gliomas (Fig. 4; Supplementary Table S6).

In the \( IDH \) mutant, astrocytoma subclass half (5 of 10) had at least 50% of tumor cells DLL3 positive, including 4 (40%) with \( \geq 80\% \) DLL3 positive tumor cells. A subset (4/10, 40%) of \( IDH \) mutant astrocytomas had little to no DLL3 expression, and 3 (75%) of these tumors were high-grade. However, there was no statistically significant difference in DLL3 expression between low-grade and high-grade astrocytomas in this subclass. Notably, there were two glioblastomas in the molecular \( IDH \)-mutant astrocytoma set, with tumor cells positive in only 5% in one and 80% in the other. Similar to the discovery set, only 2 of 25 (8%) \( IDH \)-mutant gliomas in the validation set lacked DLL3 expression, both were astrocytomas (Fig. 4; Supplementary Tables S4, S6).

\( IDH \) wild-type glioblastoma can be subclassified into two robust prognostic and biological groups based on the status of \( MGMT \) promoter methylation (36, 37). Among \( IDH \) wild-type glioblastomas in the discovery set, all 4 tumors with DLL3 expression in \( \geq 50\% \) of tumor cells were \( MGMT \) methylated. There was a trend toward lower DLL3 expression in \( MGMT \) unmethylated tumors, although this difference did not reach statistical significance (median H-score 4.5 for \( MGMT \) unmethylated vs. 27.5 for \( MGMT \) methylated, \( P = 0.11 \); and median DLL3-positive tumor cells 5% for \( MGMT \) unmethylated vs. 25% for \( MGMT \) methylated, \( P = 0.12 \)). In the validation set, there was no significant difference between \( MGMT \) methylated and \( MGMT \) unmethylated tumors in DLL3 expression; however, expression of all \( IDH \) wild-type glioblastoma was lower in the validation set compared to the discovery set (Fig. 4; Supplementary Tables S3 and S6).

In addition, the DNA methylation array classifier tool divides \( IDH \) wild-type glioblastoma into several phenotypic subclasses \( (34) \). The classifier tool subclasses identified in the discovery set of \( IDH \) wild-type glioblastoma included “RTKI” \( (n = 3) \), “RTKII” \( (n = 7) \), and “Mesenchymal” \( (n = 6) \). Because previous glioblastoma expression profiling subtyping studies have reported DLL3 overexpression in proneural tumors and no expression in Mesenchymal tumors (38, 39), we compared DLL3 expression in DNA methylation classifier-defined Mesenchymal tumors to other classifier subtypes. We found DLL3 expression, if present in \( IDH \) wild-type glioblastoma, to be generally restricted to non-Mesenchymal tumors [median H-score 0 for Mesenchymal vs. 42.5 for non-Mesenchymal, \( P = 0.019 \); median positive DLL3 tumor cells 5% for Mesenchymal vs. 35% for non-Mesenchymal, \( P = 0.0037 \)]. However, in the validation set, the H-score difference between Mesenchymal and non-Mesenchymal tumors only trended to significance (median 0.5 vs. 10, \( P = 0.08 \); Fig. 4; Supplementary Tables S5 and S6).

An anti-DLL3 antibody–drug conjugate is cytotoxic to DLL3-expressing patient-derived \( IDH \)-mutant glioma tumormesphere cultures

We then assessed whether DLL3 represents a therapeutic target in \( IDH \)-mutant glioma cells. We first confirmed by Western blot analysis that our patient-derived, endogenous \( IDH \)-mutant glioma tumormesphere lines MGG119 and MGG152 and the publicly available line BT142, expressed DLL3 (refs. 17, 40; Fig. 5A; Supplementary Fig. S1). Interestingly, we did not find that introducing \( IDH1 \) R132H into glioma cells induced DLL3 expression. Malignant glioma cells engineered to constitutively overexpress \( IDH1 \) R132H (U87-IDH) and an \( IDH \) wild-type patient-derived glioblastoma tumormesphere line engineered with tetracycline-inducible \( IDH1^{R132H} \) (MGG18-IDH; ref. 21) had no DLL3 expression by Western blot analysis (Fig. 5A).

