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ZEB1 expression is increased in *IDH1*-mutant lower-grade gliomas

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Abstract

Transcription factors that induce epithelial-mesenchymal transition (EMT) promote invasion, chemoresistance and a stem-cell phenotype in epithelial tumors, but their roles in central nervous system tumors are not well-understood. We hypothesized these transcription factors have a functional impact in grades II–III gliomas. Using the National Cancer Institute (NCI) Repository for Molecular Brain Neoplasia Data (REMBRANDT) and the Cancer Genome Atlas (TCGA) Lower-Grade Glioma (LGG) data, we determined the impact of EMT-promoting transcription factors (EMT-TFs) on overall survival in grades II–III gliomas, compared their expression across common genetic subtypes and subsequently validated these findings in a set of 31 tumors using quantitative real-time polymerase chain reaction (PCR) and immunohistochemistry. Increased expression of the gene coding for the transcriptional repressor Zinc Finger E box-binding Homeobox 1 (ZEB1) was associated with a significant increase in overall survival (OS) on Kaplan–Meier analysis. Genetic subtype analysis revealed that *ZEB1* expression was relatively increased in *IDH1/2*-mutant gliomas, and *IDH1/2*-mutant gliomas expressed significantly lower levels of many ZEB1 transcriptional targets. Similarly, *IDH1/2*-mutant tumors expressed significantly higher levels of targets of microRNA 200C (MIR200C), a key regulator of *ZEB1*. In a validation study, *ZEB1* mRNA was significantly increased in *IDH1*-mutant grades II–III

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Compliance with ethical standards

Conflict of interest

The authors have no known conflicts of interest to declare.

gliomas, and ZEB1 protein expression was more pronounced in these tumors. Our findings demonstrate a novel relationship between *IDH1/2* mutations and expression of ZEB1 and its transcriptional targets. Therapy targeting ZEB1-associated pathways may represent a novel therapeutic avenue for this class of tumors.

Keywords

IDH; Lower-grade glioma; Epithelial-mesenchymal transition; ZEB1

Introduction

Gliomas are the most common primary brain tumor in adults and one of the most common brain tumors in children [1]. Despite recent advances in extended-margin tumor resection, targeted chemotherapies and radiotherapy, prognosis of malignant gliomas remains dismal: overall survival remains about 5–10 years for World Health Organization (WHO) grade-II, 2–3 years for grade-III and 12–15 months for grade-IV gliomas [1]. Our understanding of the molecular pathogenesis of these tumors has increased dramatically over the past decade thanks to collaborative high-throughput efforts to determine the mutations, epigenetic alterations and gene expression patterns that define these tumors. Two independent workgroups recently identified multiple molecularly distinct, clinically relevant subclasses of grades II–III gliomas [2–5]. One genetic subtype is characterized by mutations in *Isocitrate Dehydrogenase 1 and 2 (IDH1/2)*, which are found in 70–80 % of grades II–III gliomas and 80–90 % of secondary glioblastomas (GBMs) but only 5–10 % of primary GBMs [6, 7]. These tumors display a hypermethylated epigenetic phenotype [2, 3, 8], are enriched in the proneural histologic subtype of glioblastoma [8], tend to be found in younger individuals and have a more favorable prognosis [6, 7]. A second genetic subtype of gliomas is characterized by mutations or amplifications in *Epidermal Growth Factor Receptor (EGFR)*, *Telomerase Reverse Transcriptase (TERT)* and the p53 regulator *MDM4* and inactivating mutations in *Phosphatase And Tensin Homolog (PTEN)*, *Neurofibromin 1 (NF1)* and *Cyclin-Dependent Kinase Inhibitor 2A (CDKN2A)*. These tumors have a less favorable prognosis and a clinical course more similar to GBMs [2–5]. Despite identifying many key molecular pathways that influence prognosis in patients with glioma, our understanding of the mechanisms of tumor initiation and maintenance is incomplete, and pharmacologic manipulation of these pathways has not yet enhanced patient survival.

