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The Tumor Suppressor Prostate Apoptosis Response-4 (Par-4) is Regulated by Mutant IDH1 and Kills Glioma Stem Cells

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Abstract

Prostate apoptosis response-4 (Par-4) is an endogenous tumor suppressor that selectively induces apoptosis in a variety of cancers. Although it has been the subject of intensive research in other cancers, less is known about its significance in gliomas, including whether it is regulated by key driver mutations, has therapeutic potential against glioma stem cells (GSCs), and/or is a prognostic marker. We found that patient-derived gliomas with mutant isocitrate dehydrogenase 1 have markedly lower Par-4 expression ($P < 0.0001$), which was validated by The Cancer Genome Atlas dataset ($P = 2.0 \times 10^{-13}$). The metabolic product of mutant IDH1, D-2-hydroxyglutarate (2-HG), can suppress Par-4 transcription *in vitro* via inhibition of promoter activity as well as enhanced mRNA degradation, but interestingly not by direct DNA promoter hypermethylation. The Selective for Apoptosis induction in Cancer cells (SAC) domain within Par-4 is highly active against glioma cells, including orthotopic xenografts of patient-derived primary GSCs ($P < 0.0001$). Among high-grade gliomas that are IDH1 wild-type, those that express more Par-4 have significantly longer median survival (18.4 versus 8.0 months, $P = 0.002$), a finding confirmed in two external GBM cohorts. Together, these data suggest that Par-4 is a significant component of the mutant IDH1 phenotype, that the activity of 2-HG is complex and can extend beyond direct DNA hypermethylation, and that Par-4 is a promising therapeutic strategy against GSCs. Furthermore, not every effect of mutant IDH1 necessarily contributes to the overall favorable prognosis seen in such tumors; inhibition of Par-4 may be one such effect.

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Conflict of Interest

The authors declare that they have no conflict of interest.

Keywords

IDH1; Glioma; Par-4; tumor prognosis; glioma stem cells

Introduction

Gliomas are the most common primary intraparenchymal brain tumors in adults, and are divided by the World Health Organization into four grades with increasing severity. Mutations in isocitrate dehydrogenase 1 and 2 (IDH1/2) are present in the majority of grades II and III infiltrative astrocytomas and oligodendrogliomas, and in the majority of grade IV glioblastomas (GBMs) that develop from those lower grade tumors (reviewed in (28)). The mutation is also present in a large proportion of chondroid tumors, cholangiocarcinomas, and acute myeloid leukemias (AMLs). With rare exceptions, IDH1/2 mutations are specific to codons 132 and 172 of IDH1 and IDH2, respectively, with R132H IDH1 being the most common mutation by far in gliomas. These codons normally encode arginine residues comprising the isocitrate substrate binding pocket, but when mutated, impart new enzyme kinetics. Instead of oxidizing isocitrate to alpha-ketoglutarate (α -KG), the neoenzyme reduces α -KG to D-2-hydroxyglutarate (2-HG)—a compound found at concentrations 10-100-fold higher in mutant tumors (18). 2-HG promotes global DNA and histone hypermethylation (16, 28), leading to inhibition of differentiation, which in turn appears to facilitate oncogenesis (9, 23, 37, 38, 47). However, the full effects of mutant IDH1 on glioma biology, including the specific ways in which it alters genes involved in tumor formation and therapeutic response, are not fully understood.

As has been shown in many solid tumors, a subset of cells within gliomas seems to be responsible for glioma growth and maintenance, with the capacity for self-renewal and (at least partial) differentiation (19). These cells, which are commonly called glioma stem cells (GSCs), are particularly resistant to apoptosis-inducing therapies like radiation (4). In fact, recent work showed that a mainstay of chemotherapy in high-grade gliomas, temozolomide, actually increases the proportion of GSCs in a tumor by inducing conversion of non-GSCs into GSCs (2). GSCs are also adept at suppressing the anticancer immune response through modulation of T-cells and macrophages/microglia (54-56). Thus, in order to more effectively treat patients with diffusely infiltrative gliomas, new therapies must be able to kill GSCs (19).

