PTEN Is a Target of Chromosome 10q Loss in Anaplastic Oligodendrogliomas and *PTEN* Alterations Are Associated with Poor Prognosis

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Allelic loss of 10q is a common genetic event in malignant gliomas, with three 10q tumor suppressor genes, *ERCC6*, *PTEN*, and *DMBT1*, putatively implicated in the most common type of malignant glioma, glioblastoma. Anaplastic oligodendroglioma, another type of malignant glioma, provides a unique opportunity to study the relevance of particular genetic alterations to chemosensitivity and survival. We therefore analyzed these three genes in 72 anaplastic oligodendrogliomas. Deletion mapping demonstrated 10q loss in 14 of 67 informative cases, with the *PTEN* and *DMBT1* regions involved in all deletions but with the *ERCC6* locus spared in two cases. Seven tumors had *PTEN* gene alterations; two had homozygous *DMBT1* deletions, but at least one reflected unmasking of a germline *DMBT1* deletion. No mutations were found in *ERCC6* exon 2. Chemotherapeutic response occurred in two of the seven tumors with *PTEN* alterations, but with unexpected short survival times. *PTEN* gene alterations were not associated with poor therapeutic response in multivariate analysis, but were independently predictive of poor prognosis even after multivariate adjustment for both 10q and 1p loss. In anaplastic oligodendroglioma, therefore, *PTEN* is a target of 10q loss, and *PTEN* alterations are associated with poor prognosis, even in chemosensitive cases. (*Am J Pathol* 2001, 159:359–367)

Oligodendrogliomas are one of the major histological subtypes of glioma, comprising up to 25% of adult gliomas.¹ In contrast to other glioma subtypes, such as astrocytomas, many oligodendrogliomas are dramatically sensitive to chemotherapy, in particular to combined che-

motherapy with procarbazine, lomustine (CCNU), and vincristine (PCV).² However, approximately one third of anaplastic oligodendrogliomas are resistant to chemotherapy, and some tumors that respond nonetheless show rapid regrowth with short patient survival.³ Anaplastic oligodendrogliomas thereby provide a unique opportunity to investigate the relevance of particular genetic alterations to chemosensitivity and survival. Recently, allelic losses of chromosomes 1p and 19q have been shown to correlate with increased chemosensitivity and better prognosis in patients with these tumors.⁴

Allelic loss of chromosome 10q is one of the most frequent genetic alterations in gliomas, and has been reported in 74 to 87% of glioblastomas,^{5–7} 37 to 75% of anaplastic astrocytomas,^{6–8} and 13 to 31% of anaplastic oligodendrogliomas.^{4,9,10} Although chromosome 10q loss in malignant gliomas usually involves all or most of the long arm, the 10q25–26 region has been suggested as the primary tumor suppressor candidate region.^{8,11–13} To date, three genes have been potentially implicated as targets of 10q loss in glioblastomas: *PTEN*, *DMBT1*, and *ERCC6*.

The *PTEN* gene at 10q23^{14–16} is mutated in multiple sporadic cancers that undergo 10q loss, including glioblastomas (28 to 46%) and anaplastic astrocytomas (5 to 23%).^{6,7,17} *PTEN* negatively regulates the phosphatidylinositol 3-kinase (PI3K)-Akt pathway, and thereby affects control of cell cycle and cell survival.^{18,19} Ectopic expression of wild-type *PTEN* in *PTEN*-mutant gliomas markedly sensitizes these cells to irradiation, but not to five chemotherapeutic drugs,²⁰ raising the possibility that *PTEN* status may relate to therapeutic sensitivity in malignant gliomas. However, mutations only rarely occur in other types of tumors that lose 10q, such as malignant meningioma and pancreatic cancer, suggesting the presence of other 10q tumor suppressors.^{21,22}

The *DMBT1* gene at 10q25.3–26.1 encodes a secreted or membrane-linked protein, which seems to participate in epithelial differentiation and in immune regulation.²³ *DMBT1* has been proposed as a candidate tumor sup-

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pressor gene for glioblastoma, medulloblastoma, lung cancer, and gastrointestinal cancers based on homozygous deletions and lack of expression in these tumors.^{24–27} *DMBT1*, however, has a repetitive genomic structure, including 14 scavenger receptor cysteine-rich domains, and is therefore potentially susceptible to chromosomal instability.²⁸ Indeed, a subset of normal individuals harbor hemizygous *DMBT1* deletions, indicating that homozygous deletions in tumors may be a result of pre-existing constitutional deletions uncovered by allelic loss.²³

