Immunocytochemical mapping of the phosphatase and tensin homolog (PTEN/MMAC1) tumor suppressor protein in human gliomas¹

Dan Fufts² and Carolyn Pedone

Department of Neurosurgery and Huntsman Cancer Institute, University of Utah Health Sciences Center, Salt Lake City, UT 84132

PTEN/MMAC1 (phosphatase and tensin homolog/mutated in multiple advanced cancers 1) is a tumor suppressor gene, the inactivation of which is an important step in the progression of gliomas to end-stage glioblastoma multiforme. We examined the distribution of PTEN protein in 49 primary human gliomas by immunocytochemistry using polyclonal antibodies that we raised against PTEN-glutathione S-transferase fusion proteins expressed in *Escherichia coli*. The study group consisted of 6 low-grade astrocytomas, 7 anaplastic astrocytomas, 21 glioblastomas multiforme, 4 low-grade oligodendrogliomas, 6 malignant oligodendrogliomas, and 5 malignant mixed oligoastrocytomas. For each tumor, we determined the percentage of tumor cells showing PTEN immunoreactivity in the most cellular regions of the tumor specimen. In both astrocytomas and oligodendrogliomas, there was an inverse relationship between the percentage of PTEN⁺ cells and malignancy grade, consistent with a role for *PTEN* as a tumor suppressor gene, the expression of which declines during glioma progression. In nonneoplastic tissue, PTEN was expressed in human fetal brain at 16, 23, and 27 weeks' gestation, but not in adult brain, indicating that PTEN is developmentally regulated in the CNS. In 21 glioblastomas multiforme, we

correlated PTEN protein expression with *PTEN* gene sequence. Although *PTEN*-mutant tumors showed significantly diminished PTEN protein expression compared with wild-type cases, suppressed expression of PTEN is more prevalent than predicted from mutation frequencies. *Neuro-Oncology* 2, 71-79, 2000 (Posted to *Neuro-Oncology* [serial online], Doc. 99-28, March 22, 2000. URL <neuro-oncology.mc.duke.edu>)

Astrocytomas, the most frequently occurring gliomas, and the less common oligodendrogliomas are thought to arise by transformation of astrocytes and oligodendrocytes, respectively. The fact that many gliomas are mixtures of astrocytic and oligodendroglial cells suggests that some gliomas may arise not from differentiated glia but from multipotential glial stem cells (Noble, 1996). Regardless of their cell of origin, gliomas all tend to progress through stages of increasing malignancy with the passage of time. Glioma progression has been studied most extensively in tumors of astrocytic lineage where at least four stages can be identified histologically: two benign stages, grade I (pilocytic) and grade II astrocytoma; an intermediate malignant stage, grade III anaplastic astrocytoma; and an end stage, grade IV GBM.³

Tumor progression is a multistep process driven by accumulation of somatic mutations in various genes controlling cell growth. Several groups of investigators have identified a tumor suppressor gene, *PTEN* (also called *MMAC1* [mutated in multiple advanced cancers 1], or *TEP1* [telomerase-associated protein 1]), located on chromosome 10, the inactivation of which is an important step in GBM formation (Li and Sun, 1997; Li et al., 1997; Steck et al., 1997). PTEN has dual-specificity phosphatase activity in vitro against peptide substrates (Myers et al., 1997). A growing body of experimental evidence

Received 25 June 1999, accepted 29 October 1999.

¹This work was supported by grants from the National Institutes of Health (CA52855) and the Huntsman Cancer Foundation.

²Address correspondence and reprint requests to Dan Fufts, Department of Neurosurgery, University of Utah Health Sciences Center, Salt Lake City, UT 84132.