Epithelial-mesenchymal transition (EMT) is the process by which a cell sheds its intercellular adhesions, loses apicobasal polarity and adopts an invasive, migratory phenotype [9, 10]. EMT is critical to normal embryonic development and the tissue response to injury but is also crucial to metastasis and the generation of cancer stem cells in carcinomas [9–11]. EMT is governed by a select set of transcription factors and microRNA (miRNA) feedback loops that are frequently dysregulated in cancer [10–12]. Similar to their roles in epithelial tumors, recent evidence implicates EMT-promoting transcription factors (EMT-TFs) in the genesis and progression of primary GBM [13–21]. In particular, the transcription factor Zinc finger E box-Binding homeobox 1 (ZEB1) promotes primary GBM tumor initiation, cell invasion and resistance to the standard-of-care drug temozolomide [17].

These findings indicate that EMT plays a crucial role in generating and maintaining the malignant features of primary GBM.

While the roles of several EMT-TFs have been explored in primary GBM, their roles in grades II–III gliomas have not been defined. As lower-grade gliomas and primary GBMs are molecularly and clinically distinct, we sought to determine whether EMT-TFs play a meaningful role in grades II–III gliomas. To that end, we assessed expression of the five canonical EMT-TFs in these tumors and quantified their effects on overall survival using large datasets, compared EMT-TF expression across different genetic subtypes of grades II–III gliomas and validated these findings using quantitative real-time PCR (qRT-PCR) and immunohistochemistry on a set of 31 tumors.

Materials and methods

Bioinformatics analysis

Microarray data of grades II–III gliomas relative to normal brain were obtained and analyzed using the Oncomine database (<http://www.oncomine.org>) [22], and the default reporter was used for each analysis. Gene expression arrays with survival data were obtained from the National Cancer Institute Repository for Molecular Brain Neoplasia Data (NCI REMBRANDT) and the Cancer Genome Atlas (TCGA) datasets. REMBRANDT data were extracted directly from the NCI REMBRANDT Web site (<https://caintegrator.nci.nih.gov/rembrandt/>) [23]. Affymetrix microarray files for all grades II–III tumors with graded histology and matching microarray files were normalized using robust multi-array (RMA) averaging in Partek Genomics Suite (Partek, St. Louis, MO). When multiple probes were aligned to the same gene, the median of all gene probes was used for expression analysis. Cancer Genome Atlas Network RNAseq and microRNA expression data were extracted from the Broad Institute Firehose Pipeline (<http://gdac.broadinstitute.org>), and genetic subtypes were sorted using cBioPortal (Memorial Sloan Kettering, New York, New York; <http://www.cbioportal.org/public-portal/>) [24]. Normalized RNAseq by Expectation-Maximization (RSEM)-normalized gene expression values and reads per million micro RNAs mapped (RPM) were used for statistical comparisons of gene expression and miRNA expression, respectively.

Human tissue acquisition and preparation

All human tissues for the validation study were acquired through the Surgical Neurology Branch at the National Institute of Neurological Disorders and Stroke (NINDS). Informed consent was obtained from all patients through Institutional Review Board-approved protocol 03-N-0164. All samples were flash-frozen at the time of surgery and confirmed to be at least 80 % tumor prior to experiments. Tumor histology was assessed by a board-certified neuropathologist who was blinded to tumor genotype.

IDH1/2 mutation assessment, DNA extraction and Sanger sequencing

IDH1/2 mutation status was determined using bidirectional Sanger sequencing of exon 4 of *IDH1* and *IDH2*. Exon 4 of *IDH1* was sequenced in all samples; exon 4 of *IDH2* was sequenced only when exon 4 of *IDH1* was wild-type. DNA was extracted from flash-frozen

tissues using a Machery-Nagel Nucleospin Tissue Kit (Bethlehem, PA). Genes were amplified using PCR Platinum Supermix (Invitrogen, Carlsbad, CA) and gene-specific primers (Sigma-Aldrich, St. Louis, MO) listed in Supplementary Table 1. Sanger sequencing was performed by MWG Operon (Huntsville, AL).

