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The contribution of tumor and host tissue factor expression to oncogene-driven gliomagenesis

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ABSTRACT

Glioblastoma multiforme (GBM) is an aggressive form of glial brain tumors, associated with angiogenesis, thrombosis, and upregulation of tissue factor (TF), the key cellular trigger of coagulation and signaling. Since TF is upregulated by oncogenic mutations occurring in different subsets of human brain tumors we investigated whether TF contributes to tumourigenesis driven by oncogenic activation of EGFR (EGFRvIII) and RAS pathways in the brain. Here we show that TF expression correlates with poor prognosis in glioma, but not in GBM. *In situ*, the TF protein expression is heterogeneously expressed in adult and pediatric gliomas. GBM cells harboring EGFRvIII (U373vIII) grow aggressively as xenografts in SCID mice and their progression is delayed by administration of monoclonal antibodies blocking coagulant (CNTO 859) and signaling (10H10) effects of TF *in vivo*. Mice in which *TF* gene is disrupted in the neuroectodermal lineage exhibit delayed progression of spontaneous brain tumors driven by oncogenic *N-ras* and *SV40* large T antigen (*SV40LT*) expressed under the control of sleeping beauty transposase. Reduced host TF levels in low-TF/SCID hypomorphic mice mitigated growth of glioma subcutaneously but not in the brain. Thus, we suggest that tumor-associated TF may serve as therapeutic target in the context of oncogene-driven disease progression in a subset of glioma.

1. Introduction

Astrocytic malignancies (gliomas) comprise a diverse cluster of primary brain tumors, of which glioblastoma multiforme (GBM) is especially aggressive [1]. GBM is also amongst the most vascular and procoagulant human malignancies [1–3] where microvascular vaso-occlusive thrombi occur regularly within tumor masses, and patients are at high risk of peripheral venous thromboembolism (VTE) [2,4]. These exclusively intracranial and lethal tumors have recently been subdivided into at least four molecular forms, including: proneural, neural, classical and mesenchymal GBM. Each of these subtypes is associated with a unique mutational and gene expression signatures, which are indicative of divergent pathogenetic mechanisms [5,6]. This diversity also includes distinctive profiles of genes related to coagulation and fibrinolytic systems (coagulome), many of which are expressed by cancer cells ectopically (e.g., FVII) [7]. In this context, the key receptor triggering the coagulation cascade, tissue factor (TF) is especially highly expressed in the classical subtype of GBM, which is also characterized by the upregulation of the oncogenic epidermal growth factor receptor (EGFR) and the expression of its transforming mutant (EGFRvIII), along with activation of the RAS signaling pathway [7,8]. This is consistent with findings suggesting that the EGFR/RAS pathways, regulate TF expression in cancer cells, including in GBM [9–12].

Abbreviations: EGFR, epidermal growth factor receptor; EGFRvIII, EGFR variant III; GBM, glioblastoma multiforme; PAI-1, plasminogen activator inhibitor 1; PAR (1–4), protease activated receptor (1–4); RAS, rat sarcoma oncogene; SCID, severe combined immunodeficiency (in mice); TF, tissue factor; VTE, venous thromboembolism.

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TF serves as the key cell-associated co-activator/receptor for soluble coagulation factor VII/VIIa. The TF/VIIa complex activates factor X to Xa, and thereby promotes generation of thrombin (IIa), fibrin deposition and activation of platelets [13]. These effects also trigger cellular signaling responses chiefly mediated by coagulation protease activated receptors 1 (PAR-1/thrombin receptor) and 2 (PAR-2) and the resulting in expression of several genes involved in proliferative, migratory, pro-inflammatory and angiogenic phenotypes of cancer and stromal cells [13–15].

Although activation of the coagulation system has traditionally been viewed as an 'unspecific' side effect of cancer, deregulation of TF by GBM-related oncogenic pathways, especially EGFR, suggest a more tumor-specific mechanism. However, the consequences of TF expression and activation during progression of glial brain tumors remains poorly studied, and so are their therapeutic implications.

