**Introduction**

Here, we report on PID1 (Phosphotyrosine Interaction Domain containing 1) in 2 important groups of brain tumors: embryonal brain tumors [medulloblastomas and atypical teratoid rhabdoid tumors (ATRT)] and gliomas. Medulloblastomas are the most common malignant brain tumors in children, where in high-risk disease prognosis remains poor. Moreover, in younger children, therapies that include whole brain irradiation are associated with serious long-term sequelae that hamper quality of life of survivors (1, 2). ATRT is another poor prognosis, highly malignant embryonal brain cancer of young children. Most ATRTs have lost expression and/or function of the INI1 (SMARCB1) tumor suppressor gene, which is part of the SWI/SNF chromatin-remodeling complex (3–5). Malignant gliomas, including glioblastoma multiforme (GBM), comprise the most common primary malignant brain tumors in adults and also carry poor prognosis (6, 7). Here, we provide novel clinical correlations of PID1 in medulloblastomas and gliomas, and demonstrate tumor-inhibitory effects of PID1 in cell lines of these 3 brain tumors.

**The PID1 gene** [also called NYGGF4 and phosphotyrosine binding (PTB)-containing, cubulin, and LRPI-interacting protein; PCL1; rhymes with "BID-1" ] was identified in 2006 based on its differential expression in adipose tissue of young children. PID1 is known to contain a PTB domain/phosphotyrosine interaction domain (PID; ref. 8), the molecular mechanism

**Purpose:** We present here the first report of PID1 (Phosphotyrosine Interaction Domain containing 1; NYGGF4) in cancer. PID1 was identified in 2006 as a gene that modulates insulin signaling and mitochondrial function in adipocytes and muscle cells.

**Experimental Design and Results:** Using four independent medulloblastoma datasets, we show that mean PID1 mRNA levels were lower in unfavorable medulloblastomas (groups 3 and 4, and anaplastic histology) compared with favorable medulloblastomas (SHH and WNT groups, and desmoplastic/nodular histology) and with fetal cerebellum. In two large independent glioma datasets, PID1 mRNA was lower in glioblastomas (GBM), the most malignant gliomas, compared with other astrocytomas, oligodendrogliomas and nontumor brains. Neural and proneural GBM subtypes had higher PID1 mRNA compared with classical and mesenchymal GBM. Importantly, overall survival and radiation-free progression-free survival were longer in medulloblastoma patients whose tumors had higher PID1 mRNA (univariate and multivariate analyses). Higher PID1 mRNA also correlated with longer overall survival in patients with glioma and GBM. In cell culture, overexpression of PID1 inhibited colony formation in medulloblastoma, atypical teratoid rhabdoid tumor (ATRT), and GBM cell lines. Increasing PID1 also increased cell death and apoptosis, inhibited proliferation, induced mitochondrial depolarization, and decreased serum-mediated phosphorylation of AKT and ERK in medulloblastoma, ATRT, and/or GBM cell lines, whereas siRNA to PID1 diminished mitochondrial depolarization.

**Conclusions:** These data are the first to link PID1 to cancer and suggest that PID1 may have a tumor inhibitory function in these pediatric and adult brain tumors. *Clin Cancer Res; 20(4); 827–36.* ©2013 AACR.
Translational Relevance

We present here the first report of PID1 (Phosphotyrosine Interaction Domain containing 1; NYGGF4) in cancer. PID1 was identified in 2006, and its role and molecular mechanism are still poorly understood. Using 6 independent datasets in 2 common brain tumors, medulloblastomas and gliomas, we show that: (i) PID1 mRNA is lowest in least favorable subgroups of medulloblastomas and gliomas; (ii) higher PID1 mRNA is directly correlated with longer patient survival; and (iii) PID1 causes increased cell death and decreased proliferation in medulloblastoma, glioma, and atypical rhabdoid tumor cell lines. These data in 2 brain tumor types suggest that it may be possible to incorporate PID1 into personalized molecular prognostic signatures that predict patient response and outcome. Ongoing work on the molecular function of PID1 intends to utilize this knowledge in the design of improved approaches to therapy.