We then tested whether patient-derived, endogenous \( IDH \)-mutant glioma tumormesphere lines could be killed with an agent

### Table 1. DLL3 IHC summary

<table>
<thead>
<tr>
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<th>H-score median (95% CI)</th>
<th>DLL3% cells median (95% CI)</th>
<th>DLL3% tumor cells</th>
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<tr>
<td></td>
<td>( n )</td>
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<tr>
<td>Discovery set</td>
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<td>( IDH ) wild-type GBM</td>
<td>17 (0–50)</td>
<td>0.0007 (all 3 groups)</td>
<td>15% (5–40%)</td>
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<td>( IDH )-mutant glioma</td>
<td>19 (50–270)</td>
<td>0.0014 (vs ( IDH )wt GBM)</td>
<td>80% (40–90%)</td>
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<td>Gloma variants</td>
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<td>0.5% (0–70%)</td>
<td>1% (0–15%)</td>
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<td>Validation set</td>
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<tr>
<td>( IDH ) wild-type GBM</td>
<td>26 (1–20)</td>
<td>5% (1–10%)</td>
<td>1% (0–5%)</td>
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<td>( IDH )-mutant glioma</td>
<td>22 (100–180)</td>
<td>60% (50–70%)</td>
<td>1% (0–5%)</td>
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<td>Recurrent ( IDH ) mutant glioma</td>
<td>14 (80–240)</td>
<td>70% (50–80%) (vs non-rec ( IDH )mut)</td>
<td>1% (0–5%)</td>
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**NOTE:** For the discovery set, immunostains were scored by two independent teams prior to determination of the tumor molecular subtype. H-scores in the table were scored by K. Isse and the percent of DLL-positive (DLL3+) tumor cells were scored by D. Zagzag and M. Snuderl. The Wilcoxon–Mann–Whitney \( U \) test was used to compare two groups and the Kruskal–Wallis test was used to compare three groups, with followup multiple comparison testing using Dunn method with false discovery rate control using the two-stage linear step-up procedure of Benjamini, Krieger, and Yekutieli. All \( P \) values were two-tailed.

Abbreviations: \( IDH \)wt, \( IDH \) wild-type; \( IDH \)mut, \( IDH \) mutant; non-rec, nonrecurrent.
that targets cell surface DLL3. We compared the effects of a DLL3-targeting ADC (SC16LD6.5, Rova-T; ref. 24) and an IgG1 control ADC (IgGLD6.5) on the cell viability of \textit{IDH}-mutant and wild-type glioma cells. In cells expressing DLL3, Rova-T is specifically internalized and trafficked to late endosomes and induces cytotoxicity (24). We found that Rova-T mediated potent and specific killing of the \textit{IDH}-mutant tumorsphere lines MGG119 and MGG152 in an antigen-dependent manner, demonstrated by concentration-dependent potency compared with lack of cell killing with control nontargeting ADC at picomolar concentrations (Fig. 5). We confirmed that Rova-T induced apoptosis in MGG119 and MGG152 by Annexin V staining (Supplementary Fig. S2).

Cells without DLL3 expression, including HEK-293T, U87 (an \textit{IDH} wild-type glioma cell line) and MGG18 (\textit{IDH} wild-type patient-derived glioblastoma tumorsphere line), were resistant to both ADCs, confirming that the cytotoxicity of Rova-T was dependent on the presence of the DLL3 antigen on the cell surface of the \textit{IDH}-mutant patient-derived glioma tumorsphere cells.

### Discussion

Here, we found that the majority of \textit{IDH}-mutant gliomas homogeneously expressed the cell surface Notch ligand DLL3 whereas expression was patchy, low, or absent in \textit{IDH} wild-type glioblastoma. In addition, we found that patient-derived endogenous \textit{IDH}-mutant glioma tumorspheres, which overexpress DLL3, were potently and selectively sensitive to the anti-DLL3 ADC Rova-T \textit{in vitro}. Therefore, we show that DLL3 is a newly identified cell surface therapeutic target in \textit{IDH}-mutant glioma.

Importantly, we found that DLL3 is rarely detected by IHC in nontumor brain samples, which confirms a previous study that did not detect DLL3 protein in human brain tissue by sensitive ELISAs (24) despite high levels of RNA being present in the brain (24, 41). These data indicate DLL3 is a tumor-associated antigen and a tractable therapeutic target in glioma.