Here we document the elevated, but heterogeneous, expression of TF mRNA and immunoreactivity in a panel of human gliomas. We suggest that at the cellular level, TF staining may correlate with the expression of EGFRvIII in GBM cells. We also demonstrate that in the EGFRvIII-driven xenograft model of GBM, targeting tumor TF activity and signaling prolongs survival of tumor bearing mice. Notably, in this setting host TF plays a minor and site-specific role. Moreover, genetic disruption of TF expression in the neuroectodermal lineage delays, but does not prevent N-*ras*-driven spontaneous brain tumourigenesis. Thus, we suggest that the inhibition of TF activation and signaling could be explored as a therapeutic target in the subset of GBMs harboring oncogenic EGFR.

2. Materials and methods

2.1. Cells, culture conditions and databases

A431 (human squamous carcinoma) EGFR-driven cell line was maintained under standard culture conditions, DMEM media (10% FBS) along with antibiotics (Penicillin–Streptomycin 1%, GIBCO). U373vIII glioma cells were maintained with the addition of geneticin/G418 (5 μg/μL, GIBCO) and hygromycin (5 μg/μL, Invitrogen), as described earlier [10,16]. REMBRANDT: National Cancer Institute (2005), (http://rembrandt.nci.nih.gov) accessed July 10 2012.

2.2. Mice, treatments and tumor analysis

Low-TF/SCID mice expressing hypomorphic human TF minigene (1% activity) on the background of mouse TF-null mutation (mTF−/−, hTF+/+) were described earlier [17,18]. Cre-Nestin/TF−/− mice express no TF in nestin-positive neuroectodermal cells (Pawlinski et al., manuscript in preparation). (i) *Subcutaneous inoculation*: Immunodeficient SCID mice (Charles River), SCID mice harboring the YFP transgene (YFP/SCID) [17] or low-TF/SCID were injected subcutaneously (s.c.) into the flank with 0.2 mL of a single cell suspension in PBS or Matrigel (BD Biosciences), with either 5×10^6 or $2–3 \times 10^6$ viable cancer cells (>90% trypan blue exclusion), or as indicated. The emerging tumors were measured with a vernier's caliper and tumor volume (TV) was calculated according to the formula: $TV = (a^2 \times b) \times 0.52$, where "*a*" and "*b*" are, the smaller and the larger perpendicular diameter, respectively. Mice were treated intraperitoneally (i.p.) with CNTO859 (500 μg or 350 μg/mouse initially followed by subsequent injections with 200 μg/mouse) or 10H10 (both from Centocor/Janssen Research and Dev.) (initially 500 μg/mouse; subsequent 200 μg/mouse or as indicated) or saline daily (Day 0–4 unless otherwise stated). In some experiments treatment was re-applied until the endpoint once tumors reached >2 mm in diameter. (ii) *Intracranial inoculation*: YFP transgenic mice and low-TF/SCID mice were injected intracranially with (5×10^4 cells/μL with total volume of 2 μL) using a Stoelting Stereotaxic Injector at the coordinates (2.5:−1.5:−3.0) of bregma and sagittal suture. All procedures involving animals were performed in accordance with the guidelines of the Canadian Council of Animal Care (CCAC) and the Animal Utilization Protocols (AUP) approved by the Institutional Animal Care Committee (ACC) at MUHC RI and McGill University. When possible, luminescence data was obtained using IVIS 200 scanner after administering D-Luciferin Firefly potassium salt (Caliper Life Science; 15 μg/mL).

2.3. Histology, immunohistochemistry and immunofluorescence

Tissue microarrays containing formalin fixed paraffin embedded specimens of normal human brain and brain tumors (204 cores) were purchased from US BioMax Inc (9 – normal; 9 – Grade I; 21 – Grade II; 16 – Grade III, 13 – Grade IV). Additional GBM blocks were sectioned using American Optical microtome into 4 μm thick tissue sections and processed for immunostaining with sheep anti-human TF (Cat SATF-IG, Affinity Biologicals, Lancaster, Ontario, Canada) and mouse anti-human EGFRvIII (Cat 08-1330, Zymed, San Francisco, Cal., USA) antibodies followed by alexa flour donkey anti-sheep 488 (A11015) and Alexa flour goat anti-mouse 594 (A11020) secondary antibodies, respectively. Slides were mounted using Vectashield Hard Set Mounting media (H-1400, Vector Laboratories, Burlingame, Cal, USA).

