Published in final edited form as:

Acta Neuropathol. 2016 December ; 132(6): 917–930. doi:10.1007/s00401-016-1620-7.

Mutant IDH1 and Thrombosis in Gliomas

Dusten Unruh, Ph.D.^{*1}, Steven R. Schwarze, Ph.D.^{*2}, Laith Khoury, M.D.³, Cheddhi Thomas, M.D.⁴, Meijing Wu, Ph.D.¹, Li Chen, Ph.D.^{5,6}, Rui Chen⁷, Yinxing Liu, Ph.D.¹, Margaret A. Schwartz, R.N.⁷, Christina Amidei, Ph.D.¹, Priya Kumthekar, M.D.⁷, Carolina G. Benjamin, M.D.⁹, Kristine Song, B.S.¹, Caleb Dawson¹⁰, Joanne M. Rispoli, M.D.¹¹, Girish Fatterpekar, M.D.¹¹, John G. Golfinos, M.D.⁹, Douglas Kondziolka, M.D.⁹, Matthias Karajannis, M.D.¹², Donato Pacione, M.D.⁹, David Zagzag, M.D., Ph.D.^{4,9}, Thomas McIntyre, Ph.D.⁸, Matija Snuderl, M.D.⁴, and Craig Horbinski, M.D., Ph.D.^{1,13}

¹Department of Neurosurgery, Northwestern University, Chicago, IL

²Department of Pathology, University of Kentucky, Lexington, KY

³Department of Neurosurgery, University of Kentucky, Lexington, KY

⁴Department of Pathology, New York University, New York, NY

⁵Department of Biostatistics, University of Kentucky, Lexington, KY

⁶Markey Cancer Center, University of Kentucky, Lexington, KY

⁷Department of Neurology, Northwestern University, Chicago, IL

⁸Department of Cellular and Molecular Medicine, Cleveland Clinic, Cleveland, OH

⁹Department of Neurosurgery, New York University, New York, NY

¹⁰University of Kentucky, Lexington, KY

¹¹Department of Radiology, New York University, New York, NY

¹²Department of Pediatrics, New York University, New York, NY

¹³Department of Pathology, Northwestern University, Chicago, IL

Abstract

Mutant *isocitrate dehydrogenase 1 (IDH1)* is common in gliomas, and produces D-2-hydroxyglutarate (D-2-HG). The full effects of *IDH1* mutations on glioma biology and tumor microenvironment are unknown. We analyzed a discovery cohort of 169 World Health Organization (WHO) grade II-IV gliomas, followed by a validation cohort of 148 cases, for *IDH1* mutations, intratumoral microthrombi, and venous thromboemboli (VTE). 430 gliomas from The Cancer Genome Atlas were analyzed for mRNAs associated with coagulation, and 95 gliomas in a tissue microarray were assessed for Tissue Factor (TF) protein. *In vitro* and *in vivo* assays evaluated platelet aggregation and clotting time in the presence of mutant IDH1 or D-2-HG. VTE occurred in 26–30% of patients with wild-type *IDH1* gliomas, but not in patients with mutant

Address correspondence to: Craig Horbinski, M.D., Ph.D., Tarry 2-705, 300 East Superior Street, Chicago, IL 60611, Phone: 312-503-6127, craig.horbinski@northwestern.edu.

*These authors contributed equally to this study.

IDH1 gliomas (0%). *IDH1* mutation status was the most powerful predictive marker for VTE, independent of variables such as GBM diagnosis and prolonged hospital stay. Microthrombi were far less common within mutant *IDH1* gliomas regardless of WHO grade (85–90% in wild-type versus 2–6% in mutant), and were an independent predictor of *IDH1* wild-type status. Among all 35 coagulation-associated genes, *F3* mRNA, encoding TF, showed the strongest inverse relationship with *IDH1* mutations. Mutant *IDH1* gliomas had *F3* gene promoter hypermethylation, with lower TF protein expression. D-2-HG rapidly inhibited platelet aggregation and blood clotting via a novel calcium-dependent, methylation-independent mechanism. Mutant *IDH1* glioma engraftment in mice significantly prolonged bleeding time. Our data suggest that mutant *IDH1* has potent antithrombotic activity within gliomas and throughout the peripheral circulation. These findings have implications for the pathologic evaluation of gliomas, the effect of altered isocitrate metabolism on tumor microenvironment, and risk assessment of glioma patients for VTE.

Keywords

Isocitrate dehydrogenase; D-2-hydroxyglutarate; thrombosis; glioma; Tissue Factor; platelet

Introduction

Diffusely infiltrative glioma is the most common primary brain tumor in adults. For over 100 years, gliomas have been classified according to their appearance under the light microscope, and for over 60 years, necrosis has been associated with aggressive behavior [40]. The World Health Organization (WHO) grades these tumors on a progressive scale of severity from I to IV (with grade I being a distinct type of non-infiltrative tumor), and necrosis is a major diagnostic feature of grade IV malignancy, a.k.a. glioblastoma (GBM) [29]. While the advent of molecular diagnostics has extended our understanding of gliomas, there remains an important role for light microscopy, because physical differences among genetically classified gliomas can provide important clues about the effects of key mutations.

Somatic point mutations of *isocitrate dehydrogenase 1 or 2 (IDH1/2)* occur in a large subset of gliomas [61]. Wild-type *IDH1/2* enzymes oxidize isocitrate into α -ketoglutarate (α -KG), but mutations in the isocitrate binding pocket unmask a latent oxidoreductase reaction, causing the enzyme to convert α -ketoglutarate (α -KG) into the rare metabolite D-2-hydroxyglutarate (D-2-HG). [13] Most infiltrative gliomas with wild-type *IDH1/2* are diagnosed as WHO grade IV GBMs, whereas the overwhelming majority of *IDH1/2* mutant gliomas are not (Supplemental Fig. 1). This suggests that *IDH1/2* mutations may inhibit development of the diagnostic features of GBM. Furthermore, necrosis is far less extensive in *IDH1/2*-mutant GBMs compared to wild-type GBMs [27, 35]. The molecular basis for this, and for the less aggressive behavior of *IDH1/2*-mutant gliomas [20], are unclear.

Studies on D-2-HG in gliomas and other *IDH1/2* mutant cancers have mostly focused on its ability to induce histone and DNA hypermethylation via competitive inhibition of α -KG-dependent demethylases [11, 14, 30, 36, 41, 50, 60]. Very little is known about other

activities of mutant IDH1 and D-2-HG, either within the cell or in the tumor microenvironment.

Many cancers cause aberrant blood coagulation, marked by deep venous thrombi (DVT) and pulmonary emboli (PE), together known as venous thromboemboli (VTE). Gliomas are one of the most at-risk cancers for VTE. This complication causes great suffering, can be difficult to manage, and contributes to cancer-associated mortality [37, 49, 56]. The Food and Drug Administration Oncologic Drug Advisory Committee, the American Society of Clinical Oncology, and the National Comprehensive Cancer Network have all expressed an urgent need to better identify which cancer patients are at greatest risk of VTE [15, 31, 34]. Tumor genotype has recently been shown to affect VTE risk in carcinomas of the lung and colon [1, 12], and others have studied prognostic biomarkers for VTE in brain cancer patients through blood-based testing [52, 63]. Advanced age and GBM diagnosis are known VTE risk factors [37], but to date, there is no data on whether glioma genotype alters VTE risk.

Materials and Methods

Patient cohorts

The discovery cohort consisted of 169 gliomas that had been tested for *IDH1/2* mutations as part of routine clinical care at the University of Kentucky (UK) from 2009–2014 (Table 1). This cohort included 15 grade II oligodendrogliomas, 14 grade II astrocytomas, 6 grade III oligodendrogliomas, 16 grade III astrocytomas, and 118 GBMs. (Some cases had originally been resected prior to 2009, but for clinical reasons required retrospective testing.) The validation cohort comprised 148 gliomas that had been tested for *IDH1/2* mutations at New York University (NYU) (Table 1), including 5 grade II astrocytomas, 7 grade II oligodendrogliomas, 11 grade III astrocytomas, 5 grade III oligodendrogliomas, and 120 GBMs. All gliomas in both cohorts were from adult patients (18+ years). A separate prospective cohort at Northwestern Memorial Hospital (NMH) consisted of 29 patients with newly diagnosed WHO II-IV gliomas, in which preoperative arterial blood was obtained (Table 2).

