[CANCER RESEARCH 57, 4183–4186, October 1, 1997]

Advances in Brief

Somatic Mutations of *PTEN* in Glioblastoma Multiforme

Steven I. Wang, Janusz Puc, Jing Li, Jeffrey N. Bruce, Paul Cairns, David Sidransky, and Ramon Parsons[1]

Departments of Pathology and Medicine [S. I. W., J. P., J. L., R. P.] and Neurological Surgery [J. N. B.], College of Physicians and Surgeons, Columbia University, West 168th Street, New York, New York 10032, and Department of Otolaryngology–Head and Neck Surgery, Head and Neck Cancer Research Division, Johns Hopkins University School of Medicine, Baltimore, Maryland 21205-2196 [P. C., D. S.]

Abstract

Alterations of the *PTEN* gene occur in glioblastoma multiforme. To determine the frequency of *PTEN* alteration, 34 consecutive glioblastomas were studied in detail. Sequencing each of the nine exons amplified from tumor DNA revealed 11 mutations. Analysis of polymorphic markers within and surrounding the *PTEN* gene identified an additional four homozygous deletion mutations. Loss of heterozygosity (LOH) was observed in 25 of 34 (74%) cases. All mutations occurred in the presence of LOH. *PTEN* was mutated in 44% (15 of 34) of all glioblastomas studied and 60% (15 of 25) of tumors with LOH on 10q. Thus, *PTEN* appears to be the major target of inactivation on chromosome 10q in glioblastoma multiforme.

Introduction

Glioblastoma multiforme is the most aggressive form of glioma, and patients diagnosed with glioblastoma typically survive less than 2 years (1). Genetic analysis of glioblastoma tumorigenesis has identified alterations of *p15/p16/Rb/CDK4, p53/MDM2,* and *EGFR* (Ref. 2 and references therein). Mutations affecting cyclin D activity are present in nearly all glioblastomas. The majority of tumors have mutations deleting *p15* and *p16* (3, 4). Tumors lacking mutations in these genes frequently harbor inactivating mutations of *Rb* or an amplification of *CDK4* (5). *p53* and *MDM2* are altered in approximately 30 and 10% of tumors, respectively (Ref. 6 and references therein). Gene amplification of *EGFR* concomitant with augmented expression of the receptor occurs in 40–50% of glioblastomas (7), and alterations of *EGFR* and *p53* are mutually exclusive in the tumor (8). Loss of chromosome 10q occurs in the vast majority of glioblastomas (9–11) and is associated with alterations of both *EGFR* and *p53* (8, 12).

The *PTEN* gene, a tumor suppressor recently discovered on chromosome 10q23, contains a phosphatase domain, and its protein product has phosphatase activity (13–15). Mutations of this gene have been detected in glioblastoma cell lines and tumors (13, 14). In addition, other types of cancer and the inherited predisposition to cancer, Cowden disease, are associated with *PTEN* mutations (13, 14, 16). To determine the frequency of *PTEN* alterations in glioblastomas, 34 normal-tumor pairs were investigated. The results showed that 74% (25 of 34) of glioblastomas demonstrated LOH[2] at the *PTEN* locus, and 60% (15 of 25) of these cases contained somatic *PTEN* mutations of both alleles, implicating the *PTEN* gene in tumor development.

Materials and Methods

Tumor Samples. Thirty-four normal-tumor pairs of glioblastoma multiforme were kindly provided by the Columbia Comprehensive Cancer Center Tumor Bank (Department of Pathology, Columbia University). Preparation of genomic DNA from blood and tumor tissues has been described previously (17).

LOH Analysis. Four highly polymorphic dinucleotide-repeat markers flanking the *PTEN* gene, *D10S532, D10S1687, D10S541,* and *D10S583* (Research Genetics, Huntsville, AL), which have been mapped on the sequence tag site (STS)-based map of the human genome (18), were used to determine allelic imbalance in this locus. Another polymorphic dinucleotide-repeat marker, *PTENCA* (forward primer, 5′-GTTAGATAGAGTACCTGCACTC-3′; reverse primer, 5′-TTATAAGGACTGAGTGAGGGA-3′) derived from a bacterial artificial chromosome (BAC) clone D that contains the 5′ end of the gene was also included in the analysis (13).[3] Each forward primer was labeled with [γ-^{32}P]ATP by T4 polynucleotide kinase (New England Biolabs), and the PCR product was analyzed by 6% PAGE. In addition, a missense polymorphism was found 32 bp from the splice donor site of intron 8, TTG(T/G)TGACTT, and was used to test for LOH by sequencing normal-tumor paired DNA. LOH was defined as a reduction of band intensity of more than 90% relative to the second allele for at least two markers. Only one case (case 18) demonstrated loss of only one marker and clearly displayed microsatellite instability at other loci.