2.4. Expression of mRNA

Cells and tissues were extracted in 1 mL of TRIzol Reagent (Invitrogen), processed and PCR amplified as described previously [16]. PCR primers used included (forward/reverse): hTF: GCTGACTTCAATCCATG/GAAGGTGCCCAGAATACCAA, hGAPDH: GAGTCAACGGATTTGGTCGT/TTGATTTTGGAGGGATCTCG; mTF: TGCTTCTCGACCACAGACAC/TAAAAACTTTGGGGCGTTTG; mPAR1: CTCCTCAAGGAGCAGACCAC/AGACCGTGGAAACGATCAAC; mFVII: TCCAGGGACCTCTAGGGACT/CCTCCGTTCTGACATGGATT; mGAPDH: AA CTTTGGCATTGTGGAAGG/ACACATTGGGGGTAGGAACA.

2.5. Data analysis

All experiments were reproduced at least twice with similar results and presented as number of replicates (n) and mean value of replicates +/−S.D. Statistical analyses were performed using JMP 10.0 (SAS Institute Inc). Differences were considered statistically significant when $P < 0.05$. A Wilcoxon & Log-rank testing were performed for all mouse experiments. Otherwise, for all other experiments statistical analysis was performed using one-tailed, unpaired *t*-test.

3. Results

3.1. TF expression correlates with poor prognosis in glioma but not in GBM

TF mRNA expression is thought to be elevated in aggressive brain tumors [7,19], but its impact on disease outcomes remains unclear. We assessed this link *in silico* using the clinically annotated public database (REMBRANDT) which contains gene expression profiles of 343 human glial brain tumors. Indeed, higher levels of TF mRNA (⩽2-fold increase) significantly correlated with poor patient survival in the whole glioma population (Fig. 1A). However, when we restricted this analysis to GBM (grade IV astrocytoma) there was only a trend toward better survival of low expressors, with no statistical significance (Fig. 1B). This could be explained by the generally poor prognosis in all GBM cases [1],