(s) underlying its activities are poorly understood. PID1 mRNA increases during differentiation of 3T3-L1 preadipocytes to adipocytes (8), and is lower in brains of patients with Alzheimer disease compared with controls (9). PID1 overexpression increases proliferation of 3T3-L1 preadipocytes, but does not alter their adipogenic differentiation (8). In NIH-3T3 cells, however, overexpression of PID1 amino acids 84-230 (aa84-230), which includes its PTB domain, causes cell-cycle arrest (10), suggesting that PID1 function may differ depending on cellular context.

Pilot microarray expression analysis identified PID1 mRNA levels as highly correlated with outcome in medulloblastomas. A similar correlation of PID1 mRNA levels in gliomas using publicly available microarray datasets prompted us to study PID1 in more detail. Analysis revealed highly significant correlations between PID1 mRNA and survival and between PID1 mRNA and subgroup, and/or grade in medulloblastomas and gliomas across 6 independent datasets. Moreover, ectopic expression of PID1 in embryonal brain tumor cell lines (medulloblastomas and ATRT) and GBM cell lines showed consistent growth-inhibitory effects. These data are the first to link PID1 to cancer in general and to brain tumors in particular, and to suggest that PID1 may have a growth-inhibitory function in medulloblastomas, glioblastomas, and ATRT.

Materials and Methods

Details for additional methods can be found in Supplementary Materials.

Patients, samples, and mRNA expression data

Medulloblastoma specimens and clinical records from 81 children diagnosed at Children’s Hospital Los Angeles (CHLA) between 1989 and 2008 were obtained according to a protocol approved by the local Institutional Review Board. Patient and sample characteristics are described in Supplementary Table S1. The Heidelberg microarray dataset includes 446 medulloblastomas, profiled on Affymetrix U133 plus2.0 arrays, and is a combination of published data on 230 patients obtained from the Gene Expression Omnibus (GSE10327, GSE12992, and GSE37418; refs. 11–13) and unpublished data on 216 additional patients from Heidelberg (M. Kool and S. Pfister; unpublished data). The published Toronto microarray dataset includes 103 medulloblastomas profiled on Affymetrix Human Exon Array data (GSE21140), and the published Boston microarray dataset includes 194 medulloblastomas profiled on Affymetrix U133A (14, 15). Molecular subgroups of medulloblastoma were identified either by using available published data, or by cluster analyses to assign molecular subgroup for the unpublished datasets (11, 12, 14), or for the CHLA analysis, by quantitative reverse transcription PCR (qRT-PCR) and a medulloblastoma gene signature derived from prior microarray studies (15, 16) and a CHLA study (manuscript in preparation). Glioma PID1 mRNA microarray data in REMBRANDT (REpository for Molecular Brain Neoplasia Data) were from Affymetrix U133 plus2.0 arrays (17). Glioblastoma PID1 mRNA data in TCGA (The Cancer Genome Atlas; The Cancer Genome Atlas Research Network, National Cancer Institute and National Human Genome Research Institute, Bethesda, MD) were from AgilentG4502A_07 microarrays; (18). For GBM clinical and PID1 correlations, publicly available data for 196 patients was obtained from TCGA and Verhaak and colleagues (19), and was accessed at https://tcga-data.nci.nih.gov/docs/publications/brain/ (files: unifiedScaled.txt and TCGA_unified_CORE_ClinicalDataMatrix.htm?mode=ApplyFilter&diseaseType=GBM (files: clinical_patient_gbm.txt and clinical_follow_up_v1.0_gbm.txt) on February 18, 2013. RNA from CHLA medulloblastoma samples was extracted and processed using a previously published method (20). PID1 mRNA levels were obtained by qRT-PCR (PID1: forward primer: 5’-GATGCGGCAACACCCTGAGTG-3’, reverse primer: 5’-AAATGAGATGGGCTGTTACATT-3’, probe: 5’-TCCAGACATCTTCTTCCACAGCT-3’) and after normalization to 3 housekeeping genes (GAPDH/SDHA/HPRT1).