Mutation screening was done via R132H IDH1 immunohistochemistry (Dianova, Hamburg). Immunonegative tumors were tested for less common *IDH1/2* mutations via pyrosequencing (UK) or by 450K methylation array profiling (NYU) as described previously [50]. Survival data was obtained for 166 cases in the UK cohort via the Kentucky Cancer Registry, but was not available for NYU cases. In the discovery cohort, the median follow-up time for *IDH1/2* wild-type gliomas was 7.6 months (with 74% having died of disease at the time of study), whereas *IDH1/2* mutant gliomas had a median follow-up time of 31 months (12% died of disease at the time of study). Data on coagulation history were available by chart review for 162 and 136 patients at UK and NYU, respectively. At least 6 months of follow-up time was obtained on each case (in the validation cohort, the median amount of tracking time in the patient charts was 9 months for *IDH1/2* wild-type gliomas and 14 months for mutant gliomas). For the prospective NMH cohort, coagulation history was also determined by chart review, with an average length of follow-up of 3.5 months (median=3.9 months, range=0.3–6.7 months), and an average time from surgery to first VTE of 0.71 months (median=0.45

months). The glioma tissue microarray (TMA) cohort containing 95 grade II-IV gliomas has been previously described [28]. Institutional Research Board approval was obtained at UK, NYU, and NMH prior to study initiation.

Platelet free plasma (PFP) collection and preparation

Blood was collected from an arterial line immediately prior to surgery and placed in a vacutainer (Becton Dickinson, Franklin Lakes, New Jersey) containing 3.2% sodium citrate (Fisher Scientific, Waltham, MA). Whole blood was centrifuged at $2,500 \times g$ for 15 min at room temperature. The supernatant was transferred to a new 2 mL tube (Eppendorf, Hauppauge, NY) and spun under the same previous conditions. Supernatant (PFP) was transferred into cryovials and stored at -80°C until use.

Tissue Factor microparticle (TF-MP) procoagulant activity assay

Circulating TF-MP procoagulant activity was measured using the chromogenic reporter substrate (Pefachrome FXa 8595, Enzyme Research Laboratories, South Bend, IN). Briefly, 250 μL of human platelet free plasma was diluted with 1 mL of HBSA (137 mM NaCl, 5.4mM KCl, 5.6 mM Glucose, 10 mM HEPES, 0.1% bovine serum albumin, pH 7.4) and centrifuged at $21,000 \times g$ for 15 min at 4°C to pellet MPs. Pelleted MPs were washed once with HBSA and resuspended in 160 μL HBSA. 50 μL of MP suspensions were then incubated in 50 μL of HBSA containing 73.2 nM FX (Enzyme Research Laboratories), 2.4 nM FVIIa (Enzyme Research Laboratories), and 10 mM CaCl_2 for 2 hours at 37°C in a 96-well plate. The reaction was stopped with 25 μL of HBSA containing 25 mM EDTA. Then, 25 μL of FXa substrate (Pefachrome FXa, 4.0 mM) was added to the reaction and incubated for 15 min at 37°C , and OD 405 nm recorded using Synergy 2 Multi-Mode Microplate Reader (BioTek). To calculate TF-MP procoagulant activity, a standard curve was generated using recombinant relipidated TF (0–30 pg/mL, Haematologic Technologies, Essex Junction, VT).

Pathologic evaluation

All pathologic slides on each case in the discovery (UK) and validation (NYU) cohorts were collected and analyzed for this study. Cases were scored by light microscopic examination while blinded to *IDH1/2* status (CH at UK, MS and CT at NYU) for key histologic features, including necrosis, microvascular proliferation (MVP), and microthrombi. Necrosis and MVP were scored as present or absent, in accordance with standard WHO grading. Microthrombi were scored according to the average number of microthrombi per tissue block. Cutoff Finder was used for finding the optimal cutoff value to discriminate between mutant *IDH1/2* and wild-type *IDH1/2* gliomas in the discovery cohort [5].

Tissue factor (TF) immunohistochemistry (IHC) was performed on 4 μm -thick sections cut from a separate cohort of formalin fixed paraffin embedded TMA slides according to routine histology protocols.[28] Anti-human TF antibody (catalog #4509, Sekisui, Stamford, CT) was diluted 1:500 in TBS-Tween and applied to slides at room temperature for 1 hour. TMA cores were evaluated while blinded to *IDH1/2* status according to previously published methods [28]. Briefly, each core was scored on a semiquantitative scale as follows:

0=negative, 1=weak, 2=moderate, 3=strong. Three cores per tumor were randomly scattered on the TMA blocks and were averaged to produce a final score for that tumor.

Statistical evaluation

Statistical significance of differences between groups was determined via two-sample t-test, one-way ANOVA with post hoc Tukey's test, Fisher's exact test, or log-rank test as appropriate using GraphPad software (La Jolla, CA). The Bonferroni method was used to adjust for multiple comparisons in the analysis of the association between *F3* promoter methylation and *IDH1/2* status. Odds ratios were calculated based on exact logistic regression. Hazard ratios were calculated based on Cox proportional hazards models or competing risk regression. Multivariable analyses were performed using competing risk regression based on Fine and Gray's proportional subhazards model with Firth's correction [24], logistic regression models with Firth's correction or exact logistic regression, as appropriate, when there were rare or no events in one group, using SAS 9.4 software. Model fit parameter C index for Cox proportional hazards models and competing risk regression, or C statistics for logistic regression, were provided where applicable. Optimal cutoffs for TF IHC and *F3* mRNA from TMA and TCGA datasets were objectively determined using recursive partitioning analysis and Cutoff Finder [5], respectively. In all analyses, the significance target was $P = 0.05$.

See the Electronic Supplementary Data for additional methods.

Results

We observed systemic coagulation (including DVT and PE) in 30/117 (26%) *IDH1/2* wild-type glioma patients from the discovery cohort (Table 1), consistent with results reported by others [37]. In striking contrast, none of the 45 (0%) patients with mutant *IDH1/2* glioma presented with or developed any VTE ($P < 0.001$). The validation cohort produced similar findings, wherein 31/105 (30%) *IDH1/2* wild-type glioma patients developed VTE but none of the 31 (0%) *IDH1/2* mutant glioma patients did ($P < 0.001$). Of the patients who had VTEs, the median time from surgery to first VTE episode was 1.0 months in the discovery cohort (Q1=0.1 months, Q3=3.0 months) and 3.5 months in the validation cohort (Q1=0.3 months, Q3=8.0 months). Univariable and multivariable analysis of clinical variables often associated with VTE risk, including GBM diagnosis, KPS score, and length of hospital stay, showed a potential link between *IDH1/2* status and VTEs in the discovery and validation cohorts (OR=0.043, 95% CI=0.002–0.78, $P=0.033$ in the discovery cohort; OR=0.052, 95% CI=0.003–0.91, $P=0.043$ in the validation cohort) (Supplemental Table 1). A competing risk analysis of VTE with death in the discovery cohort showed a trend toward mutant *IDH1/2* as an independent prognosticator for VTE (HR=0.059, 95% CI=0.003–1.1, $P=0.059$) (Supplemental Table 2). Preoperative prothrombin and partial thromboplastin times showed no difference in mutant versus wild-type *IDH1/2* patients (Supplemental Fig. 2), suggesting no functional differences in circulating clotting factors I, II, V, VII, VIII, IX, X, XI, & XII.

