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Advances in Brief

PTEN/MMAC1 Mutations and *EGFR* Amplification in Glioblastomas[1]

Wanguo Liu, C. David James,[2] Lori Frederick, Benjamin E. Alderete, and Robert B. Jenkins

Department of Laboratory Medicine and Pathology, Division of Experimental Pathology, Mayo Clinic and Foundation, Rochester, Minnesota 55905

Abstract

Loss of heterozygosity (LOH) from chromosome 10 is a hallmark of glioblastoma, the most malignant (grade IV) form of glioma. A candidate tumor suppressor gene, *PTEN/MMAC1*, that may be targeted for deletion in association with chromosome 10 LOH has recently been identified. Here we have investigated 63 glioblastomas for *PTEN/MMAC1* alterations and identified DNA sequence changes that would affect the encoded protein in 17 (27%) tumors. Microsatellite analyses of normal-tumor DNA pairs were performed on 14 of these cases and revealed LOH at locations flanking and/or near *PTEN/MMAC1* in all but 1 instance, suggesting that deletion of the remaining wild-type allele had occurred in the large majority of tumors with *PTEN/MMAC1* mutations. Competitive PCR assays were developed to address the possible occurrence of *PTEN/MMAC1* homozygous deletions in glioblastomas, and this analysis identified three samples having loss of both *PTEN/MMAC1* alleles. *EGFR* amplification was determined to occur at similar frequencies among cases with or without *PTEN/MMAC1* homozygous deletions or mutations, suggesting that a growth-promoting effect resulting from amplification-associated increases in epidermal growth factor receptor signaling is not necessarily dependent on the inactivation of *PTEN/MMAC1*.

Introduction

Chromosome 10 deletions represent one of the most common karyotypic abnormalities in malignant gliomas (1). The tumor suppressor gene or genes marked for inactivation in association with such deletions have been sought for several years (2–8), and information presented in two recent reports suggests that one target gene has been identified (9, 10).

The gene, *PTEN/MMAC1*, encodes a tyrosine phosphatase (11) and is located in band q23 of chromosome 10. *PTEN/MMAC1* has been shown to be inactivated in glioblastomas through deletion combined with mutation of the remaining allele (9) and by mutation or homozygous deletion in glioblastoma cell lines (9, 10). The activity of the *PTEN/MMAC1* protein is of particular interest, because the importance of RTK[3] activity to glial tumor development has been well established.

Among the genes encoding RTKs, *EGFR* is particularly important, due to its amplification in a significant percentage of glioblastomas (12–14). Given their opposing enzymatic activities, the question of whether the protein product of *PTEN/MMAC1* acts antagonistically to the tyrosine kinase signaling of EGFR is a matter of some interest. Were this so, one might expect to find *EGFR* amplification and *PTEN/MMAC1* mutations in distinct subsets of glioblastomas. One subset would have amplification-associated overexpression of EGFR, whose cumulative signaling would overcome the negative regulatory effect of the protein encoded by *PTEN/MMAC1*, and the other group would lack this negative regulatory activity due to *PTEN/MMAC1* deletion/mutation; increased expression of EGFR as a result of gene amplification may not be necessary among the latter subset of tumors.

An alternative possibility is that inactivation of *PTEN/MMAC1* may be necessary for any level of EGFR expression to have a growth-promoting effect. In such an event, mutation of *PTEN/MMAC1* might be expected to have occurred in all tumors with *EGFR* amplification.

Here we have investigated a large panel of glioblastomas as well as a smaller number of grade III gliomas for *PTEN/MMAC1* deletions and mutations and for amplification of the *EGFR* gene. Our results indicate significant frequencies of *PTEN/MMAC1* and *EGFR* alterations among the glioblastomas and indicate that these changes seem to occur independently of one another.