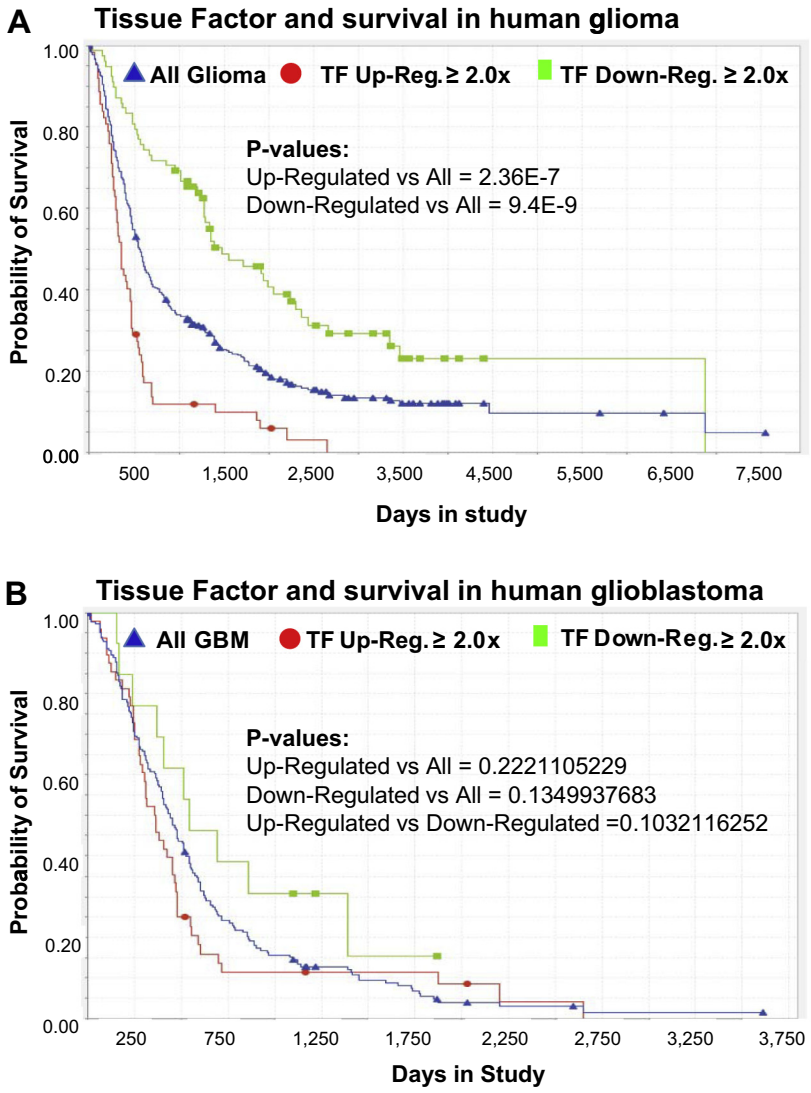


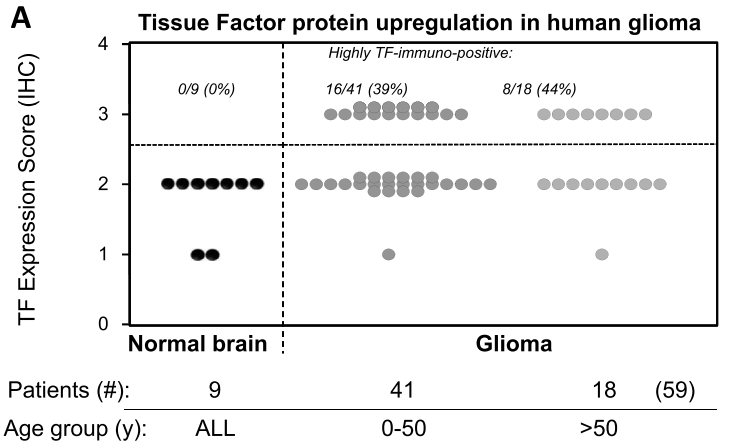
Fig. 1. The link between tissue factor expression and prognosis in glioma and GBM patients. (A) The link between TF mRNA levels and prognosis of all patients with glioma included in the publically available Repository of Molecular Brain Neoplasia Data (REMBRANDT). (A) Kaplan Meier curves of all glioma cases (astrocytoma, GBM, mixed and oligodendroglioma) suggest survival advantage in patients with low levels of TF expression. (B) Analysis focusing on patients with GBM reveals no prognostic significance. The relationship between TF levels and survival is depicted by the respective lines: blue – all glioma, red – glioma with over 2-fold upregulation of TF mRNA versus all glioma, green – gliomas with over 2-fold down regulation of TF. Statistical significance of differences was calculated as indicated by the P^* values.

but may also suggest that in this setting TF levels are more heterogeneous than expected thereby blurring the existing correlation.

3.2. Tissue factor protein expression in glioma is heterogeneous and may associate with oncogenic EGFR

To explore the aforementioned question further we focused on TF protein expression, which has rarely been studied in GBM. To accomplish this we conducted a semi-quantitative analysis of TF immunoreactivity using tissue microarrays (TMA) containing samples of 59 glioma patients (177 cores) and 9 tumor-free controls (Fig. 2A). A computerized image analysis revealed that TF was ubiquitously expressed across the spectrum of normal and glioma specimens, including both adult (56 cases over 18 yrs of age) and pediatric (3 cases under 18 years of age) patients, most of which represented high grade tumors (including: adults (>18 years of age): normal – 8; GBM – 56 and pediatric patients (<18 years): normal – 1; GBM – 3). While some high grade tumors in all age groups exhibited a comparable TF signal to the normal brain, a subset of these GBMs (39–44%) was markedly more positive than any tumor-free brain tissue analyzed. Interestingly, the TF signal was detected primarily in the tumor cell compartment, while only some blood vessels contained TF-expressing endothelial cells (data not shown). This is consistent with our earlier findings suggesting that TF is a target of GBM – related oncogenic events operative in cancer cells [12]. In particular, we reported that high TF levels are found in the classical GBM and in conjunction with oncogenic EGFR/EGFRvIII, but not (less so) in other molecular subtypes of this disease [7].

This correlation prompted us to analyze the distribution of cells positive for TF and EGFRvIII in GBM samples expressing this oncogene (Fig. 2B). Indeed, double immunofluorescent staining revealed intratumoural heterogeneity of both TF and EGFRvIII signals, which mostly colocalized to the same regions or cells. This pattern extends the aforementioned mRNA studies to the realm of protein expression *in situ*, and is consistent with both, TF regulation by EGFRvIII [12] and the published heterogeneity of EGFRvIII distribution in GBM tissues [20].