Our findings have potentially significant clinical impact. Rova-T is in clinical development for other cancers, including phase III clinical trials (NCT03334487; refs. 24, 28); therefore, our findings...
could be immediately translated to clinical trials in *IDH*-mutant glioma. In addition, the cell surface localization of DLL3 and its homogeneous expression in *IDH*-mutant glioma potentially opens the door for the development of novel therapeutic strategies that exploit clonal cell surface antigens, such as adoptive cell transfer and other antibody-based targeting strategies. Furthermore, the selectivity of DLL3 expression in gliomas may also provide insight into the developmental origins of glioma biological subgroups. In early brain development, DLL3 expression is dynamic and occurs after neuroepithelial cells have ceased proliferating and before terminal differentiation into neurons. Therefore, DLL3-expressing cells may represent a population of postmitotic neuroblasts on the verge of terminal neuronal differentiation (42, 43). It is intriguing to speculate that the progenitor cell of *IDH*-mutant gliomas is a DLL3-expressing low proliferation cell (44–46) prevents terminal differentiation of neuroblasts into neurons. Our finding that introduction of mutant *IDH1* into established cancer cells could not induce DLL3 expression while patient-derived *IDH*-mutant glioma stem-like tumorspheres (17) and nearly all *IDH*-mutant glioma tumors overexpressed DLL3 would be consistent with such a model where DLL3 expression precedes *IDH* mutation.

**Figure 5.** Patient-derived endogenous *IDH*-mutant glioma tumorspheres overexpress DLL3 and are sensitive to an antibody drug conjugate targeting DLL3 (Rova-T). A, Western blot using antibodies against DLL3, the *IDH1 R132H*-mutant enzyme, and GAPDH (loading control). Lane labels: U87-GFP, an *IDH* wild-type glioma cell line (U87) engineered to overexpress GFP; U87-IDH: U87 constitutively overexpressing *IDH* R132H; MGG18-IDH (-dox): An *IDH* wild-type patient-derived glioblastoma tumorsphere line (MGG18) engineered with a tetracycline-inducible *IDH* R132H gene (MGG18-IDH), cultured without doxycycline; MGG18-IDH (+-dox): MGG18-IDH cultured with doxycycline for 72 hours; MGG18: An *IDH* wild-type patient-derived glioblastoma tumorsphere line; MGG52: A patient-derived glioblastoma tumorsphere line with endogenous *IDH* R132H; MGG19: A patient-derived glioblastoma tumorsphere line with endogenous *IDH* R132H. NCI-H211: cell line known to lack DLL3 expression (negative control). NCI-H69: Cell line with low to medium DLL3 expression (control). SHP77: Cell line with high DLL3 expression (positive control). B to F, Cells treated with a DLL3-targeting antibody–drug conjugate (ADC) SC16LD6.5 (Rova-T, red) or a nontargeting ADC (IgGLD6.5, black) and then analyzed with CellTiter-Glo cell viability assay (Promega) 8 days after treatment. **Figure 5.** Patient-derived endogenous *IDH*-mutant glioma tumorspheres overexpress DLL3 and are sensitive to an antibody drug conjugate targeting DLL3 (Rova-T). A, Western blot using antibodies against DLL3, the *IDH1 R132H*-mutant enzyme, and GAPDH (loading control). Lane labels: U87-GFP, an *IDH* wild-type glioma cell line (U87) engineered to overexpress GFP; U87-IDH: U87 constitutively overexpressing *IDH* R132H; MGG18-IDH (-dox): An *IDH* wild-type patient-derived glioblastoma tumorsphere line (MGG18) engineered with a tetracycline-inducible *IDH* R132H gene (MGG18-IDH), cultured without doxycycline; MGG18-IDH (+-dox): MGG18-IDH cultured with doxycycline for 72 hours; MGG18: An *IDH* wild-type patient-derived glioblastoma tumorsphere line; MGG52: A patient-derived glioblastoma tumorsphere line with endogenous *IDH* R132H; MGG19: A patient-derived glioblastoma tumorsphere line with endogenous *IDH* R132H. NCI-H211: cell line known to lack DLL3 expression (negative control). NCI-H69: Cell line with low to medium DLL3 expression (control). SHP77: Cell line with high DLL3 expression (positive control). B to F, Cells treated with a DLL3-targeting antibody–drug conjugate (ADC) SC16LD6.5 (Rova-T, red) or a nontargeting ADC (IgGLD6.5, black) and then analyzed with CellTiter-Glo cell viability assay (Promega) 8 days after treatment. B, HEK-293T cells (no DLL3 expression; ref. 24). C, U87-GFP (no DLL3 expression). D, MGG18, an *IDH*-wild-type patient-derived GBM tumorsphere line with no to minimal DLL3 expression. E, MGG18, an *IDH*-wild-type patient-derived GBM tumorsphere line with high DLL3 expression. F, MGG19, an endogenous *IDH*-mutant patient-derived GBM tumorsphere line with medium to high DLL3 expression. All assays were performed in triplicate and repeated at least once (*, *P < 0.01; **, *P < 0.001; *** *P < 0.000001; t test, two-tailed).
Evidence for the similarity of IDH-mutant gliomas and neuroblasts exists in prior literature. In 2006, prior to the identification of cancer-associated IDH1/2 mutations, Phillips and colleagues described high-grade glioma subtypes by expression profiling, including "proneural" and "mesenchymal" subtypes. High DLL3 expression was the signature marker of proneural tumors (38), and later studies identified the major feature of the proneural gene expression subtype to be IDH mutation (39). Intriguingly, Phillips and colleagues noted that proneural and mesenchymal subtypes paralleled stages of neurogenesis in the adult forebrain, with proneural tumors resembling committed neuronal precursors such as low proliferation rate neuroblasts while mesenchymal tumors resembled neural stem cells and/or transit-amplifying cells (38). Similarly, we find that DLL3 was overexpressed in the majority of IDH-mutant gliomas, which have a proneural gene expression signature, whereas IDH wild-type glioblastoma mostly lacked DLL3 expression, particularly the Mesenchymal subtype.