Annexin V, cell proliferation, and mitochondrial depolarization

Assays were performed on green fluorescent protein (GFP)-positive cells 24 hours after transfections. Annexin V staining was done by flow cytometry using the APC Annexin V Kit (Cat. No. 550474; BD Pharmingen) according to manufacturer’s instructions. Cell proliferation and viability were assessed by flow cytometry using the APC BrdUrd Flow Kit (Cat. No. 552598; BD Pharmingen). Mitochondrial depolarization was measured by flow cytometry using the MitoProbe DiIC1 (5) Assay Kit for flow cytometry (Cat. No. M34151; Invitrogen).
Cell culture
The cell lines used were: GBM (U87, U251, LN18, LN229, CHLA-07-B5GBM), medulloblastoma (D283MED, D425MED, UW-228-2, CHLA-259, CHLA-01-MED, CHLA-01R-MED), and ATRT (BT-12, CHLA-05-ATRT, CHLA-06-ATRT). Details for their culture are in Supplementary Materials.

Plasmids
PID1 variant 1 (NM_017933.4; PID1-1) and variant 2 (NM_001100818.1; PID1-2) human cDNA ORFs with a turbo-GFP (tGFP) C-terminal tag in pCMV6-AC-tGFP (pCMV6-AC-PID1-tGFP) were from OriGene (Cat. No. PS00010, RG212451, RG212505). PID1 in the pCLS-2A-tGFP plasmid (ref. 21; from Dr. M.A. Lawlor, University of Michigan, Ann Arbor, MI) was expressed S' to the foot and mouth disease virus-derived 2A self-cleaving peptide sequence. In pCIGENs (a pcDNA3.1-based CMV promoter-driven expression vector that also expresses eGFP via an EMCV IRES), PID1 (variant 1) was expressed from the CMV promoter.

Statistical analysis
Details of the statistical analysis of patient-related information can be found in the Supplementary Materials.

In vitro experiments were analyzed using GraphPad Prism version 5.0 for MAC (GraphPad Software; www.graphpad.com). Results are depicted as mean ± SEM from at least 3 independent experiments unless stated otherwise. P values represent unpaired 2-sided Student t-test unless stated differently.

Results

**PID1 mRNA is higher in favorable medulloblastomas and correlates with longer r-F-PFS and OS in patients with medulloblastoma**
qR-PCR of 81 pediatric medulloblastoma tumors showed that mean PID1 mRNA levels were significantly higher in medulloblastomas with desmoplasic/nodular histology compared with those with anaplastic histology (P < 0.001; Fig. 1A; patient characteristics in Supplementary Table S1). Supporting this, analysis of PID1 in the 4 medulloblastoma core molecular subgroups (11, 22, 23) showed that SHH group medulloblastomas had higher mean PID1 mRNA compared with the less favorable subgroup, groups 3 and 4 (Fig. 1B). Microarray data from 3 other independent medulloblastoma datasets similarly revealed higher mean PID1 mRNA in the more favorable subgroups (SHH and WNT) compared with medulloblastomas in groups 3 and 4 (Fig. 1C and Supplementary Fig. S1A; refs. 11–13). Mean expression of PID1 mRNA was higher in fetal cerebellum compared with groups 3 and 4 medulloblastomas, and was similar to PID1 mRNA in the WNT and SHH groups (Fig. 1C).

In univariate analysis of the CHLA medulloblastoma patients (n = 81; Supplementary Table S1), r-F-PFS was significantly longer in children whose tumor PID1 mRNA was higher than the median of the cohort, compared with those with tumor PID1 mRNA lower than median (r-F-PFS 73% ± 11% vs. 11% ± 10%, respectively; P < 0.001; Fig. 1D). Difference in r-F-PFS also remained significant when dividing the group into equal tertiles according to PID1 mRNA (Supplementary Fig. S1B). In the non-CHLA medulloblastoma patients from Fig. 1C for whom survival data were available, overall survival (OS) was significantly longer in patients with higher tumor PID1 mRNA compared with those with lower PID1 mRNA (Fig. 1E), supporting the findings in the CHLA cohort (Fig. 1D). In multivariate analysis of the CHLA cohort, risk of radiation-free disease progression was significantly higher in patients with lower-than-median PID1 mRNA compared with those with higher-than-median PID1 mRNA (Table 1).

