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IDH mutation status is associated with distinct vascular gene expression signatures in lower-grade gliomas

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Abstract

Background. Vascular gene expression patterns in lower-grade gliomas (LGGs; diffuse World Health Organization [WHO] grades II–III gliomas) have not been thoroughly investigated. The aim of this study was to molecularly characterize LGG vessels and determine if tumor isocitrate dehydrogenase (IDH) mutation status affects vascular phenotype.

Methods. Gene expression was analyzed using an in-house dataset derived from microdissected vessels and total tumor samples from human glioma in combination with expression data from 289 LGG samples available in the database of The Cancer Genome Atlas. Vascular protein expression was examined by immunohistochemistry in human brain tumor tissue microarrays (TMAs) representing WHO grades II–IV gliomas and nonmalignant brain samples. Regulation of gene expression was examined in primary endothelial cells in vitro.

Results. Gene expression analysis of WHO grade II glioma indicated an intermediate stage of vascular abnormality, less severe than that of glioblastoma vessels but distinct from normal vessels. Enhanced expression of laminin subunit alpha 4 (LAMA4) and angiopoietin 2 (ANGPT2) in WHO grade II glioma was confirmed by staining of human TMAs. IDH wild-type LGGs displayed a specific angiogenic gene expression signature, including upregulation of ANGPT2 and serpin family H (SERPINH1), connected to enhanced endothelial cell migration and matrix remodeling. Transcription factor analysis indicated increased transforming growth factor beta (TGFβ) and hypoxia signaling in IDH wild-type LGGs. A subset of genes specifically induced in IDH wild-type LGG vessels was upregulated by stimulation of endothelial cells with TGFβ2, vascular endothelial growth factor, or cobalt chloride in vitro.

Conclusion. IDH wild-type LGG vessels are molecularly distinct from the vasculature of IDH-mutated LGGs. TGFβ and hypoxia-related signaling pathways may be potential targets for anti-angiogenic therapy of IDH wild-type LGG.

Key words

angiogenesis | ANGPT2 | glioma | IDH | tumor vessel

Importance of the study

Whereas vascular abnormalities in glioblastomas have been well characterized, potential molecular alterations in vessels in LGGs (WHO grades II–III) have not been thoroughly investigated. This study demonstrates that the gene expression signature of WHO grade II glioma vessels represents an intermediate stage of vascular abnormality, falling between non-tumor brain vessels and glioblastoma vessels. The vasculature in IDH wild-type LGG harbors a distinct vascular gene expression pattern compared with IDH-mutated LGG, indicative of ongoing angiogenesis and vascular remodeling. This indicates that IDH wild-type LGG vessels are functionally distinct and may respond differently to anti-angiogenic therapy. TGFβ and hypoxia signaling were identified as contributing to the altered vascular gene expression and may represent potential targets for normalizing the vasculature in IDH wild-type LGG.

Diffuse gliomas are malignant brain tumors that arise predominantly in the cerebral hemisphere in adults.[1] Based on integrative molecular profiling, lower-grade gliomas (LGGs; World Health Organization [WHO] grades II–III) have been classified into 3 molecular subgroups: isocitrate dehydrogenase (IDH) wild-type, IDH-mutated with 1p/19q codeletion (IDH mut-codel), and IDH-mutated without 1p/19q codeletion (IDH mut-noncodel). Gliomas that lack IDH mutations are molecularly and clinically similar to primary glioblastomas (WHO grade IV). IDH mut-noncodel gliomas are diagnosed as astrocytomas, whereas IDH mut-codel are oligodendrogliomas and associated with a more favorable prognosis.[2] With progress in surgery techniques and more effective chemoradiotherapy regimes, the survival of patients with LGG has been improved substantially.[3–6] However, all tumors eventually relapse and progress to higher malignancy grades, and the patients die as a consequence of their disease.[7] The relapses are attributed to the extreme invasiveness of the tumor and the existence of drug-resistant glioma stemlike cells (GSCs).[8]

The glioma vasculature provides a specialized microenvironment for the GSCs by creating a vascular niche.[9] Moreover, glioma cells reside close to blood vessels and use the perivascular space for invasion.[10] Therefore, the tumor vasculature has been identified as a target for glioma therapy.[11] Based on a prolonged progression-free survival and improved quality of life, bevacizumab, a humanized anti–vascular endothelial growth factor (VEGF) monoclonal antibody, was approved by the FDA for the treatment of recurrent glioblastoma.[12,13] Bevacizumab in combination with the topoisomerase 1 inhibitor irinotecan improved symptoms and stabilized disease in over 80% of children with recurrent pediatric LGGs who failed standard chemotherapy.[14,15] It is likely that the benefit of anti-angiogenic therapy will differ between patients with glioblastoma and LGG, at least partially due to differences in tumor vascular phenotype. Blood vessels in glioblastoma are abnormal and display a distinct gene expression signature compared with vessels in normal brain.[16–18] Vascular abnormalization in glioblastoma is connected to high expression of angiogenic factors, including VEGF, transforming growth factor (TGF) β2, and pleiotrophin (PTN).[18–21] The vasculature in LGG has not been thoroughly investigated, and potential differences in gene expression compared with normal brain vessels have not yet been identified.

Here, we have employed bioinformatics analysis of gene expression to characterize LGG vessels. We demonstrate that blood vessels in LGG display alterations in gene expression that partially overlap with changes previously identified in glioblastoma vessels. IDH wild-type glioma vessels have a distinct vascular gene expression pattern associated with vascular remodeling. Transcriptional profiling of vascular genes specifically expressed in IDH wild-type glioma indicated increased SMAD and hypoxia-inducible factor 1 alpha (HIF1α) signaling, and a subset of these genes were induced in endothelial cells after stimulation with TGFβ2, VEGF, or cobalt(II) chloride ($CoCl_2$) in vitro. Hereby, we identify molecular alterations of blood vessels in LGGs and uncover signaling pathways and growth factors that may contribute to a distinct vascular gene expression signature in IDH wild-type LGG.