Interestingly, the DLL3 gene maps to 19q13, a region lost during whole-arm chromosome 19q loss of heterozygosity in the 1p/19q codeleted subset of IDH-mutant gliomas. In our study, IDH-mutant, 1p/19q codeleted gliomas universally expressed DLL3, with the majority showing highly homogeneous and intense expression. This suggests the hallmark genomic alteration of this glioma subtype and DLL3 expression may be functionally linked. Given that DLL3 expression is dynamic during neurogenesis, investigating the effect of DLL3 loss of heterozygosity on DLL3 regulation may provide clues to the cell of origin and pathogenesis of 1p/19q codeleted tumors.

Our study would benefit from independent validation of DLL3 expression by IHC and in vivo validation of Rova-T in an IDH-mutant glioma model. In addition, although DLL3 was expressed in the majority of tumor cells in IDH-mutant gliomas in our validation set, median H scores were lower compared with the discovery set. We suspect the age of the NYU archival tumor blocks might account for some of the observed difference. This is supported by the fact that we observed generally higher levels of expression in more recently resected tumors, although this difference was not statistically significant. In addition, it will be important to examine the level and pattern of DLL3 expression in a set of tumors recurrent after known adjuvant therapy as most of our paired tumors (10 paired tumors) received no treatment other than resection, and the adjuvant treatment status was unknown for 3 others.

In conclusion, we have identified DLL3 as tumor-associated antigen and a novel cell surface therapeutic target in IDH-mutant gliomas. Our finding has potentially rapid clinical application and has implications for the development of novel therapeutic strategies that exploit cell surface tumor-associated antigens. In addition, the tight association between DLL3 expression and IDH-mutant glioma raises interesting questions regarding the cell of origin of IDH-mutant glioma given the essential role of DLL3 in cell fate decisions during neurogenesis. Further investigation of the role of DLL3 in IDH-mutant glioma development may provide additional clues to gliomagenesis as well as novel therapeutic targets.

Disclosure of Potential Conflicts of Interest

E.P. Sulman reports receiving commercial research grants from and is a consultant/advisory board member for Novocure and AbbVie, and reports receiving speakers bureau honoraria from Merck. D.P. Cahill is a consultant/advisory board member for Lilly. K. Isse reports receiving other remuneration from AbbVie-Stemcentrix. L. R. Saunders is an employee of and holds ownership interest (including patents) in AbbVie. No potential conflicts of interest were disclosed by the other authors.

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References


Surface DLL3 is a Therapeutic Target in IDH-mutant Glioma


Cell Surface Notch Ligand DLL3 is a Therapeutic Target in Isocitrate Dehydrogenase–mutant Glioma

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