RNA extraction and quantitative real-time polymerase chain reaction

Total RNA was extracted from flash-frozen tissues using a Qiagen RNeasy Mini Kit (Valencia, CA). RNA was converted to cDNA using Superscript III qRT-PCR Supermix (Invitrogen). Quantitative real-time PCR was performed using SYBR Select Master Mix (Invitrogen) and gene-specific primers (Sigma-Aldrich) on a ViiA 7 Real Time PCR System (Applied Biosystems, Foster City, CA). Primer sets are listed in Supplementary Table 2. Gene expression relative to the housekeeping gene *ACTB* was calculated using the 2^{-Ct} method, and error is represented using standard deviations from the mean.

Immunohistochemistry

Frozen tissue blocks were sectioned at a width of 8 μ m. All staining was performed using a BOND-MAX automatic tissue stainer (Leica Biosystems, Buffalo Grove, IL). ZEB1 expression was assessed using a rabbit polyclonal ZEB1 antibody (Sigma-Aldrich) at 1:200 dilution and visualized using antibody horseradish peroxidase and 3,3'-diaminobenzidine (DAB). ZEB1 expression was labeled as being negative, weak, moderate or strong as determined by the number of positive cells in the specimen. Staining intensity was determined by a board-certified neuropathologist who was blinded to tumor genotype.

Statistical comparisons

Between-group comparisons of gene expression were made using paired or unpaired student's *t* tests where appropriate. Gene expression correlations were performed using linear regression with Pearson coefficients. The effect of gene expression on overall survival was determined using Kaplan–Meier analysis and formally compared using Kaplan–Meier log-rank tests and Cox regression analysis where appropriate. In the Cox regression analysis, EMT-TF expression was treated as a continuous variable, and tumor grade and KPS score were treated as ordinal variables. Age was treated as a continuous variable in the TCGA dataset, but due to restrictions in data recording, age was treated as an ordinal variable in the REMBRANDT dataset. In both the REMBRANDT and TCGA LGG datasets, multivariate Cox regression analysis was performed on a complete-case basis, i.e., only cases with available time to follow-up, tumor grade, age and Karnofsky Performance Status (KPS) score were included in the analysis. Log-rank tests were performed only on cases in which tumor grade and time to follow-up were available. All comparisons between high- and low-expressing tumors using log-rank tests were made by separating tumors into groups of two equal sizes at the median expression value. A significance threshold of $p < 0.05$ was set for all comparisons with Bonferroni corrections made for multiple simultaneous comparisons (i.e., if five comparisons were run in parallel, the statistical threshold was set to $p < 0.01$). Statistical comparisons were made using SPSS version 22 (International Business Machines Corporation, Armonk, NY) and GraphPad Prism (Prism Software Corp., Irvine, CA).

Results

ZEB1 expression is increased in lower-grade gliomas and is correlated to an increase in overall survival

The canonical EMT-TFs SNAIL (*SNAIL*), SLUG (*SNAIL2*), TWIST (*TWIST1*), ZEB1 (*ZEB1*) and ZEB2 (*ZEB2*) have previously been shown to impact the phenotype of primary GBM [13–21]. To determine the relative expression of EMT-TFs in grades II–III gliomas, we first queried gene expression of these five EMT-TFs against normal brain across all publicly available microarray datasets of grades II–III gliomas with normal brain comparisons catalogued in the Oncomine database. Our search detected five relevant microarray datasets [25–29], which are shown in Supplementary Table 3. Relative to normal brain, expression levels of *SNAIL2*, *TWIST1* and *ZEB1* were significantly increased in at least one dataset. None of the queried targets were significantly decreased in any dataset. *SNAIL2* was up-regulated 1.509–2.403-fold in grade-III tumors in two datasets and *TWIST1* was up-regulated 1.406-fold in grade-III oligodendrogliomas in one dataset. Of the three up-regulated EMT-TFs, *ZEB1* demonstrated the most robust increase in expression: *ZEB1* was increased 2.892-fold in grade-II astrocytomas and 3.037–5.254-fold in grade-III gliomas across four datasets.

To determine whether expression of EMT-TFs impacts survival in grades II–III gliomas, we compared relative expression of each EMT-TF against overall survival in grades II–III gliomas using the NCI REMBRANDT and TCGA LGG datasets. First, expression of each EMT-TF was factored into a multivariate Cox proportional hazards model with age, KPS and tumor grade as covariates (Table 1). Surprisingly, *ZEB1* expression was independently associated with an increase in overall survival in the REMBRANDT dataset (HR 0.668, 95 % CI 0.496–0.898, $p=0.008$), but this was not observed in the TCGA dataset (HR 0.814, 95 % CI 0.584–1.135, $p=0.225$). No significant effect on OS was observed with any other EMT-TF.