Microscopic analysis of tissue sections in the discovery cohort showed that a cutoff of 0.15 microthrombi/block optimally discriminated between mutant *IDH1/2* and wild-type *IDH1/2* gliomas (AUC=0.92, $P < 0.001$) This was slightly adjusted to a working cutoff of 0.25

microthrombi per block, so as to improve its universal practicality for routine neuropathological examination. The adjusted cutoff only altered assignment of one case, yielding similar overall results (AUC=0.92, $P<0.001$). Gliomas with microthrombi were nearly always *IDH1/2* wild-type (100 of 117=85%, see Table 1, Fig. 1, and Supplemental Fig. 3). In contrast, only a single *IDH1/2*-mutant glioma displayed microthrombi (1 of 52=1.9%) ($P<0.001$, Fisher's exact test). This inverse relationship between *IDH1/2* mutations and microthrombi held across all glioma grades in discovery and validation cohorts (Table 1, Fig. 1a–f). Others have suggested that necrosis in a glioma may be caused by microthrombi.[42] Yet in our discovery cohort, whereas 96% (94 of 98) of necrotic *IDH1/2* wild-type tumors also contained microthrombi, only 10% (1 of 10) of necrotic *IDH1/2*-mutant gliomas did. The necrotic gliomas in the validation cohort showed similar results, with microthrombi in 92% (97 of 106) and 8% (1 of 13) of wild-type and mutant tumors, respectively ($P<0.001$ for each cohort, Fisher's exact test, Fig. 1g–h).

Receiver operating characteristic (ROC) analysis showed that, although all variables produced statistically significant areas under the curve (AUCs), the presence of microthrombi (AUC=0.92, 95% CI=0.88–0.95, $P<0.001$) was superior to advanced age (AUC=0.83, 95% CI=0.77–0.89, $P<0.001$), necrosis (AUC=0.82, 95% CI=0.76–0.89, $P<0.001$), and MVP (AUC=0.82, 95% CI=0.88–0.95, $P<0.001$) as a predictor of *IDH1/2* mutation status in the discovery cohort (microthrombi versus age: $\chi^2=5.02$, $P=0.025$; versus necrosis: $\chi^2=10.21$, $P=0.001$; versus MVP: $\chi^2=8.06$, $P=0.0045$.) (Supplemental Fig. 4). The validation cohort confirmed this, as all variables had significant AUCs (microthrombi AUC=0.92, 95% CI=0.87–0.97, $P<0.001$; patient age AUC=0.71, 95% CI=0.62–0.80, $P<0.001$; necrosis AUC=0.77, 95% CI=0.69–0.86, $P<0.001$; MVP AUC=0.72, 95% CI=0.63–0.81, $P<0.001$), but the microthrombi AUC was stronger than the others (microthrombi versus age: $\chi^2=20.01$, $P<0.001$; versus necrosis: $\chi^2=9.23$, $P=0.024$; versus MVP: $\chi^2=20.20$, $P<0.001$). On a multivariable analysis of key variables correlating with *IDH1/2* status, microthrombi remained a significant independent predictor in both cohorts (Supplemental Table 3).

In the past, microthrombi had been proposed as another diagnostic criterion for GBM, in addition to necrosis and MVP [38, 51]. For the tumor series examined here, univariate analysis revealed that the presence of microthrombi (log-rank $P<0.001$; HR=5.1, 95% CI=2.9–9) was comparable to necrosis (log-rank $P<0.001$; HR=4.0, 95% CI=2.3–6.9) and MVP (log-rank $P<0.001$; HR=6.4, 95% CI=3.3–12) in prognostic stratification, and predicted a much worse prognosis for grade III gliomas (log-rank $P=0.008$; HR=9.0, 95% CI=1.2–65) (Supplemental Fig. 5). On multivariable analysis of these histologic variables, only microthrombi (HR=2.7, 95% CI=1.2–5.8, $P=0.01$) and MVP (HR=4.2, 95% CI=1.6–10.7, $P=0.003$) proved to be significant, not necrosis (Supplemental Table 4).

We next sought to identify possible mechanisms for the inverse relationship between mutant *IDH1/2* and thrombosis in gliomas. In The Cancer Genome Atlas (TCGA), mRNA levels of 35 coagulation-associated genes were examined with respect to glioma grade and *IDH1/2* status. Among procoagulant genes, *F3* showed the strongest inverse association with tumor *IDH1/2* mutation status. The mean transcript level for *F3*, encoding Factor III (also known as Tissue Factor (TF)), was 75% lower in mutant gliomas across all tumor grades (95% CI=67–

84, $P=1.1\times 10^{-52}$) (Fig. 2 and Supplemental Fig. 6). TF is a transmembrane glycoprotein that is expressed at variable levels in many tumors [2, 16]. In its membrane-bound form, TF initiates coagulation. Mutant *IDH1/2* suppresses expression of many genes by inducing hypermethylation [11, 14, 30, 36, 41, 50, 58, 60], so we investigated *F3* promoter methylation among TCGA gliomas. Of 17 CpG sites within the *F3* promoter, 8 had significantly higher levels of methylation in *IDH1/2* mutant tumors, compared to wild-type tumors, across all glioma grades (Fig. 3a, Supplemental Table 6). TF immunoreactivity was lower in *IDH1/2* mutant gliomas compared to wild-type gliomas (Fig. 3b & c), consistent with the TCGA *F3* mRNA data. In wild-type gliomas there was a greater range of TF expression, and in those tumors, low TF expression correlated with longer survival (log-rank $P=0.049$; HR=2.0, 95% CI=0.99–4.0) (Supplemental Fig. 7), a finding independently confirmed by others [17].

Many cancers release TF-containing microparticles (TP-MPs) into the circulation, an event which has been repeatedly linked to cancer-induced VTE [3, 7, 10, 32, 43–45, 55]. To further establish a link between *IDH1/2* mutations, TF, and VTE in gliomas, we analyzed circulating TF-MP activity in platelet-free plasma, isolated from preoperative arterial blood in a prospective cohort of 29 newly-diagnosed glioma patients (Table 2). Average circulating TF-MP activity, quantified as picograms of Factor Xa generated per ml plasma, was elevated in patients whose gliomas were *IDH1/2* wild-type, compared to patients with *IDH1/2* mutant gliomas (1.2 ± 0.21 pg/ml versus 0.49 ± 0.19 pg/ml, $P=0.036$, Fig. 3d). Furthermore, there was a positive correlation between the degree of preoperative circulating TF-MP activity and development of VTE (Spearman coefficient $\rho(27)=0.45$, 95% CI=0.089–0.71, $P=0.014$) (Table 2).

The major metabolic consequence of *IDH1/2* mutation is elevated production of D-2-HG [13]. The effect of this metabolite on platelet aggregation, a key event in coagulation, has not yet been investigated. By adding D-2-HG to human platelets isolated from whole blood, we observed a rapid, potent, and concentration-dependent suppression of platelet aggregation (Fig. 4a & b, Supplemental Fig. 8a & b). D-2-HG inhibited iCa^{++} accumulation in platelets by 72% (Fig. 4c, Supplemental Fig. 8c), and this inhibitory effect was prevented by adding the calcium ionophore A23187 to platelet-D-2-HG mixtures (Fig. 4d, Supplemental Fig. 8d). D-2-HG directly bound iCa^{++} ($K_d=48.9\pm 3.5$ mM, $R^2=0.99913$) (Supplemental Fig. 9), reduced the levels of free Ca^{++} in human serum (Fig. 4e), and delayed clotting of human blood (Fig. 4f). Finally, glioma xenografts expressing R132H *IDH1* lowered free serum Ca^{++} in mice by 28% (Fig. 4g) and markedly delayed clotting after tail snip (Fig. 4h, Supplemental Fig. 10).

Discussion

To date, studies of mutant *IDH1/2* have been tumor cell-centric: i.e., they have focused on what the mutation does inside the tumor cell. Our data suggest this mutation also exerts profound effects outside the tumor cell, and not only within the immediate tumor microenvironment, but systemically. This greatly advances our understanding of the significance of this mutation in gliomas, and accounts for some features of mutant *IDH1/2* that have proved puzzling. For example, most mutant *IDH1/2* gliomas do not present with

necrosis, and even when they do, it is much less extensive than in wild-type tumors [27, 35]. For the first time, our demonstration of a relationship between mutant *IDH1/2*, reduced TF expression, impaired platelet activity, and the absence of microthrombi within mutant tumors provides a plausible explanation for the striking paucity of necrosis in this subset of gliomas.