Finally, the most centromeric 10q candidate, the *ERCC6* gene at 10q11.2–21.2, is responsible for complementation group B of Cockayne syndrome, an autosomal recessive disorder characterized by postnatal growth failure, mental retardation, and cutaneous photosensitivity.²⁹ *ERCC6* is involved in a subpathway of nucleotide excision repair (transcription-coupled repair) for preferential repair of damage to the transcribed strand of active genes.^{30,31} Of note, mutations in exon 2 of the *ERCC6* gene have been noted in 17.5% of high-grade gliomas.³²

To date, no extensive analysis of these 10q candidate glioma suppressor genes has been reported for anaplastic oligodendrogliomas, which undergo molecular alterations that are often distinct from astrocytic malignant gliomas such as glioblastoma.³³ Moreover, because our previous studies suggested that chromosome 10q loss may denote tumors that respond less often to chemotherapy,⁴ the identification of a 10q anaplastic oligodendroglioma gene could provide biological information relevant to understanding either chemoresistance or overall tumor behavior. We therefore analyzed the *PTEN*, *DMBT1*, and *ERCC6* genes as well as the regional pattern of 10q loss in a large series of anaplastic oligodendrogliomas, and addressed the relevance of the genetic alterations to pathogenesis, chemosensitivity, and prognosis.

Materials and Methods

Tissues and Clinical Parameters

Seventy-two anaplastic (grade III) oligodendrogliomas were classified and graded according to World Health Organization criteria³⁴ by at least two neuropathologists, and cases with definite astrocytic components were excluded. Of the 72 patients, 47 were newly diagnosed patients who underwent chemotherapy as an integral part of initial treatment strategy; 45 patients received the PCV regimen of procarbazine, lomustine (CCNU), and vincristine, one received carmustine (BCNU) and one received temozolamide. Thirty-seven of these 47 patients received radiation therapy after completing a chemotherapy program or at the time of tumor recurrence after chemotherapy. Twenty-five of the 72 patients were treated with chemotherapy at recurrence after initial treatment with radiation therapy. Neuroradiological responses to chemotherapy were noted in 24 of the 36 evaluable (ie, with neuroradiologically assessable residual disease after surgery) tumors (67%) treated with chemotherapy as an initial treatment regimen, and in 23 of the 24 evaluable

Figure 1. Deletion map of chromosome 10q in anaplastic oligodendrogliomas. Fourteen tumors with allelic loss of chromosome 10q (ie, all tumors with 10q loss except for case 1896 that has nonspecific distal loss) had deletions involving both *PTEN* and *DMBT1* loci. However, the *ERCC6* locus was spared in cases 1826 and 1968. The data from tumor 1722, which had a *PTEN* mutation without allelic loss, is also shown. The order and chromosomal locations of the markers and the genes were determined according to the draft sequence of the human genome (<http://www.ncbi.nlm.nih.gov/genome/guide/human/>), the Human Genome Browser at the University of California, Santa Cruz (<http://genome.ucsc.edu/goldenPath/octTracks.html>), the Marshfield linkage map (<http://research.marshfieldclinic.org/genetics/>), and the Genome Data Base (<http://www.gdb.org/>). **Filled squares** indicate allelic loss. **Open squares** indicate retention of both alleles. **Vertical lines** indicate not informative or not tested. P, cases with *PTEN* mutation or deletion; D, cases with homozygous deletion of *DMBT1*.

tumors (96%) treated with chemotherapy at recurrence. These investigations have been approved by the Massachusetts General Hospital Subcommittee on Human Studies and the Review Board for Health Science Research Involving Human Subjects at the University of Western Ontario. Tumor DNA was extracted from formalin-fixed, paraffin-embedded sections.³⁵ Constitutional DNA was extracted from blood leukocytes or from formalin-fixed, paraffin-embedded sections of adjacent, uninvolved brain or other tissues.

Analysis of Allelic Loss of Chromosome 10q

Allelic loss of chromosome 10q was assessed at 14 polymorphic loci, with particular emphasis on the *ERCC6*, *PTEN*, and *DMBT1* regions: at D10S196, D10S109, D10S1687, D10S608, D10S215, D10S2491 (~20 kb from the 5' end of the *PTEN* gene³⁶), D10S583, D10S185, D10S88, D10S187, D10S587 (near *DMBT1*²⁴), D10S1723, and D10S169 (from centromeric to telomeric, Figure 1) using microsatellite analysis;³⁵ and at the *ERCC6* locus with a single nucleotide polymorphism in exon 2 (135G/C, Leu45Leu) using single-strand conformation polymorphism (SSCP) analysis (see below).