Materials and Methods

Datasets

Internal dataset

We employed an Affymetrix microarray dataset of gene expression in total tissue and blood vessels isolated by laser capture microdissection (LMD) from normal brain, WHO grade II glioma, and glioblastoma.[18]

External datasets

The LGG dataset including patient's clinical information and processed RNA-sequencing data were downloaded from the database of The Cancer Genome Atlas (TCGA) (http://cancergenome.nih.gov). IDH mutation status, presence of 1p/19q codeletions, combination of trisomy of whole chromosome 7 and loss of chromosomal arm 10q (+7/–10q), telomerase reverse transcriptase gene promoter (TERTp) mutation status, BRAF and H3F3A mutation status were downloaded from the data portal (http://gliovis.bioinfo.cnio.es/) or extracted from a previous study.[2] The processed Gravendeel dataset was downloaded from the GlioVis database (http://gliovis.bioinfo.cnio.es/).

Identification of Vascular Enriched Genes and Correlation Analysis

Pairwise comparison of LMD samples with corresponding total tissue was done using the Student's *t*-test method. The cutoff for inclusion was set as a fold change >2 compared

with total tissue and $P < 0.05$. The average fold change in gene expression in LMD vessels of grade IV glioma versus control and LMD vessels in grade II glioma versus control was calculated. These values were correlated to each other to compare alteration of gene expression in tumor vessels in WHO grades II and IV gliomas.

Functional Annotation of Genes

Functional annotation of genes was performed using the Gene Ontology tool (http://geneontology.org), including terms for biological processes only. Terms with a $P < 0.05$ were considered significantly enriched.

Ethical Considerations

Use of anonymized biobank material and tumor tissue microarrays was granted by Uppsala County's ethical committee (Ups 03-412/2003-10-02, Dnr 2010/291/2010-11-17, Dnr Ups 02-330, Ups 06-084, Dnr Ki 02-254).

Tumor Tissue Microarrays and Image Analysis

Tumor tissue microarrays (TMAs) of human brain tumors collected retrospectively at Uppsala University Hospital were used. The TMAs contained duplicate tissue cores (1 mm diameter) of WHO grade II gliomas ($n = 70$, $n = 64$ IDH1 mutation status available), WHO grade III gliomas ($n = 34$, $n = 28$ IDH1 mutation status available), WHO grade IV gliomas ($n = 78$), and nonmalignant control brain tissue ($n = 4$). Tumors were originally classified according to the 2007 WHO classification and further characterized for the presence of 1p/19q codeletions and a series of immunohistochemical markers, including IDH1-R132H, as described.[22,23] Cores were chosen to represent characteristic areas of the tumors. Protein expression patterns were analyzed by immunohistochemistry-based protein profiling as described[18] using antibodies listed in Supplementary Table S1. Scoring of vascular staining was blinded.

Microvasculature Signature Score, Survival Analysis, and Unsupervised Clustering

Each vascular enriched gene from the LGG TCGA dataset was standardized using the z-score method. For each gene, the average gene expression was subtracted and the resulting value was divided by the standard deviation. After transformation, the values of most genes were distributed around the [–1, 1] region, and genes with a higher expression than the mean received a positive value. For each sample, the standardized values of all 456 vascular enriched genes were added to yield a microvasculature (MV) signature score (an MV score), as described.[24]

Gliomas from the database of TCGA were dichotomized into high MV or low MV subgroups (median cutoff). Survival curves were plotted by the Kaplan–Meier method. Univariate test (log-rank) or multivariate test (Cox proportional hazards model) was used to compare survival times of 2 groups.

Unsupervised clustering of LGG samples from the database of TCGA was based on the vascular enriched genes using Euclidean distance and average linkage method in R software (www.r-project.org).

To identify differences in gene expression between IDH wild-type LGG and IDH-mutated LGG samples, the DESeq2 software was used. Genes more than 2-fold enriched in IDH wild-type LGG samples and with adjusted P-value <0.05 were identified as significantly upregulated in IDH wild-type LGG.

Transcription Factor Analysis

The TFacts database (www.tfacts.org) was used to predict transcription factors differentially activated in IDH wild-type LGG vessels compared with IDH-mutated LGG vessels.[25] The analysis was based on genes specifically upregulated in IDH wild-type LGG vessels compared with IDH-mutated LGG vessels.

In Vitro Stimulation of Endothelial Cells

Murine brain endothelial cells (bEND.3) were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco) supplemented with 10% fetal calf serum (FCS) (Sigma). Human dermal blood endothelial cells (HDBECs) were maintained up to passage 8 on gelatinized culture plates in endothelial basal medium (EBM-MV2; PromoCell) with supplements. The bEND.3 cells and HDBECs were starved in DMEM with 1% FCS (Sigma) or EBM-MV2 with 1% FCS (PromoCell), respectively, overnight and stimulated with TGFβ2 (2 ng/mL; Peprotech), VEGFA (50 ng/mL; Peprotech), or cobalt chloride (400 μM $CoCl_2$; Sigma Aldrich) diluted in EBM-MV2 with1% FCS for 8 hours and 24 hours, respectively. RNA was isolated as described.[26]

Complementary DNA Synthesis and Quantitative PCR

Complementary DNA from total RNA was synthesized using random hexamer primers and SuperScript III reverse transcriptase (ThermoFisher). Quantitative (q)PCRs were done using SYBR Green PCR Master Mix (ThermoFisher). Relative gene expression was normalized to HPRT (hypoxanthine-guanine phosphoribosyltransferase) according to the following formula: Relative expression $_{\text{gene x}} = 2^{-}(CT_{HPRT} - CT_{\text{gene x}})$. Primer sequences are listed in Supplementary Table S2.

Statistical Analysis and Software

Statistical analysis was performed using GraphPad Prism 6.0 software and R software. The Mann–Whitney test or the Kruskal–Wallis test was performed to determine statistically significant differences. All statistical tests were 2-sided and P-values smaller than 0.05 were considered statistically significant.