One such strategy could involve Prostate apoptosis response-4 (Par-4), which is a ~40 kDa tumor suppressor protein encoded by the *PAWR* gene on chromosome 12q21. Par-4 promotes apoptosis in a variety of cancerous cells, but not in normal mature cells. Par-4 kills human carcinoma cells from the prostate (10, 49), kidney (32, 33), pancreas (3), lung (10), cervix (10), endometrium (48), breast (43), and colon (50). It also has activity against melanoma and leukemia (8, 36, 39, 49). Par-4 can bind and inactivate protein kinase C- ζ (13, 20), leading to inhibition of the prosurvival NF- κ B pathway (12). Intracellular Par-4 can also directly downregulate Bcl2 (44) and indirectly inhibit the activity of mitogen-activated protein kinase and Akt (7, 30, 33). Extracellular Par-4 triggers cancer-selective extrinsic apoptosis via binding to cell membrane GRP78, a stress response protein that is in

the endoplasmic reticulum but also translocates to the cell membrane (10). Inhibition of Par-4 is both necessary and sufficient for breast cancer recurrence in mice (1). In humans, low intratumoral expression of Par-4 is an adverse prognostic marker in breast and oral squamous carcinomas (1, 17, 41).

Within Par-4 is a 59 amino acid SAC domain that is sufficient to induce cancer-selective apoptosis, even in many cancer cells that are resistant to full-length Par-4 (21). Like full-length Par-4, it triggers extrinsic apoptosis via binding to cell membrane GRP78 (10), but unlike full-length Par-4, SAC cannot be inactivated by pAkt (26, 51). Remarkably, transgenic mice overexpressing SAC in all tissues throughout their bodies are highly resistant to carcinogenesis, even to the point where engraftment of human cancers into such mice is difficult (57, 58). In fact, simply transplanting bone marrow from a SAC-transgenic mouse to a wild-type mouse confers cancer resistance (57).

Despite the therapeutic promise that Par-4 has shown in other cancers, not nearly as much is known regarding Par-4 in gliomas. Induced expression of full-length Par-4 kills many glioma cell lines (53). It is also critical for the apoptotic response to chemotherapy, both in human GBM cell lines (59) and in GSCs that have been passaged many times in serum (29). Yet its full impact in gliomas remains unknown, including whether it is regulated by any driver mutations, its activity against patient-derived GSCs *in vivo*, and its significance as a prognostic marker.

In our prior work, we found that mutant IDH1 and its 2-HG product are capable of suppressing apoptosis in human glioma cells (25). Further investigation into the effects of mutant IDH1 on proapoptotic pathways in gliomas led to the discovery of a novel and powerful link between mutant IDH1 and Par-4. Herein we describe that link, as well as demonstrate the potential of Par-4-based therapy against GSCs.

Materials and Methods

Cell culture and viability assays

D-2-hydroxypentanedioic acid disodium (D-2-hydroxyglutaric acid disodium salt, D-2-HG) was acquired from Sigma-Aldrich (H8378) and PepTech Corporation (RC402) and was solubilized in water. U87MG (HTB-14), U138MG (HTB-16) and LN18 (CRL-2610) glioma cell lines were purchased directly from ATCC. All cell lines were cultured in RPMI 1640 (11875), 10% fetal bovine serum (10082-147), and 1% penicillin streptomycin (15070-063) purchased from Life Technologies. Cells were cultured in humidified conditions at 37°C and 5% CO₂. Vector and R132H mutant IDH1 stable U87MG, U138MG, and LN18 cell lines were generated as previously described (25). (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTS) cell viability assays were performed as previously described (25).

Patient-derived primary GBM stem cells (08-387) were generously gifted by Drs. Jeremy Rich and Monica Venere (Cleveland Clinic). The CD133+ stem cells were enriched and isolated from flank xenografts and sorted by FACS as described previously (4). Briefly, tumors were dissociated using Worthington Papain Dissociation System (Lakewood, NJ).