Mutational Analysis. Intronic primer pairs were designed to amplify and sequence each exon, including the splice junctions (16). Forward and reverse primer sequences for exon 2 are 5′-GTTTGATTGCTGCATATTTCAG-3′ and 5′-GGCTTAGAAATCTTTTCTAAATG-3′, respectively; for exon 3, 5′-AATGACATGATTACTACTCTA-3′ and 5′-TTAATCGGTTTAGGAATACAA-3′, respectively; and for exon 4, 5′-CATTATAAAGATTCAGGCAATG-3′ and 5′-GACAGTAAGATACAGTCTATC-3′, respectively. The sequencing primer for exon 2 is 5′-TCTAAATGAAAACACAACATGAA-3′ (antisense); for exon 4, 5′-GATTCAGGCAATGTTTGTTAG-3′ (sense). The reverse PCR primer for exon 3 was used as a sequencing primer for the same exon. All primers used in this study were purchased from DNAgency (Malvern, PA). Ten to 50 ng of genomic DNA were amplified at 94°C for 30 s, 54°C for 1 min, and 72°C for 1 min for 35 cycles. Prior to sequencing reactions, PCR products were treated with 10 units of exonuclease I and 2 units of shrimp alkaline phosphatase for 25 min at 37°C and incubated at 80°C for 15 min. Cycle sequencing (Amersham Life Science) was performed for 25 cycles. Sequencing reactions were resolved on 5% polyacrylamide gels buffered with taurine. Each mutation was verified independently at least twice.

Results

Somatic *PTEN* Mutations. Glioblastoma multiforme samples from 34 tumor cases were sequenced throughout all 9 exons of the *PTEN* gene, and mutations were found in 11 cases (Table 1). Frameshift mutations (Fig. 1*A*) that resulted in premature translational terminations were detected in three cases (cases 2, 19, and 21). In case 2, an A insertion at cysteine 136 resulted in a subsequent termination at codon 146; in case 19, an AT deletion at nucleotide positions 227–228 converted tyrosine 76 to a stop codon; in case 21, a 4-bp deletion from nucleotide positions 1011–1014 at codon 337 resulted in a subsequent termination at codon 343. Nonsense mutations were detected at tyrosine 225, arginine 335, and tyrosine 336 in cases 10, 6, and 8, respectively (Fig. 1*B*). Five missense mutations were found

Received 7/21/97; accepted 8/15/97.

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[1] To whom requests for reprints should be addressed, at Department of Pathology and Medicine, Columbia University, 630 West 168th Street, New York, NY 10032. Phone: (212) 305-1058; Fax: (212) 305-1735.

[2] The abbreviation used is: LOH, loss of heterozygosity.

[3] Cairns, P., Evron, E., Okami, K., Halachmi, N., Bose, S., Wang, S. I., Parsons, R., and Sidransky D. Mutation analysis of PTEN/MMAC1 in primary bladder and renal cell cancers, submitted for publication.

Table 1 *Summary of PTEN mutations in primary glioblastoma multiforme*

Case no.	Codon change[a]	Mutation	Exon	Predicted alteration[b]
2	$TGT_{408} \rightarrow ATGT_{408}$	Frameshift	5	C136fs
6	$CGA_{1005} \rightarrow TGA_{1005}$	Nonsense	8	R335Opal
8	$TAC_{1008} \rightarrow TAG_{1008}$	Nonsense	8	Y336Amber
10	$TAT_{675} \rightarrow TAG_{675}$	Nonsense	7	Y225Amber
15	$GGA_{495} \rightarrow AGA_{495}$	Missense	6	G165R
19	$TAT_{228} \rightarrow \Delta[AT]_{227-228}$	Frameshift	4	Y76fs
20	$GGA_{387} \rightarrow AGA_{387}$	Missense	5	G129R
21	$TTT_{1012} \rightarrow \Delta[TTCT]_{1011-1014}$	Frameshift	8	F337fs
26	$GAT_{321} \rightarrow TAT_{321}$	Missense	5	D107Y
29	$CTG_{1035} \rightarrow CAG_{1035}$	Missense	9	L345Q
30	$GCA_{363} \rightarrow CCA_{363}$	Missense	5	A121P

[a] Mutated nucleotides are in boldface type.
[b] Positions refer to the deduced PTEN protein sequence (13); fs, frameshift.