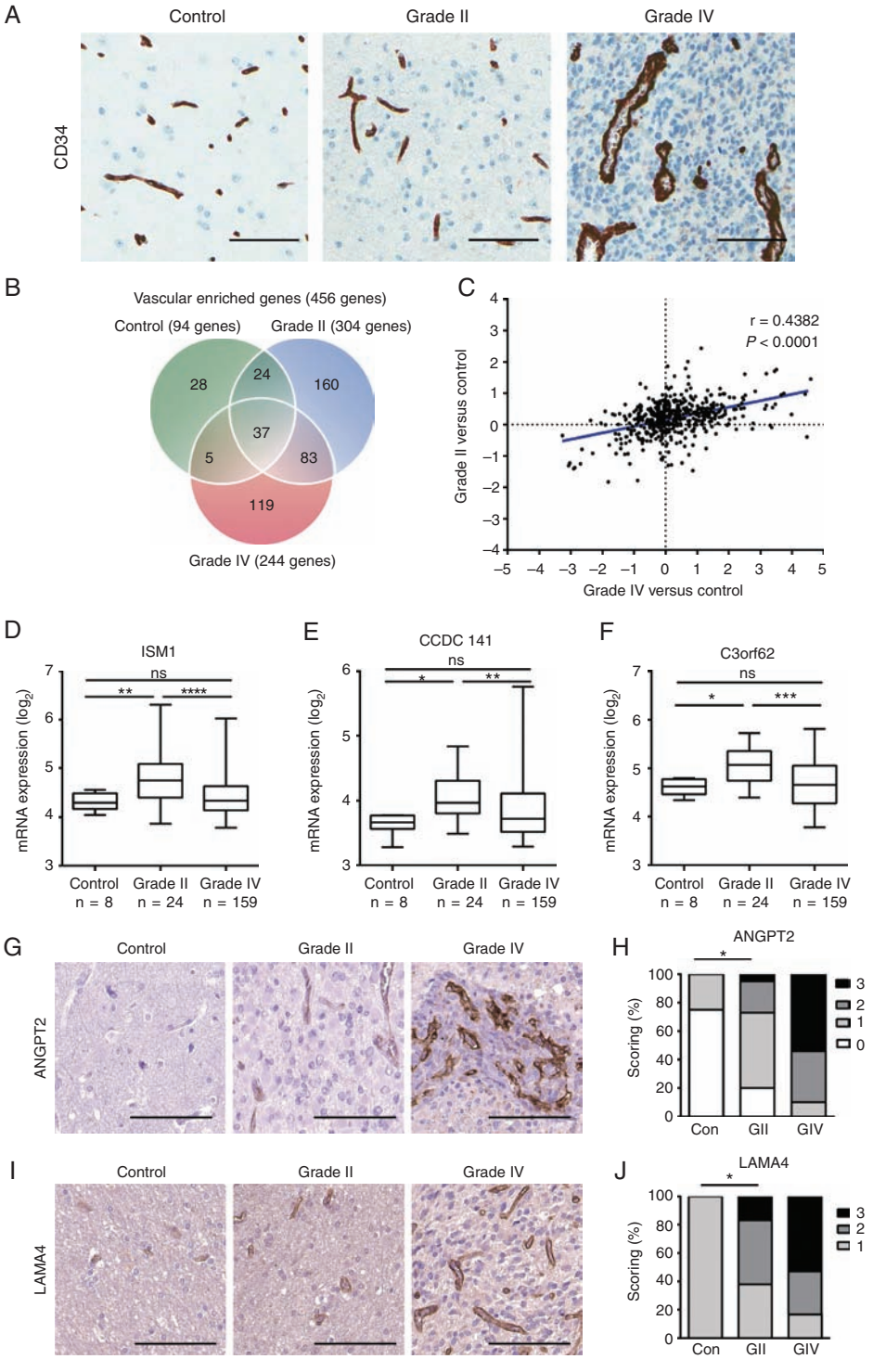

Fig. 1 Changes in gene expression in grade II glioma vessels partially overlap with those observed in grade IV glioma vessels. (A) Immunohistochemical staining with anti-CD34 to visualize tumor vessels in nonmalignant brain, grade II glioma, and grade IV glioma. (B) Four hundred fifty-six genes were identified as vascular enriched by pairwise comparison. (C) Plot of fold change (FC) of the 456 vascular enriched genes in grade II vasculature versus control vasculature and FC of grade IV vasculature. RNA levels of ISM1 (D), CCDC141 (E), and C3orf62 (F) in control, WHO grade II glioma, or WHO grade IV glioma from the Gravendeel dataset (mean ± SD; $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, ns = nonsignificant, one-way ANOVA with Dunnett's posttest). (G, I) Immunohistochemical staining of a TMA showing expression of ANGPT2 (G) and LAMA4 (I) in representative samples of nonmalignant brain, grade II glioma, and grade IV glioma, respectively. Vascular staining intensity of ANGPT2 (H) and LAMA4 (J) was scored semi-quantitatively on scale from 0 to 3 (0, no staining; 1, weak staining; 2, medium staining; 3, strong staining) (scale bar =100 μm).

Results

Identification of Genes Differentially Expressed in Vessels of WHO Grade II Gliomas

Glioblastoma vessels are highly abnormal, while vessels in WHO grade II gliomas are morphologically more similar to normal brain vessels (Fig. 1A). To map gene expression patterns of vessels in WHO grade II and grade IV glioma, we analyzed transcriptome data derived from blood vessels microdissected from human brain specimens and the corresponding total tumors or brain tissue.[18] We have previously used this dataset to characterize gene expression in glioblastoma vessels. By pairwise comparison of gene expression in the microdissected samples with their corresponding total tissue, we identified 456 genes that were preferentially expressed in the vasculature (Fig. 1B, Supplementary Table S3). Gene ontology (GO) analysis of the vascular enriched genes revealed notable enrichment of terms related to angiogenesis/vasculature development/endothelium development (Supplementary Table S4).