Next, tumors were divided into two equal-sized groups based on their relative expression of each EMT-TF, and OS was compared between groups using the Kaplan–Meier logrank test (Fig. 1). Relatively high levels of *ZEB1* expression were robustly associated with longer OS in both the REMBRANDT dataset ($\chi^2 = 22.560$, $p < 0.0001$) and TCGA dataset ($\chi^2 = 11.940$, $p = 0.0006$). There was a trend toward shorter OS in tumors expressing relatively high levels of *SNAIL2*, but this comparison did not reach statistical significance in either dataset (for REMBRANDT $\chi^2 = 3.094$, $p = 0.0786$; for TCGA $\chi^2 = 2.897$, $p = 0.0887$). Similarly, there was a trend toward shorter OS in tumors expressing relatively high expression of *TWIST1* in the TCGA dataset only, but this did not reach statistical significance ($\chi^2 = 4.660$, $p = 0.0309$). Given that *ZEB1* is known to confer invasive and stem-cell properties to GBM cells but was associated with a significant increase in overall survival in our analysis, we chose to focus on *ZEB1* for further analyses.

ZEB1 is preferentially up-regulated in IDH1/2-mutant grades II–III gliomas, and IDH1/2-mutant tumors express higher levels of MIR200C targets

Given the association between *ZEB1* expression and OS was most robust when tumors were divided into two subgroups based on *ZEB1* expression, we postulated that *ZEB1* is preferentially up-regulated in one or more genetic subtypes of grades II–III gliomas. As mentioned previously, mutations in *IDH1* and *IDH2* are present in 70–80 % of grades II and III gliomas and, when present, are believed to be the earliest genetic event in gliomagenesis [30]. Recent efforts have further defined genetic subtypes of *IDH1/2*-mutant tumors and their associations with pathologic phenotype. In gliomas with mutations in *IDH1* or *IDH2*, subsequent alterations in *TP53* and *ATRX* are associated with astrocytic differentiation [31] whereas co-deletion of chromosome arms 1p and 19q (1p19q co-deletion), mutations in *FUBP1* (on chromosome 1p), *CIC* (on chromosome 19q), *NOTCH1* and *PIK3CA* are associated with oligodendroglial differentiation [2, 3, 32, 33]. Interestingly, it has been previously shown that the most common *IDH1/2* mutations induce a ZEB1-dependent EMT in the colon and breast adenocarcinoma cell lines [34], but these cancers typically do not contain mutations in *IDH1/2*, and increased expression of ZEB1 has never been demonstrated in *IDH1/2*-mutant tumors.

To determine if *ZEB1* is preferentially over-expressed in tumors with specific genetic alterations, we compared *ZEB1* expression across these genetic alterations in grades II–III gliomas using the TCGA dataset (Fig. 2). Overlapping data on genotype and gene expression were available for 288 patients. *ZEB1* expression was significantly higher in tumors carrying mutations in *IDH1* or *IDH2* ($p < 0.0001$). However, within *IDH1/2*-mutant tumors, additional 1p19q co-deletion or mutations in *ATRX*, *TP53*, *CIC*, *FUBP*, *NOTCH* or *PIK3CA* were not associated with a significant change in *ZEB1* expression. To determine if *IDH1/2* mutations contributed to the observed increase in overall survival in tumors expressing relatively high levels of *ZEB1*, we performed a multivariate Cox regression analysis with *IDH1/2* mutation status as well as age, tumor grade and pre-operative KPS on the TCGA LGG dataset. After inclusion of *IDH1/2* mutation status as a covariate in this analysis, *ZEB1* expression was not independently associated with overall survival in this dataset (HR 1.605, 95 % CI 0.851–3.028, $p = 0.144$).