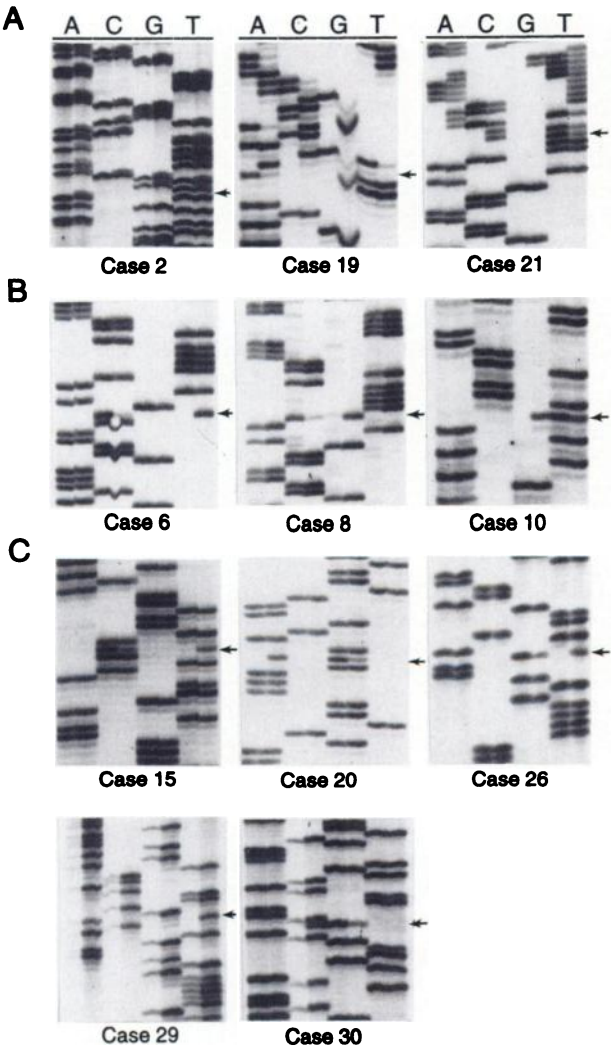

Fig. 1. Illustration of codon mutations found in the glioblastoma cases. *A*, frameshift mutations; *B*, nonsense mutations; *C*, missense mutations. Case numbers and mutated positions refer to Table 1. *Arrows*, mutated codons. Lanes were loaded in the normal-tumor order. All cases were sequenced with sense primers except for cases 15 and 29, for which antisense primers were used. Nucleotide sequences shown for each case: case 2, 393–447; case 6, 1001–1019; case 8, 1007–1025; case 10, 668–686; case 15, partial intron 5 and 493–507; case 19, 220–241; case 20, 374–397; case 21, 1007–1025; case 26, 308–331; case 29, partial intron 8 and 1050–1027; and case 30, 346–381.

(Fig. 1*C*). A G to T mutation in case 26 resulted in a glutamate 107 to tyrosine alteration, and a G to C change in case 30 resulted in a change of alanine 121 to proline. Glycine 129 and glycine 165 were mutated to arginine in cases 20 and 15, respectively. In case 29, leucine 345 was mutated to glutamine. No mutations were detected in the blood-derived DNA, indicating that all mutations were somatic.

LOH at the *PTEN* Locus. Four microsatellite markers flanking the *PTEN* gene, *D10S532*, *D10S1687*, *D10S541*, and *D10S583*, were initially used to evaluate allelic loss in this locus (Fig. 2). Individually, the frequency of LOH was 83% (19 of 23) for *D10S532*, 81% (17 of 21) for *D10S1687*, 57% (12 of 21) for *D10S541*, and 64% (18 of 28) for *D10S583*. Tumors with microsatellite instability were detected in