Next, we analyzed the overall alterations in vascular gene expression by comparing the fold change of the 456 vascular enriched genes in WHO grade II or grade IV glioma vasculature versus control brain vessels (Fig. 1C, Supplementary Table S3). Correlation analysis revealed that vascular enriched genes in WHO grade II and WHO grade IV gliomas were regulated in a similar manner ($r = 0.4382$, $P < 0.0001$) (Fig. 1C). Ninety-four vascular enriched genes were enhanced more than 1.5-fold in WHO grade II glioma vessels compared with controls (Supplementary Table S5). Functional annotation of the 94 upregulated genes resulted in significant enrichment of terms connected to angiogenesis, such as "collagen fibril organization," "glomerulus development," and "angiogenesis" (Supplementary Table S6). Similar GO terms were enriched in grade IV vessels (Supplementary Table S6). Only 6 genes (DENND2C, C3orf62, C10orf113, ISM1, CCDC141, and OR52B6) were upregulated more than 2-fold in grade II glioma vessels compared with control brain vessels but were not upregulated in grade IV glioma vasculature (Supplementary Table S3). Specific upregulation of ISM1, CCDC141, and C3orf62 in WHO grade II glioma vessels was confirmed using a publicly available glioma dataset (Gravendeel)[27] (Fig. 1D–F). These results indicate that WHO grade II gliomas have an intermediate stage of vascular abnormality between WHO grade IV glioma vessels and control brain vasculature.

ANGPT2 and LAMA4 Are Upregulated in WHO Grade II Glioma Vasculature

To determine if the observed changes in gene expression are reflected by differential protein expression, eight candidate genes were selected for validation. Angiopoietin 2 (ANGPT2), kinase insert domain receptor (KDR), laminin subunit alpha 4 (LAMA4), solute carrier organic anion transporter family, member 2A1 (SLCO2A1), E26 transformation-specific 1 (ETS1), laminin subunit beta 1 (LAMB1), purine nucleoside phosphorylase (PNP), and serpin family H (SERPINH1) are either transmembrane or secreted and may serve as targets for therapy. ETS1 is a key transcriptional regulator in endothelial cells. Protein expression in tumor vessels was analyzed by immunohistochemical staining of TMAs containing human control brain, WHO grade II gliomas and WHO grade IV gliomas. All proteins had vascular staining patterns (Figure 1G, I and Supplemental Figure S1A). ANGPT2, KDR, LAMA4, LAMB1, PNP, and SERPINH1 were semi-quantitatively scored according to the intensity of staining on a scale from 0 to 3. SLCO2A1 and ETS1 stainings were scored based on the fraction of positively stained vessels on a scale from 0 to 2. Whereas all examined proteins were highly expressed in WHO grade IV glioma vessels (Fig. 1G–J and Supplementary Figure S1A), only ANGPT2 and LAMA4 were significantly upregulated in WHO grade II vessels compared with blood vessels from nonmalignant brain (Fig. 1G–J, Table 1). However, KDR, LAMB1, PNP, SERPINH1, ETS1, and SLCO2A1 showed clear trends toward upregulation in WHO grade II vessels (Supplementary Figure S1A, B). These results identify ANGPT2 and LAMA4 as upregulated in tumor vessels in WHO grade II gliomas, and indicate that further changes occur to a variable extent.

IDH Wild-Type LGGs Display a Higher Microvascular Score and Show a Distinct Vascular Gene Expression Signature

The potential association between tumor vascular density and survival of patients with LGG has not been investigated. To this end, we evaluated the levels of the 456 vascular enriched genes in the LGG dataset in the database of TCGA (http://cancergenome.nih.gov). Gene expression profiles and clinical data of 289 patients with LGG were extracted from the database. We generated an MV score by summarizing the standardized mRNA expression levels for the vascular enriched genes as described.[24] By

Table 1 Summary of TMA quantification of ANGPT2 and LAMA4 staining in human glioma

ANGPT2 Staining				
		Control	WHO Grade II	WHO Grade IV
	Total	4	61	70
Score	0	3	20	
	1	1	25	7
	2		13	25
	3		3	38
LAMA4 Staining				
		Control	WHO Grade II	WHO Grade IV
	Total	4	64	71
Score	1	4	24	12
	2		29	21
	3		11	38

*Control: nonmalignant brain tissue.

dichotomizing patients into 2 groups of equal size according to their MV scores, we found that a high MV score was significantly associated with shorter survival (P = 0.0265, log-rank test) (Fig. 2A, Supplementary Tables S7 and S8). The MV score did not significantly correlate to survival when correcting for IDH mutation status (n = 287, n = 2 data missing) in a multivariate analysis (Supplementary Table S9) and there was no correlation between MV score and survival within the IDH-mutated or IDH wild-type LGG subgroups (Supplementary Figure S2A, B).

To investigate potential differences in tumor angiogenesis in molecular subgroups of LGG, we compared the MV score in each subgroup by combining the dataset from the database of TCGA with published information regarding the presence of IDH mutations and 1p/19q codeletions in these tumors (http://gliovis.bioinfo.cnio.es/).[2] The MV score was similar in the IDH mut-codel subgroup (oligodendrogliomas) and IDH mut-noncodel subgroup (astrocytomas) (Fig. 2B). However, IDH wild-type gliomas showed a significantly higher MV score than both subtypes harboring IDH mutations (Fig. 2B). We therefore conclude that a high MV score is a feature of IDH wild-type LGG but that the MV score is not an independent prognostic marker in these tumors.

When analyzing IDH-mutated and wild-type LGG groups separately, we did not observe any significant difference in MV scores between WHO grade II and WHO grade III tumors (Fig. 2C). However, IDH wild-type WHO grade III LGGs displayed significantly higher MV scores compared with IDH mutated WHO grade III LGGs (Fig. 2C).