To determine whether increased *ZEB1* expression has a meaningful effect on gene expression patterns in *IDH1/2*-mutant tumors, we determined the relative expression of the MIR200BC family targets using the TCGA LGG dataset (Fig. 3). The MIR200BC family of miRNAs, which includes MIR200B, MIR200C and MIR429, plays a key role in negatively regulating *ZEB1* [12]. MIR200C accounts for the majority of miRNA in this family in grades II–III gliomas (Fig. 3a). Consistent with *ZEB1* up-regulation in *IDH*-mutant tumors, MIR200C was relatively down-regulated in *IDH1/2*-mutant grades II–III gliomas ($p < 0.0001$, Fig. 3b). We next determined the relative expression of 51 genes known to be associated with ZEB1 and MIR200C (Fig. 3c). These included 36 genes associated with a differentiated epithelial phenotype that are direct transcription targets of ZEB1 [35]; three genes up-regulated by ZEB1 in GBM that are associated with a stem-cell phenotype (*OLIG2*), invasion (*ROBO1*) and chemoresistance (*MYB*) [17]; four genes coding for cyclin-dependent kinase inhibitors associated with ZEB1-associated cell-cycle progression

[36]; and a polycomb ring-finger oncogene induced by *ZEB1* (*BMII*) [37]. Six additional genes coding for histone- and DNA-modifying enzymes (*TET1*, *TET2*, *TET3*, *JMJD1C*, *PHF2* and *CTCF*) were identified as *ZEB1* expression correlates in a screen of all genes associated with *ZEB1* expression using the TCGA dataset [24] and were included in comparisons. Consistent with *ZEB1* up-regulation, 12 of 15 (80 %) *MIR200C* targets and *ZEB1* correlates were significantly up-regulated and 19 of 36 (52.8 %) *ZEB1* targets were significantly down-regulated in *IDH*-mutant tumors.

Given our observation that *ZEB1* and targets of *MIR200C* were relatively up-regulated in *IDH1/2*-mutant grades II/III gliomas, we next asked whether *ZEB1* is up-regulated in other tumor types that frequently contain mutations in *IDH1* or *IDH2*. To answer this question, we compared *ZEB1* expression across *IDH1/2*-wild type and *IDH1/2*-mutant WHO grade-IV gliomas and acute myeloid leukemia (AML) samples using the TCGA GBM and Acute Myeloid Leukemia (LAML) datasets (Supplementary Figs. 1, 2). Similar to grades II–III gliomas, *ZEB1* expression was significantly higher in *IDH*-mutant grade-IV gliomas ($p < 0.0001$). However, there was no association between overall survival and *ZEB1* expression in the TCGA GBM dataset (Cox regression HR 0.745, 95 % CI 0.518–1.071, $p = 0.112$; log-rank $\chi^2 = 0.0218$, $p = 0.8827$). In the TCGA LAML dataset, there was no significant difference in *ZEB1* expression between *IDH*-mutant and *IDH*-wild type tumors ($p = 0.557$), but *MIR200C* expression was significantly decreased in *IDH*-mutant tumors ($p = 0.0148$). *ZEB1* expression did not significantly impact overall survival in AML (univariate Cox regression HR 0.995, 95 % CI 0.866–1.143, $p = 0.943$; logrank $\chi^2 = 0.3981$, $p = 0.5281$). These findings demonstrate that *ZEB1* expression may be up-regulated in *IDH1/2*-mutant GBM, but *ZEB1* mRNA expression is not associated with overall survival in these datasets.