These analyses demonstrate that the clinically favorable medulloblastomas (desmoplastasic/nodular histology, or WNT and SHH molecular subgroups) had higher mean PID1 mRNA compared with clinically unfavorable medulloblastomas (anaplastic histology or groups 3 and 4), and that higher medulloblastoma PID1 mRNA correlated with longer r-F-PFS and OS.

**GBM have lower PID1 mRNA compared with other gliomas and nontumor brains; higher PID1 mRNA correlates with longer overall survival in patients with glioma**
Analysis of PID1 mRNA data using the REMBRANDT glioma clinical genomics database (17) revealed that PID1 mRNA levels were lower in the highest grade gliomas, GBMs, compared with nontumor brains, astrocytomas, and oligodendrogliomas (Fig. 2A). The TCGA glioma dataset (18) similarly showed that mean PID1 mRNA in GBM was significantly lower than its level in nontumor brains [95% confidence interval (CI), 0.34–0.38; P < 0.0001; Fig. 2B]. For patients with GBM for whom tumor molecular subtype (classical, mesenchymal, neural, proneural) and clinical data were available and unified PID1 mRNA expression values from 3 platforms in TCGA were analyzed (see Materials and Methods), there were significant differences in mean PID1 mRNA among the molecular subtypes (P < 0.0001; Supplementary Fig. S2A and Table S2). Neural and proneural GBM subtypes showed higher PID1 mRNA compared with classical and mesenchymal subtypes. All paired comparisons between GBM with known subtypes were also significantly different (after Bonferroni adjustment for multiple comparisons) with the exception of the neural versus proneural pairing (P = 0.88). Differences in PID1 mRNA between GBM subtypes remained significant even after adjusting for age at diagnosis (P < 0.0001).

Survival analysis using the REMBRANDT dataset revealed significant differences in OS among patients who were stratified according to PID1 mRNA. PID1 mRNA was directly correlated to OS in patients with any diagnosis of glioma, or within the subset of astrocytomas that does not include GBMs (Fig. 2C and D, Supplementary Fig. 2B–E; using 2 available PID1 probesets). For GBMs in the TCGA dataset, PID1 mRNA was univariately significantly associated with survival (Cox regression analysis, P = 0.031; Fig. 2E), but the REMBRANDT dataset did not reveal such correlation (not shown). Although the PID1 mRNA association with OS in GBM (TCGA) was no
longer significant after adjusting for molecular subtype ($P = 0.15$), the relative failure rate decrease per unit increase in $\text{PID1 mRNA}$ was only slightly attenuated in our analysis ($0.83 \pm 0.071$ univariate, $0.85 \pm 0.094$ multivariate). In Cox multivariate analysis, with age at diagnosis, and $\text{PID1 expression and subtype as variables (TCGA)}$, $\text{PID1 was marginally significant} (P = 0.056)$. The relative failure rate decrease per unit increase in $\text{PID1 mRNA}$ was $0.81 \pm 0.091$.

Thus, similar to our findings in medulloblastomas, the most malignant gliomas (i.e., GBMs) had lower $\text{PID1 mRNA}$ compared with other gliomas or with nontumor brains. In addition, higher tumor $\text{PID1 mRNA}$ correlated with longer OS in patients with glioma.