Although mutant *IDH1/2* clearly promotes gliomagenesis, it may also limit the development of the histologic characteristics of GBM. While histologic features are obviously the result of many diverse mechanisms, blocking the development of intratumoral thrombi, and its resultant necrosis, potentially could help explain why most mutant *IDH1/2* gliomas are not grade IV, whereas most wild-type gliomas are (Supplemental Fig. 1). We also show that microthrombi may be comparable to MVP and necrosis in prognostic stratification, though additional prospective data are needed to verify this. Indeed, the entire WHO grading system can be regarded as a set of histologic features that act as surrogate markers for specific underlying gene alterations. It is little surprise, then, that molecular data usually provide more accurate diagnostic and prognostic information for gliomas than traditional histotyping [39]. Diffusely infiltrative gliomas that lack mutant *IDH1/2* have the same aggressive behavior as GBM, even when current WHO histologic criteria for a GBM diagnosis are not met [4, 39]. Because the presence of microthrombi is the strongest histologic predictor that an infiltrative glioma lacks mutant *IDH1/2* (Supplemental Fig. 4, Supplemental Table 3), and because it is an even stronger correlate of shorter survival than necrosis (Supplemental Fig. 5, Supplemental Table 4), microthrombi may prove to be a useful histologic criterion for GBM. Although upfront screening for mutant *IDH1/2* is routine in more developed parts of the world, access to advanced molecular diagnostics can be difficult in many other areas. Even in those developed parts of the world, cost containment is a growing concern in medicine. When a glioma is immunonegative for the most common *IDH1/2* mutation, R132H *IDH1*, screening for microthrombi could help decide when it is worthwhile to employ additional tests to detect less frequent *IDH1/2* mutations.

VTE risk is known to be higher in patients diagnosed with a GBM or those of advanced age, compared to patients with grade II-III gliomas [9, 48]. Our data suggest that this may be due to the fact that *IDH1/2* mutations are less common in older patients and in GBMs. A recent paper showed that *KRAS* mutations increase TF expression and VTE risk in colorectal carcinomas [1], but the current study is the first example, in any cancer, of a specific mutation conferring reduced VTE risk. This could therefore help stratify glioma patients into low- and high-risk groups for prospective trials, potentially avoiding thromboprophylaxis in patients at very low risk of this dangerous complication.

Thus far, there are two potential complementary mechanisms whereby mutant *IDH1/2* suppresses thrombosis (Fig. 5). The first is methylation-based, wherein mutant *IDH1/2* causes *F3* promoter hypermethylation [57], leading to reduced *F3* mRNA transcription and TF protein expression. Indeed, the original study that showed global CpG hypermethylation in mutant *IDH1/2* GBMs included *F3* among the top 50 hypermethylated and downregulated genes in that GBM subset [36]. Many cancers release significant amounts of biologically active TF into the systemic circulation in the form of membrane-bound vesicles, called TF-containing microparticles (TF-MPs), which have been repeatedly linked to cancer-induced VTE [3, 7, 10, 32, 43–45, 55]. Such a connection is still equivocal in gliomas, as some

studies have shown elevated circulating TF-MPs in glioma patients with VTE [44, 45], but not others [52–54]. Our data supports a role for TF-MPs in glioma-induced VTE (Fig. 3d, Table 2). Differences in methods and sampling may account for these conflicting reports and, as has been previously observed, makes comparisons between studies difficult [19]. In contrast to prior work, we analyzed TF-MP activity in peripheral arterial plasma rather than venous plasma. Other groups often included patients with recurrent and treated tumors, whereas we included only preoperative blood obtained from new, untreated glioma patients. Our data therefore suggest that a relative lack of TF-MP production and activity could contribute not only to the absence of microthrombi within a mutant *IDH1/2* tumor (Fig. 1–Fig. 3), but also to the absence of distal VTE (Fig. 3d, Table 1–Table 2.).

The second potential mechanism is a novel, rapid, calcium-dependent inhibition of human platelet aggregation and clotting by D-2-HG (Fig. 4). Homogenates of mutant *IDH1/2* gliomas contain up to 30 mM D-2-HG [13], and cells expressing mutant IDH1 release D-2-HG into culture medium [13, 18]. A precise estimate of the concentration of D-2-HG in the immediate tumor microenvironment is difficult to calculate, but concentrations exceeding 100 μ M D-2-HG have recently been reported in the circulating cerebrospinal fluid of mutant *IDH1/2* glioma patients [23], meaning that the levels of D-2-HG within the immediate tumor microenvironment may frequently be in the millimolar range. Glioma xenografts expressing R132H IDH1 showed severely reduced coagulation following tail snip of host mice, thereby suggesting that an intact tumor can release sufficient D-2-HG to affect clotting. Of note, in the model shown in Fig. 4, R132H IDH1 did not alter TF expression in U87MG cells (not shown) or significantly alter circulating TF-MP activity in the periphery (Supplemental Fig. 11). R132H IDH1 typically requires sustained expression (>30 cell passages) to induce DNA hypermethylation [57]. This is much longer than what was done for the current model (<10 passages), which was specifically designed to study the effect of R132H IDH1 on *in vivo* platelet activity, not TF expression and thrombosis. In higher-grade gliomas, where the blood-brain barrier breaks down and is most conducive to thrombosis, D-2-HG could enter the blood and exert a local suppressive effect on platelet aggregation within the tumor vascular bed (Fig. 5). However, mutant *IDH1/2* gliomas only achieve low micromolar concentrations of D-2-HG in the serum of patients [8], so platelet activity and coagulation are unlikely to be significantly affected in the periphery. To our knowledge, no data exists on the level of D-2-HG that is reached in the interstitial and intravascular compartments within a mutant *IDH1/2* glioma, though it is likely to be much higher than that observed in the peripheral circulation. D-2-HG could also exert paracrine effects on the behavior of other nonneoplastic cells in and around the tumor, including another hallmark of GBM, endothelial cell proliferation (Supplemental Fig. 12).

This antiplatelet activity of D-2-HG cannot be related to methylation, because the effect is too rapid, and because platelets lack DNA. The most well-studied mechanism of D-2-HG activity is competitive inhibition of demethylase dioxygenases that require alpha-ketoglutarate as a cofactor [20]. But in platelets the only alpha-ketoglutarate -dependent dioxygenases are procollagen-lysine, 2-oxoglutarate 5-dioxygenases 1 and 3 [6]. These enzymes crosslink collagen chains to each other and are involved in neither aggregation nor methylation. 12/15 lipoxygenase is involved in platelet aggregation, but does not depend on alpha-ketoglutarate. Our data therefore suggest an entirely new mechanism of action by D-2-

HG, and its perturbation of calcium signaling could affect numerous pathways and activities in and around mutant *IDH1/2* glioma cells.

The anticoagulation effect of mutant *IDH1/2* and D-2-HG is consistent with prior studies. First, well before the discovery of *IDH1/2* mutations, Kondziolka et al. reported that intratumoral hemorrhage was significantly more frequent in grade II-III gliomas (18/135 cases, 13.3%) than GBMs (17/264, 6.4%) ($P=0.025$ via Fisher's exact test) [25]. Furthermore, they observed the highest levels of intratumoral hemorrhages in oligodendrogliomas, which are now known to be almost invariably driven by *IDH1/2* mutations. Second, brain bleeding can occur in patients with the rare metabolic disorder D-2-HG aciduria [26, 59]. Third, mice constitutively expressing mutant *IDH1* in the brain die of perinatal intracerebral hemorrhage [46].