³Abbreviations used are as follows: GBM, glioblastoma multiforme; GFP, green fluorescent protein; GST, glutathione S-transferase; LOH, loss of heterozygosity; PTEN, phosphatase and tensin homolog.

indicates that PTEN inhibits cell growth via the protein kinase B (PKB, Akt) signal transduction pathway. PTEN dephosphorylates the lipid second messenger, phosphatidylinositol 3,4,5-trisphosphate (PIP3) (Maehama and Dixon, 1998). Mutations that eliminate the lipid phosphatase activity of PTEN also reduce its ability to suppress cell growth (Furnari et al., 1998). Glioblastoma cells with *PTEN* mutations and thymic lymphomas that arise in *Pten*^{+/-} transgenic mice show increased activity of protein kinase B and elevated levels of phosphatidylinositol 3,4,5-trisphosphate (Haas-Kogan et al., 1998; Suzuki et al., 1998).

The N-terminal half of PTEN shares close sequence homology with the cytoskeletal proteins tensin and auxilin, suggesting a role in adhesion-dependent cell signaling. Transfection experiments have shown that overexpression of PTEN reduces cell migration, cell spreading, focal adhesion formation, and actin stress fiber formation (Tamura et al., 1998).

Sequence analysis of the *PTEN* coding region has shown that mutations are present in 24% to 44% of primary GBMs (Fults et al., 1998; Liu et al., 1997; Rasheed et al., 1997; Wang et al., 1997). The fact that the frequency of LOH on chromosome 10 reported in GBM patients (>70%) is much higher than the above-described frequency of *PTEN* gene mutation suggests that other tumor suppressor genes exist on chromosome 10 or that *PTEN* expression is lost in tumors without a coding sequence mutation. A study using reverse transcription-polymerase chain reaction and immunocytochemistry showed *PTEN* expression in GBMs to be diminished or absent in 69% of cases—higher than mutation frequencies reported previously (Sano et al., 1999). In that study, *PTEN* expression was detected in vascular endothelial cells and neurons. That observation implies that the overall level of mRNA or protein in a tumor sample may not accurately reflect gene expression in the tumor cell population.

To examine the distribution of PTEN protein in tumor cells, we carried out an immunocytochemistry study of 49 primary human gliomas, including 21 GBMs with a known *PTEN* gene sequence. In both astrocytomas and oligodendrogliomas, PTEN expression was lower in high-grade tumors, consistent with its role as a tumor suppressor. PTEN was expressed in human fetal brain but not in adult brain, indicating that PTEN is developmentally regulated in the CNS. Correlation of *PTEN* genotype and expression in GBMs showed that suppressed expression of PTEN was common, even in cases without a *PTEN* gene mutation.

Materials and Methods

Preparation of Antisera

We expressed two regions of wild-type, human *PTEN* cDNA in *E. coli* as GST fusion proteins using the bacterial expression vector pGEX-2T (Pharmacia Biotech, Piscataway, N.J.). These regions corresponded to N-terminal amino acids M35-L100 and C-terminal amino acids Y336-V403. Polyclonal antisera were raised in rabbits against affinity-purified fusion proteins (Cov-

ance Research Products, Inc., Denver, PA). Two rabbits were immunized with each antigen. We tested immune antisera obtained from each rabbit 72 days postvaccination (after the primary immunization and three boosts) by immunoblotting against purified fusion proteins. Antibodies directed against GST were blocked by preadsorbing immune sera with sonicated extracts of bacteria carrying the nonrecombinant pGEX-2T vector (no *PTEN* cDNA insert present) and expressing only GST. Each immune serum recognized a protein of the same molecular weight as the original antigen. To verify that an immune response was occurring specifically against the PTEN sequence, we digested the fusion proteins with thrombin protease to cleave off the GST peptide segment and analyzed the cleavage products on Western blots with the immune antisera. In each case, the PTEN cleavage fragments were recognized by immune antisera, and no immunoreactivity was seen when we probed with preimmune sera.