SSCP Analysis

The entire coding sequence and intron/exon borders of *PTEN* were screened for mutations using SSCP.^{37,38} Forward primers to amplify exon 1 and exon 3 were redesigned to avoid primer overlap and to allow amplification

of DNA extracted from archival materials (ex 1f: 5'-CATCCTGCAGAAGAAGCCCC-3', 182 bp; ex 3f: 5'-TTGTTAATGGTGGCTTTTGTG-3', 169 bp). In tumors with 10q loss, the 5' UTR 252 bp upstream of the open reading frame was also examined.³⁸ The primers are intronic (except for exon 8-2) and therefore do not amplify the processed *PTEN* pseudogene.^{39,40} Because mobility shifts caused by *PTEN* mutations could be subtle because of high A-T content,⁷ most polymerase chain reaction (PCR) fragments were examined with more than one gel condition. For SSCP of *ERCC6* exon 2, novel primer sets were designed to amplify three overlapping fragments covering 91% of the coding region of exon 2, including all reported mutation sites.³² *ERCC6* products were separated on 8% and/or 10% nondenaturing polyacrylamide gel (AA:BIS 19:1) containing 10% glycerol at room temperature at 6 to 8 W. Tumors with altered *PTEN* or *ERCC6* migration patterns were cycle-sequenced (fmol DNA cycle sequencing system; Promega, Madison, WI) bidirectionally using separately amplified PCR products.

Reverse Transcriptase-PCR

Two glioma cell lines (Gli13, Gli46) that have a T insertion in a stretch of 15 T before exon 8 of *PTEN* were investigated by reverse transcriptase-PCR for expression of an aberrant transcript. Gli46 was heterozygous, whereas Gli13 was homozygous, for the insertion. Total RNA was extracted from these two cell lines as well as from two cell lines (Gli36, Gli49) with wild-type sequence, using TRI reagent (Molecular Research Center, Inc., Cincinnati, OH). Reverse transcription with Superscript II and the oligo (dT) primer (Life Technologies, Inc., Rockville, MD) was followed by 35 cycles of PCR using ex 7-2f and ex 9-2r as primers.³⁸ PCR products were separated on 1.2% Tris borate-ethylenediaminetetraacetic acid (TBE)-agarose gel and visualized by ethidium bromide staining.

Multiplex PCR

Homozygous deletions of the *PTEN* gene were assayed using a modification of a comparative multiplex PCR approach described previously.³⁸ Two sets of oligonucleotide primers were used to amplify a 165-bp fragment of the 3' part of *PTEN* exon 5 (ex 5-2f, ex 5-2r), and a 171-bp fragment of intron 7 of the *desmin* gene (in7f, in7r) as a control. The *desmin* gene is on chromosome 2q, a site rarely altered in oligodendrogliomas.¹⁰ PCR was performed in a final volume of 10 μ L containing 1 to 10 ng genomic DNA, 50 mmol/L KCl, 1.5 mmol/L MgCl₂, 10 mmol/L Tris-HCl, pH 8.3, 0.2 mmol/L of each dNTP, 1 μ mol/L of each primer, 0.5 U *Taq* polymerase (Fisher Scientific, Pittsburgh, PA). PCR conditions consisted of initial denaturation at 95°C for 5 minutes, 31 cycles of 95°C for 30 seconds, 52°C for 30 seconds, and 72°C for 40 seconds, and a final extension step of 10 minutes at 72°C. PCR products were separated on 4% TBE-agarose gel and visualized by ethidium bromide staining. Quantitation of the bands was performed with the Image-Pro Plus program (Media Cybernetics, Silver Spring, MD),

and the *PTEN:desmin* ratio was calculated. Because DNA extracted from paraffin-embedded tissues tended to show lower *PTEN:desmin* ratios than DNA extracted from blood, titration assays using serial mixtures of normal DNA and a *PTEN* homozygously deleted cell line DNA was not applicable. Instead, homozygous deletions were scored by comparison to the ratios of tumors with *PTEN* mutations accompanied by allelic loss.