IDH wild-type gliomas can be subdivided into 3 subgroups with different prognoses: combination of trisomy

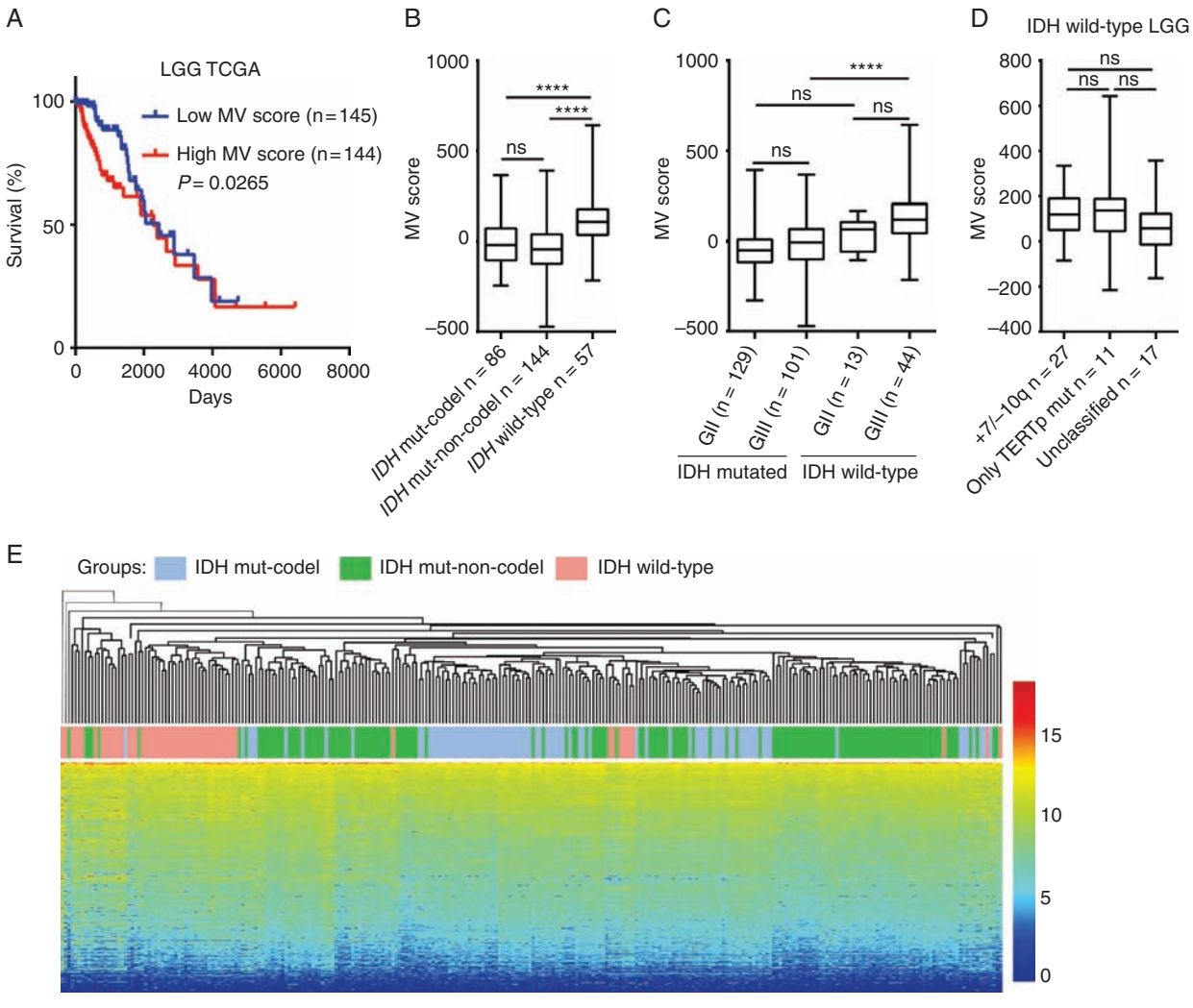

Fig. 2 IDH wild-type LGGs have a distinct vascular gene expression signature compared with IDH-mutated LGGs. (A) Survival plot for patients with LGG with low microvasculature (MV) score or with high MV score (B) Quantification of MV scores in IDH mut-codel, IDH mut-noncodel, and IDH wild-type subtypes of LGG, respectively. (C) Quantification of MV score in WHO grade II and WHO grade III in IDH mutated and IDH wild-type glioma, respectively (GII: WHO grade II glioma, GIII: WHO grade III glioma). (D) Quantification of MV score in different subtype of IDH wild-type LGG. (E) Unsupervised clustering and heat map of LGG samples based on vascular enriched genes.

of whole chromosome 7 and loss of chromosomal arm 10q (+7/–10q), only TERTp mutated (TERTp mut), and unclassified.[28] We did not observe any significant differences in MV scores between +7/–10q (n = 27), TERTp mut (n = 11), or unclassified subgroups (n = 17) (n = 3 data missing) (Fig. 2D).

Unsupervised clustering of the 456 vascular enriched genes showed that a majority of the IDH wild-type samples clustered together, while IDH mut-codel and IDH mut-noncodel samples were similar to each other and differed from IDH wild-type samples (Fig. 2E). This result demonstrates that there is a distinct pattern of