**$\text{PID1 confers growth disadvantage in brain tumor cell lines}$**

We next asked if the clinical correlations we found for $\text{PID1 mRNA pointed to a possible biological role for PID1 in brain tumors. To evaluate a role in growth, we assessed the effect of PID1 on colony formation in U251 GBM, Figure 1. Groups 3 and 4 medulloblastomas have lower $\text{PID1 mRNA than WNT and SHH groups; higher PID1 mRNA correlates with longer rf-PFS and OS in medulloblastomas. A, relative mRNA expression (real-time qRT-PCR, $-\Delta CT$) of PID1 according to medulloblastoma histology (desmoplastic/nodular, classic, anaplastic) in 81 CHLA patients. B, relative PID1 mRNA ($-\Delta CT$) of 66 of the tumors described in A for which molecular subgroup classification (WNT, SHH, group 3, and group 4) by qRT-PCR was available. Lines depict mean mRNA for each group; gray dashed line represents median PID1 mRNA of all samples. C, PID1 mRNA in medulloblastomas from the Heidelberg series, analyzed on Affymetrix U133 plus2.0, according to molecular classification. This series ($n = 446$) is a combination of published data (11–13) on 230 patients and unpublished data on 216 additional patients from Heidelberg (M. Kool and S. Pfister, unpublished data). The medulloblastoma subgroup distribution in this cohort is: 54 WNT, 123 SHH, 98 group 3, and 171 group 4. These were compared with 5 fetal cerebella and 13 adult cerebella. P-values comparing the groups are listed above the panels and are color-coded according to the groups compared. mRNA values are expressed in arbitrary units, using the MA55.0 normalized Affymetrix values; D, radiation-free progression-free survival (rf-PFS; Kaplan–Meier curve) according to PID1 mRNA (variant 1, NM_017933.4; real-time qRT-PCR) in 81 CHLA medulloblastomas. Green curve, patients with above-the-median PID1 mRNA; orange curve, patients with below-the-median PID1 mRNA. E, Kaplan–Meier curve of OS for 182 patients with medulloblastoma from the series shown in (C) for whom OS data were available, plotted according to PID1 mRNA above 100 (high PID1; blue) or below 100 (low PID1; red). Molecular subgrouping for the tumors of patients in the high PID1 and low PID1 curves are detailed in the table left of panel.**
LN229 GBM, D283MED medulloblastoma, and CHLA-06-ATRT brain tumor cell lines as follows: cells were transfected with PID1-tGFP or tGFP control for 24 hours, tGFP-expressing cells were sorted by flow cytometry and plated at equal numbers, and colonies were allowed to form over 2 weeks without further selection. Each of the cell lines formed significantly fewer colonies when transfected with PID1-tGFP than did cells transfected with the control tGFP vector, despite an equal number of sorted tGFP$^+$ cells plated for the tGFP control and PID1-tGFP transfected cells (Fig. 3A and B). Both variants of PID1 (variant 1: NM_017933, variant 2: NM_001100818) showed similar effects in this assay. Similar inhibition of colony formation was also observed using the self-cleaving bicistronic pCLS-PID1-2A-eGFP compared with its control vector, pCLS-2A-eGFP (21) as well as with the bicistronic pCIENS-PID1 compared with pCIENS control vector (Supplementary Fig. S3A and S3B). Expression of PID1 at the time of flow sorting and plating of the tGFP-expressing cells was verified by Western blotting (24 hours after transfection; Supplementary Fig. S3C). These data indicate that PID1 confers growth and/or survival disadvantage upon brain tumor cell lines ectopically expressing PID1.

**PID1 inhibits proliferation and promotes cell death of cultured brain tumor cell lines**

To characterize the PID1-induced growth disadvantage, we analyzed proliferation and cell death, measuring BrdUrd/7AAD uptake in tGFP-positive cells 24 hours after transient transfection with pCMV6-PID1-tGFP or pCMV6-tGFP control. GBM (U251) and medulloblastoma (UW-228-2) cell lines as well as primary medulloblastoma cells (CHLA-259) expressing PID1-tGFP had significantly more cells in the sub-G0–G1 phase compared with cells expressing tGFP$^+$ control vector (Fig. 4A and B and Supplementary Fig. S4), indicating that PID1 increased cell death. PID1-tGFP also decreased proliferation, as indicated by the decreased proportion of cells in S-phase (Fig. 4A and B). Apoptosis accounted for at least some of the cell death, as annexin V binding was higher in PID1-tGFP–expressing cells compared with tGFP controls (Fig. 4C). There were very few 7AAD-positive/annexin V–negative cells, indicating that necrosis was only minimal. As mitochondria are involved in metabolism and several forms of cell death and PID1 has been reported to induce mitochondrial dysfunction in adipocytes and myocytes (24–28), we examined the effect of PID1 on mitochondrial depolarization. U251 GBM, D283MED medulloblastoma, and CHLA-06-ATRT cell lines transfected with PID1-tGFP showed increased depolarization of mitochondrial membrane potential compared with tGFP-transfected cells 24 hours after transfection (Fig. 4D). Conversely, siRNA knockdown of PID1 in U87 GBM (U87 express PID1 protein; Supplementary Fig. S5) diminished baseline depolarization of the mitochondrial membrane potential compared with nonsilencing siRNA control (Fig. 4E). siPID1 knockdown similarly diminished baseline