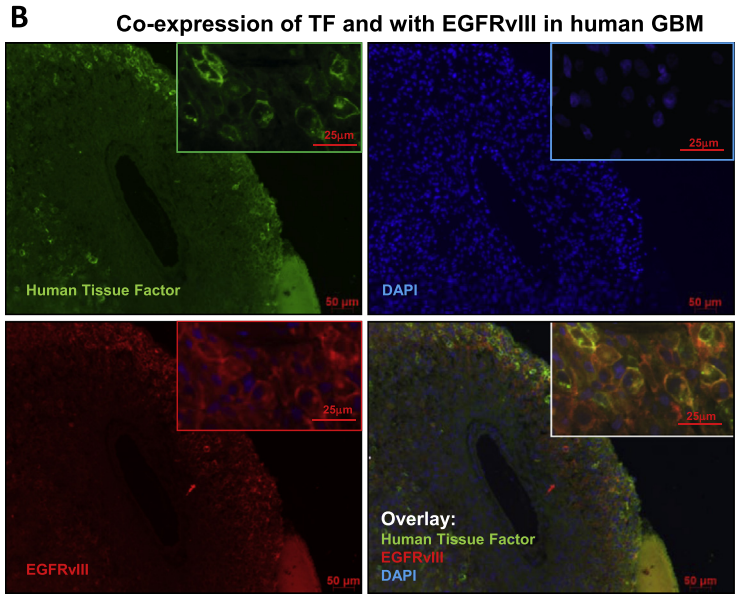


Fig. 2. Tissue factor protein expression in human glioma. (A) Expression of TF immunoreactivity in tissue sections of human glioma. Tissue Microarray (TMA) including glioma of all grades were stained for TF, and the signal intensity was ranked as 0 (no staining), 1+ (low intensity), 2+ (medium intensity), 3+ (high intensity), according to measurements acquired by Aperio's ImageScope (20×). TF expression values were plotted according to age groups of glioma patients, as indicated, versus a series of normal brain tissues. Each patient sample was represented by 3 TMA cores, each of which was scored across 3 random fields (only intact tissue) and measured by Aperio's ImageScope (a total of 207 cores). (B) Colocalization between EGFRvIII and TF in GBM tissue sections. Immunofluorescent staining for TF and EGFRvIII in human GBM (WHO IV) specimens reveals regions of double positivity; red – EGFRvIII, green – TF; blue – DAPI. Size bars as indicated.

3.3. TF contributes to oncogene-driven gliomagenesis

Given the co-expression and regulation of TF by the EGFRvIII oncogene we assessed the functional consequences of this link using human glioma xenograft model (U373vIII) [12,16,21]. U373vIII cells were inoculated subcutaneously into YFP/SCID mice at numbers indicated and the animals subsequently received a short course of intraperitoneal injections of unique neutralizing, anti-human-TF antibodies with either anticoagulant (CNTO 859) or selectively anti-signaling (10H10) activities [10,22]. Remarkably, while control groups reached their clinical endpoint within 50–70 days, and more rapidly when the initial inoculum was larger, mice treated with either of the anti-TF antibodies survived significantly longer (1 month or more; Fig. 3A and B). Similar outcomes were observed for an unrelated EGFR-driven carcinoma cell line (A431), where we observed long term survivors (Fig. 3C). The mechanisms by which non-anticoagulant 10H10 antibody exerts its effects are not fully elucidated, but may include interference with invasion, migration or angiogenesis [22,23].

TF is also upregulated by the activated RAS pathway [9], frequently triggered in GBM [8]. To interrogate the role of TF in this setting we generated spontaneous primitive brain tumors (PBTs) in mouse neonates, through intracranial delivery of oncogenic *N-ras* and *SV40LT* sequences using the Sleeping Beauty transposase system [24,25]. This led to formation of aggressive PBTs in both C57BL/6 mice and in their counterparts lacking neuroectodermal TF expression (Cre-nestin-TF−/− backcrossed). While both strains are phenotypically normal, suggesting that TF is not essential for the normal development of the neuronal lineage (Pawlinski – manuscript in preparation), PBT growth was delayed in the absence of TF. This is evident from the extended survival of mice deficient for brain TF expression and harboring the PBTs. While this effect is not dramatic, it suggests the involvement of TF in the progression of an independent, spontaneous and highly aggressive brain tumor type.