Cells were labeled with CD133 antibody conjugated with APC and sorted to enrich CD133+ stem cells by FACS. The CD133+ cells were then cultured in Neurobasal Media supplemented with B27 without vitamin A (Invitrogen), 10 ng/mL bFGF (Life Technologies, PHG0026), and 10 ng/mL EGF (Life Technologies, PHG0313).

Immunoblotting

SDS-PAGE and Western immunoblotting were done on cell lysates as previously described (25) using the following antibodies: anti-tubulin, (Cell Signaling, 2125), anti R132H IDH1 (Dianova, DIA-H09), and anti-Par-4 (Santa Cruz, sc-1807).

mRNA experiments

After cells were passaged and treated with 2-HG 24 hrs later, cells were lysed in TRIzol (Life Technologies, 15596018) at indicated times and stored at -80°C until further processing. Total RNA was isolated using PureLink RNA Mini kit (Life Technologies, 12183018A), incorporating the On-Column PureLink DNase Treatment (Life Technologies, 12185010). 1 μg of total RNA was used to synthesize cDNA using the High Capacity RNA-to-cDNA Kit (Applied Biosystems, 4387406). Real-time PCR was conducted with Power SYBR Green PCR Master Mix (Applied Biosystems, 4367659) and primer sets directed against *PAWR* (Fwd: AGATCGAGAAGAGGAAGCTG and Rev: CTCGTTTCCGCTCTTTCTG) and *I8S* (Fwd: CGCCGCTAGAGGTGAAATTCT and Rev: CGAACCTCCGACTTTCGTTCT). A 3 step protocol was used (40x: 30s at 95°C , 30s at 60°C , and 1:30 min at 72°C). *PAWR* data were analyzed using the comparative Ct method and were normalized to expression levels of *I8S*.

For cycloheximide (CHX) experiments, cells were treated with 10 μM CHX (Sigma-Aldrich, C7698) or ethanol alone for one hour before 2-HG treatment. Three days after adding 2-HG, samples were collected with TRIzol and following steps proceeded as detailed above. No effect of cycloheximide was seen on *I8S* ribosomal RNA levels within the timeframe of these experiments (data not shown). For actinomycin experiments, cells were passaged and treated with 2-HG (30 mM) or water (0 mM). Six days after 2-HG treatment, actinomycin D (Enzo Life Sciences, BML-GR300-0005, solubilized in DMSO) was added at 5 $\mu\text{g/mL}$ of culture medium. At indicated times, cells were lysed, stored, and processed as above. The combination of cycloheximide and actinomycin D assays are commonly used to evaluate mRNA stability (6).

Methylation assay

The human *PAWR* CpG assay (Cat. No. PM00160664) was purchased from Qiagen (Valencia, CA). Methylation assay was performed using EpiTect Methyl II PCR Assay (Qiagen, Valencia, CA, Cat. No. 335002) according the manufacturer's instructions. Briefly, U87 cells were harvested after treatment with or without 2-HG; cells stably expressing either vector-GFP or R132H IDH1-GFP were also harvested. The genomic DNAs were extracted from the cells using a DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA, Cat. No. 65904). The genomic DNAs were digested using the EpiTect Methyl II DNA Restriction Kit (Qiagen, Valencia, CA, Cat. No. 335452) and real-time PCR was performed using RT SYBR

Green ROX qPCR Mastermix (Qiagen, Valencia, CA, Cat. No 330520). The data was analyzed using the methylation assay software provided by Qiagen.

Luciferase reporter assay

Cells were dual-transfected with a firefly luciferase reporter construct driven by a fragment of the Par-4 promoter (52) and with a renilla luciferase construct using Lipofectamine 2000 (1:5 DNA:Lipofectamine ratio; Life Technologies). 24 hours later, 2-HG was added to cells. Six days post-2HG treatment, luciferase assays were performed using the Dual Glo Luciferase Assay System (Promega, E2920) according to manufacturer's instructions.

Lentivirus SAC domain experiments

GFP alone or the SAC domain of Par-4 fused with GFP was sub-cloned into lentivirus vectors and packaged in HEK293T cells. The virus-containing medium was filtered with a 0.22 μ m filter and concentrated by centrifugation. The titer of the concentrated virus was estimated using multiplicity of infection (MOI). Cells were passaged and 24 hours later the appropriate lentivirus construct (5 μ l) was added. Three days later, caspase 3/7 activity was measured using Caspase 3/7 Glo Assay according to manufacturers' instructions (Promega, G8090).