Validation of RNASeq data by quantitative real-time PCR and immunohistochemistry

We next assessed transcript levels of *ZEB1* using qRT-PCR in a set of 31 grades II–III gliomas and temporal lobe white matter from two non-neoplastic controls to validate our findings in the TCGA dataset (Fig. 4). Mutations in *IDH1* were detected in 28 tumors (90 %; 27 R132H, 1 R132L), and no tumors carried a mutation in *IDH2*. The *IDH1*-wild-type tumors included 1 grade-II astrocytoma, 1 grade-III astrocytoma and 1 grade-III oligoastrocytoma. The *IDH1*-mutant tumors included 2 grade-II oligoastrocytomas, 16 grade-III astrocytomas, 7 grade-III oligoastrocytomas and 3 grade-III oligodendrogliomas. *IDH1*-mutant grades II–III gliomas expressed significantly higher levels of *ZEB1* relative to *IDH*-wild-type tumors (average 3.19-fold vs. 1.28-fold increase relative to non-neoplastic brain, $p = 0.0278$). We next compared transcript levels of *ZEB1* to those of ten selected *MIR200C* targets or *ZEB1* correlates and two *ZEB1* targets with relatively high transcript levels in the TCGA LGG dataset (*F11R* and *PMEPA1*) using qRT-PCR (Fig. 5). *ZEB1* expression was robustly associated with expression of *ROBO1* ($R^2 = 0.5750$, $p < 0.0001$), *MYB* ($R^2 = 0.5107$, $p < 0.0001$) and *BMII* ($R^2 = 0.4443$, $p = 0.0001$) as well as six genes coding for epigenetic modifying enzymes. An association was also observed with *OLIG2*, but this did not meet the threshold of statistical significance after p value correction ($R^2 = 0.2282$, $p = 0.0101$). Surprisingly, *ZEB1* was also associated with expression of two genes associated with epithelial differentiation, *F11R* ($R^2 = 0.5329$, $p < 0.0001$) and *PMEPA1* ($R^2 = 0.5778$, $p < 0.0001$).

To further validate these findings, ZEB1 protein expression was assessed using immunohistochemistry in these samples (Fig. 6). Tissue sections were available for 30 of 31 tumor sections used in the qRT-PCR dataset and for both of the controls. ZEB1 expression was negative in both controls. In *IDH1/2*-wild-type tumors, ZEB1 expression was weakly present in two of three tumors (67 %): sporadic, weakly staining ZEB1-positive cells were noted in one grade-III oligodendroglioma and present in one small area of a grade-III astrocytoma. In IDH-mutant tumors, ZEB1 was expressed in 12 of 27 specimens (44 %). In ZEB1-positive, *IDH1/2*-mutant tumors, ZEB1 staining was moderate in five tumors and weak in seven specimens. Thirteen ZEB1-positive specimens (93 %) were grade-III tumors, 10 (71 %) were astrocytomas and four (29 %) were oligoastrocytomas. When present, ZEB1 staining was largely confined to tumor cells; focal lymphocyte positivity was noted in only one specimen (7 % of all tumor specimens staining positive for ZEB1).

Discussion

These data demonstrate that ZEB1 is preferentially overexpressed in *IDH1/2*-mutant grades II–III gliomas and *ZEB1* expression is associated with expression of multiple genes that are known to play important roles in tumor initiation, invasion, resistance to therapy and epigenetic modifications. These results suggest that ZEB1 up-regulation and concomitant up-regulation of MIR200C targets may be important to the biology of *IDH1/2*-mutant tumors.

Epithelial-mesenchymal transition was originally described as the phenomenon by which a cell loses apicobasal polarity and adopts a migratory phenotype [9]. EMT is induced by a handful of transcription factors including, but not limited to, ZEB1, ZEB2, SNAIL, SLUG and TWIST. These transcription factors are post-transcriptionally regulated by several families of miRNAs leading to reciprocal feedback loops that regulate EMT [12]. Similar to their role in epithelial cells, these transcription factors have been observed to promote cellular invasion in primary GBM [13–21]. Although classically defined as a process occurring in metastatic cancer cells, EMT is also critical to the generation of cancer stem cells [38], highlighting the importance of this phenomenon in coordinating the hallmarks of cancer. While cancer stem cells were once believed to reside in a stem cell niche that subsequently generated more differentiated cancer cells, recent work demonstrates that a subpopulation of non-stem cancer cells gives rise to cancer stem cells, and this process depends on ZEB1 [38].

ZEB1 is involved downstream of multiple pathways that are deranged in glioblastoma including the receptor tyrosine kinase-RAS-RAF-MEK-ERK [36, 37, 39, 40], Transforming Growth Factor-Beta (TGF- β) [41], β -catenin [42] and PTEN-PI3K-Akt pathways [43]. The MIR200BC family of miRNAs is the major regulator of ZEB1 [12], and loss of MIR200C disinhibits expression of multiple factors that maintain the stem-cell phenotype including the polycomb protein BMI1 [37] (Fig. 7). MicroRNAs that regulate EMT are frequently down-regulated in multiple cancers including GBM [44, 45], suggesting that therapies targeting these miRNAs may be of potential benefit in abrogating cancer cell invasion and stem cell generation. Our findings extend the spectrum of ZEB1-associated pathways to include

mutations in *IDH1* and *IDH2* and suggest that further investigation is warranted regarding the use of ZEB1-targeted therapy for grades II–III gliomas.