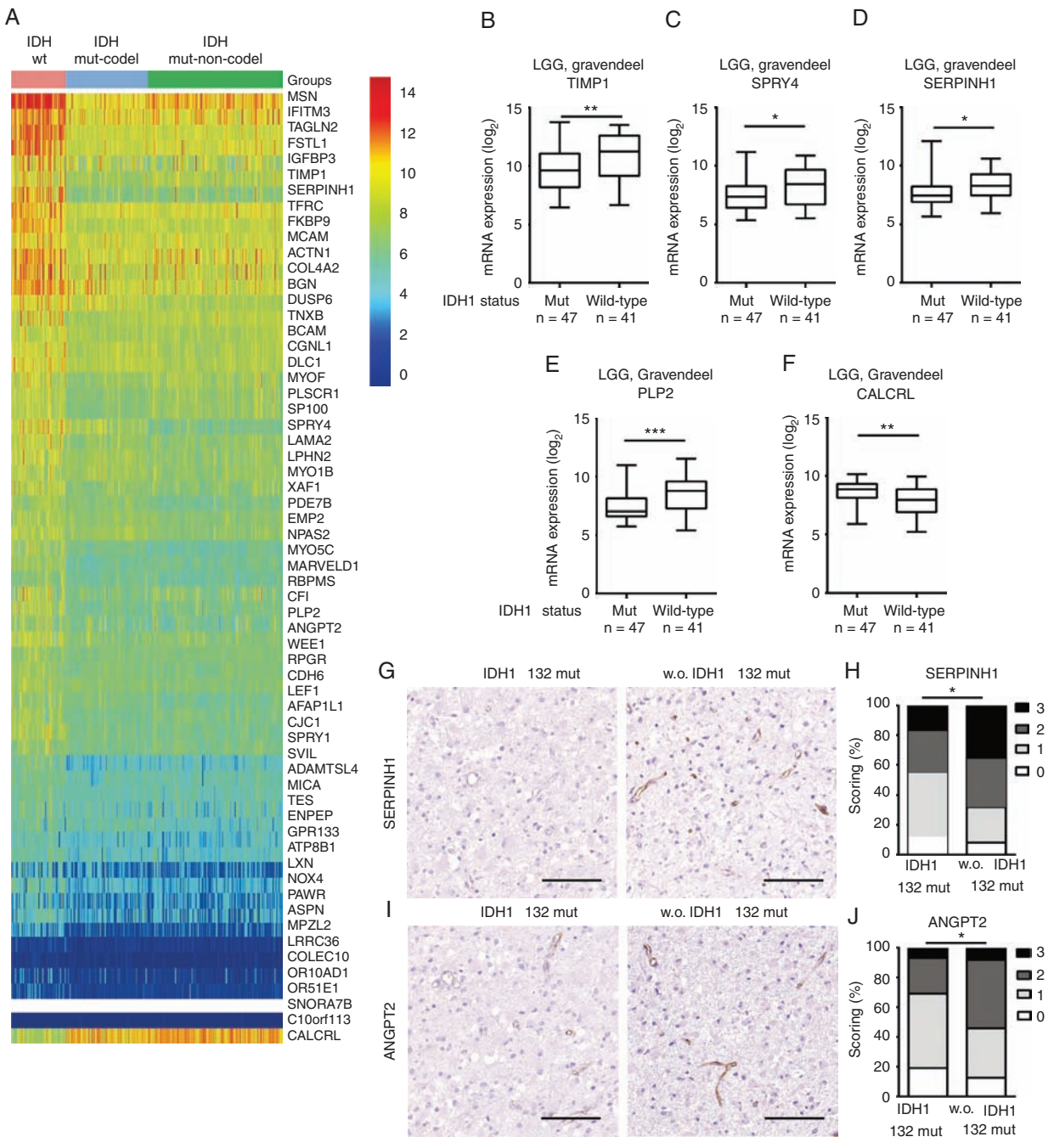

Fig. 3 Genes differentially expressed in IDH wild-type LGGs. (A) Heat map of 59 vascular enriched genes upregulated in IDH wild-type LGGs compared with LGGs with IDH mutations. RNA level of (B) TIMP1, (C) SPRY4, (D) SERPINH1, (E) PLP2, and (F) CALCRL in LGG samples from the Gravendeel dataset. (G–J) Immunohistochemical staining and quantification of SERPINH1 (G–H) and ANGPT2 (I–J) in LGGs with or without the IDH-R132H mutation (scale bar =100 μm).

vascular gene expression in IDH wild-type LGG vessels. By comparing vascular gene expression in IDH wild-type and IDH-mutated LGG, we identified 61 differentially expressed genes, 59 genes being significantly upregulated and 2 genes (*C10orf113*, *CALCRL*) downregulated in IDH wild-type LGG (Fig. 3A, Supplementary Table S10). This finding was further supported by analysis of the Gravendeel dataset, in which representative genes (*TIMP1, SPRY4, PLP2, SERPINH1*, and *CALCRL*) were confirmed to be regulated specifically in IDH wild-type LGG (Fig. 3B–F).

Functional annotation of the 59 upregulated genes revealed significant enrichment of terms connected to "biological adhesion/cell adhesion/cell migration," reflecting processes coupled to angiogenesis and vascular remodeling in the IDH wild-type glioma vasculature (Supplementary Table S11).

Increased Expression of ANGPT2 and SERPINH1 in IDH Wild-Type Glioma Vessels

To validate our findings, we stratified the LGGs included in the TMAs according to the IDH mutation status of the tumor (64 IDH1-R132H immunopositive gliomas; 49 WHO grade II and 15 WHO grade III gliomas versus 28 IDH1-R132H immunonegative gliomas: 15 WHO grade II and 13 WHO grade III gliomas). ANGPT2 and SERPINH1 were selected for validation, since they have been suggested as targets for anti-angiogenic therapy of glioma.[29,30] In agreement with the gene expression analysis, we found that antibodies recognizing ANGPT2 and SERPINH1 specifically stained tumor vessels and that their respective staining scores were significantly higher in samples that lacked the IDH1-R132H mutation than in IDH-R132H mutated samples (Table 2, Fig. 3G–J). The IDH1-R132H mutation accounts for nearly 80% of all IDH mutations,[31] and by staining of IDH1-R132H, we cannot exclude the presence of other, less common IDH1 mutations or IDH2 mutations. However, our results are consistent with the bioinformatic analysis, showing significantly higher levels of ANGPT2 and SERPINH1 in IDH wild-type glioma.

Table 2 Summary of TMA quantification of ANGPT2 and SERPINH1 staining in IDH wild-type and mutated LGG

ANGPT2 Staining			
		IDH Mutated LGG	IDH Wild-Type LGG
	Total	58	24
Score	0	11	3
	1	29	8
	2	14	11
	3	4	2
SERPINH1 Staining			
		IDH Mutated LGG	IDH Wild-Type LGG
	Total	60	25
Score	0	7	2
	1	26	6
	2	17	8
	3	10	9

Signaling Pathways that Alter Vascular Gene Expression in IDH Wild-Type LGG Vessels

The TFacts tool was employed to predict transcription factors that regulate vascular gene expression in IDH wild-type LGG vessels, based on the 59 genes that were significantly upregulated in IDH wild-type LGG in the dataset of TCGA. Transcription factors involved in TGF and hypoxia signaling pathways were significantly enriched, such as SMAD3 and HIF1α (Supplementary Table S12). Consistently, the expression of TGFβ2 was significantly higher in IDH wild-type LGG than IDH-mutated gliomas (Fig. 4A). The hypoxia-related transcription factor signature is consistent with a previous study showing that IDH wild-type gliomas display higher levels of hypoxia- and angiogenesis-related gene sets compared with IDH-mutated gliomas.[32] VEGF, which is induced under hypoxic conditions, was significantly higher in IDH wild-type gliomas (Fig. 4B). These results indicate that enhanced TGFβ2, VEGF, and hypoxia-related signaling underlie alterations in vascular gene expression in IDH wild-type LGG.