To demonstrate the specificity of the antisera against PTEN, we coupled 100 µg (2.8 nmol) of affinity-purified antigen (39-kDa PTEN-GST fusion protein containing the C-terminal portion of PTEN fused to GST) to glutathione Sepharose-4B beads for 20 min. The beads were washed three times with phosphate-buffered saline, then collected by centrifugation. Antiserum was diluted 1:100 in phosphate-buffered saline, and 100 µl of diluted antiserum (10 µg of IgG, 0.063 nmol) was incubated with the Sepharose-coupled PTEN-GST antigen for 60 min on ice. Antigen-antibody complexes, coupled to the Sepharose beads, were removed by centrifugation, and the supernatant was diluted 1:40 with phosphate-buffered saline. This adsorbed antiserum was then applied to Western transfer filters to demonstrate complete blocking of immunoreactivity against the 77-kDa PTEN-GFP fusion protein expressed by COS-7 cells (Fig. 1C). The final antiserum dilution was 1:4000, identical to that used for immunostaining the tissue sections. In control adsorptions, we repeated the above-described protocol using 100 µg (3.8 nmol) of affinity-purified GST or 20 µl of phosphate-buffered saline instead of PTEN-GST.

Transient Transfection of COS-7 Cells

For each transfection, COS-7 cells (2×10^6) were plated in medium containing 10% fetal bovine serum and incubated at 37°C until cell density reached 70% confluence. Expression plasmid DNA (16 µg in 800 µl OptiMEM medium) was mixed with 15 µl LipofectAMINE (GibcoBRL, Grand Island, N.Y.) and incubated at room temperature for 30 min. Recipient cells were washed in serum-free medium, and the LipofectAMINE/DNA mixture was added. After the cells were incubated at 37°C for 5 h in the absence of serum, an equal volume of growth medium containing 20% fetal bovine serum was added, and the transfected cells were incubated for an additional 48 h. In each experiment, we assessed transfection efficiency by transfecting a control plate of cells with pEGFP-C1 (Clontech, Palo Alto, CA), a reporter vector expressing GFP. By counting the number of GFP cells, we estimated an average transfection efficiency of 30%. The PTEN expression plasmids we

Fig. 1. Western analysis of COS-7 cells. A and B. COS-7 cells overexpressing PTEN-GFP fusion protein (A) or GFP monomer (B). Anti-PTEN antisera (1:4000) detected a 77-kDa fusion protein in cells expressing PTEN-GFP, but not in cells expressing GFP alone. Anti-GFP antisera (1:1000) detected the 77-kDa PTEN-GFP fusion protein and the 27-kDa GFP moiety. C. Immunoreactivity against the 77-kDa fusion protein was completely blocked by preadsorption with the original immunizing antigen (PTEN-GST), but not with GST alone or phosphate buffered saline (0).

tested were as follows: pEGFP-PTEN containing full-length, wild-type, human PTEN cDNA fused 5' to GFP in expression vector pEGFP-C1; and pEFm-PTEN containing the same PTEN cDNA with 5' CMYC epitope tag in expression vector pEFm (gifts from Dr. Andrew Thorburn, University of Utah).

Patient Study Group

Tumor samples were obtained from the surgical pathology archives of the University of Utah Health Sciences Center. The tumor tissue had been fixed in formalin at the time of surgery, then embedded in paraffin. Tumors were classified according to the World Health Organization international histologic classification (Kleihues et al., 1993). Patient selection was restricted to cases where an extensive tumor resection had been performed so as to minimize the sampling error inherent in needle biopsy procedures. The age and sex of the 49 patients are shown in Table 1. All tumors were removed at surgery, prior to radiation or chemotherapy, except in 9 patients (A36, A34, U48, U71, U58, O18, O76, O22, A26) who had received prior radiation and/or chemotherapy.

When subdivided according to tumor histopathology, the patient study group had 6 low-grade astrocytomas (pilocytic astrocytomas and grade 2 fibrillary astrocytomas), 7 anaplastic astrocytomas, 21 GBMs, 4 low-grade oligodendrogliomas, 6 malignant oligodendrogliomas, and 5 malignant mixed oligoastrocytomas. Among these subgroups, there was no evidence that the groups differed with respect to sex or history of prior radiation/chemotherapy ($P = 0.38$ and $P = 0.45$, respectively, using the Fisher-Freeman exact test). The small

size of the patient subgroups may have masked actual differences in sex or in treatment history. However, the age distribution was significantly different, with higher age associated with more advanced tumors ($P = 0.0003$, using Kruskal-Wallis test). In every GBM patient, the sequence of all nine coding exons of the *PTEN* gene was determined, and LOH analysis was performed for multiple loci on chromosome 10 as reported by us previously (Fults and Pedone, 1993; Fults et al., 1998).

