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ORIGINAL PAPER

Mutant IDH1 and thrombosis in gliomas

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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 *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

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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 Figure 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], is 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]. Yet the full extent of mutant *IDH1/2* activities within the cell, and in the tumor microenvironment, are not known.

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 have been proposed as 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 to 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 450 K 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

Table 1 Patient characteristics for the discovery and validation cohorts, stratified by *IDH1/2* mutation status

Characteristic	No. of patients	Discovery Cohort ($N = 169$)			Validation Cohort ($N = 148$)		
		IDH1/2 Wild-type, no. (%)	*IDH1/2* Mutant, no. (%)	*P*	*IDH1/2* Wild-type, no. (%)	*IDH1/2* Mutant, no. (%)	*P*
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	<0.001	64.4 ± 1.3	46.0 ± 2.1	<0.001
Gender							1.0
Male	175	61 (52.1)	27 (51.9)	0.98	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)	<0.001	0 (0.0)	12 (35.3)	<0.001
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)	<0.001	114 (100.0)	22 (64.7)	<0.001
Oligodendroglioma	34	0 (0.0)	21 (40.4)		0 (0.0)	12 (35.3)	
Necrosis							
Yes	227	98 (83.8)	10 (19.2)	<0.001	106	13	<0.001
No	90	19 (16.2)	42 (80.8)		8	21	
Intratumoral microthrombi							
Yes	206	100 (85.5)	1 (1.9)	<0.001	103	2	<0.001
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*							
VTE present	61	30 (25.6)	0 (0.0)	<0.001	31 (29.5)	0 (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)	<0.001	23 (24.5)	14 (46.7)	0.02
<90	152	57 (49.1)	8 (17.4)		71 (75.5)	16 (53.3)	
Surgery type*							
GTR	117	35 (29.9)	20 (38.5)	0.15	44 (44.0)	18 (58.1)	0.33
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)	<0.001	29 (43.9)	18 (62.1)	0.10
>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)	0.01	5 (5.7)	0 (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* Karnofsky 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	*IDH1/2*	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*
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)

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, USA). Whole blood was centrifuged at 2500×*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, 5.4 mM KCl, 5.6 mM Glucose, 10 mM HEPES, 0.1 % bovine serum albumin, pH 7.4) and centrifuged at 21,000×*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 50µL of HBSA containing 73.2 nM FX

(Enzyme Research Laboratories), 2.4 nM FVIIa (Enzyme Research Laboratories), and 10 mM $CaCl_2$ for 2 h 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 h. 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 $P \leq 0.05$.

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 VTE, 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 VTE 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 Figure 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