In summary, our study has new implications for several aspects of glioma research and clinical practice, including understanding the histologic and biologic differences between mutant *IDH1/2* and wild-type *IDH1/2* gliomas, demonstrating a novel methylation-independent mechanism of action by mutant *IDH1/2*, and more effectively identifying glioma patients at low risk of VTE. This creates many new opportunities to advance our understanding of the relationships between glioma mutations and tumor biology, and to ultimately improve personalized medicine in glioma patients.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

CH was supported by the National Cancer Institute (K08CA155764). The authors thank Michael Gallagher for Fig. 5, Bernice Slone from the Kentucky Cancer Registry for outcome data, Dana Napier for histotechnical support, Sarah Langford for assistance with ionized calcium measurements, Dr. Sidney Whiteheart for advice on tail vein bleeding assays, Dr. Andrew Lane for the NMR experiment and manuscript comments, Kathleen McCartney for collecting the blood samples used in Figure 3b and Table 2, and Dr. C. David James for manuscript comments. The University of Kentucky Biospecimen and Tissue Procurement, Biostatistics, and Redox Metabolism Shared Resource Facilities are supported by the Markey Cancer Center (P30CA177558). The Northwestern Nervous System Tumor Bank is supported by the Department of Neurological Surgery. Methylation profiling of brain tumors at NYU is supported by The Friedberg Charitable Foundation.

References

1. Ades S, Kumar S, Alam M, Goodwin A, Weckstein D, Dugan M, Ashikaga T, Evans M, Verschraegen C, Holmes CE. Tumor oncogene (KRAS) status and risk of venous thrombosis in patients with metastatic colorectal cancer. *J Thromb Haemost.* 2015; 13:998–1003. [PubMed: 25809746]
2. Anand M, Brat DJ. Oncogenic regulation of tissue factor and thrombosis in cancer. *Thromb Res.* 2012; (129 Suppl 1):S46–S49.
3. Bastida E, Ordinas A, Escolar G, Jamieson GA. Tissue factor in microvesicles shed from U87MG human glioblastoma cells induces coagulation, platelet aggregation, and thrombogenesis. *Blood.* 1984; 64:177–184. [PubMed: 6733271]
4. Brat DJ, Verhaak RG, Aldape KD, Yung WK, Salama SR, Cooper LA, Rheinbay E, Miller CR, Vitucci M, Morozova O, et al. Comprehensive, Integrative Genomic Analysis of Diffuse Lower-Grade Gliomas. *The New England journal of medicine.* 2015; 372:2481–2498. [PubMed: 26061751]

5. Budeczies J, Klauschen F, Sinn BV, Gyorffy B, Schmitt WD, Darb-Esfahani S, Denkert C. Cutoff Finder: a comprehensive and straightforward Web application enabling rapid biomarker cutoff optimization. *PLoS One*. 2012; 7:e51862. [PubMed: 23251644]
6. Burkhart JM, Vaudel M, Gambaryan S, Radau S, Walter U, Martens L, Geiger J, Sickmann A, Zahedi RP. The first comprehensive and quantitative analysis of human platelet protein composition allows the comparative analysis of structural and functional pathways. *Blood*. 2012; 120:e73–e82. [PubMed: 22869793]
7. Butenas S, Bouchard BA, Brummel-Ziedins KE, Parhami-Seren B, Mann KG. Tissue factor activity in whole blood. *Blood*. 2005; 105:2764–2770. [PubMed: 15604222]
8. Capper D, Simon M, Langhans CD, Okun JG, Tonn JC, Weller M, von Deimling A, Hartmann C, German Glioma N. 2-Hydroxyglutarate concentration in serum from patients with gliomas does not correlate with IDH1/2 mutation status or tumor size. *Int J Cancer*. 2011; 131:766–768. [PubMed: 21913188]
9. Chaichana KL, Pendleton C, Jackson C, Martinez-Gutierrez JC, Diaz-Stransky A, Aguayo J, Olivi A, Weingart J, Gallia G, Lim M, et al. Deep venous thrombosis and pulmonary embolisms in adult patients undergoing craniotomy for brain tumors. *Neurol Res*. 2013; 35:206–211. [PubMed: 23336127]
10. Chou J, Mackman N, Merrill-Skoloff G, Pedersen B, Furie BC, Furie B. Hematopoietic cell-derived microparticle tissue factor contributes to fibrin formation during thrombus propagation. *Blood*. 2004; 104:3190–3197. [PubMed: 15280200]
11. Chowdhury R, Yeoh KK, Tian YM, Hillringhaus L, Bagg EA, Rose NR, Leung IK, Li XS, Woon EC, Yang M, et al. The oncometabolite 2-hydroxyglutarate inhibits histone lysine demethylases. *EMBO Rep*. 2011; 12:463–469. [PubMed: 21460794]
12. Corrales-Rodriguez L, Soulieres D, Weng X, Tehfe M, Florescu M, Blais N. Mutations in NSCLC and their link with lung cancer-associated thrombosis: a case-control study. *Thromb Res*. 2014; 133:48–51. [PubMed: 24290524]
13. Dang L, White DW, Gross S, Bennett BD, Bittinger MA, Driggers EM, Fantin VR, Jang HG, Jin S, Keenan MC, et al. Cancer-associated IDH1 mutations produce 2-hydroxyglutarate. *Nature*. 2009; 462:739–744. [PubMed: 19935646]
14. Figueroa ME, Abdel-Wahab O, Lu C, Ward PS, Patel J, Shih A, Li Y, Bhagwat N, Vasanthakumar A, Fernandez HF, et al. Leukemic IDH1 and IDH2 mutations result in a hypermethylation phenotype, disrupt TET2 function, and impair hematopoietic differentiation. *Cancer Cell*. 2010; 18:553–567. [PubMed: 21130701]
15. Food and Drug Administration Oncologic Drug Advisory Committee Wednesday. [Accessed July 10, 2015] Jun 20. morning session <http://www.fda.gov/downloads/AdvisoryCommittees/CommitteesMeetingMaterials/Drugs/OncologicDrugsAdvisoryCommittee/UCM314708.pdf>
16. Garnier D, Milsom C, Magnus N, Meehan B, Weitz J, Yu J, Rak J. Role of the tissue factor pathway in the biology of tumor initiating cells. *Thromb Res*. 2010; (125 Suppl 2):S44–S50. [PubMed: 20434004]
17. Gerber NK, Goenka A, Turcan S, Reyngold M, Makarov V, Kannan K, Beal K, Omuro A, Yamada Y, Gutin P, et al. Transcriptional diversity of long-term glioblastoma survivors. *Neuro Oncol*. 2014; 16:1186–1195. [PubMed: 24662514]
18. Gilbert MR, Liu Y, Neltner J, Pu H, Morris A, Sunkara M, Pittman T, Kyprianou N, Horbinski C. Autophagy and oxidative stress in gliomas with IDH1 mutations. *Acta neuropathologica*. 2014; 127:221–233. [PubMed: 24150401]
19. Hisada Y, Alexander W, Kasthuri R, Voorhees P, Mobarrez F, Taylor A, McNamara C, Wallen H, Witkowski M, Key NS, et al. Measurement of microparticle tissue factor activity in clinical samples: A summary of two tissue factor-dependent FXa generation assays. *Thromb Res*. 2016; 139:90–97. [PubMed: 26916302]
20. Horbinski C. What do we know about IDH1/2 mutations so far, and how do we use it? *Acta neuropathologica*. 2013; 125:621–636. [PubMed: 23512379]
21. Hu J, Yan J, Rao G, Latha K, Overwijk WW, Heimberger AB, Li S. The Duality of Fgl2 - Secreted Immune Checkpoint Regulator Versus Membrane-Associated Procoagulant: Therapeutic Potential and Implications. *Int Rev Immunol*. 2014; 2014:26.