Immunocytochemistry

Tissue sections were cut 6- μ m thick, mounted on glass slides, deparaffinized with toluene, and then hydrated through a descending series of ethanol. For each tumor, sections were treated with and without an antigen unmasking protocol (Vector Laboratories, Burlingame, CA) in which slides were exposed to two power bursts in a microwave oven (Sears Kenmore, Chicago, Ill.) at full power (750 W) for 5 min each. The antigen unmasking protocol heightened the intensity of the specific immunoperoxidase staining. In no case did this protocol reveal immunoreactivity staining that was not visible at lower intensity levels in untreated sections.

Sections were treated with H_2O_2 (1% vol/vol) for 10 min to quench endogenous peroxidase activity. After immersion in normal goat serum (2%), sections were incubated with primary antibody (antiserum, diluted 1:4000–1:2000, or purified IgG, 3–6 μ g/ml) in a humid chamber at 4°C overnight. The avidin-biotin complex technique (Hsu et al., 1981) was used to localize PTEN immunoreactivity. Sections were developed with diaminobenzidine (0.075% wt/vol)- H_2O_2 (0.002% vol/vol), then counterstained with toluidine blue (0.1% wt/vol). To demonstrate competition, we preincubated 100 μ l anti-serum (1 mg of IgG, 6.3 μ mol) with 300 μ g (7.7 μ mol) antigen (39-kDa PTEN-GST fusion protein containing the C-terminal portion of PTEN coupled to GST) for 1 h before diluting and applying it to the slides.

For each patient, the percentage of tumor cells showing PTEN immunoreactivity was counted in three, 40 $\times$ microscope fields in the most cellular region of the tissue section. Some tumors rated at 0% contained scattered foci of positive cells remote from the tumor center. The PTEN immunoreactivity data shown in Table 1 was derived from tissue sections treated with the unmasking protocol because of the more obvious differences between PTEN⁺ and PTEN⁻ tumor cells.

Results

Characterizing the Immune Antisera

PTEN was overexpressed in COS-7 cells as a fusion protein coupled with GFP by transient transfection of cDNA using expression plasmid pEGFP-C1. On immunoblots, antisera directed against two different PTEN epitopes (peptide segments M35-L100 and Y336-V403) detected a 77-kDa fusion protein in cells transfected with the fusion construct (pEGFP-PTEN) but not in cells transfected with control plasmid (pEGFP) containing GFP