For homozygous deletions of *DMBT1*, two multiplex PCR assays were used. Because most reported homozygous deletions (23 of 27) within the *DMBT1* gene involved the g14ext/g14 locus in intron 18,²⁴⁻²⁷ these two closely positioned STSs were used as target sequences. A 190-bp sequence from chromosome 8 (c12) and a 187-bp sequence of the *APEX* nuclease gene on chromosome 14q were used as controls for g14ext and g14, respectively. All primer sequences, except the forward primer to amplify STS g14 (5'-ATTAGGGCTGCTGAGCAAAG-3'), have been published.^{24,41} PCR was performed for 29 cycles at annealing temperature of 60°C to amplify g14ext/c12, and the annealing temperature was gradually decreased from 63°C to 56°C with 30 cycles in total for amplification of g14/*APEX*. The products were separated on 3% TBE-agarose gels. Homozygous deletions were scored by comparing the *DMBT1*:control ratio of the test tumor to that of mixture consisting of 30% constitutional DNA of each case and 70% U343MG cell line DNA, which has a homozygous deletion at the g14ext/g14 locus.

Long-Range PCR

Long-range PCR was used to evaluate constitutional *DMBT1* deletions. The extent of the deletion was initially estimated based on published Southern blot data of a constitutional hemizygous deletion involving the g14ext/g14 locus in a normal individual (G3 configuration),²³ and was further defined by several PCR amplifications with unique sequence primers for each exon/intron using the homozygously deleted U343 cell line. The primers were designed in *DMBT1* intron 10 (L1595: 5'-CTGCTGAGCATTGCCTGTGTTCTA-3', 32921 to 32944) and intron 26 (L1629: 5'-TCAAAGTCAAAGAAGCAGTGACACCCTA-3', 51129 to 51102) (referenced sequence: GenBank AJ 243211). PCR was performed with 20 ng of blood DNA as template and the Expand Long Template PCR system (Roche Molecular Biochemicals, Mannheim, Germany). PCR conditions consisted of initial denaturation at 94°C for 2 minutes, 28 cycles of 94°C for 30 seconds, 66°C for 30 seconds, and 68°C for 6 minutes, and a final extension step of 5 minutes at 68°C. The products were separated on 0.8% Tris acetate-ethylenediaminetetraacetic acid (TAE)-agarose gels. The sequences of PCR products from case 1968 and other deletion cases (not included in this study) were confirmed for more than 300 bp of both 5' and 3' ends, as well as for unique sequences flanking the deletion. Sensitivity and specificity of the assay was verified by comparison to Southern blot data with probe *DMBT1*/sr1sid2 (generous gift from Dr. Jan Mollenhauer, Heidelberg, Germany) and by long-range PCR data

using a different antisense primer (L1591: 5'-GTCATAT-CAGCTCTGAATAGAAAAGTGC-3', 49875 to 49848). Long-range PCR amplifying 12.3 kb of *DMBT1* sequence (primers: L1547, 5'-GACTTTAGCCATTAGGACGTGC-3', within the deletion; L1550, 5'-AACAGGATTCCACGG-GAGAC-3', outside the deletion) was used to confirm DNA quality.

Analysis of the other Molecular Genetic Factors

Tumors included in this study were also analyzed for other molecular genetic changes common to malignant gliomas: allelic losses of 1p and 19q, mutations of exons 5 to 8 of *TP53*, homozygous deletions of *CDKN2A*, and *EGFR* amplification. These assays and their results are detailed elsewhere.⁴²

Statistical Analysis

To avoid the potential selection bias associated with the group of 25 patients treated with chemotherapy at recurrence, statistical analysis was performed only on the 47 patients who were initially treated with chemotherapy. Odds ratios were used to measure the pair-wise associations among genetic alterations. Response to chemotherapy was defined radiographically, as a decrease in tumor size of 50% or greater. Survival time was measured from diagnosis, and censored at the time of last follow-up. Logistic regression was used to model response, and Cox proportional hazard regression to model survival. Univariate models and multivariate models that adjusted for 1p loss and 10q loss were examined. Survival distributions were estimated using Kaplan-Meier curves.