22. Isaka T, Yoshimine T, Maruno M, Kuroda R, Ishii H, Hayakawa T. Altered expression of antithrombotic molecules in human glioma vessels. *Acta neuropathologica*. 1994; 87:81–85. [PubMed: 8140898]
23. Kalinina J, Ahn J, Devi NS, Wang L, Li W, Olson JJ, Glantz M, Smith T, Kim EL, Giese A, et al. Selective detection of the D-enantiomer of 2-Hydroxyglutarate in the CSF of glioma patients with mutated Isocitrate Dehydrogenase. *Clin Cancer Res*. 2016 In Press.
24. Kohl M, Plischke M, Leffondre K, Heinze G. PSHREG: a SAS macro for proportional and nonproportional subdistribution hazards regression. *Comput Methods Programs Biomed*. 2015; 118:218–233. [PubMed: 25572709]
25. Kondziolka D, Bernstein M, Resch L, Tator CH, Fleming JF, Vanderlinden RG, Schutz H. Significance of hemorrhage into brain tumors: clinicopathological study. *J Neurosurg*. 1987; 67:852–857. [PubMed: 3316531]
26. Kwong KL, Mak T, Fong CM, Poon KH, Wong SN, So KT. D-2-Hydroxyglutaric aciduria and subdural haemorrhage. *Acta Paediatr*. 2002; 91:716–718. [PubMed: 12162609]
27. Lai A, Kharbanda S, Pope WB, Tran A, Solis OE, Peale F, Forrest WF, Pujara K, Carrillo JA, Pandita A, et al. Evidence for sequenced molecular evolution of IDH1 mutant glioblastoma from a distinct cell of origin. *J Clin Oncol*. 2011; 29:4482–4490. [PubMed: 22025148]
28. Liu Y, Gilbert MR, Kyprianou N, Rangnekar VM, Horbinski C. The tumor suppressor prostate apoptosis response-4 (Par-4) is regulated by mutant IDH1 and kills glioma stem cells. *Acta neuropathologica*. 2014; 128:723–732. [PubMed: 25135281]
29. Louis, DN., Ohgaki, H., Wiestler, OD., Cavenee, WK. WHO Classification of Tumors of the Central Nervous System. In: Ohgaki, H., editor. World Health Organization Classification of Tumors. 4. IARC, City: 2007.
30. Lu C, Ward PS, Kapoor GS, Rohle D, Turcan S, Abdel-Wahab O, Edwards CR, Khanin R, Figueroa ME, Melnick A, et al. IDH mutation impairs histone demethylation and results in a block to cell differentiation. *Nature*. 2012; 483:474–478. [PubMed: 22343901]
31. Lyman GH, Bohlke K, Khorana AA, Kuderer NM, Lee AY, Arcelus JJ, Balaban EP, Clarke JM, Flowers CR, Francis CW, et al. Venous thromboembolism prophylaxis and treatment in patients with cancer: american society of clinical oncology clinical practice guideline update 2014. *J Clin Oncol*. 2015; 33:654–656. [PubMed: 25605844]
32. Milsom C, Yu J, May L, Meehan B, Magnus N, Al-Nedawi K, Luyendyk J, Weitz J, Klement P, Broze G, et al. The role of tumor-and host-related tissue factor pools in oncogene-driven tumor progression. *Thromb Res*. 2007; (120 Suppl 2):S82–S91. [PubMed: 18023719]
33. Nadir Y, Vlodavsky I, Brenner B. Heparanase, tissue factor, and cancer. *Semin Thromb Hemost*. 2008; 34:187–194. [PubMed: 18645924]
34. National Comprehensive Cancer Network Cancer-Associated Venous Thromboembolic Disease (version 2.2014). [Accessed August 2, 2015] http://www.nccn.org/professionals/physician_gls/f_guidelines.asp
35. Nobusawa S, Watanabe T, Kleihues P, Ohgaki H. IDH1 mutations as molecular signature and predictive factor of secondary glioblastomas. *Clin Cancer Res*. 2009; 15:6002–6007. [PubMed: 19755387]
36. Noshmehr H, Weisenberger DJ, Diefes K, Phillips HS, Pujara K, Berman BP, Pan F, Pelloso CE, Sulman EP, Bhat KP, et al. Identification of a CpG island methylator phenotype that defines a distinct subgroup of glioma. *Cancer Cell*. 2010; 17:510–522. [PubMed: 20399149]
37. Perry JR. Thromboembolic disease in patients with high-grade glioma. *Neuro Oncol*. 2012; (14 Suppl 4)
38. Prayson NF, Koch P, Angelov L, Prayson RA. Microscopic thrombi in anaplastic astrocytoma predict worse survival? *Ann Diagn Pathol*. 2011; 15:389–393. [PubMed: 21849253]
39. Reuss DE, Sahm F, Schrimpf D, Wiestler B, Capper D, Koelsche C, Schweizer L, Korshunov A, Jones DT, Hovestadt V, et al. ATRX and IDH1-R132H immunohistochemistry with subsequent copy number analysis and IDH sequencing as a basis for an “integrated” diagnostic approach for adult astrocytoma, oligodendroglioma and glioblastoma. *Acta neuropathologica*. 2015; 129:133–146. [PubMed: 25427834]

40. Ringertz N. Grading of gliomas. *Acta Pathol Microbiol Scand*. 1950; 27:51–64. [PubMed: 15406242]
41. Rohle D, Popovici-Muller J, Palaskas N, Turcan S, Grommes C, Campos C, Tsoi J, Clark O, Oldrini B, Komisopoulou E, et al. An inhibitor of mutant IDH1 delays growth and promotes differentiation of glioma cells. *Science*. 2013; 340:626–630. [PubMed: 23558169]
42. Rong Y, Durden DL, Van Meir EG, Brat DJ. 'Pseudopalisading' necrosis in glioblastoma: a familiar morphologic feature that links vascular pathology, hypoxia, and angiogenesis. *J Neuropathol Exp Neurol*. 2006; 65:529–539. [PubMed: 16783163]
43. Rong Y, Post DE, Pieper RO, Durden DL, Van Meir EG, Brat DJ. PTEN and hypoxia regulate tissue factor expression and plasma coagulation by glioblastoma. *Cancer Res*. 2005; 65:1406–1413. [PubMed: 15735028]
44. Sartori MT, Della Puppa A, Ballin A, Campello E, Radu CM, Saggiorato G, d'Avella D, Scienza R, Cella G, Simioni P. Circulating microparticles of glial origin and tissue factor bearing in high-grade glioma: a potential prothrombotic role. *Thromb Haemost*. 2013; 110:378–385. [PubMed: 23803674]
45. Sartori MT, Della Puppa A, Ballin A, Saggiorato G, Bernardi D, Padoan A, Scienza R, d'Avella D, Cella G. Prothrombotic state in glioblastoma multiforme: an evaluation of the procoagulant activity of circulating microparticles. *J Neurooncol*. 2011; 104:225–231. [PubMed: 21104297]
46. Sasaki M, Knobbe CB, Itsumi M, Elia AJ, Harris IS, Chio II, Cairns RA, McCracken S, Wakeham A, Haight J, et al. D-2-hydroxyglutarate produced by mutant IDH1 perturbs collagen maturation and basement membrane function. *Genes Dev*. 2012; 26:2038–2049. [PubMed: 22925884]
47. Sciacca FL, Ciusani E, Silvani A, Corsini E, Frigerio S, Pogliani S, Parati E, Croci D, Boiardi A, Salmaggi A. Genetic and plasma markers of venous thromboembolism in patients with high grade glioma. *Clin Cancer Res*. 2004; 10:1312–1317. [PubMed: 14977830]
48. Smith TR, Lall RR, Graham RB, McClendon J Jr, Lall RR, Nanney AD, Adel JG, Zakarija A, Chandler JP. Venous thromboembolism in high grade glioma among surgical patients: results from a single center over a 10 year period. *J Neurooncol*. 2014; 120:347–352. [PubMed: 25062669]
49. Sorensen HT, Mellekjær L, Olsen JH, Baron JA. Prognosis of cancers associated with venous thromboembolism. *The New England journal of medicine*. 2000; 343:1846–1850. [PubMed: 11117976]
50. Sturm D, Witt H, Hovestadt V, Khuong-Quang DA, Jones DT, Konermann C, Pfaff E, Tonjes M, Sill M, Bender S, et al. Hotspot mutations in H3F3A and IDH1 define distinct epigenetic and biological subgroups of glioblastoma. *Cancer Cell*. 2012; 22:425–437. [PubMed: 23079654]
51. Tehrani M, Friedman TM, Olson JJ, Brat DJ. Intravascular thrombosis in central nervous system malignancies: a potential role in astrocytoma progression to glioblastoma. *Brain Pathol*. 2008; 18:164–171. [PubMed: 18093251]
52. Thaler J, Ay C, Kaider A, Reitter EM, Haselbock J, Mannhalter C, Zielinski C, Marosi C, Pabinger I. Biomarkers predictive of venous thromboembolism in patients with newly diagnosed high-grade gliomas. *Neuro Oncol*. 2014; 16:1645–1651. [PubMed: 24987133]
53. Thaler J, Ay C, Mackman N, Bertina RM, Kaider A, Marosi C, Key NS, Barcel DA, Scheithauer W, Kornek G, et al. Microparticle-associated tissue factor activity, venous thromboembolism and mortality in pancreatic, gastric, colorectal and brain cancer patients. *J Thromb Haemost*. 2012; 10:1363–1370. [PubMed: 22520016]
54. Thaler J, Preusser M, Ay C, Kaider A, Marosi C, Zielinski C, Pabinger I, Hainfellner JA. Intratumoral tissue factor expression and risk of venous thromboembolism in brain tumor patients. *Thromb Res*. 2013; 131:162–165. [PubMed: 23084660]
55. Thomas GM, Panicot-Dubois L, Lacroix R, Dignat-George F, Lombardo D, Dubois C. Cancer cell-derived microparticles bearing P-selectin glycoprotein ligand 1 accelerate thrombus formation in vivo. *J Exp Med*. 2009; 206:1913–1927. [PubMed: 19667060]
56. Timp JF, Braekkan SK, Versteeg HH, Cannegieter SC. Epidemiology of cancer-associated venous thrombosis. *Blood*. 2013; 122:1712–1723. [PubMed: 23908465]
57. Turcan S, Rohle D, Goenka A, Walsh LA, Fang F, Yilmaz E, Campos C, Fabius AW, Lu C, Ward PS, et al. IDH1 mutation is sufficient to establish the glioma hypermethylator phenotype. *Nature*. 2012:2012.