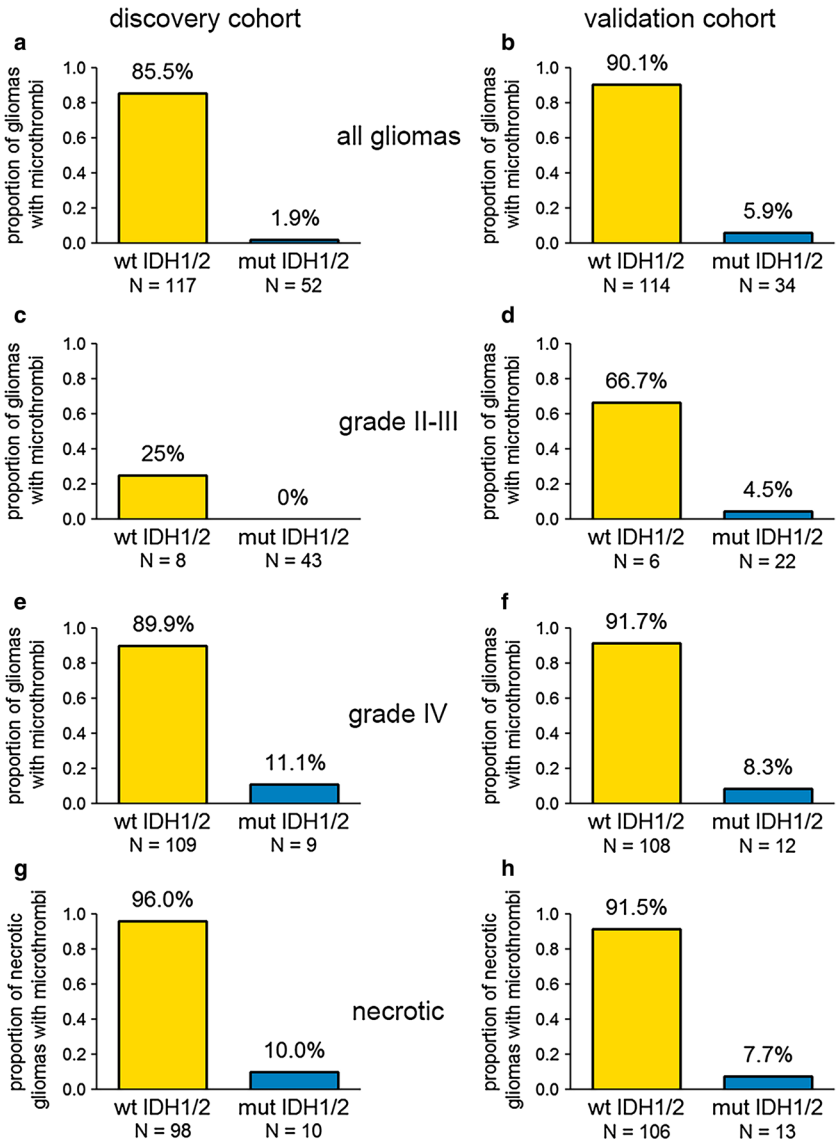

Fig. 1 Microthrombi and *IDH1/2* mutations in gliomas. Frequency of intratumoral microthrombi according to *IDH1/2* mutation status in discovery (*left*) and validation (*right*) cohorts, in all WHO grade II–IV gliomas (**a**, **b**), grade II–III (**c**, **d**), grade IV GBM (**e**, **f**), and necrotic gliomas (**g**, **h**). $P < 0.001$ in **a**, **b** and **e**–**h**; $P = 0.02$ in **c** and 0.0034 in **d**. All *P* values were calculated by Fisher's exact test

microthrombi were nearly always *IDH1/2* wild-type (100 of 117 = 85.5 %, see Table 1; Fig. 1, and Supplemental Figure 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 Figure 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 Figure 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 Figure 6). TF is a transmembrane glycoprotein that is expressed 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 Figure 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 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 Figures 8a and b). D-2-HG inhibited iCa^{++} accumulation in platelets by 72 % (Fig. 4c, Supplemental Figure 8c), and this inhibitory effect was prevented by adding the calcium ionophore A23187 to platelet-D-2-HG mixtures (Fig. 4d, Supplemental Figure 8d). D-2-HG directly bound iCa^{++} ($K_d = 48.9 \pm 3.5$ mM, $R^2 = 0.99913$) (Supplemental Figure 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 Figure 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 puzzling features of mutant *IDH1/2*. 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