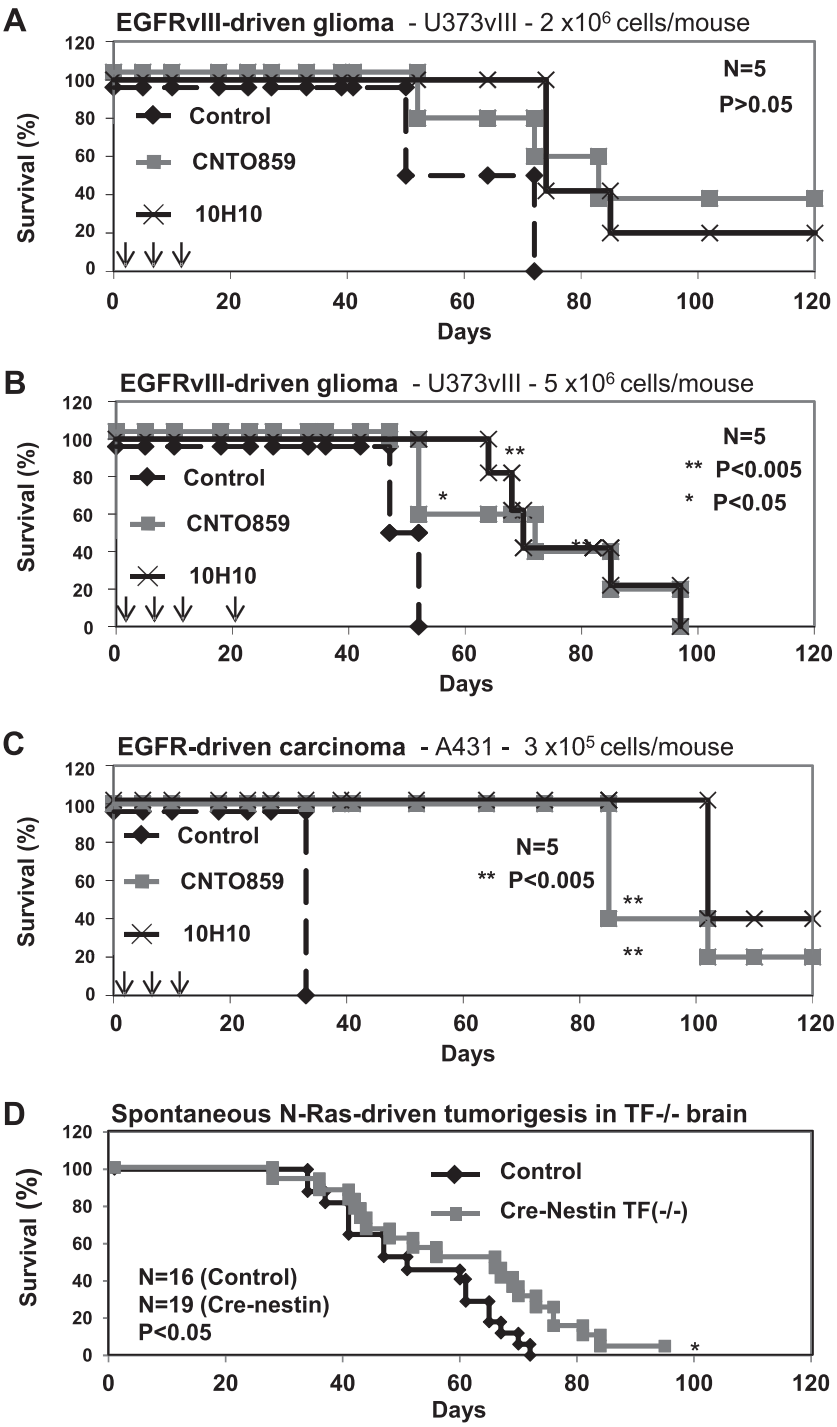


Fig. 3. Contribution of tissue factor to oncogene-driven gliomagenesis in mice. (A) U373vIII cells (harboring EGFRvIII) were injected subcutaneously at 2×10^6 or (B) 5×10^6 into SCID mice, and treated with anti-TF antibodies at 500 μg/mouse on day 0 and 200 μg/mouse on days 1, 4 & 32 ($N = 5$). Wilcoxon test; $P < 0.05^*$; $P < 0.005^{**}$ (C) Prolongation of survival in mice harboring A431 s.c. (3×10^5 cells injected; 500 μg/mouse of either CNTO or 10H10, on days 0, 1 & 4 at ($N = 5$). (D) Disruption of the TF/F3 gene in the neuroectodermal lineage results in delayed progression of spontaneous aggressive brain tumors. Neonatal C57BL/6 mice either proficient or deficient for neuroectodermal TF expression (Cre/Nestin/TF−/− mice) were injected into the right lateral ventricle with construct containing NRASV12 and SC40-LgT plasmids. TF-proficient C57BL/6 mice ($N = 16$) were compared to the Cre/Nestin (−/−) strain ($N = 19$). The latter mice exhibit increased survival (Log Rank Test < 0.05*).

3.4. Site-specific contribution of host TF to gliomagenesis

We observed that mouse TF (mTF) and mouse PAR1/2 (mPAR1/2) mRNA is detectable in subcutaneous xenografts of human U373vIII glioblastoma in SCID mice. This pool of TF coagulation effectors would not have been affected by the aforementioned anti-human TF monoclonal antibodies (Fig. 4A, data