**Table 1. Cox proportional hazards model for progression-free survival (patients who received irradiation as part of the primary treatment were censored at time of irradiation)**

<table>
<thead>
<tr>
<th>Variables</th>
<th>Univariate analyses</th>
<th>Multivariate analyses</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HR$^a$  95% CI$^a$</td>
<td>P$^a$</td>
</tr>
<tr>
<td>Age at diagnosis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Relative risk increase per 1 year</td>
<td>1.2 (1.05, 1.4)</td>
<td>0.017</td>
</tr>
<tr>
<td>≥3years vs. &lt;3 years</td>
<td>3.5 (1.3, 9.1)</td>
<td>1.2 (0.36, 3.8)</td>
</tr>
<tr>
<td>Stage$^d$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>M0</td>
<td>1.0</td>
<td>—</td>
</tr>
<tr>
<td>M$^-$</td>
<td>4.7 (1.4, 15)</td>
<td>16 (2.0, 128)</td>
</tr>
<tr>
<td>Histology$^d$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Classic</td>
<td>1.0</td>
<td>—</td>
</tr>
<tr>
<td>Desmoplastic/nodular</td>
<td>0.42 (0.09, 2.0)</td>
<td>3.8 (0.29, 49)</td>
</tr>
<tr>
<td>Anaplastic/large cell</td>
<td>2.0 (0.54, 7.6)</td>
<td>0.35 (0.06, 1.9)</td>
</tr>
<tr>
<td>Molecular subgroup$^d$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SHH/WNT</td>
<td>1.0</td>
<td>—</td>
</tr>
<tr>
<td>Group 3</td>
<td>6.2 (1.5, 26)</td>
<td>2.7 (0.17, 44)</td>
</tr>
<tr>
<td>Group 4</td>
<td>3.2 (0.58, 18)</td>
<td>2.1 (0.12, 37)</td>
</tr>
<tr>
<td>PID1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>High</td>
<td>1.0</td>
<td>—</td>
</tr>
<tr>
<td>Low</td>
<td>6.8 (2.2, 21)</td>
<td>21 (1.9, 221)</td>
</tr>
</tbody>
</table>

$^a$HR and CI from univariate Cox models.

$^b$HR, CI, and P-values from the Cox model that included age, stage, histology, molecular subgroup, and PID1 expression.

$^c$HR, CI, and P-values from the Cox model that included stage and PID1 expression.

$^d$Missing data for these variables were included in the models as a "missing" category.

BrdUrd/7AAD uptake in tGFP-positive cells 24 hours after transient transfection with pCMV6-PID1-tGFP or pCMV6-tGFP control. GBM (U251) and medulloblastoma (UW-228-2) cell lines as well as primary medulloblastoma cells (CHLA-259) expressing PID1-tGFP had significantly more cells in the sub-G0–G1 phase compared with cells expressing tGFP$^+$ control vector (Fig. 4A and B and Supplementary Fig. S4), indicating that PID1 increased cell death. PID1-tGFP also decreased proliferation, as indicated by the decreased proportion of cells in S-phase (Fig. 4A and B). Apoptosis accounted for at least some of the cell death, as annexin V binding was higher in PID1-tGFP–expressing cells compared with tGFP controls (Fig. 4C). There were very few 7AAD-positive/annexin V–negative cells, indicating that necrosis was only minimal. As mitochondria are involved in metabolism and several forms of cell death and PID1 has been reported to induce mitochondrial dysfunction in adipocytes and myocytes (24–28), we examined the effect of PID1 on mitochondrial depolarization. U251 GBM, D283MED medulloblastoma, and CHLA-06-ATRT cell lines transfected with PID1-tGFP showed increased depolarization of mitochondrial membrane potential compared with tGFP-transfected cells 24 hours after transfection (Fig. 4D). Conversely, siRNA knockdown of PID1 in U87 GBM (U87 express PID1 protein; Supplementary Fig. S5) diminished baseline depolarization of the mitochondrial membrane potential compared with nonsilencing siRNA control (Fig. 4E). siPID1 knockdown similarly diminished baseline...
mitochondrial depolarization in D283MED medulloblastoma cells (Fig. 4E). These data demonstrate that the inhibitory effect of PID1 on brain tumor cell line growth is the result of both inhibition of proliferation and increased cell death, and is associated with depolarization of mitochondrial membrane potential.