Table 1. PTEN expression in 49 human gliomas

Patient	Tumor type	Sex/age	% PTEN + cells	LOH 10	PTEN genotype
L9	PA	F/36	100	N	
L27	PA	M/25	100		
L28	PA	M/22	100		
L29	PA	M/26	100		
A29	AII	F/25	83	N	
L30	AII	M/26	100		
A28	AA	M/28	62	N	
A27	AA	F/24	0	N	
A21	AA	F/29	63	N	
A36	AA	F/24	26	N	
A31	AA	M/60	33	N	
A38	AA	M/16	50	N	
A34	AA	F/42	65	N	
U49	GBM	M/68	0	N	Y16 > Stop (exon 1)
U48	GBM	M/33	0	N	S229 > Stop (exon 7)
U72	GBM	M/59	0	N	R335 > Stop (exon 8)
U42	GBM	M/82	0	N	Frameshift (exon 2)
U71	GBM	M/40	0	N	Splice variant (intron 4)
U23	GBM	F/62	0	N	Splice variant (intron 6)
U52	GBM	M/55	0	N	H61 > R (exon 3)
U80	GBM	M/56	0	N	L152 > V (exon 5)
U50	GBM	F/58	0	N	R173 > C (exon 6)
U15	GBM	M/71	31	N	M35 > R (exon 2)
U58	GBM	F/47	0	N	Wild type
U60	GBM	F/58	32	N	Wild type
U68	GBM	M/55	0	N	Wild type
U61	GBM	M/40	23	N	Wild type
U57	GBM	M/60	0	N	Wild type
U73	GBM	M/49	0	N	Wild type
U70	GBM	M/62	0	N	Wild type
U78	GBM	M/48	10	N	Wild type
U69	GBM	M/52	10	N	Wild type
U46	GBM	M/28	67	N	Wild type
U79	GBM	M/67	55	Y	Wild type
O18	LGO	F/33	100		
O19	LGO	M/34	52		
O20	LGO	F/39	37		
O21	LGO	M/56	60		
O56	MO	F/45	0	N	
O5	MO	M/34	0	N	
O11	MO	F/56	11	Y	
O14	MO	M/43	7		
O15	MO	M/69	16		
O76	MO	M/45	19	N	
O22	MOA	M/25	10		
O23	MOA	M/29	31		
O1	MOA	M/40	22	N	
A26	MOA	F/55	50	N	
A19	MOA	F/37	53	N	

Abbreviations: PA, pilocytic astrocytoma; AII, grade II astrocytoma; AA, anaplastic astrocytoma; GBM, glioblastoma multiforme; LGO, low grade oligodendroglioma; MO, malignant oligodendroglioma; MOA, malignant mixed oligoastrocytoma.

alone (Fig. 1A). Control antisera against GFP detected the 77-kDa PTEN-GFP fusion protein and the 27-kDa GFP moiety in cells transfected with pEGFP-PTEN and pEGFP, respectively (Fig. 1B). The anti-GFP serum detected an additional band in the 65- to 70-kDa size range in COS-7 cells transfected with PTEN-GFP, GFP, or salmon sperm DNA. This band most likely represents cross reactivity with an unknown COS-7 cell protein unrelated to PTEN.

To control for effects on epitope presentation by the 27-kDa GFP fusion partner, we transfected COS-7 cells with full-length *PTEN* cDNA carrying a short CMYC epitope tag (10 amino acid residues). On immunoblots, all four anti-PTEN antisera and a control monoclonal antibody specific for the CMYC epitope detected a 50-kDa PTEN-CMYC fusion protein (data not shown).

Immunoreactivity against the 77-kDa PTEN-GFP fusion protein expressed by COS-7 cells was completely blocked by preadsorption of antiserum with the original immunizing antigen. Fig. 1C shows the results of an adsorption (competition) experiment using antibody UT139 directed against a 39-kDa PTEN-GST fusion protein containing the C-terminal portion of PTEN. We concluded from these results that our antisera recognized PTEN specifically.

PTEN Immunocytochemistry in Human Gliomas

We examined PTEN protein expression in a series of 49 human gliomas by immunoperoxidase staining of formalin-fixed tissue sections. For each tumor, we determined the percentage of tumor cells showing PTEN immunoreactivity in the most cellular regions of the tumor specimen. The data shown in Table 1 were obtained following antigen unmasking. Among gliomas of the astrocytic lineage, there was an inverse relationship between the percentage of PTEN⁺ tumor cells and malignancy grade. Low-grade astrocytomas (4 pilocytic astrocytomas and 2 grade II astrocytomas) showed immunoreactivity in almost every tumor cell, especially in the dense glial fibers in the pilocytic astrocytomas (Fig. 2A). PTEN immunoreactivity was completely blocked by preincubation of the antibody with purified antigen. The anaplastic astrocytomas showed variable PTEN expression (0%–65% positive cells) (Fig. 2B). No PTEN immunoreactivity was detectable in 14 of 21 GBMs (67%) (Fig. 2C).