Patient-derived tissues and Immunohistochemistry

Deidentified tissue microarrays (TMAs) were constructed from archival formalin-fixed, paraffin-embedded (FFPE) gliomas as previously described (25). A total of 95 cases comprised the TMAs, including 9 World Health Organization (WHO) grade II astrocytomas, 11 grade III astrocytomas, 12 anaplastic oligodendrogliomas, 16 grade II oligodendrogliomas, and 47 grade IV glioblastomas (GBMs). Institutional Research Board (IRB) approval was obtained prior to collecting the archival tissues for TMA construction.

Immunohistochemistry of paraffin-embedded xenografts and TMAs was performed as previously described (25). Primary antibodies used included R132H IDH1 (1:20, Dianova, DIA-W09) and par-4 (1:250, Santa Cruz, sc-1807); secondary antibodies were EnVision labeled polymer-HRP (horseradish peroxidase) anti-mouse or anti-rabbit as appropriate. Staining was visualized using 3, 3'-diaminobenzidine (DAB) chromogen (Dako, K8000).

Par-4 immunohistochemical results on TMAs were semiquantified on a relative scale from 0 to 3, with 0 = negative and 3 = strongest (see Supplemental Figure 1). Each tumor was represented by 3 separate cores on 3 separate blocks; scoring was done while blinded to IDH1 mutation status.

The Cancer Genome Atlas (TCGA) dataset was accessed through UC Santa Cruz Cancer Browser (<https://genome-cancer.ucsc.edu/proj/site/hgHeatmap/>). The National Cancer Institute (NCI) REpository for Molecular BRAin Neoplasia DaTa (REMBRANDT, <https://caintegrator.nci.nih.gov/rembrandt/>) dataset was also used as an external validation cohort for the TMA outcome data.

Xenograft experiments

CD133+ primary GBM stem cells (08-387) were pretreated with lentivirus containing either GFP alone or the SAC domain of Par-4 tagged with GFP (MOI = 50). One day later, after verifying expression via inverted fluorescent microscopy, 10^5 cells were implanted into the right frontal lobes of 5 week-old female NU/J nude mice as previously described (4). Survival was tracked thereafter, euthanizing mice when weight loss exceeded 10% and/or neurologic signs of impending death were observed, in accordance with a protocol approved by the University of Kentucky Institutional Animal Care and Use Committee.

Statistical analyses

Differences between groups were analyzed via two-tailed t-test, one-way ANOVA with post hoc Tukey's test, or two-way ANOVA where appropriate. All *in vitro* experiments were repeated at least 3 times, with representative data shown in the Results. TCGA methylation data were analyzed in conjunction with mutant IDH1/2 status and WHO grade via multiple regression. Survival differences were compared via log-rank (Mantel-Cox) test. Multivariate survival analysis was done via Cox proportional hazards survival regression. Significance was reached when $P < 0.05$. All analyses were done via GraphPad software (La Jolla, CA), except for Cox proportional hazards survival regression, which was done using <http://statpages.org/>.

Results

Par-4 immunohistochemistry on a glioma TMA showed a strong inverse relationship between mutant IDH1 and Par-4 in grades II-IV gliomas (Figure 1a and b, $P < 0.0001$). A similar analysis of mRNA in TCGA grades II-IV gliomas showed dramatically lower Par-4 mRNA in mutant IDH1/2 gliomas compared to their wild-type counterparts (Figure 1c, $P = 2.0 \times 10^{-13}$). A direct link between mutant IDH1/2 and Par-4 was established via stable overexpression of the most common *IDH1* mutation, R132H IDH1, in human glioblastoma (GBM) cell lines. Consistent with the patient-derived data, R132H IDH1 suppressed Par-4 protein levels in U87MG and U138MG cells (Figure 1d and Supplemental Figure 2a). Similarly, treatment with exogenous 2-HG suppressed Par-4 in wild-type U87MG cells and trended towards suppression in U138MG cells (Figure 1e and Supplemental Figure 2c). Interestingly, though, neither R132H IDH1 overexpression nor exogenous 2-HG had an effect on Par-4 in LN18 cells (Supplemental Figure 2b and 2d). Together these data indicate a strong inverse relationship between mutant IDH1/2 and the Par-4 tumor suppressor, and that some glioma cells better recapitulate this relationship *in vitro* than others.