Regulation of Endothelial Gene Expression by TGFβ2, VEGF, and Hypoxia

To determine if VEGF-, TGFβ2-, and hypoxia-related signaling are sufficient to cause changes in gene expression noted in IDH wild-type glioma vasculature, endothelial cells (bEND.3 and HDBEC) were stimulated with TGFβ2, VEGFA, or $CoCl_2$, which induces hypoxia by inhibiting the prolyl hydroxylase domain–containing enzymes leading to stabilization of HIF1α.[33] Expression of candidate genes induced in IDH wild-type gliomas (*ANGPT2, SPRY4, PLP2, SERPINH1, TIMP1, MCAM, TFRC, IGFBP3*) was analyzed by qPCR. Exposure of murine brain endothelial cells (bEND.3) to VEGF was associated with upregulation of *Angpt2*, *Serpinh1*, *Timp1*, and *Igfbp3* (Fig. 4C, D), while TGFβ2 stimulation induced expression of *Angpt2, Serpinh1*, *Timp1*, and *Mcam* (Fig. 4D). Under $CoCl_2$-induced hypoxic conditions, *Serpinh1*, *Spry4*, and *Tfrc* were upregulated in bEND.3 cells (Fig. 4D). In HDBECs, *Angpt2* and *Spry4* were upregulated upon either VEGF or TFGβ2 stimulation, *Plp2* was upregulated by TGFβ2 or CoCl2, and *Serpinh1* was upregulated by $CoCl_2$ (Supplementary Figure S3A, B). Taken together, it is likely that VEGFA, TGFβ2, and hypoxia contribute to the specific vascular gene expression signature of IDH wild-type LGG.

Multivariate analysis revealed that *ANGPT2*, *SERPINH1*, *SPRY4*, and *PLP2* expression does not correlate with survival of LGG patients when adjusted for IDH mutation status (Supplementary Table S13). However, high SPRY4 expression significantly correlated with poor survival in IDH wild-type LGGs (Fig. 4E), and high ANGPT2 expression significantly correlated with poor survival in IDH-mutated tumors (Fig. 4F). These genes may have prognostic values for LGG and can be further explored as prognostic biomarkers.