Among tumors of oligodendroglial lineage, a similar pattern of PTEN expression was observed, with 37% to 100% positive cells (median = 56%) in low-grade oligodendrogliomas (Fig. 2D) and 0% to 19% (median = 9%) in malignant oligodendrogliomas (Fig. 2E). In five cases of malignant, mixed oligoastrocytomas, cytoplasmic staining was present in tumor cells with morphologic features of immature oligodendroglia (small round nuclei; scant cytoplasm) as well as in those with astrocytic features (elliptical nuclei; short cytoplasmic processes) (Fig. 2F).

Fig. 3 shows a dot plot of the percentage of PTEN⁺ cells in the six subclasses of gliomas. We compared the distributions of these percentages using an exact version of the Wilcoxon rank-sum test. According to the hypoth-

esis that an inverse relationship exists between tumor grade and PTEN expression, we performed one-sided tests to compare low-grade astrocytoma versus anaplastic astrocytoma ($P = 0.0006$), anaplastic astrocytoma versus GBM ($P = 0.0042$), and low-grade oligodendroglioma versus malignant oligodendroglioma ($P = 0.0048$). All these differences are significant at the 0.05 level. The less meaningful comparison of low-grade astrocytoma versus malignant mixed oligoastrocytoma ($P = 0.0022$) showed a significant difference, but the low-grade oligodendroglioma versus malignant mixed oligoastrocytoma comparison ($P = 0.056$) did not quite reach significance. The proportions of PTEN⁺ cells among the six glioma subclasses were compared using analysis of covariance (ANCOVA) on the logit-transformed proportions. The effects of the covariates—age, gender, and treatment history—were not significant for any of these comparisons.

We observed the same pattern and specificity of staining with antisera raised against the N-terminal portion of the PTEN molecule and against the C-terminal portion. The results shown in Table 1 were obtained using high-titer, C-terminal antibody UT139. Taken together, these comparisons show an inverse relationship between PTEN immunoreactivity and malignancy grade in both astrocytomas and oligodendrogliomas, consistent with a role for *PTEN* as a tumor suppressor gene, the expression of which declines during glioma progression.

PTEN Immunocytochemistry in Nonneoplastic Brain Tissue

To examine the distribution of PTEN expression in nonneoplastic brain tissue, we performed immunoperoxidase staining of brain sections from the following sources: hippocampus and temporal neocortex removed from an adult patient during surgery for epilepsy, putamen obtained at autopsy from a patient with Alzheimer's Disease, frontal lobe autopsy specimen from a patient with multiple sclerosis, and three human fetal brain specimens (frontal lobe) at 16, 23, and 27 weeks' gestation.

PTEN immunoreactivity was not detectable in either neurons or glia in the adult brain sections. (Fig. 2G shows results in the dentate gyrus of hippocampus.) In fetal brain, however, PTEN immunoreactivity was present. In the 27-week specimen, PTEN immunoreactivity was present throughout fetal layer II, the most superficial part of the cortical plate (Mrzijk et al., 1988). In addition, PTEN immunoreactivity was visible in the cell bodies and apical dendrites of pyramidal neurons in fetal layer V. Only subpopulations of these neurons were positive (Fig. 2H). At 16 and 23 weeks' gestation, PTEN immunoreactivity was present throughout the cortical plate region (Fig. 2I). Faint brown staining was visible in the marginal, subplate, and intermediate zones, indicating that PTEN expression was absent there, or very low. No PTEN expression was seen in the primitive neuroepithelial cells of the ventricular germinal matrix present in the 16-week fetal brain specimen. These results indicate that PTEN is developmentally regulated in the human cerebrum.