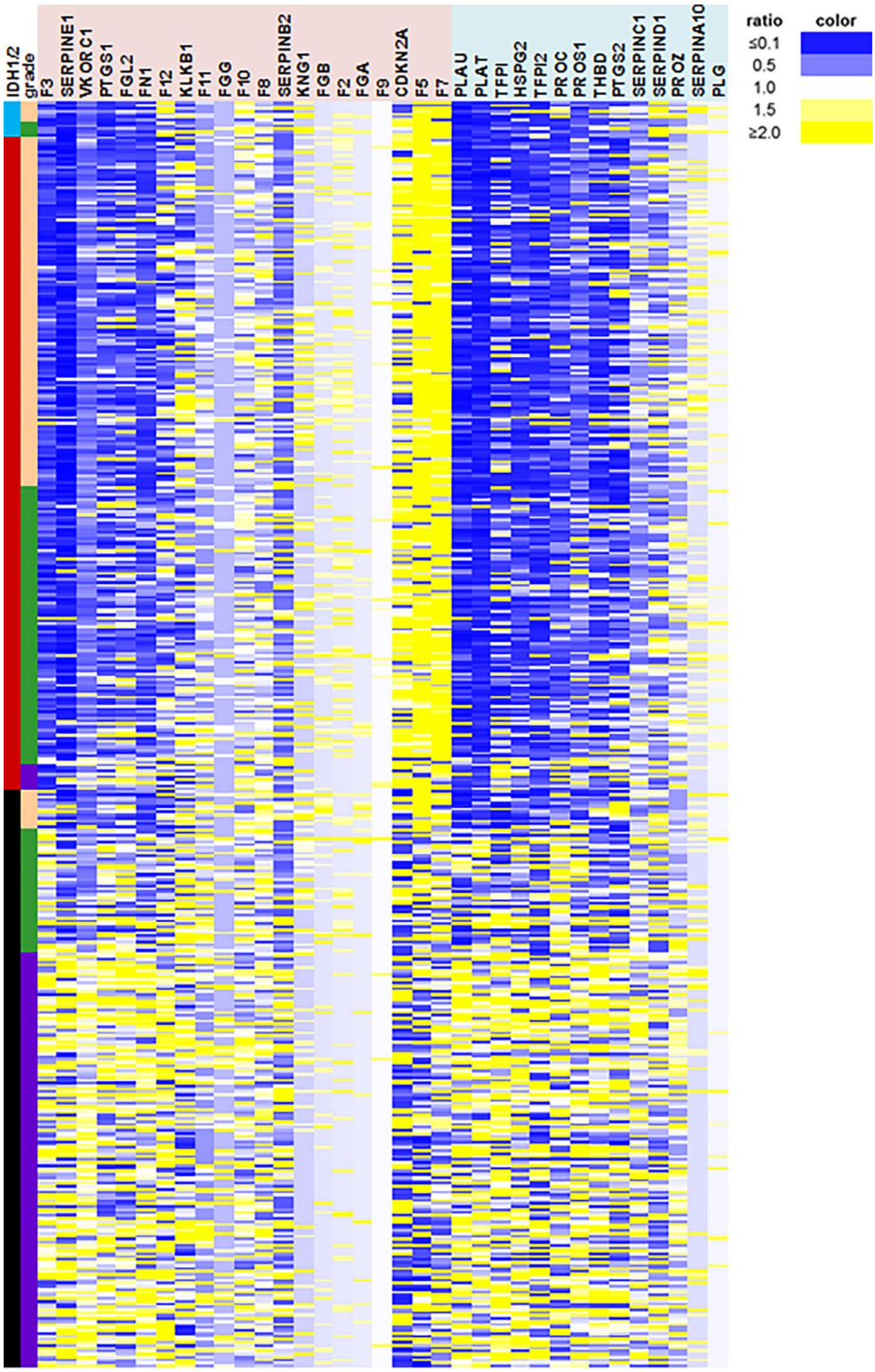

Fig. 2 Coagulation-associated genes according to *IDH1/2* status in gliomas. Expression of key genes known to be involved in clotting and glioma-associated thrombosis [21, 22, 33, 45, 47, 62] (Supplemental Table 5) was assessed via Illumina HiSeq 2000 RNA Sequencing platform in grade II-IV TCGA gliomas. *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* down-regulated, *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

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 Figure 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