Knowledge of the molecular characteristics of grades II–III gliomas has increased dramatically in the past several years owing to the discovery of *IDH1* and *IDH2* mutations in the majority of these tumors. Mutations in exon 4 of either *IDH1* or *IDH2* code for a neomorphic enzyme incapable of converting isocitrate to alpha-ketoglutarate (α -KG) [46, 47]. This mutant enzyme forms a heterodimer with its wild-type counterpart and instead reduces α -KG to enantiomeric-specific D-2-hydroxyglutarate (D-2-HG) [46, 47]. D-2-hydroxyglutarate is a competitive inhibitor of α -KG-dependent dioxygenases including Ten Eleven Translocase (TET) DNA demethylases and Jumonji C domain-containing (JmjC) histone demethylases, thus inhibiting demethylation of cytosine residues in DNA and histone lysine residues, respectively [48, 49]. The net effect of mutant IDH is a stem-cell phenotype secondary to a block in progenitor cell differentiation [50]. One possible explanation for the role of *IDH1/2* mutations in cancer is that stem cells maintained by high levels of D-2-HG subsequently become cancerous following accumulation of alterations to other oncogenes and tumor suppressor genes. Relevant to this hypothesis, ZEB1 is expressed in subventricular zone neural progenitor cells [16, 51], is critical for neuronal survival following hypoxic injury [52] and maintains a neural stem cell niche in the spinal cord [53]. While we did not directly assess the mechanism of *ZEB1* up-regulation in glioma, it is possibly secondary to specific chromatin configurations at the *ZEB1* promoter that are specified by D-2-HG. Others have shown such configurations at the *ZEB1* promoter to be necessary for cancer stem cell generation [38]. Similar to this role, it may be that ZEB1 plays a key role in generating cancer stem cells from *IDH1/2*-mutant neural stem cells. Future investigations are necessary to explore this possibility.

Others have found that ZEB1 is up-regulated in primary GBM and is not associated with *IDH1/2* mutation status in GBM [17]. At first glance, this would seem paradoxical given that *IDH1/2*-wild-type grades II–III gliomas have a molecular signature similar to that of primary GBM. As evidenced by the TCGA LGG and GBM datasets, the molecular features of *IDH1/2*-wild-type grades II–III glioma and primary GBM are not identical but instead overlapping [2–5]. It has been postulated that this difference is related to the development of a hypoxic microenvironment, which is exclusive to GBM [2]. To that end, others have recently shown that ZEB1 is up-regulated under hypoxia in glioma neurospheres and its inhibition blunts hypoxia-induced cell invasion [16]. It would be tempting to speculate that so-called *IDH1/2* mutation-induced pseudohypoxia secondary to inhibition of prolyl-4-hydroxylase domain-containing enzymes [54] may contribute to *ZEB1* up-regulation in IDH-mutant tumors. However, the most recent evidence suggests that transcriptional targets of hypoxia-inducible factors (HIFs) are, in fact, not up-regulated at biologically relevant (low-millimolar) concentrations of D-2-HG, which is consistent with the lack of angiogenesis in these tumors [55]. Intriguingly, ZEB1 expression in primary glioblastoma is associated with a low-proliferating, chemoresistant and invasive phenotype in primary GBM [17]. Expression of R132H-mutant IDH1 in a model of glioblastoma has been shown to decrease the proliferation of glioma cells in vitro and vivo [56].