not shown). To explore the functional consequences of host TF expression in the U373vIII GBM model we used low-TF/SCID mice as tumor recipients. In these mice TF is expressed at 1% of its wild type levels owing to the insertion of the human hypomorphic minigene (hTF^{low}) [17,18].

Interestingly, subcutaneous growth of U373vIII tumors was modestly, but significantly delayed in low-TF/SCID mice vs their

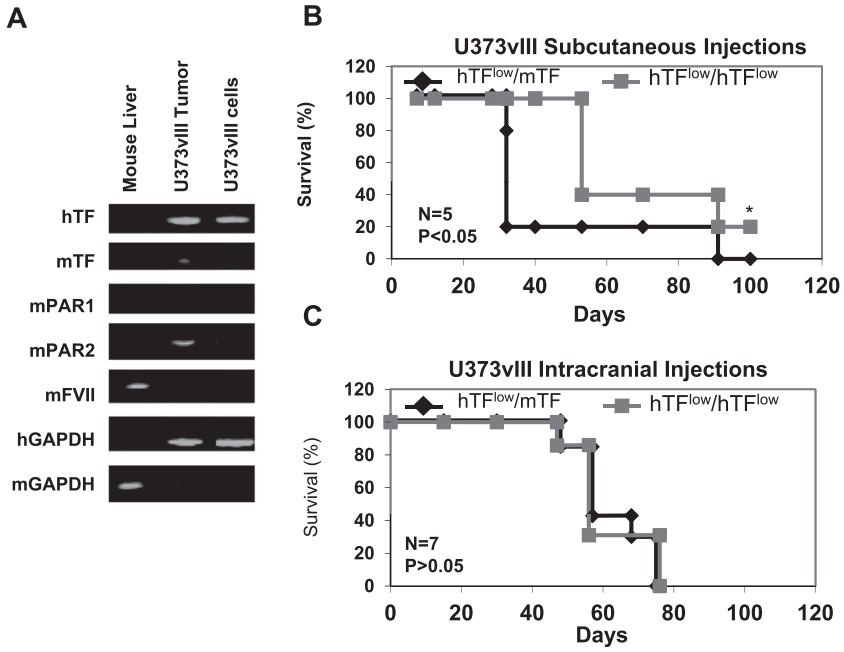


Fig. 4. The site-specific role of host TF in gliomagenesis. (A) Detection of host (mouse) TF and PARs in U373vIII xenografts. RT-PCR demonstrates mouse TF and PAR1/2 (mTF, mPAR1/2) mRNA in tumors. (B) Growth of subcutaneous U373vIII tumors in wild type and TF-deficient SCID mice. Kaplan–Meier curves demonstrate differential survival of mice harboring U373vIII tumors (5×10^6 cells per mouse) in SCID mice heterozygous (hTF+/mTF+ depicted as hTF^{low}/mTF) or homozygous for the hypomorphic human TF allele (hTF+/hTF+/mTF−/mTF− depicted as hTF^{low}/hTF^{low}). The graph shows a representative experiment ($N = 5$), which has been repeated with 6 additional mice with similar results ($P < 0.05^*$). (C) Unchanged growth of intracranial U373vIII xenografts in TF-proficient and -deficient SCID mice (U373vIII 5×10^4 cells per inoculum) grafted orthotopically into either hTF^{low}/hTF^{low} mice or hTF^{low}/mTF mice, as indicated ($N = 7$).