58. Wang P, Dong Q, Zhang C, Kuan PF, Liu Y, Jeck WR, Andersen JB, Jiang W, Savich GL, Tan TX, et al. Mutations in isocitrate dehydrogenase 1 and 2 occur frequently in intrahepatic cholangiocarcinomas and share hypermethylation targets with glioblastomas. *Oncogene*. 2012;315.
59. Wang X, Jakobs C, Bawle EV. D-2-Hydroxyglutaric aciduria with absence of corpus callosum and neonatal intracranial haemorrhage. *J Inherit Metab Dis*. 2003; 26:92–94. [PubMed: 12872850]
60. Xu W, Yang H, Liu Y, Yang Y, Wang P, Kim SH, Ito S, Yang C, Xiao MT, Liu LX, et al. Oncometabolite 2-Hydroxyglutarate Is a Competitive Inhibitor of alpha-Ketoglutarate-Dependent Dioxygenases. *Cancer Cell*. 2010; 19:17–30.
61. Yan H, Parsons DW, Jin G, McLendon R, Rasheed BA, Yuan W, Kos I, Batinic-Haberle I, Jones S, Riggins GJ, et al. IDH1 and IDH2 mutations in gliomas. *The New England journal of medicine*. 2009; 360:765–773. [PubMed: 19228619]
62. Zerrouqi A, Pyrzynska B, Brat DJ, Van Meir EG. P14ARF suppresses tumor-induced thrombosis by regulating the tissue factor pathway. *Cancer Res*. 2014; 74:1371–1378. [PubMed: 24398474]
63. Zwicker JJ, Liebman HA, Bauer KA, Caughey T, Campigotto F, Rosovsky R, Mantha S, Kessler CM, Eneman J, Raghavan V, et al. Prediction and prevention of thromboembolic events with enoxaparin in cancer patients with elevated tissue factor-bearing microparticles: a randomized-controlled phase II trial (the Microtec study). *Br J Haematol*. 2013; 160:530–537. [PubMed: 23240761]

Fig. 1.

Microthrombi and *IDH1/2* mutations in gliomas. Frequency of intratumoral microthrombi according to *IDH1/2* mutation status in discovery (left) and validation (right) cohorts, in all WHO grade II-IV gliomas (a & b), grade II-III (c & d), grade IV GBM (e & f), and necrotic gliomas (g & h). $P < 0.001$ in panels a–b and e–h; $P = 0.02$ in panel c and 0.0034 in panel d. All P values were calculated by Fisher's exact test.

Fig. 2. Coagulation-associated genes according to *IDH1/2* status in gliomas. Expression of key genes known to be involved in clotting and glioma-associated thrombosis [21, 22, 33, 45, 47, 62] (Supplemental Table 5) was assessed via Illumina HiSeq 2000 RNA Sequencing platform in grade II-IV TCGA gliomas. Each row represents a single tumor, columns indicate either *IDH1/2* status (aqua = *IDH2* mutant, red = *IDH1* mutant, black = *IDH1/2* wild-type); WHO grade (peach = II, green = III, purple = IV); or gene expression as a ratio of the individual tumor mRNA relative to the mean of all *IDH1/2* wild-type tumors for that

gene (blue = downregulated, yellow = upregulated, white = no change). Genes in the pink shaded area are procoagulant; genes in the light blue shaded area are anti-coagulant. N = 196 *IDH1/2* wild-type and 234 mutant *IDH1/2* gliomas.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Fig. 3.

TF by *IDH1/2* status in gliomas. (a) Mean increase in methylation beta value at each CpG site in the *F3* promoter of mutant *IDH1/2* gliomas, relative to wild-type gliomas. CpG sites are grouped according to their location within and around the CpG island of the *F3* promoter. * $P < 0.01$ in all 3 grades of glioma. See Supplemental Table 6 for additional details. (b) Representative H&E photomicrographs of *IDH1/2* wild-type and mutant GBMs from the TMA cohort, and their corresponding tissue factor IHC. Scale bar in lower right panel = 50 μ m. (c) TF IHC scores according to *IDH1/2* mutation status in high-grade (III–IV) gliomas. (d) Preoperative TF-MP activity, measured in pg Factor Xa generation per ml in platelet-free plasma, obtained from the arterial blood of patients with *IDH1/2* wild-type and mutant gliomas (see Table 2). Dotted line represents the cutoff for “strong TF activity” in cancer patients, as published previously [19]. In (c) and (d), each point represents a single tumor, and red triangles in (d) represent patients who developed VTE. Bars = mean $\pm$ SD.

Fig. 4. Platelet activity, calcium, and clotting time in the presence of mutant IDH1 and D-2-HG. Washed human platelets (2×10^8 /mL) from 3 distinct donors were stimulated by (a) 1 μ g/mL collagen or (b) 0.1 U/mL thrombin in the presence or absence of D-2-HG. Platelet aggregation was measured by turbidity (Chronolog) with stirring (1000 rpm). (c) Platelets were labeled with Fura2-AM and iCa^{++} levels were determined via 340nm/380nm excitation ratio under the specified conditions. (d) Washed human platelets were stimulated by thrombin or the calcium ionophore A23187 in the presence or absence of D-2-HG. (e)

Human serum levels of free Ca^{++} in the presence of D-2-HG (note, SD bars small and not visible). (f) Clotting time of human blood in the presence of D-2-HG. Data are expressed as clotting time beyond matched untreated controls (mean baseline = 190 seconds) $\pm$ SD. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. (g) Free Ca^{++} levels in serum from mice bearing flank xenografts of human gliomas with and without R132H IDH1. (h) Tail vein bleeding times for mice bearing flank xenografts of human gliomas with and without R132H IDH1.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Fig. 5.