Materials and Methods

Heteroduplex and Sequence Analysis. All tumors used in this study were obtained from patients undergoing surgical treatment at the Mayo Clinic. DNAs from peripheral blood leukocytes and snap-frozen tumor tissue were isolated and purified as described previously (2). Samples used for mutation screening and sequencing were generated in 50-μl reaction volumes containing 10–100 ng of genomic DNA, 20 pmol of forward and reverse primers for one each of *PTEN/MMAC1* exons 1–9 (9), 200 μm of deoxynucleotide triphosphates (Perkin-Elmer Corp., Foster City, CA), 1.25 units of Taq polymerase (AmpliTaq Gold; Perkin-Elmer Corp.), 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, and 0.001% gelatin. PCR amplifications were for 35 cycles: 95°C for 30 s; 55°C for 30 s; and 72°C for 1 min (final extension at 72°C for 10 min) after sample denaturation at 95°C for 9 min. Synthesis of appropriately sized PCR reaction products was confirmed by agarose gel electrophoresis.

Reaction products were examined for heteroduplex content by subjecting 7 μl of each sample containing approximately 100 ng of *PTEN/MMAC1* exon sequence to DHPLC (15, 16). Individual samples displaying heteroduplex signature patterns were sequenced to confirm and identify specific sequence alterations. For sequencing, 10-μl solutions were prepared that contained 10–20 ng of product from the previous PCR reaction; 50 mM Tris-HCl (pH 8.3); 50 mM KCl; 2.5 mM $MgCl_2$; 10% DMSO; 400 μM ddATP; 600 μM ddTTP; 60 μM ddGTP; 200 μM ddCTP; 10 μM each dATP, dTTP, and dCTP; 20 μM 7-deaza-dGTP (Boehringer Mannheim); 0.05 μM [γ-^{32}P]ATP end-labeled sequencing primer; and 0.05 unit of Taq polymerase. Sequencing reactions were carried out for 30 cycles at 95°C for 20 s, 58°C for 30 s, and 72°C for 1 min using a 1-min ramp time between the annealing and elongation phases. After sample denaturation, reaction products were loaded onto a 6% sequencing gel (19:1 acrylamide, 7 M urea, 0.5× Tris-borate EDTA, and 15% formamide). Electrophoresis was at 75 W at room temperature for 1–3 h, after which the gels were dried and exposed to X-ray film.

Microsatellite Analysis. PCR reactions for determination of tumor LOH contained approximately 10 ng of genomic DNA; 8–10 pM forward and reverse primers for either the *D10S541* or *D10S1765* locus (Research Genetics, Huntsville, AL); 0.8 uCi of [α-^{32}P]dCTP; and 0.2–0.35 unit of Taq polymerase in 10–15 μl of buffer containing 200 μM dGTP, dATP, and dTTP; 25–34 μM dCTP; 50 mM KCl; 10 mM Tris-HCl (pH 9.0); 0.1% Triton X-100; and 2.5 mM $MgCl_2$. Samples were placed in 96-well plates, overlaid with mineral oil, and amplified at 95°C denaturation (30 s), 55°C annealing (30 s), and 72°C extension (1 min) for 43 cycles. At completion of PCR, an equal volume of denaturing buffer containing 95% formamide, 10 mM EDTA, 1 mg/ml bromphenol blue, and 0.03% xylene cyanol was added to each reaction. Samples

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[2] To whom requests for reprints should be addressed, at Mayo Clinic and Foundation, 200 First Street, S.W., Hilton Building, Room 820-D, Rochester, MN 55905. Phone: (507) 284-8989; Fax: (507) 266-5193; E-mail: james.charles@mayo.edu.

[3] The abbreviations used are: RTK, receptor tyrosine kinase; LOH, loss of heterozygosity; EGFR, epidermal growth factor receptor; DHPLC, high-performance liquid chromatography under conditions of partial denaturation; dd, dideoxy.

were then heated to 95°C and quenched on ice. Two μl of each sample were applied to 4 or 6% acrylamide sequencing gels containing 8 M urea and 15% formamide and electrophoresed for 1.5–3 h at 75 W. Gels were dried and exposed to Kodak XAR film for 4–48 h.