The findings of this study should be considered in light of several limitations. First, although we used two separate databases to assess the impact of EMT-TF expression on overall survival, the TCGA LGG workgroup is still accruing data, and most study participants have not yet reached the primary endpoint (i.e., the data are heavily censored). We attempted to counteract this by using only complete cases in the Cox regression analysis, but additional clinical data are necessary to determine whether these findings hold for the entire TCGA LGG patient population. Second, experimental data are required to determine the effect of ZEB1 expression on grades II–III glioma phenotype. We analyzed expression of established ZEB1 and MIR200C targets to verify that *ZEB1* overexpression led to expected changes in expression of its downstream targets, but development of appropriate experimental models of IDH-mutant grades II–III gliomas is necessary to further assess the functional impact of ZEB1 in *IDH1/2*-mutant LGGs. Finally, our validation study was relatively small and skewed toward *IDH1*-mutant tumors slightly more than what has been observed in larger datasets, and this likely decreased our power to detect changes in ZEB1 expression between *IDH1/2*-mutant and *IDH1/2*-wild-type tumors. Thus, the conclusions presented here would benefit from further validation studies analyzing additional tumor specimens.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

Kaplan–Meier analysis of the effect of EMT-TF expression on overall survival in the Cancer Genome Atlas Lower Grade Glioma (TCGA LGG) and Repository for Molecular Brain Neoplasia Data (REMBRANDT) datasets. Cases were divided at the median expression level for each gene of interest and compared using the Kaplan–Meier log-rank test

Fig. 2.
ZEB1 expression by common genetic subtypes of lower-grade glioma using the TCGA LGG dataset. Groups were compared using a 2-tailed unpaired student's *t* test

Fig. 3.

Expression of ZEB1 and MIR200C targets by IDH-subtype in the TCGA LGG dataset. Relative abundance of the microRNA (MIR) 200BC family members MIR200B, MIR200C and MIR429 (a). Relative expression of MIR200C by *IDH* mutation status (b). Expression of 51 ZEB1 transcriptional targets, MIR200C targets and ZEB1 expression correlates by *IDH* mutation status (c)

Fig. 4.

Evaluation of relative *ZEB1* expression in a set of 31 grades II–III gliomas and two specimens of non-neoplastic brain using quantitative real-time PCR (qRT-PCR). Comparisons of relative *ZEB1* expressions by *IDH* mutation status were performed using 2-tailed unpaired student's *t* tests

Fig. 5.

Correlation of *ZEB1* expression with 12 genes associated with *ZEB1* or *MIR200C* in a set of 28 *IDH1*-mutant grades II/III gliomas using qRT-PCR. Linear regression was used to quantitatively assess associations between gene expression

Fig. 6.
Light microscopic images of immunohistochemistry for ZEB1 expression in non-neoplastic brain, *IDH1/2*-wild-type and *IDH1/2*-mutant grades II–III gliomas. Magnification $\times 400$ for all images

Fig. 7.

Schematic illustrating molecular pathways influenced by *ZEB1* and their functional significance in glioma. *ZEB1* and *MIR200C* mutually repress one another in a negative feedback loop. *ZEB1* functions as a transcriptional repressor and, thus, directly represses expression of its transcriptional targets. *MIR200C* represses translation of multiple transcripts of genes involved in maintaining a stem cell phenotype, promoting cell invasion and regulating the epigenetic milieu of the cell. Mutations in *IDH1* and *IDH2* increase *ZEB1* expression and therefore disinhibit expression of multiple genes involved in cancer initiation, progression and maintenance

Table 1

Correlation of epithelial-mesenchymal transition-promoting transcription factor (EMT-TF) expression to overall survival using the TCGA and REMBRANDT datasets

	Multivariate Cox regression			TCGA LGG		
	HR	95 % CI	P	HR	95 % CI	P
<i>SNAI1</i>	1.859	0.344–10.042	0.471	1.219	1.036–1.435	0.017
<i>SNAI2</i>	1.338	0.973–1.841	0.073	0.903	0.746–1.094	0.297
<i> Twist1</i>	0.925	0.669–1.279	0.638	1.108	0.936–1.312	0.234
<i>ZEB1</i>	0.668	0.496–0.898	0.008	0.814	0.584–1.135	0.225
<i>ZEB2</i>	1.473	0.534–4.062	0.454	0.991	0.719–1.366	0.956

Each EMT-TF was factored into a multivariate Cox regression analysis adjusting for tumor grade, Karnofsky performance status (KPS) score and age. Analysis was performed on a complete-case basis; data for all covariates were available for 67 patients in the REMBRANDT dataset and 291 patients in the TCGA LGG dataset

HR hazard ratio, CI confidence interval