Results

Deletion Mapping of Chromosome 10q

The D10S196, D10S608, D10S2491, D10S185, D10S187, D10S587, and D10S1723 loci were studied in all cases; the other loci were studied in 49 to 91% of cases. Five tumors showed equivocal results and were excluded from deletion mapping. Fourteen of the remaining 67 tumors demonstrated allelic loss (Figure 1); this included 12 of 45 tumors initially treated with chemotherapy. Case 1896 showed loss at only the most distal marker, which was considered nonspecific. All 14 cases with allelic loss had deletions that involved both the *PTEN* and *DMBT1* regions. The *ERCC6* region appeared spared in cases 1826 and 1968. Cases 1760 and 1948 demonstrated retention of heterozygosity flanked by allelic loss at adjacent markers (Figure 2), which often indicates homozygous deletion.⁴³

Mutation Screening of *PTEN* and *ERCC6*

All fragments covering the entire coding sequence of *PTEN* and *ERCC6* exon 2 were successfully analyzed in all 72 anaplastic oligodendrogliomas. Six tumors showed

Figure 2. Loss of heterozygosity studies in case 1760 showing apparent retention of heterozygosity at D10S2491. Apparent retention of heterozygosity flanked by loss of heterozygosity at adjacent markers often suggests homozygous deletion (see Figure 3). The bars indicate each allele, with arrows indicating lost alleles. N, normal DNA; T, tumor DNA.

tumor-specific SSCP migration shifts of *PTEN*. Sequencing demonstrated two tumors with small deletions resulting in frame shifts and predicted premature protein termination, one tumor with mutation at a splice donor site, and three tumors with missense mutations (Table 1). Five of six tumors with mutations had allelic loss of 10q, whereas a single tumor (case 1722) retained heterozygosity at all informative loci including those flanking *PTEN* (Figure 1). Case 1722 had combined allelic losses of 1p and 19q, but lacked *TP53* mutation, *CDKN2A* deletion, and *EGFR* amplification. Seventeen tumors had an insertion of a T into a 15 T stretch at the splice acceptor site before exon 8 [IVS7(-3) to (-2)insT]; this insertion was also present in corresponding constitutional DNA. Because an alternative *PTEN* transcript excluding exon 5 has been reported in a tumor in which the only sequence change identified (in the tumor and corresponding blood) was a T insertion into a polyT tract in intron 4,⁷ the possible expression of splice variants was investigated by reverse transcriptase-PCR in two glioma cell lines with the T insertion at the splice acceptor site in intron 7. Reverse transcriptase-PCR to amplify 3' part of exon 7, exon 8, and the coding region of exon 9 failed to reveal any aberrant transcript, suggesting that the T insertion before exon 8 does not affect exon 8 splicing (data not shown). No tumor-specific SSCP migration shifts were noted in exon 2 of the *ERCC6* gene.

Homozygous Deletions of *PTEN* and *DMBT1*

All 72 anaplastic oligodendrogliomas were assayed for homozygous *PTEN* and *DMBT1* deletions. For the *PTEN* homozygous deletion assay, *PTEN:desmin* ratios were

Table 1. *PTEN* Mutations in Anaplastic Oligodendrogliomas

Case ID	Exon/intron	Sequence change*†	Predicted consequence†
1722	Exon 1	c.17-18delAA	Lys6fsX9
1724	Exon 8	c.955-958delACTT	Thr319X
1738	Exon 3	c.170T>C	Leu57Ser
1742	Intron 3	IVS3+1G>C	Splice variant
1756	Exon 8	c.977A>G	Asp326Gly
1798	Exon 5	c.302T>C	Ile101Thr

*Reference sequences: Genbank U93051, AF067844.

†Nomenclature following published recommendations.⁵⁴

Figure 3. Multiplex PCR demonstrating homozygous deletions of PTEN and DMBT1. **A:** Amplification of PTEN ex 5-2 in case 1760 is significantly less than paired constitutional DNA and representative loss of heterozygosity cases. **B:** Amplification of intragenic sequence (g14ext, 156 bp) of DMBT1 in cases 1828 and 1968 is significantly less than paired constitutional DNA, 30% constitutional DNA/70% U343 DNA, and loss of heterozygosity cases. Thirty percent normal DNA/70% U343 DNA represents homozygous deletion contaminated with 30% of normal cells. N, paired constitutional DNA; T, tumor DNA; HD, homozygous deletion.

equivalent in nine normal DNA samples extracted from paraffin-embedded tissues, and *PTEN:desmin* ratios were constant over a range of 0.05 to 5 ng/ μ l of template DNA concentration. *PTEN:desmin* ratios in tumors with mutations accompanied by allelic losses ranged from 0.55 to 0.72, with a mean value of 0.65. Case 1760, which had findings suggestive of homozygous *PTEN* deletion on loss of heterozygosity studies (see above), demonstrated a markedly lower ratio (0.16 to 0.29, over repeated experiments) than those tumors with mutations and was therefore considered to have homozygous *PTEN* deletion (Figure 3A). The other cases with allelic loss showed ratios indistinguishable from those of the mutation cases, and no other tumor had decrease of *PTEN* amplification comparable to case 1760.