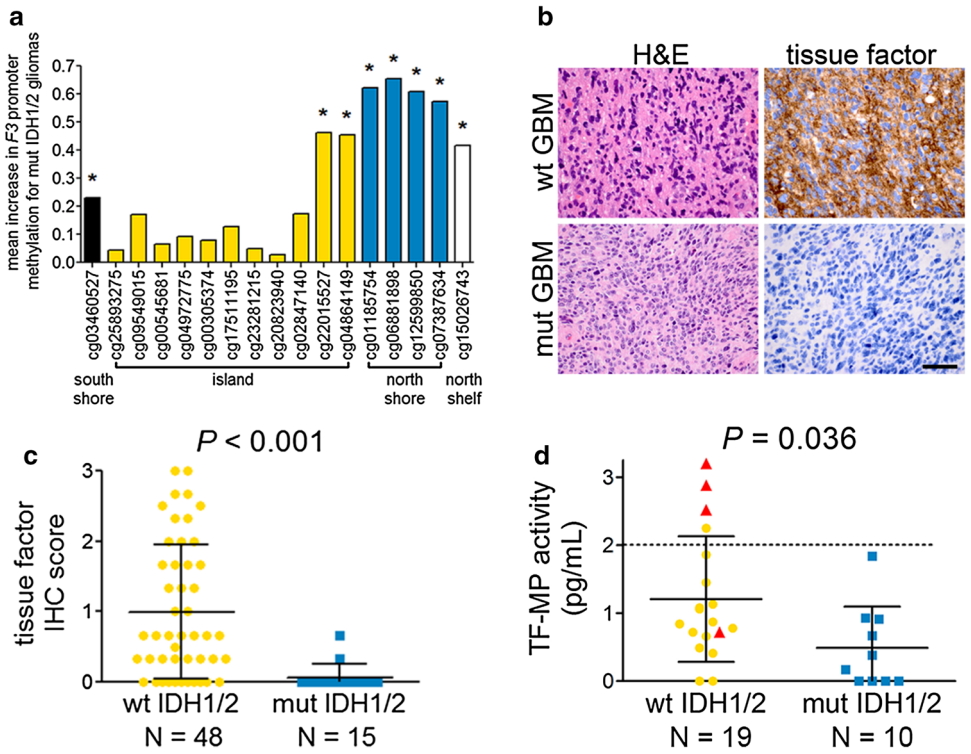

Fig. 3 TF by *IDH1/2* status in gliomas. **a** Mean increase in methylation beta value at each CpG site in the *F3* promoter of mutant IDH1/2 gliomas, relative to wild-type gliomas. CpG sites are grouped according to their location within and around the CpG island of the *F3* promoter. **P* < 0.01 in 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 μm. **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 ± SD

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 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 Figure 4, Supplemental Table 3), and because it is an even stronger correlate of shorter survival than necrosis (Supplemental Figure 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 is challenging in many other areas. Furthermore, even in more 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 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 unnecessary thromboprophylaxis in patients at very low risk of VTE.

Thus far, our data suggest two 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