To begin exploring the mechanism of mutant IDH1-mediated Par-4 downregulation, steady-state Par-4 mRNA levels were measured via real-time PCR in U87MG cells stably overexpressing R132H IDH1. Mutant IDH1 decreased Par-4 mRNA levels (Figure 2a). Luciferase reporter assays showed that exogenous 2-HG potently decreased activity at the Par-4 promoter (Figure 2b), indicating that 2-HG decreases Par-4 expression through transcriptional means. Although mutant IDH1 and 2-HG are well known to upregulate global gene methylation (28), neither stable R132H IDH1 overexpression nor 2-HG led to an increase in Par-4 promoter methylation (Supplemental Table 1). Similarly, in TCGA

gliomas, the only variable that significantly and independently correlated with Par-4 mRNA levels was IDH1/2 mutation status, not methylation of CpG sites within the Par-4 promoter (Table 1). These data indicate that mutant IDH1 and 2-HG can downregulate Par-4 expression through transcriptional means, independent of direct promoter hypermethylation.

To investigate whether decreased steady-state mRNA levels of Par-4 might also be the result of post-transcriptional regulation, cycloheximide was used to inhibit polypeptide chain elongation, thus trapping the ribosomes on the mRNA and physically blocking mRNA degradation (6). Exogenous 2-HG caused a decrease in Par-4 mRNA levels in U87MG cells treated with vehicle (ethanol), while blocking mRNA degradation with CHX prevented the 2-HG-mediated decrease in Par-4 mRNA (Figure 2c). Independently confirming the cycloheximide experiments, 2-HG also reduced the half-life of Par-4 mRNA when transcription was blocked with actinomycin D (Figure 2d). Together, these data indicate that 2-HG can also downregulate Par-4 expression post-transcriptionally.

Prior studies demonstrated that the SAC domain of Par-4 triggers apoptosis in carcinomas (10, 57, 58), yet its effect in gliomas is unknown. In U87MG cells, delivering SAC-GFP via lentivirus caused a nearly 40% decrease in cell viability after just 3 days (Figure 3a and b) and an increase in caspases 3/7 activity (Supplemental Figure 3). Importantly, SAC-GFP showed potent activity against primary patient-derived CD133+ GSCs (IDH1/2 wild-type), with a 50% decrease in viability after 6 days *in vitro* (Figure 3c). Those same GSCs showed essentially no baseline Par-4 expression (Supplemental Figure 2d). SAC-GFP was also able to significantly extend the survival of GSC xenografted mice by 42% even without any other adjuvant therapy (Figure 3d, 27 days versus 19 days, $P < 0.0001$).

Regarding Par-4 and patient outcome, mean Par-4 expression was higher in grade IV GBMs compared to grades II-III gliomas in the TMA, with no significant difference between grades II and III tumors (Figure 4a). However, it was clear that only a subset of GBMs had appreciable levels of Par-4. That subset of IDH1 wild-type GBMs with above-average levels of Par-4 protein expression also showed longer median survival (18.4 months versus 8.0 months, $P = 0.002$) (Figure 4b). This was confirmed by GBM cohorts from the NCI REMBRANDT database (Figure 4c) and TCGA (Supplemental Figure 4). On multivariate analysis of all TMA grades II-IV gliomas, low levels of Par-4 protein expression trended toward being an adverse prognostic variable (Supplemental Table 2).