For the *DMBT1* homozygous deletion assay, only tumors with ratios lower than mixtures of 30% constitutional DNA of each case and 70% U343 DNA were judged to have homozygous deletions because the population of nonneoplastic cells in the areas for DNA extraction were considered to be <30%. With these criteria, two tumors (cases 1828 and 1968) had homozygous deletions (Figure 3B). These two tumors had allelic loss of chromosome 10q, but did not have detectable *PTEN* mutations or deletions (Figure 1). The homozygous deletions in these two tumors were confirmed by the g14:APEX multiplex assay (data not shown).

Figure 4. Constitutional hemizygous *DMBT1* deletion. **Top:** Long-range PCR flanking the deletion shows the truncated 5.5-kb product in normal leukocyte DNA from patient 1968 and in the homozygously deleted U343 cell line. The wild-type PCR product would be 18.2 kb, and therefore only truncated products are amplified (see Materials and Methods). **Bottom:** Long-range PCR with primers within and outside the deletion (see Materials and Methods). A 12.3-kb PCR product is amplified from the wild-type allele of normal leukocyte DNA from patient 1968, but not from the homozygously deleted U343 cell line. M 1, Lambda DNA *Hind*III digest; M 2, lambda DNA *Bst*EII digest; N, normal leukocyte DNA.

Constitutional Hemizygous *DMBT1* Deletion

The possibility of constitutional hemizygous deletion of the g14ext/g14 locus was investigated by long-range PCR in six patients for whom blood DNA was available and whose tumors had chromosome 10q loss. Because the wild-type PCR product would be greater than 18 kb, only the allele with the intragenic deletion is amplified. Long-range PCR demonstrated constitutional deletion within the *DMBT1* gene in patient 1968; the homozygous deletion in the tumor thus arose after allelic loss of the wild-type allele (Figure 4). Partial sequencing and exon by exon (or intron) investigation by PCR unique to each exon/intron showed that the deletion spanned 12.7 kb from intron 14 to exon 25 (or exon 14 to intron 24; exon 14 and exon 25 are identical). Homologous recombination between *DMBT1* repeat 2-3 and 2-7 was most likely responsible for the deletion.²⁸ Unfortunately, blood DNA was not available on case 1828.

Chemotherapeutic Response and Survival

Twelve of the 14 cases with 10q loss were newly diagnosed tumors for which chemotherapy was planned at the time of diagnosis, and were included in the statistical analysis. Allelic loss of chromosome 10q was negatively associated with 1p loss (odds ratio = 0.024, $P < 0.001$), the strong predictor for increased chemosensitivity and longer survival⁴ found in 27 of the 47 cases included in the statistical analysis. Accordingly, response to chemotherapy was obtained in only 2 of 10 evaluable cases with allelic loss of 10q. Loss of 10q was negatively associated with chemotherapeutic response ($P = 0.002$), but was no longer predictive of poor response among patients whose tumors did not have 1p loss ($P = 1.00$). The patients whose tumors had 10q loss had significantly

Table 2. Cox Proportional Hazards Models with All Combinations of *PTEN* Alteration, 10qLOH, and 1pLOH as Predictors

Model	Predictors	Hazard ratio	P value
1	10qLOH	8.470	<0.001
2	10qLOH	5.631	0.003
	1pLOH	0.138	0.001
3	<i>PTEN</i> alteration	8.787	<0.001
4	<i>PTEN</i> alteration	6.869	0.001
	1pLOH	0.122	<0.001
5	<i>PTEN</i> alteration	5.472	0.006
	10qLOH	5.778	0.003
6	<i>PTEN</i> alteration	4.687	0.029
	10qLOH	3.555	0.081
	1pLOH	0.147	0.001

Cox proportional hazards models for survival with all combinations of *PTEN* alteration, 10q LOH and 1p LOH as predictors (no other predictors are included in these models). (See Materials and Methods.)

shorter survival times than patients whose tumors had both 10q alleles ($P < 0.001$; median survival with 10q loss = 15.3 months; without 10q loss = 123.4 months), and allelic loss of chromosome 10q was significantly associated with decreased survival time even after adjusting for 1p loss (Table 2, models 1 and 2).