Finally, to begin investigating the molecular mechanism of PID1, we examined its effect on serum-mediated phosphorylation of AKT and ERK, 2 effectors that are central to a number of proliferation and survival signaling pathways. LN229 GBM cells transiently transfected with PID1, serum starved overnight and acutely stimulated with 20% FBS showed decrease in the serum-induced phosphorylation of AKT compared with empty vector-transfected cells (Fig. 4F). In addition, transient transfection of PID1 into D283MED medulloblastoma cells grown in FBS-containing medium caused decrease in phosphorylation of both AKT and ERK (Fig. 4G and Supplementary Fig. S6). This indicates that...
PID1 inhibits serum-mediated signaling pathways, which involve AKT and ERK.

In summary, using 6 independent datasets, we have shown that PID1 mRNA is lower in the more aggressive medulloblastomas and gliomas compared with their relatively more favorable counterparts, and that higher PID1 mRNA correlates with longer rf-PFS and OS in patients with medulloblastoma and longer OS in patients with glioma and GBM. We also showed that PID1 conferred a growth-inhibitory effect on cell lines of 3 types of brain tumors (medulloblastoma, GBM, and ATRT), which manifested as decreased proliferation and/or increased cell death. Finally, PID1 induced mitochondrial depolarization and inhibition of serum-mediated phosphorylation of AKT and ERK. Taken together, these findings suggest that PID1 is a novel growth inhibitor in gliomas and embryonal brain tumors.

Discussion

The small number of articles reporting on PID1 to date have mostly been in the context of obesity and diabetes in adipocytes and muscle cells (8, 28–30) and in Alzheimer’s brains (9). Our work here is the first to report on PID1 in cancer. Our clinical findings for PID1 using 6 independent datasets comprising 2 different types of brain cancers emphasize the validity of the association between lower PID1 mRNA and less favorable tumor subgroups. The in vitro experiments suggest that PID1 confers growth suppression because of combined inhibition of proliferation and increase in cell death. Regulation of PID1 expression in embryonal brain tumors and gliomas is currently unknown.

The molecular mechanism by which PID1 affects growth of brain tumor cells in culture is also unknown at this time. Our finding on depolarization of mitochondrial membrane potential by PID1 in brain tumor cell lines is consistent with reports that PID1 disrupts mitochondrial function in adipocytes and muscle cells (24–28). In adipocytes and muscle cells, PID1 also inhibits insulin-mediated phosphorylation of IRS-1 and AKT, and insulin-mediated translocation of the GLUT-4 glucose transporter to the membrane, resulting in decreased glucose uptake (28–30). Our experiments showing that PID1 inhibits phosphorylation of AKT and ERK suggest that PID1 may modulate signaling pathways involved in cell proliferation and survival. Although insulin signaling is less likely to be a target of PID1 modulation in brain tumor cell lines because of their low and/or infrequent expression of the insulin receptor, it will be interesting to examine if PID1 modulates signaling through the insulin-like growth factor receptor (IGF1R), which is important in many brain tumors (31–40). The inhibition of insulin receptor signaling in adipocytes was hypothesized to be mediated via interaction of the PID1 PTB domain with the NPXY motif in the cytoplasmic tail of the insulin receptor (29); however, co-immunoprecipitation of the 2 proteins has not been reported yet. Using yeast 2 hybrid screens and pull-down experiments, several groups reported direct interaction between the PTB domain of overexpressed PID1 and the NPXY motif of low-density lipoprotein receptor-like-1 (LRP1; refs. 9 and 10). However, it is unlikely that LRP1 is a major player in the PID1 (aa84-230)-induced cell-cycle arrest in NIH-3T3, because the arrest could not be overcome by overexpression of LRP1 (10).