Postulated mechanism of mutant *IDH1/2* suppression of local and systemic thrombosis. (a) Wild-type *IDH1/2* glioma cells produce tissue factor-containing microparticles (TF-MPs, blue dots), which promote microthrombus formation within the tumor (yellow and brown objects = platelets). TF-MPs from the glioma also circulate throughout the body, increasing the risk of DVTs and PEs. (b) In contrast, mutant *IDH1/2* gliomas make very little TF, and consequently release very little TF-MPs, but do produce D-2-HG (pink triangles). In the local tumor vascular bed, where permeability increases in higher-grade tumors (arrows), the

reduced numbers of TF-MPs, plus the antiplatelet activity of D-2-HG, combine to prevent intratumoral thrombosis. In the systemic circulation, D-2-HG is not present at sufficient concentrations to inhibit peripheral platelet aggregation, but the lack of glioma-derived TF-MPs still results in low risk for VTE.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Table 1
Patient Characteristics for the Discovery and Validation Cohorts, Stratified by *IDH1/2* Mutation Status.

Characteristic	Discovery Cohort (N = 169)				Validation Cohort (N = 148)			
	No. of Patients	<i>IDH1/2</i> Wild-type, No. (%)	<i>IDH1/2</i> Mutant, No. (%)	<i>P</i>	<i>IDH1/2</i> Wild-type, No. (%)	<i>IDH1/2</i> Mutant, No. (%)	<i>P</i>	
Total	317	117 (69.2)	52 (30.8)	—	114 (77.0)	34 (23.0)	—	
Mean age at diagnosis, years	317	60.6 ± 1.1	39.4 ± 1.6	< .001	64.4 ± 1.3	46.0 ± 2.1	< .001	
Gender								1.0
Male	175	61 (52.1)	27 (51.9)	.98	67 (58.8)	20 (58.8)		
Female	142	56 (47.9)	25 (48.1)		47 (41.2)	14 (41.2)		
WHO grade								
II	41	1 (0.9)	28 (53.8)	< .001	0 (0.0)	12 (35.3)	< .001	
III	38	7 (6.0)	15 (28.8)		6 (5.3)	10 (29.4)		
IV	238	109 (93.2)	9 (17.3)		108 (94.7)	12 (35.3)		
Subtype								
Astrocytoma	283	117 (100.0)	31 (59.6)	< .001	114 (100.0)	22 (64.7)	< .001	
Oligodendroglioma	34	0 (0.0)	21 (40.4)		0 (0.0)	12 (35.3)		
Necrosis								
Yes	227	98 (83.8)	10 (19.2)	< .001	106	13	< .001	
No	90	19 (16.2)	42 (80.8)		8	21		
Intratumoral microthrombi								
Yes	206	100 (85.5)	1 (1.9)	< .001	103	2	< .001	
No	111	17 (14.5)	51 (98.1)		11	32		
Preexisting history of VTE								
Yes	2	2 (1.7)	0 (0.0)	1.0	0 (0.0)	0 (0.0)	1.0	
No	315	115 (98.3)	52 (100.0)		114 (100.0)	34 (100.0)		
VTE status*								

	Discovery Cohort (N = 169)				Validation Cohort (N = 148)			
Characteristic	No. of Patients	IDH1/2 Wild-type, No. (%)	IDH1/2 Mutant, No. (%)	P	IDH1/2 Wild-type, No. (%)	IDH1/2 Mutant, No. (%)	P	
VTE present	61	30 (25.6)	0 (0.0)	< .001	31 (29.5)	0 (0.0)	< .001	
VTE absent	237	87 (74.4)	45 (100.0)		74 (70.5)	31 (100.0)		
Preoperative KPS *								
90	134	59 (50.9)	38 (82.6)	< .001	23 (24.5)	14 (46.7)	.02	
< 90	152	57 (49.1)	8 (17.4)		71 (75.5)	16 (53.3)		
Surgery type *								
GTR	117	35 (29.9)	20 (38.5)	.15	44 (44.0)	18 (58.1)	.33	
STR	109	48 (41.0)	24 (46.2)		29 (29.0)	8 (25.8)		
Biopsy only	74	34 (29.1)	8 (15.4)		27 (27.0)	5 (16.1)		
Length of hospital stay *								
3 days	148	62 (54.4)	39 (86.7)	< .001	29 (43.9)	18 (62.1)	.10	
> 3 days	106	52 (45.6)	6 (13.3)		37 (56.1)	11 (37.9)		
Adjuvant therapy *								
RT only	23	9 (7.7)	5 (10.6)	.01	5 (5.7)	0 (0.0)	.24	
TMZ only	17	5 (4.3)	9 (19.1)		1 (1.1)	2 (6.9)		
RT + TMZ	138	56 (47.9)	21 (44.7)		42 (47.7)	14 (48.3)		
RT + TMZ + BV	67	21 (17.9)	1 (2.1)		20 (22.7)	5 (17.2)		
RT + TMZ + BV + other	20	6 (5.1)	3 (6.4)		8 (9.1)	3 (10.3)		
RT + other	14	4 (3.4)	1 (2.1)		8 (9.1)	1 (3.4)		
none	32	16 (13.7)	7 (14.9)		4 (4.5)	4 (13.8)		

WHO = World Health Organization; VTE = venous thromboemboli; KPS = Kamofsky performance score; GTR = gross total resection; STR = subtotal resection; RT = radiotherapy, TMZ = temozolomide, BV = bevacizumab. *P* values for WHO grade, subtype, necrosis, intratumoral microthrombi, preexisting history of VTE, VTE status, and adjuvant therapy were calculated via Fisher's exact test. *P* values for the remaining characteristics, other than patient age, were calculated via chi square. Patient age was calculated by t-test.

* The majority of patients in the discovery and validation cohorts, respectively, had sufficient clinical details available to determine VTE status (162 and 136), preoperative KPS (162 and 124), surgery type (169 and 131), length of hospital stay (159 and 95), and adjuvant therapy regimen (164 and 117).

Table 2
Preoperative circulating TF-MP activity in glioma patients according to *IDH1/2* mutations.

Age (years)	Gender	Diagnosis	<i>IDH1/2</i>	TF-MP activity (pg/mL)	VTE
65	male	GBM	wild-type	0.00	no
72	female	GBM	wild-type	0.00	no
36	male	anaplastic oligodendroglioma	mutant	0.00	no
35	male	oligodendroglioma II	mutant	0.00	no
33	male	anaplastic oligodendroglioma	mutant	0.00	no
41	female	anaplastic astrocytoma	mutant	0.00	no
29	male	anaplastic astrocytoma	mutant	0.17	no
30	female	oligodendroglioma II	mutant	0.38	no
57	female	GBM	wild-type	0.41	no
77	male	GBM	wild-type	0.49	no
70	male	GBM	wild-type	0.66	no
35	female	GBM	mutant	0.67	no
45	female	anaplastic astrocytoma	wild-type	0.72	no
67	male	GBM	wild-type	0.72	yes
85	female	GBM	wild-type	0.78	no
53	female	GBM	wild-type	0.84	no
63	male	GBM	wild-type	0.87	no
41	female	anaplastic astrocytoma	mutant	0.91	no
30	male	GBM	mutant	0.93	no
33	female	GBM	wild-type	1.06	no
81	male	GBM	wild-type	1.08	no
64	male	GBM	wild-type	1.13	no
62	male	GBM	wild-type	1.45	no
31	female	anaplastic astrocytoma	mutant	1.84	no
73	male	GBM	wild-type	1.86	no
62	male	GBM	wild-type	2.25	no
69	male	GBM	wild-type	2.52	yes

Age (years)	Gender	Diagnosis	IDH1/2	TF-MP activity (pg/mL)	VTE
69	male	GBM	wild-type	2.88	yes
72	male	GBM	wild-type	3.20	yes

Twenty-nine patients with WHO grade II-IV gliomas had Tissue Factor microparticle (TF-MP) activity analyzed in platelet-free plasma isolated from arterial blood before their initial surgery. Patients in *italics* experienced venous thromboemboli (VTE).