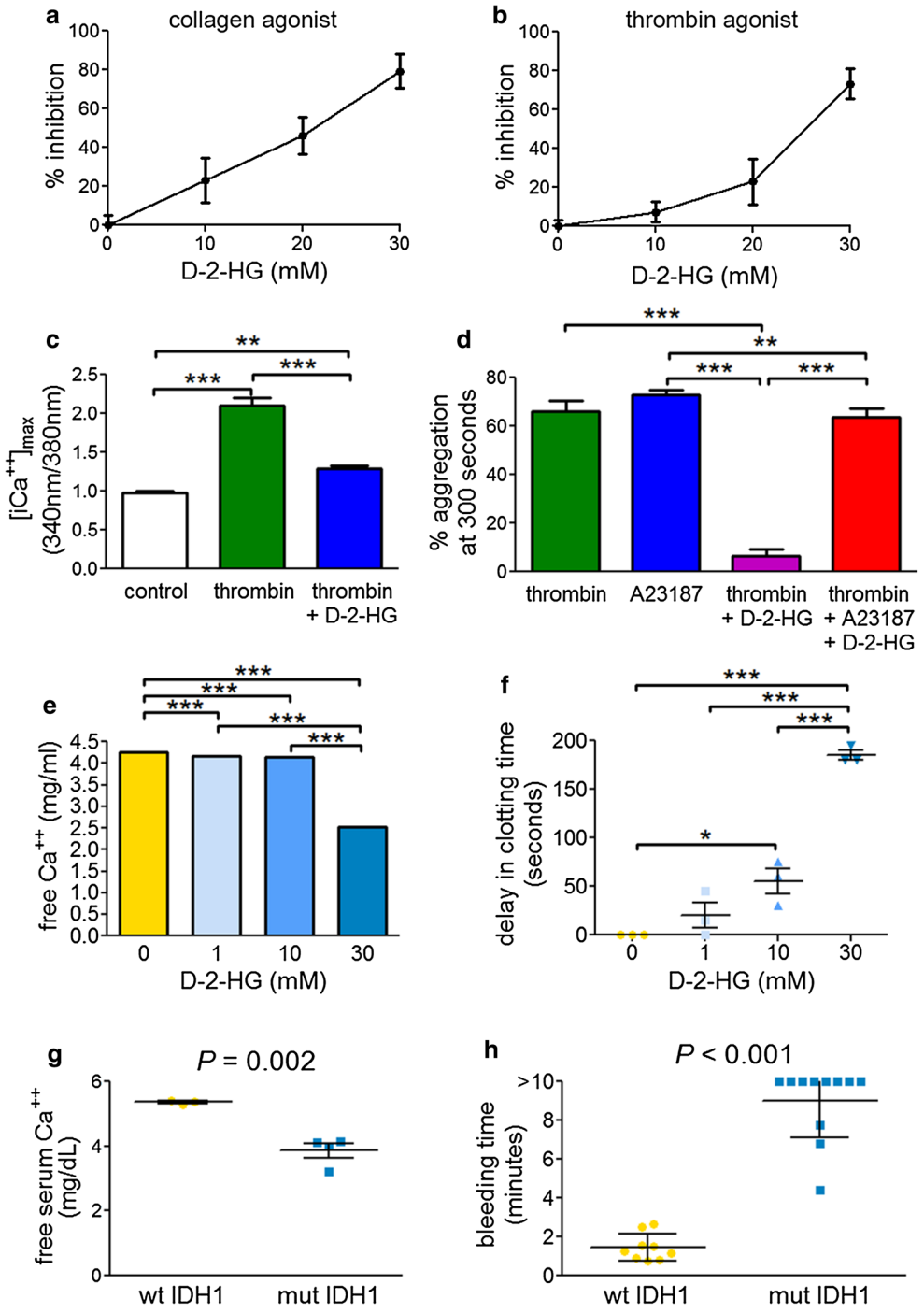

Fig. 4 Platelet activity, calcium, and clotting time in the presence of mutant IDH1 and D-2-HG. Washed human platelets (2×10^8/mL) from 3 distinct donors were stimulated by **a** 1 μg/mL collagen or **b** 0.1 U/mL thrombin in the presence or absence of D-2-HG. Platelet aggregation was measured by turbidity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