Discussion

Very little work on Par-4 in gliomas has been done; so far all of it has been strictly *in vitro* using cell lines and/or high-passage patient-derived tumor cells grown in serum (29, 53, 59). In those studies, Par-4 mediated some of the toxic activity of temozolomide (29, 59), and forced overexpression of full-length Par-4 was sufficient to kill glioma cell lines (53). Very little is known about whether any key driver mutations in gliomas can affect Par-4, the prognostic significance of Par-4, or whether its SAC domain can kill GSCs. The current study therefore contains several novel findings, including: 1.) Mutant IDH1 can suppress Par-4 expression; 2.) The activity of mutant IDH1 and its 2-HG oncometabolite in downregulating Par-4 involves novel mechanisms beyond direct promoter DNA

hypermethylation; 3.) Increased Par-4 expression correlates with longer survival in IDH1 wild-type GBMs; 4.) The SAC domain of Par-4 can effectively kill GSCs and inhibit their growth *in vivo*.

Mutant IDH1/2 is a powerful favorable prognostic marker in gliomas and is known to have far-reaching effects on cell biology (28), but the full range of those effects are not yet clear. Although mutant IDH1 is the focus of much attention in cancer research, few proteins whose expression is downregulated by it have been specifically examined in detail. Most that have been studied thus far are involved in cellular metabolism (14, 15, 40). But considering the impact of Par-4 in other cancers, the fact that mutant IDH1 is capable of robustly inhibiting this powerful tumor suppressor is a strong clue that it is important in glioma biology.

While an inverse relationship between mutant IDH1 and Par-4 may seem counterintuitive at first, the mutation clearly must have progliomagenic effects or it would not be so prevalent. In other words, not all the consequences of mutant IDH1 are deleterious to the tumor. For example, mutant IDH1 is now known to impede cellular differentiation (47), which is itself an adverse event. Mutant IDH1 may also promote resistance to temozolomide (42). And mutant IDH1 gliomas are still lethal, albeit generally taking longer to kill compared to wild-type gliomas. Thus, like many other mutations, the effects of mutant IDH1 are complex and consist of a mixture of pro- and anti-tumor activities. While Par-4 suppression by mutant IDH1 clearly does not negate other “favorable” side effects of the mutation on patient survival (e.g. increased sensitivity to radiation (34)), and is certainly not the only activity of mutant IDH1 that contributes to the overall phenotype of these tumors, it is likely an important component. In other settings, Par-4 has been shown to be a potent tumor suppressor, as its absence contributes to oncogenesis and is associated with carcinoma progression (5, 22). Par-4 knockout mice have higher rates of neoplasia in the lung, liver, bladder, prostate, and endometrium (24), and fibroblasts from knockout mice are resistant to apoptosis (31). Furthermore, knockdown of Par-4 facilitates the transformation of NIH 3T3 fibroblasts (27, 45). It is therefore possible that, in addition to inhibiting differentiation via global hypermethylation (28), Par-4 suppression also plays a role in IDH1/2-mutant gliomas.

Of note, in both our TMA and the TCGA dataset, roughly half of wild-type IDH1 gliomas have protein and mRNA levels within the range seen in mutant IDH1 tumors (Figures 1b and 1c). This suggests that a large proportion of wild-type gliomas also manage to suppress Par-4 even without mutant IDH1, albeit by other as-of-yet-unidentified means. Since wild-type GBMs that do not suppress Par-4 have a better prognosis compared to wild-type GBMs that do (Figure 4), inducing expression of Par-4 (or just its SAC domain) in otherwise low-expressing gliomas is an attractive strategy for improving survival.

Even though most effects of mutant IDH1 described to date have involved hypermethylation (28), our data indicate that mutant IDH1 suppresses Par-4 at the transcriptional level, independent of Par-4 DNA promoter methylation (Figure 2, Supplemental Table 1). Indeed, TCGA data showed that mutant IDH1/2 status, not the degree of Par-4 promoter methylation, correlates best with Par-4 mRNA levels (Table 1). The concentration of 2-HG used in our experiments (Figures 1e and 2b-d) is within the range of what has been reported for endogenous mutant IDH1 gliomas in patients (18), and is also similar to that used by