SCID (TF wild type) littermates. However, such effect was not observed when the tumors were implanted orthotopically into the brains of these respective strains (Fig. 4B and C). These results enforce the notion that while host cells may have some role in contributing the TF activity to the growing tumor mass, this effect is modest and site-specific.

4. Discussion

Our study presents the first piece of formal evidence that in subsets of GBMs where the expression of TF is under the control of oncogenic pathways, such as EGFRvIII and RAS, this coagulant receptor may play a functionally important role in tumourigenesis. This effect is primarily linked to TF signaling, and can be therapeutically opposed without anticoagulant side effects. This is documented in two different tumor models (U373vIII and A431) driven by two different oncogenic EGFR mutations (deletion and amplification, respectively) where a robust antitumor activity was achieved following treatment with the 10H10 antibody, which lacks the ability to interfere with TF-induced clotting [22].

This is of note as the highly angiogenic and procoagulant nature of high grade brain tumors has not been fully explored as a therapeutic target due concerns over the possibility of intracranial bleeding [2]. Our observation that a non-anticoagulant anti-TF antibody can prolong survival of mice harbourng EGFR-driven tumors may suggest that such agents could hold promise in GBM, which is presently incurable. We postulate that approaches targeting TF could be more likely effective in tumors driven by the EGFR-related oncogenic pathway.

Interestingly, in a limited number of cases of pediatric GBM available to our immunostaining analysis, TF expression was detected, often at relatively high levels. While TF is often discussed in the context of hypercoagulability (VTE), the risk of systemic thrombosis is low in pediatric GBM patients [26] in spite of vascular features of these tumors strikingly similar to those of their highly procoagulant adult counterparts [3]. Thus, it would be of interest to explore whether TF plays an alternative biological role in pediatric settings, and whether this presents new therapeutic opportunities.

We suggest that the primary sources of the biologically active TF in EGFRvIII-driven GBMs are tumor cells themselves. Indeed, intracranial growth of U373vIII xenografts was unaffected in low-TF/SCID mice, which are hypomorphic for TF activity [17]. While tumor growth was somewhat delayed at the subcutaneous site in this model, the magnitude of this effect was less pronounced than that resulting from targeting human TF expressed by cancer cells. Prior studies have explored the effects of the selective host TF depletion in various tumor types, and the effects were, for the most part, rather modest [18,27,28], with one study suggesting the requirement for host TF during formation of TF-deficient teratoma [18]. However, the presence of host TF has not been previously investigated in the context of brain tumors.

It would be of interest to examine whether different known site-specific effects of the coagulation system [29] could be extended to intracranial and extracranial metastasis of visceral malignancies. Since TF is thought to regulate haemostasis in a site specific manner [30], it would be relevant to examine whether other processes involving this receptor, e.g., in tumor growth, angiogenesis and metastasis are also site-specific, and why.

The RAS pathway upregulates TF [9] and is activated by oncogenic EGFR [31]. This pathway is also constitutively triggered in GBM [8] including pediatric GBM [32]. Our model of spontaneous, murine GBM-like PBT [24], driven by N-*ras* and *SV40LT* in neonatal mice, approximates these latter settings [24]. Notably, this approach shows that tumourigenesis in the brain is delayed in the absence of TF in the neuroectodermal lineage. While this effect is modest in magnitude, the high level of cellular aggressiveness observed in PBTs may present a different biological and molecular context than endogenous (non-mutational) activation of RAS in

human GBM cells [8]. It is tempting to speculate that the role of TF in brain tumors may be as dependent on the disease context and oncogenic pathway activation, as is the TF expression [7,16,33].

Overall, our study strengthens the rationale for targeting TF in the context of GBM, a disease that is associated with extreme vascular perturbations and procoagulant phenotype [2], and where any therapeutic progress is desperately needed [1]. However, we suggest that a possible exploration of this strategy should, perhaps, be considered in the context of specific molecular subtypes of GBM, where TF is expressed in cancer cells under the influence of defined oncogenic drivers, such as EGFR, and where the mechanistic links can be rationally explored.

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