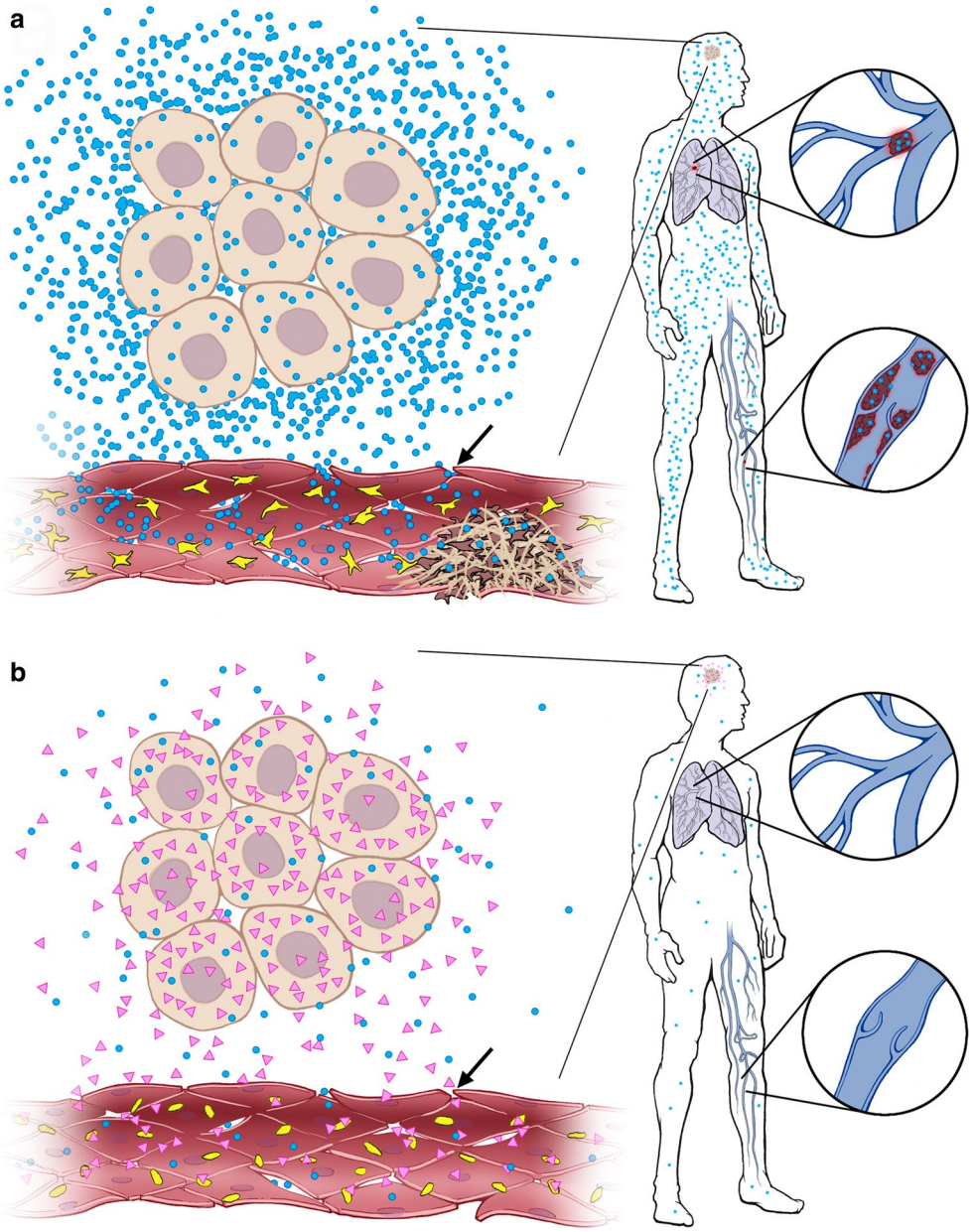

Fig. 5 Postulated mechanism of mutant *IDH1/2* suppression of local and systemic thrombosis. **a** Wild-type *IDH1/2* glioma cells produce tissue factor-containing microparticles (TF-MPs, *blue dots*), which promote microthrombus formation within the tumor (*yellow and brown* objects = platelets). TF-MPs from the glioma also circulate throughout the body, increasing the risk of DVT and PE. **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

genes in that subset of GBMs [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 has been 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 only studied 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 (Figs. 1, 2, 3), but also to the absence of distal VTE (Fig. 3d; Tables 1, 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 μM 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 Figure 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 other nonneoplastic cells in and around the tumor, including another hallmark of GBM, endothelial cell proliferation (Supplemental Figure 12).

The antiplatelet activity of D-2-HG cannot be due 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.

Our discovery that mutant *IDH1/2* and D-2-HG have anticoagulant properties 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.

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