Fig. 2. Immunoperoxidase staining of PTEN in human gliomas (A–F) and nonneoplastic brain tissue (G–I). A. Pilocytic astrocytoma (case L28). Inset shows competitive blocking of PTEN immunoreactivity by preadsorption of antibody with PTEN-GST fusion peptide. Elliptical structures are granular bodies. B. Anaplastic astrocytoma (case A34). C. Glioblastoma multiforme (case U42). A focus of tissue necrosis is visible in the upper right corner. D. Low-grade oligodendroglioma (case O18). E. Malignant oligodendroglioma (case O11). Two mitotic cells are visible in the upper part of the micrograph. F. Malignant mixed oligoastrocytoma (case A26) with PTEN immunoreactivity in both astrocytic component (upper panel) and oligodendroglial component (lower panel). G. Absence of PTEN immunoreactivity in dentate gyrus of adult hippocampus (left panel). Adjacent section stained with anti-gliofibrillary acidic protein antibody (right panel). H. Low power view of 27-week cerebral cortex showing PTEN immunoreactivity in fetal layer II (external granular layer) and in subpopulations of pyramidal neurons (arrows) of layer IV (right panel). Roman numerals indicate cortical layers (Mrzijk et al., 1988). High power view of PTEN⁺ pyramidal neurons in layer IV (left panel). I. PTEN immunoreactivity in the cortical plate of 23-week fetal brain. A portion of the marginal layer is visible at the upper edge of the micrograph. Scale bars, 10 μ m.

Fig. 3. PTEN protein expression in 49 gliomas. Dot plot shows the distribution of percentages of PTEN⁺ tumor cells in six glioma subclasses: LGA, low-grade astrocytoma (pilocytic and grade 2 astrocytomas); AA, anaplastic astrocytoma; GBM, glioblastoma multiforme; LGO, low-grade oligodendroglioma; MO, malignant oligodendroglioma; MOA, malignant mixed oligoastrocytoma. The median values for each glioma type are indicated by vertical bars.

Correlation of PTEN Genotype and Expression in GBM

In 21 GBM cases, the genotype of the *PTEN* coding region and LOH status were known (Table 1). Six cases (U49, U48, U72, U42, U71, U23) had LOH on 10q and a chain-terminating mutation in the *PTEN* gene (three stop codons, one frameshift, and two splice donor site variants). No PTEN immunoreactivity was found in these cases, the expected result for null mutations. Four cases (U52, U80, U50, U15) had LOH on 10q and a *PTEN* missense mutation. Three of these missense mutants showed no protein expression in the tumor cells. One GBM (U15) with a missense mutation (an M>R amino acid substitution at codon 35 in exon 2) did show PTEN protein expression in 31% of tumor cells. Among the 11 GBMs with wild-type *PTEN*, tumor cell protein was absent in 5 cases and present in 6 cases. We compared the distributions of the percentages of PTEN⁺ tumor cells in GBMs with wild-type and mutant *PTEN* using the Wilcoxon rank-sum test. *PTEN*-mutant tumors showed significantly diminished PTEN protein expression compared with wild-type cases ($P = 0.00423$).

Discussion

In gliomas of astrocytic lineage, the inverse relationship we observed between PTEN protein expression and histopathology grade is consistent with a role for *PTEN* as a tumor suppressor gene that becomes inactivated during progression to end-stage GBM. The variation in PTEN expression among anaplastic astrocytomas suggests that PTEN loss may be a marker for tumors that have already made this transition. Furthermore, the presence of peripherally located subpopulations of PTEN⁺ tumor cells in some GBM cases supports the idea that loss of PTEN expression confers a growth advantage to the mutant cells and permits their clonal expansion.

In a previously reported sequence analysis, *PTEN* gene mutations were not found in oligodendrogliomas, either benign or malignant (Rasheed et al., 1997). In the present study, we found PTEN protein expression to be diminished in malignant oligodendrogliomas compared with low-grade oligodendrogliomas, a pattern similar to that found in astrocytomas. Taken together, these findings suggest that down regulation of PTEN is a factor in malignant progression of oligodendrogliomas, even without *PTEN* gene mutation.