other groups (46). As we reported previously, this concentration of 2-HG does not have severe toxic effects within this timeframe *in vitro*, even though it is quite capable of entering the cells exogenously (25). Thus, these data are unlikely to be the result of nonspecific toxicity, but rather indicate that the effects of 2-HG are diverse and profound. However, our data do not exclude indirect epigenetic effects of 2-HG on Par-4 expression, as genes encoding one or more intracellular Par-4 regulators could be hypermethylated. Similarly, specific histones within the Par-4 promoter could be hypermethylated rather than the DNA itself. The kinetics of histone hypermethylation are faster than global DNA hypermethylation, which would be consistent with the relatively rapid Par-4 suppression we see in our cells. However, that would still not explain the destabilizing effect of 2-HG on Par-4 mRNA. And although 2-HG competitively inhibits certain α -ketoglutarate-dependent enzymes like dioxygenases (28), none of the enzymes known to be affected by 2-HG appear to have any direct connection to Par-4 or to post-transcriptional regulation. Efforts are ongoing to determine exactly how 2-HG decreases both Par-4 transcription and stability of its mRNA. We are also exploring the possibility that the suppression of Par-4 by mutant IDH1 might be relieved when treating cells with mutant IDH1 inhibitors. Moreover, at this point we cannot rule out the possibility that 2-HG affects post-translational protein stability.

It is interesting to note that not all glioma cell types appear to recapitulate the relationship between mutant IDH1 and Par-4 seen in patient-derived tumors (Supplemental Figure 2b & 2d). This indicates that the presence of other accompanying mutations and/or cell signaling characteristics probably alters the effects of mutant IDH1 on cell biology. And even though Par-4 Western blot conditions were identical throughout all experiments, the primary IDH1/2 wild-type GSC 08-387 cells showed essentially no detectable Par-4 expression, whereas IDH1/2 wild-type cell lines did. This is not surprising for two reasons: 1.) Even many GBMs show very low to no Par-4 expression (Figure 4a); 2.) Because GSCs tend to be apoptosis-resistant, they might have very low baseline Par-4 expression. Additional work focused on more GSCs of varying genetic backgrounds, and comparisons between GSCs and their more differentiated progeny, are underway.

GSCs are notoriously resistant to therapies like radiation and temozolomide, and are believed to be responsible for post-therapeutic glioma recurrence (4). The SAC domain of Par-4 was previously shown to be sufficient to induce carcinoma-selective apoptosis in transgenic mice (10, 57, 58). Our data show that, even in the absence of any other treatment, the SAC domain of Par-4 can kill patient-derived GSCs *in vitro* and inhibit their growth *in vivo*. To our knowledge, this is the first time that SAC has been directly studied in any cancer stem cells, much less those of the brain. Since GSCs are a major driver of glioma growth and recurrences, yet are very difficult to kill (11, 35), this represents a promising new avenue for antiglioma therapeutics. The suppression of Par-4 by mutant IDH1 raises the question as to whether this glioma subset might be especially sensitive to SAC-based therapy. Our data in U87MG cells stably expressing R132H IDH1 suggests that there may be increased sensitivity, but cells that were already overexpressing GFP-labeled constructs proved highly sensitive to additional infection by lentivirus (not shown). Efforts are therefore ongoing to assess SAC efficacy in cells containing endogenous mutant IDH1 versus other driver mutations.

Finally, and consistent with prior studies in other cancers (1, 17, 41), we showed for the first time that low Par-4 correlates with worse survival in GBMs (Figure 4). In our univariate analysis only wild-type GBMs were included in order to avoid the potential confounding effect of mutant IDH1/2 (Figure 4b, Supplemental Figure 4). But even in our multivariate analysis, including other variables known to be significant prognostic markers in gliomas like WHO grade, IDH1 status, and adjuvant therapy, low Par-4 still trended toward a higher risk of death (Supplemental Table 1).