All seven cases with *PTEN* alterations were tumors treated with chemotherapy at the time of diagnosis, and were included in the statistical analysis. Response to chemotherapy was obtained in only two of the seven cases with *PTEN* alterations; *PTEN* alterations were negatively associated with response ($P = 0.029$), but were no longer significantly predictive of poor response after adjusting for either 10q or 1p loss ($P = 0.956, 0.556$, respectively). The two tumors with *PTEN* alterations that responded to chemotherapy had complete or near-complete responses: case 1756, with an Asp326Gly mutation, showed a 90% response to PCV; and case 1722, with a deletion mutation but without 10q loss, had a complete response to PCV. Given these marked responses, these two patients had unexpectedly short survival times because of tumor recurrence: case 1756 died 19 months after diagnosis and patient 1722 died 55 months after diagnosis, compared with a median survival time of >123.4 months for cases whose tumors had no *PTEN* alterations. Case 1722 was yet more remarkable because this tumor also had combined allelic losses of 1p and 19q, a molecular signature typically associated with a median survival time of >10 years.⁴ Both patients underwent radiation therapy at the time of recurrence. Overall, the survival time of all seven patients with *PTEN* alterations was markedly shorter than those of the other 40 patients (Figure 5), and *PTEN* alterations were independently predictive of decreased survival time even after adjusting for 10q and 1p loss (Table 2, models 3 to 6). The two tumors that had homozygous deletions within *DMBT1* gene did not respond to PCV chemotherapy, and had short survival times (16 months and 49 months).

Discussion

PTEN alterations were detected in seven of 72 anaplastic oligodendrogliomas (7 of 47 tumors treated with chemo-

Figure 5. Kaplan-Meier estimates of the survivor distributions for the 47 patients with anaplastic oligodendrogliomas treated with chemotherapy at the time of diagnosis. Those patients whose tumors had alterations in the *PTEN* gene have markedly shorter survival times ($P < 0.001$; median survival for *PTEN* alteration, 14.8 months; for without *PTEN* alteration, 123.4 months).

therapy at the time of diagnosis) and were accompanied by 10q allelic loss in six of the seven cases. Biallelic *PTEN* inactivation was present in 43% of tumors with 10q loss, a similar percentage to that noted in glioblastomas (35 to 53%).^{6,7,21} The Asp326Gly missense mutation in the C-terminal half of *PTEN* has, to our knowledge, not been reported to date, but is of particular interest. Missense mutations of the C-terminal half are rare, with most mutations in the C-terminal half leading to premature termination. The C-terminal half of *PTEN* is a membrane-associated C2 domain that may optimally position the catalytic region to membrane-bound phosphatidylinositol(3, 4,5)-triphosphate through an extensive interface between phosphatase and C2 domains.⁴⁴ Asp326 is one of most highly conserved amino acids in the C2 domain, and participates in a hydrogen bond network to maintain this interface.⁴⁴ Therefore, the Asp326Gly mutation could greatly reduce *PTEN* enzymatic activity.

Because *PTEN* negatively regulates the PI3K-Akt pathway, and Akt suppresses apoptosis and promotes cell survival by phosphorylating and inhibiting inducers of apoptosis,¹⁹ the relationship between *PTEN* status and therapeutic sensitivity is of interest. Gene transfer of wild-type but not mutant *PTEN* markedly sensitizes *PTEN*-deleted glioma cells to irradiation and CD95 ligand-induced apoptosis, whereas *PTEN* gene transfer has no

effect on chemosensitivity to vincristine, cytarabine, teniposide (VM26), cisplatin, and BCNU.²⁰ On the other hand, adenovirus-mediated transfer of wild-type PTEN restores doxorubicin sensitivity in bladder cancer cell lines.⁴⁵ In the present study, *PTEN* alterations were negatively associated with response to chemotherapy, but were no longer predictive of poor response after adjusting for either 10q loss or 1p loss. Moreover, the presence of *PTEN*-mutated tumors that showed complete or near-complete responses, including the case with the Asp326Gly mutation, suggests that PTEN is not likely to have a major regulatory role in chemosensitivity to procarbazine, lomustine, and vincristine in gliomas.

The prognostic relevance of PTEN inactivation seems to vary according to tumor type. For instance, *PTEN* mutation in endometrial cancer has been associated with favorable clinical and pathological characteristics, whereas *PTEN* mutation in pediatric malignant astrocytomas has been associated with decreased survival.^{46,47} In glioblastomas, tumors with a universally poor prognosis, the issue remains controversial.^{38,48} In this study of anaplastic oligodendrogliomas, *PTEN* alterations were associated with decreased survival, even after adjusting for 10q and 1p loss (Table 2, model 6). Notably, even the two tumors with *PTEN* mutations that responded markedly to chemotherapy had unexpected short survival times. Overall, therefore, *PTEN* gene alterations clearly have a role in the pathogenesis of some anaplastic oligodendrogliomas and are associated with an aggressive tumor phenotype regardless of chemosensitivity. The mechanism for this association is unclear, but could relate to abrogating PTEN function in the control of cellular proliferation.