Our observation that PTEN is expressed in fetal cerebral cortex, coupled with the known inhibitory effect of overexpressed PTEN on cell migration in wound healing assays (Tamura et al., 1998), suggests that PTEN may play a role in migration of differentiating neurons and/or glia from the ventricular germinal matrix to the neocortex during embryogenesis. In support of this concept, germline mutations in *PTEN* occur in patients with Lhermitte-Duclos disease, a condition wherein hamartomatous masses of hypertrophic ganglion cells replace the normal layers of granule and Purkinje neurons in the cerebellum (Liaw et al., 1997). The absence of PTEN expression in the proliferative neuroepithelium of the 16-week brain suggests that PTEN may be more important in regulating cell migration than in regulating proliferation, at least during neural development. Somatic acquired *PTEN* mutations encoding inactive versions of PTEN would likely contribute to the well-known hypermotility of tumor cells in human gliomas.

The prognosis for patients with gliomas correlates tightly with histopathologic tumor grade (reviewed in Kleihues and Cavenee, 1997). A study of *PTEN* expression, as measured by reverse transcriptase-polymerase chain reaction, in 89 gliomas showed that survival times were significantly longer in patients whose tumors expressed high levels of *PTEN* mRNA (Sano et al., 1999). Although our study lacked sufficient statistical power to establish PTEN as an independent predictor of clinical outcome, the close correlation we found between PTEN immunoreactivity and tumor grade indicates that

loss of PTEN expression is a marker for increased aggressiveness in both oligodendrogliomas and astrocytomas.

Correlation of PTEN immunoreactivity with *PTEN* genotype in 21 GBMs revealed that cell protein levels were undetectable or markedly diminished, not only in *PTEN*-mutant tumors, but also in most wild-type *PTEN* tumors. One genetic mechanism that may account for decreased or absent *PTEN* expression in tumors with wild-type *PTEN* sequence is haploinsufficiency. Because all but one of our wild-type *PTEN* GBMs had LOH on 10q, gene transcription from the single remaining allele may have dropped cell protein levels below the level of immunodetection. According to a theoretical model of stochastic gene expression, inactivation of a single allele in diploid cells decreases the probability of gene expression from the remaining allele (Cook et al., 1998).

Alternatively, these PTEN⁻ tumors may have arisen from glial cells that originally expressed only one of two existing *PTEN* alleles, the other having been functionally silenced during the course of embryonic development (monoallelic expression). Monoallelic expression of the insulin-like growth factor type 2 (*IGF2*) gene was demonstrated in a study of Wilms' tumors using allele-specific, in situ hybridization probes (Ohlsson et al., 1998). Within individual tumors, some cells preferentially expressed one insulin-like growth factor type 2 allele, while other cells expressed the other allele, exclusively. If a population of glioma cells expressed *PTEN* in a monoallelic fashion and the expressed allele became deleted (chromosome instability may have arisen from previously acquired mutations in other genes), then PTEN protein would disappear altogether. This series of events might confer a growth advantage, leading to the

clonal expansion of glioma cells devoid of PTEN protein yet still retaining the wild-type *PTEN* gene sequence.

Another mechanism of ablating PTEN protein expression in cells without a coding sequence mutation is by methylation of the promoter. In support of this concept, one group of investigators restored *PTEN* mRNA expression in a prostate carcinoma xenograft by treatment with 5-azadeoxycytidine, a demethylating agent (Whang et al., 1998). PTEN expression may also be down regulated by transacting factors like transforming growth factor (TGF)- β , which is produced by glioma cells and which decreases *PTEN* gene expression (Li and Sun, 1997).

We cannot exclude the possibility that the *PTEN* mis-sense mutants were expressed but remained unrecognized by our antibodies, which were raised against wild-type proteins. Nevertheless, our immunocytochemistry results indicate that suppressed expression of *PTEN* in GBM is more prevalent than predicted from mutation frequencies. A broader implication of this work is that a low mutation frequency in primary tumors does not exclude a candidate gene as a physiologically relevant tumor suppressor.

Acknowledgments

The authors thank Patricia Maness (University of North Carolina) and Scott Rogers (University of Utah) for helpful advice on immunology, David James (Mayo Clinic) for providing cDNAs, Jeanette Townsend, Tom Parks (University of Utah), and Eric Holland (University of Texas) for critical discussions, and the Biostatistics Resource Core Facility (Huntsman Cancer Institute) for statistical analysis.

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