In sum, the present data indicates a new function of mutant IDH1 in gliomas—the suppression of Par-4 expression. Interestingly, this suppression appears to involve mechanisms other than promoter DNA hypermethylation, thus opening up new areas of research into the effects of mutant IDH1 and its 2-HG oncometabolite in cancer biology. Par-4 may also represent a novel therapeutic strategy in GSCs regardless of IDH1/2 mutation status. Our understanding of gliomas has clearly improved a great deal in recent years; it seems only a matter of time until treatment also improves.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1. Par-4 protein expression in gliomas according to mutant IDH1/2 status

a Immunohistochemistry from two patient-derived GBMs in the TMA cohort, one with only wild-type IDH1 (left panels) and one with mutant IDH1 (right panels). Scale bar = 50 μm in lower right subpanel. **b** IHC quantification of Par-4 in WHO grade II-IV gliomas, according to mutant IDH1/2 status. Each point represents a single tumor. **c** Comparison of Par-4 mRNA in grades II-IV gliomas according to IDH1/2 mutation status in the TCGA cohort. **d** Par-4 protein expression in human U87MG and U138MG GBM cells stably overexpressing either empty vector-GFP or R132H IDH1-GFP, 6 days after plating. **e** Par-4 protein expression in U87MG and U138MG cells treated with exogenous 2-HG for 6 days.

Fig. 2. Effect of mutant IDH1 and its 2-HG oncometabolite on Par-4 mRNA

a Par-4 mRNA levels in human U87MG GBM cells stably overexpressing R132H IDH1, 6 days after plating ($N = 6$). **b** Par-4 promoter activity via renilla luciferase and firefly luciferase assay ($N = 6$). **c** Par-4 mRNA levels in U87MG cells 3 days after 2-HG treatment in the absence (ethanol) or presence of CHX ($N = 5$). **d** U87MG cells treated with 2-HG for 6 days before adding actinomycin D, followed by serial Par-4 mRNA measurements via PCR ($n = 3$ per timepoint) P value denotes the effect of 2-HG in all timepoints after actinomycin D treatment, as determined by two-way ANOVA. Data in all panels are expressed as mean $\pm$ SEM.

Fig. 3. Par-4 SAC domain activity against GBM

a Phase contrast (left panels) and fluorescent micrographs (right panels) of U87MG GBM cells 3 days after infection with lentivirus containing vector-GFP or SAC-GFP. Scale bar = 50 μ m in lower right panel. **b** MTS viability assay of U87MG cells 3 days after transfection with vector-GFP or SAC-GFP ($n = 6$). **c** MTS viability assay of patient-derived IDH1/2 wild-type GBM stem cells in response to vector-GFP or SAC-GFP ($n = 6$; $*P < 0.05$, $***P < 0.0001$ via one-way ANOVA with post-hoc Tukey's test). Data in **b** and **c** are shown as mean $\pm$ SEM. **d** Kaplan-Meier curves of nude mice orthotopically xenografted with patient-derived IDH1/2 wild-type GBM stem cells that had been pre-infected with lentivirus containing either vector-GFP ($N = 15$) or SAC-GFP ($N = 13$).

Fig. 4. Par-4 as a biomarker in gliomas

a Expression of Par-4 via IHC according to WHO grade in a TMA of gliomas. Bars = mean $\pm$ SEM; * $P < 0.001$, ** $P < 0.0001$ via one-way ANOVA and post-hoc Tukey's test. Grade IV GBMs are color-coded to match their corresponding survival curves in **b**, see below. **b** Kaplan-Meier curves of grade IV GBMs with Par-4 score < 1 ($N = 23$) or ≥ 1 ($N = 20$). **c** Kaplan-Meier curves from an external cohort of GBMs (NCI REMBRANDT) with low

Par-4 mRNA (downregulation 2 fold relative to nonneoplastic tissue, N = 24) versus normal or high Par-4 mRNA (all remaining cases, N = 157).

Table 1

Multivariate analysis of factors related to Par-4 mRNA expression in TCGA gliomas. Variables associated with Par-4 mRNA were compared by multiple regression. Par-4 promoter CpG islands include cg09418283 and cg00864867; methylation was measured by Illumina Infinium Human Methylation450. Data was obtained from UCSC Cancer Browser, based on the TCGA dataset. N = 128.

variable	t ratio	P
<i>IDH1/2 mut versus wt</i>	3.47	0.0007
GBM vs. grades II-III	0.53	0.60
cg09418283 methylation	0.028	0.98
cg00864867 methylation	0.68	0.50