Homozygous deletions and lack of expression of *DMBT1* have suggested *DMBT1* as a tumor suppressor gene in glioblastoma, medulloblastoma, lung cancer, and gastrointestinal cancers.²⁴⁻²⁷ However, two concerns give pause to accepting *DMBT1* as a tumor suppressor: lack of experimental evidence linking *DMBT1* to tumorigenesis, and constitutional hemizygous deletions in a subset of normal individuals.²³ These constitutional hemizygous deletions are germline polymorphisms; their presence indicates that homozygous deletions in tumors could arise when allelic loss of the chromosome 10q bearing an intact *DMBT1* gene occurs in the presence of a constitutionally deleted *DMBT1* allele. In the present study, the two anaplastic oligodendrogliomas that had homozygous *DMBT1* deletions were not accompanied by *PTEN* alterations, which could support the hypothesis that *DMBT1* is a second target of 10q loss in anaplastic oligodendrogliomas. However, at least one of these two homozygous *DMBT1* deletions simply reflects allelic loss unmasking a constitutional deletion polymorphism, which would argue against *DMBT1* as a classical tumor suppressor gene. Nonetheless, Mollenhauer and colleagues²³ proposed a possible role for *DMBT1* in cancer immune surveillance, as reported for another scavenger receptor cysteine-rich protein, Mac-2-bp/90K,^{23,49} and so the functional significance of *DMBT1* deletions remains unclear at the present time.

Mutations of exon 2 of the *ERCC6* gene have been reported in 7 of 40 (17.5%) malignant astrocytic tumors.³² These findings, as well as the role of *ERCC6* in nucleotide excision repair, prompted us to study *ERCC6* in anaplastic oligodendrogliomas. Deletion mapping showed that the *ERCC6* locus was lost in most of the tumors with 10q loss, but was not preferentially deleted relative to the *PTEN* and *DMBT1* loci. Mutation analysis of *ERCC6* exon 2 failed to demonstrate mutation in all 72 anaplastic oligodendrogliomas, suggesting that *ERCC6* exon 2 is not a mutational target in anaplastic oligodendrogliomas. Cockayne syndrome is an autosomal recessive disorder and, despite both alleles being affected by severe alterations leading to protein truncation in many patients,^{50,51} there is no increased frequency of skin cancers. On the other hand, mice homozygously mutant for the murine *ERCC6* homologue are more susceptible to skin cancer than are wild-type mice.⁵² Mutation analysis of the entire coding sequence and study of *ERCC6* expression in a large number of gliomas will be needed to fully evaluate *ERCC6* in glioma tumorigenesis.

The present study documents allelic loss of chromosome 10q in 27% of anaplastic oligodendrogliomas treated with chemotherapy at the time of diagnosis, and that 10q and 1p losses are inversely related to one another. In contrast to anaplastic oligodendrogliomas with 1p loss, tumors with 10q loss were associated with poor chemosensitivity and short survival. These observations suggest similarities between anaplastic oligodendrogliomas with 10q loss and malignant astrocytomas with 10q loss, including glioblastomas, which are universally associated with poor prognosis. In addition to our previous studies,⁴ Smith and colleagues⁵³ observed loss of 1p in 73%, and combined losses of 1p and 19q in 64%, of oligodendrogliomas with consensus histological diagnosis by at least two of the three neuropathologists. Such findings suggest that anaplastic oligodendrogliomas with 10q loss or without 1p loss do not simply reflect misdiagnosis of glioblastoma. Indeed, retrospective histological review confirmed that the diagnoses of the 10q-deleted anaplastic oligodendrogliomas in this study were consistent with current criteria for anaplastic oligodendrogliomas,³⁴ and some of the tumors with 10q loss were histologically indistinguishable from those with combined losses of 1p and 19q. The present observations and the literature thus emphasize the therapeutic and prognostic importance of molecular classification in the assessment of malignant gliomas. Once all of these molecular assays, including the more laborious screens for *PTEN* mutations, become clinically practical in terms of cost and efficiency, genetic testing should become a routine component of anaplastic oligodendroglioma management.

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