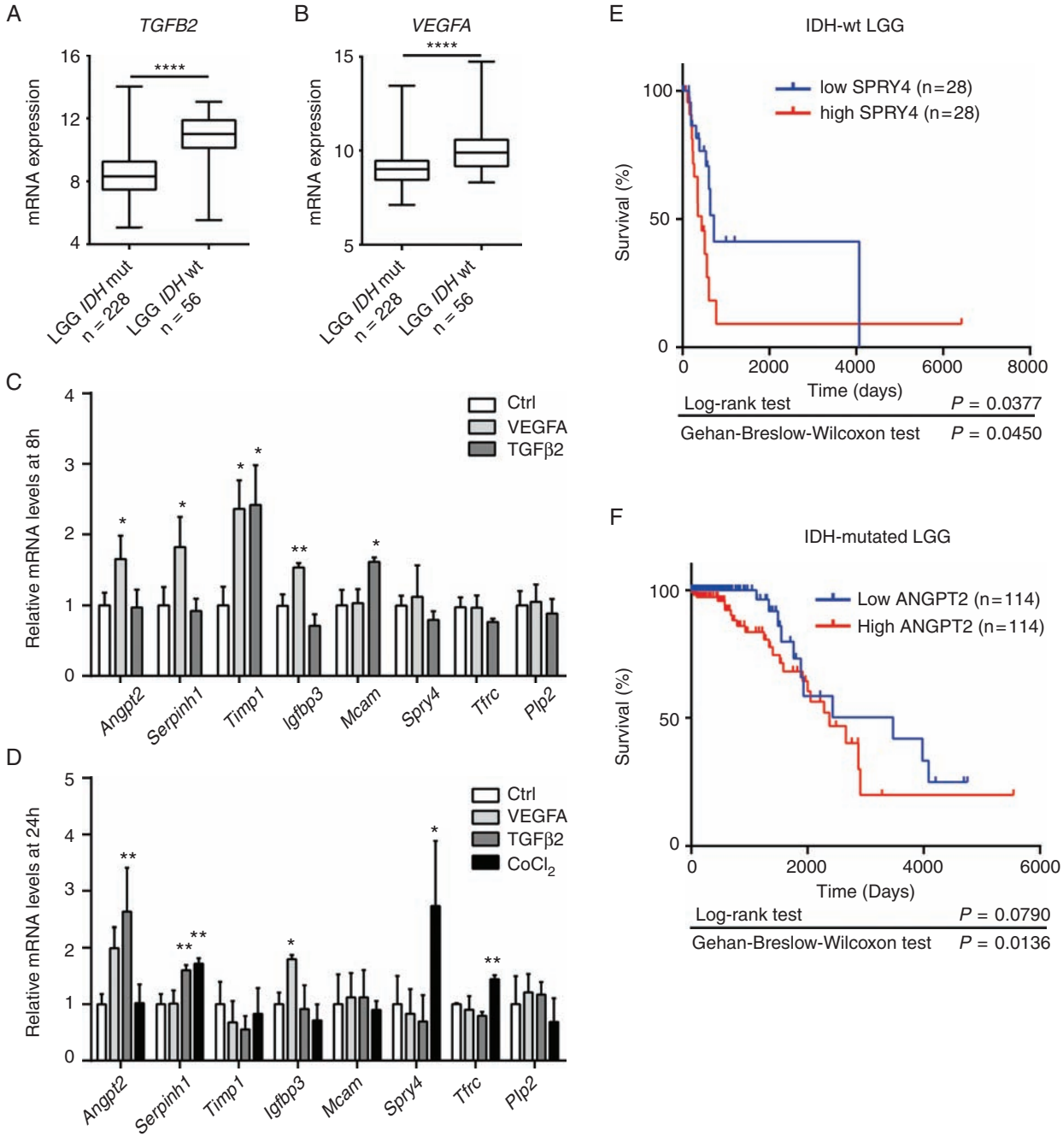

Fig. 4 The gene expression signature in IDH wild-type LGGs is partially due to hypoxia and expression of TGFβ2 and VEGF in the tumor microenvironment. RNA levels of (A) TGFβ2 or (B) VEGFA in LGG samples form the database of TCGA with or without IDH mutations. (C) Quantitative PCR data of gene induction in bEND.3 cells stimulated with VEGFA or TGFβ2 for 8 hours. (D) Quantitative PCR data of gene induction in bEND.3 cells stimulated with VEGFA, TGFβ2, or $CoCl_2$ for 24 hours. Graphs depict fold change versus unstimulated control (mean ± SD; $*P < 0.05$, $**P < 0.01$, one-way ANOVA with Dunnett's posttest). (E) Survival plot for patients with IDH wild-type LGGs with low *SPRY4* or with high *SPRY4* expression. (F) Survival plot for patients with IDH mutated LGGs with low *ANGPT2* or with high *ANGPT2* expression.

Discussion

Our results demonstrate that WHO grade II glioma vessels show incipient changes in gene expression, corresponding to an intermediate stage of vascular abnormality. This is in contrast to our previous investigation, where we focused on glioblastoma vessels and did not observe significant changes in gene expression between vessels in control samples and WHO grade II gliomas.[18] Since this study was performed by global clustering methods in LMD vessels from a small number of samples, we hypothesized that subtle changes in gene expression would not be detected. Therefore, we re-analyzed the LMD and total tissue dataset to identify vascular enriched genes and correlated the relative changes in expression of these genes in WHO grade II glioma and glioblastoma vessels compared with control. We identified mild changes in gene expression in WHO grade II gliomas, including upregulation of ANGPT2 and LAMA4, which corresponded to the pronounced alterations in gene expression observed in glioblastomas. ANGPT2 is an angiogenic growth factor which is upregulated in glioblastoma vessels and may mediate resistance to bevacizumab.[18,29,34] ANGPT2 generally acts as an antagonist to ANGPT1 signaling, decreasing activation of Tie2, which leads to vascular destabilization. An antibody that binds to ANGPT2 and converts it into an activator of Tie2 can normalize tumor vasculature, reduce hypoxia, and increase the delivery of chemotherapy drugs.[35] *LAMA4* codes for laminin subunit alpha 4, an extracellular matrix glycoprotein that is a component of the laminin complex and is consistently expressed by tumor blood vessels.[36] We have previously demonstrated that melanoma cell adhesion molecule (MCAM), a binding partner of LAMA4, is upregulated in glioblastoma vessels.[18]

Our finding that MV scores were increased in IDH wild-type LGG indicates that these tumors are more angiogenic than IDH-mutated LGG. This is in line with a previous report demonstrating that the global gene signature of IDH wild-type LGG is indicative of angiogenic activation and associated with increased relative cerebral blood volume.[32] MV score did not correlate with survival within the IDH wild-type or IDH-mutated subgroups or when correcting for IDH mutation status in a multivariate analysis. Although these results are limited by the composition of the vascular gene signature and the number of tumors available for analysis, the data indicate that a high MV score is a feature of IDH wild-type LGG and not an independent prognostic marker. We identified 59 genes with increased expression in IDH wild-type glioma vessels compared with the vasculature of IDH-mutated gliomas. Many of these have been implicated in regulation of angiogenesis (eg, ANGPT2, MCAM),[29,37] or shown to be upregulated in glioblastoma vasculature (eg, ANGPT2, MCAM, WEE1, SPRY1, NOX4, SERPINH1).[18] Gene ontology analysis revealed enrichment of processes related to cell migration and adhesion, indicating enhanced angiogenesis/vascular remodeling in IDH wild-type LGG. Notably, transcription factors involved in TGF and hypoxia signaling pathways such as SMAD3 and HIF1α were significantly enriched in IDH wild-type LGG. A recent study showed that IDH-mutated glioblastomas have smaller blood vessels and are associated with less hypoxia compared with IDH wild-type glioblastoma.[38] However, this study did not investigate if alterations in the angiogenic signaling pathways were associated with the different blood vessel patterns. Therefore, it is still unclear whether this is mediated by similar mechanisms as in LGG.

Increased hypoxia is associated with increased tumor invasiveness, a shift in cancer cell metabolism, tumor stem cell maintenance, therapy resistance, and angiogenesis.[39] Hypoxia triggers the expression of VEGF and is a key mediator of vascular abnormalization in glioblastoma.[40] VEGF can also be expressed by perivascular cells, which may enhance vascular abnormalities in glioma.[21] TGFβ signaling is activated in glioblastoma vasculature, and has been implicated in vascular abnormalization.[18] In addition, TGFβ2 can be transcriptionally regulated in glioma cells, and is involved in downregulation of endothelial tight junctions leading to impairment of the blood–brain barrier.[41–43] Given the high expression of TGFβ2 and VEGF in glioma cells, it is likely that TGFβ2 and VEGF exert their functions mainly through paracrine signaling. Inhibition of TGFβ signaling altered vascular morphology in mouse models of glioma, and combined TGFβ and VEGF inhibition suppressed tumor angiogenesis and prolonged survival in GL261 glioma.[44] Our results suggest that angiogenic signaling in IDH wild-type LGG occurs through similar mechanisms as in glioblastoma.

Our data indicate that VEGF, TGFβ, and hypoxia-related signaling contribute to the distinct vascular gene signature of IDH wild-type LGG. Differences in vascular gene expression are likely to affect vascular function and the response to anti-angiogenic drugs. Further studies are required to clarify if IDH mutation status may be a predictive biomarker for response to anti-angiogenic therapy.

Supplementary Material

Supplementary material is available at *Neuro-Oncology* online.

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Authorship

L.Z. designed and performed research; collected, analyzed, and interpreted data; and wrote the manuscript; L.H. performed research; collected, analyzed, and interpreted data; and wrote the manuscript; R.L. performed research and interpreted data; K.R. performed research; M.B. contributed tissue microarrays; A.S. contributed tissue microarrays and interpreted data;

A.D. designed research, analyzed and interpreted data, wrote the manuscript, and supervised the study.

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