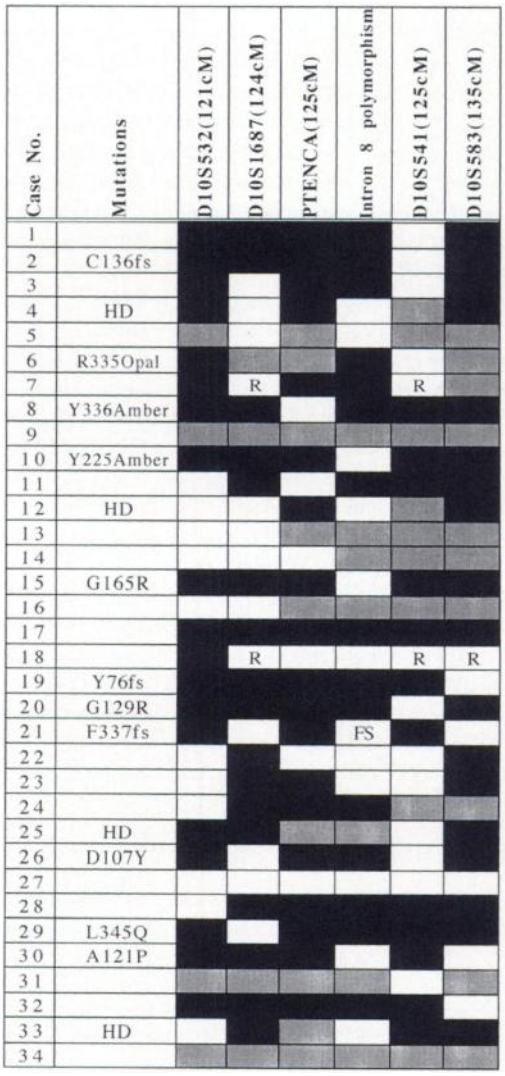

Fig. 2. LOH analysis for 34 glioblastoma cases. ■, LOH; ▨, no allelic loss; □, not informative; *R*, replication error. FS, frameshift; HD, homozygous deletion. Cases with codon mutations are indicated (Table 1).

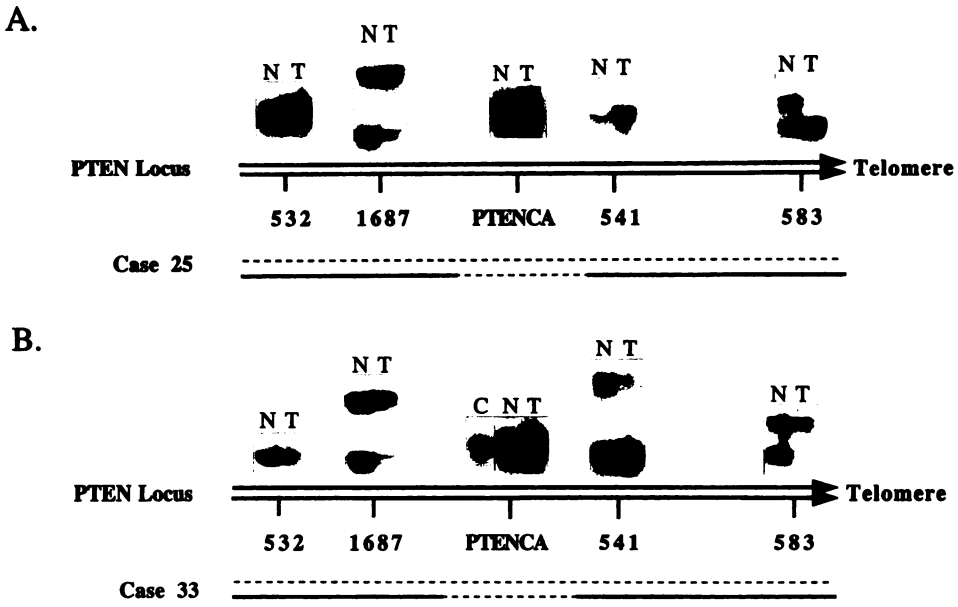

Fig. 3. Potential homozygous deletion at *PTEN*. Cases 25 and 33 are presented. Dinucleotide repeat markers are indicated with the prefix "D10S" left off. *C*, control, shown from case 22, which is not informative at *PTENCA*. The potential homozygous deletion region was proposed based on the retention of heterozygosity at *PTENCA*. Cases 25 and 33 are not informative at *D10S541* and *D10S532*, respectively. - - - - -, missing allele.

two cases (cases 7 and 18). Furthermore, there were eight cases with no detectable chromosomal lesions. To identify polymorphisms within the *PTEN* gene, a BAC spanning the 5′ end was probed with a poly(CA)$_n$ oligonucleotide to identify novel CA repeats.[3] A highly polymorphic marker, *PTENCA*, was obtained that is located within the *PTEN* gene. This marker was informative for 82% (28 of 34) of samples. LOH was detected in 68% (19 of 28) of the samples, and all these cases were also hemizygously deleted in at least one of the flanking loci. A point mutation polymorphism in intron 8 of *PTEN* demonstrated LOH in 68% (15 of 22) of informative samples. Of 34 glioblastoma cases analyzed with the above markers, 25 cases (74%) showed loss of heterozygosity at two or more of these loci (Fig. 2).