Cellular effects of PID1 may differ between cell types. In 3T3-L1 preadipocytes, PID1 increased proliferation (8), but in NIH3T3 cells a PID1 fragment that includes the PTB domain (aa84-230) caused cell-cycle arrest (10). Our findings that PID1 has a growth-suppressive effect in multiple brain tumor cell lines are consistent with the latter.
Examination of the Catalogue of Somatic Mutations in Cancer (COSMIC; ref. 41) revealed that among 7,055 tumors reported to date (July 18, 2013), there were only 27 mutations, of which 15 were nonsynonymous. No mutations or copy number variations of PID1 were found in the 510 brain tumors reported. The lack of PID1 copy number variations and PID1 mutations in brain tumors suggests that PID1 does not function as a classical tumor suppressor gene. The direct correlation of PID1 mRNA and survival in medulloblastoma and gliomas suggests that PID1 may render these brain tumors more susceptible to therapy, accounting for the better outcome in patients whose tumors expressed higher PID1 mRNA. This possibility is currently under investigation in our laboratory.

In summary, this is the first report to link PID1 to cancer and to brain tumors, to demonstrate correlation between PID1 mRNA level and survival in medulloblastomas and gliomas, and to show growth-inhibitory effect of PID1 in cultured medulloblastoma, GBM, and ATRT cell lines. Our data therefore suggest that PID1 may have a growth-modulating function in brain tumors. It will now be important to further investigate the molecular mechanism(s) of PID1’s effects in pediatric and adult malignant brain tumors.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: A. Erdreich-Epstein, J. Xu, G.M. Shackleford
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): A. Erdreich-Epstein, X. Ren, H. Zhou,

Figure 4. Overexpression of PID1 diminishes proliferation, increases cell death, and causes mitochondrial depolarization in brain tumor cell lines. A, U251 GBM cells transiently transfected for 24 hours with pCMV6-AC-GFP or pCMV6-AC-PID1-GFP (PID1 variants 1 or 2) were analyzed by flow cytometry for BrdUrd uptake and 7AAD content in the GFP-expressing cells. Left, typical flow cytometry plot demonstrating decreased BrdUrd uptake and increased sub-G0–G1 in PID1-GFP cells compared with controls. Right, mean/SEM (n = 3) from 1 of 3 similar experiments in U251 cells; B, BrdUrd and 7AAD uptake performed similarly to (A) using U228-2 medulloblastoma cells; n = 3; C, annexin V and 7AAD staining in the GFP-expressing U251 GBM cells 24 hours after transfection as in (A). Right shows representative panels, left shows mean ± SEM of 3 independent experiments; D, mitochondrial depolarization measured by flow cytometry using MitoProbe assay in GFP-expressing brain tumor cells 24 hours after transfection with GFP control or PID1-GFP; n = 3 to 8 repeats. Plasmids were all pCMV6-AC-GFP versus pCMV6-AC-PID1-GFP; E, mitochondrial depolarization, measured as in D, in FAM-labeled U87 GBM and D283MED medulloblastoma cells treated for 48 hours with nonsilencing control siRNA (C) or siPID1; F, LN229 GBM cells transiently transfected with pCIENS (control) or pCIENS-PID1 (PID1) and serum-starved overnight were stimulated for 15 minutes with medium containing 20% FBS. Phosphorylation of AKT was measured by flow cytometry in the eGFP-expressing cells. Shown are means/SEM of triplicate measurements from 1 of 2 similar experiments and representative flow cytometry tracings; G, D283MED medulloblastoma cells grown in FBS-containing conditions were transiently transfected with pCIENS (control) or pCIENS-PID1 overnight (PID1). Phosphorylation of AKT and ERK was measured in the eGFP-expressing cells by flow cytometry. Shown are means/SEM of 1 of 3 similar experiments performed in duplicate or triplicate. Supplementary Fig. S6 shows a representative flow cytometry tracing of the phospho-AKT and phospho-ERK.
Study supervision: Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases)

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References

Correction: PID1 (NYGGF4), a New Growth-Inhibitory Gene in Embryonal Brain Tumors and Gliomas

In this article (Clin Cancer Res 2014;20:827–36), which was published in the February 15, 2014, issue of Clinical Cancer Research (1), an author’s name was misspelled. The corrected name should read as follows: "Shahab Asgharzadeh." The authors regret this error.

Reference

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PID1 (NYGGF4), a New Growth-Inhibitory Gene in Embryonal Brain Tumors and Gliomas

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