Homozygous Deletions of *PTEN*. Four cases (cases 4, 12, 25, and 33) were identified which contained homozygous deletions in the *PTEN* locus (Fig. 3), as determined by apparent retention of heterozygosity flanked by LOH (19). The results revealed that the retention was present between *D10S1687* and *D10S583* in cases 25 and 33 and between *PTENCA* and *D10S583* in cases 4 and 12 (Figs. 2 and 3). In cases 25 and 33, the homozygous deletions appear to target *PTENCA*, whereas in cases 4 and 12, they appear to target the 3′ end of the gene and include *D10S541*.

Discussion

Overall, 15 cases were mutated for both alleles of *PTEN* (Fig. 1). Mutations were found in exons 4–9; no mutations could be found in exons 1–3. Exon 5, which codes for a phosphatase domain, and exon 8 were the most frequently mutated. In addition, we have demonstrated that one copy of *PTEN* is lost through LOH for 74% (25 of 34) of glioblastomas (Fig. 2). Moreover, 60% (15 of 25) of the cases with LOH carried somatic mutations of the second copy of *PTEN*, including 11 codon mutations (Fig. 1) and 4 homozygous deletions (Figs. 2 and 3). Interestingly, our previous study has shown that 63% (5 of 8) of glioblastoma cell lines contain mutations (13), but in this study only 44% (15 of 34) of primary glioblastoma cases were mutated (Fig. 2). The mutation rate of *PTEN* in this study could be underestimated due to our inability to detect all homozygous deletions or alterations of the promoter.

Previous glioblastoma studies have shown that the LOH frequency on chromosome 10q ranges from 83–95%, with the epicenter of loss located at 10q24–25 (9–11). The epicenter of LOH on 10q24–25 may be a summation of deletions targeting *PTEN* and random partial chromosomal losses distal to the *PTEN* locus. Consistent with the thought that *PTEN* is the major target of deletion, all examples of LOH indicate chromosomal break points that are centromeric to *PTEN* (Fig. 2). These data indicate that LOH in this region typically includes the *PTEN* gene. Alternatively, another tumor supressor may exist distal to *PTEN*.

The genetic analysis of glial tumor development clearly implicates chromosome 10q and therefore *PTEN* in the transition from anaplastic astrocytoma to glioblastoma multiforme (2). Of the genetic alterations identified in gliomas, only *p53* is altered in all grades of astrocytoma-derived tumors, suggesting that *p53* inactivation is an early step in glial tumor formation (6). Progression to anaplastic astrocytoma is associated with mutations of *p15/p16/Rb/CDK4* (4, 5). The transition to glioblastoma correlates with loss of chromosome 10q and an increase in the frequency of alterations affecting the cyclin D pathway (4, 5). Many glioblastomas are diagnosed in the absence of a prior, lower-grade glial tumor, however. In these cases, *p53* mutations are not commonly observed; rather, *EGFR* amplifications are seen at high frequency along with alterations of 10q and the cyclin D pathway. Interestingly, *p53* and *EGFR* alterations are not found in the same tumor (8). Thus, at a minimum, individual tumors accumulate alterations in the cyclin D regulatory pathway, chromosome 10q, for which *PTEN* is the likely target, and either *p53* or *EGFR*.

Note Added in Proof

The *PTEN* gene has been recently demonstrated to encode a dual-specificity phosphatase (Myers, M. P., Stolarov, J. P., Eng, C., Li, J., Wang, S. I., Wigler, M. H., Parsons, R., and Tonks, N. K. *PTEN*, the tumor suppressor from human chromosome 10q23, is a dual-specificity phosphatase. Proc. Natl. Acad. Sci. USA, *94:* 9052–9057, 1997).

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Cancer Res 1997;57:4183-4186.