Determination of PTEN/MMAC1 Dosage in Tumor DNAs. Competitive (multiplex) PCR reactions were carried out in a volume of 50 μl. Reaction conditions were as described above for the preparative PCR reactions used for mutation screening and sequencing, except that amplifications were for 30 cycles. Reaction mixtures contained two sets of primers, one specific for either *PTEN/MMAC1* exon 2, 6, or 7 (9), and the other specific for a reference locus, either STS marker D2S2589 or D14S610 (primers obtained from Research Genetics), or the *CPT1* gene used previously as a reference in the screening of gliomas for $p16^{INK4}$/*CDKN2* homozygous deletions (17). PCR reaction products were resolved in 2.0% agarose gels. After electrophoresis, gels were stained in 0.5 μg/ml ethidium bromide for 30 min at room temperature and then destained in deionized water for 30 min before quantitation of band intensities using the Gel Doc 1000 photodocumentation system (Bio-Rad) and its associated software (Molecular Analyst). Each set of reactions contained a series of samples with varying proportions of normal DNA mixed with DNA from a glioblastoma cell line having a *PTEN/MMAC1* homozygous deletion (SW1088; American Type Culture Collection). The *PTEN/MMAC1* signal intensities associated with the samples in this series were used to generate a standard curve for assessment of *PTEN/MMAC1* content in the tumor DNAs.

Analysis of EGFR Amplification. Tumor DNAs were digested with *Hin*dIII restriction enzyme, and the resulting restriction fragments were resolved by electrophoresis in a 0.8% agarose gel. Conditions for Southern transfer as well as for filter hybridization and filter washing were as described previously (2). After exposure to X-ray film, filters were stripped of *EGFR* probe (probe pE7; American Type Culture Collection) in 0.4 M NaOH at room temperature and rehybridized with reference probes from nonamplified, nonsyntenic loci. Autoradiograms were scanned with an imaging densitometer (GS-700; Bio-Rad), and *EGFR* signal intensities were normalized against corresponding signal intensities from reference probes.

Results

Nine segments of genomic DNA containing one each of the *PTEN/MMAC1* exons were amplified from each of 73 malignant gliomas (63 glioblastomas, 4 grade III astrocytomas, 3 grade III oligoastrocytomas, and 3 grade III oligodendrogliomas). The fragments were screened for the presence of mutations by DHPLC, and this procedure identified 19 PCR products for which there was evidence of heteroduplex formation. Sequencing of these fragments identified alterations that would affect the *PTEN/MMAC1* protein in 17 instances; among these were 2 splice junction, 6 frameshift, and 9 missense mutations

A

MTAIIKEIVS RNKRRYQEDG FDLDLTYIYP NIIAMGFPAE RLEGVYRNNI DDVVRFLDSK

HKNHYKIYNL CAERHYDTAK FNCRVAQYPF EDHNPPQLEL IKPFCEDLDQ WLSEDDNHVA

AIHCKAGKGR TGVMICAYLL HRGKFLKAQE ALDFYGEVRT RDKKGVTIPS QRRYVYYYSY

LLKNHLDYRP VALLFHKMMF ETIPMFSGGT CNPQFVVCQL KVKIYSSNSG PTRREDKFMY

FEFPQPLPVC GDIKVEFFHK QNKMLKKDKM FHFWVNTFFI PGPEETSEKV ENGSLCDQEI

DSICSIERAD NDKEYLVLTL TKNDLDKANK DKANRYFSPN FKVKLYFTKT VEEPSNPEAS

SSTSVTPDVS DNEPDHYRYS DTTDSDPENE PFDEDQHTQI TKV

B

Tumor	LOH	PTEN mutation	Consequence
01	yes	exon 1, base 69, A -> C	Leu -> Phe
02	yes	exon 1, base 70, A insertion	frame shift
03	yes	exon 3 splice donor, T insertion	aberrant splicing
04	yes	exon 5, base 279, T -> G	His -> Gln
05	yes	exon 5, base 302, T -> C	Ile -> Thr
04	NT	exon 5, bases 364-368, deletion,	frame shift
07	NT	exon 5, base 370, deletion,	frame shift
08	yes	exon 5, base 376, G -> A	Ala -> Thr
09	NT	exon 5, base 404, T -> A	Ile -> Lys
10	yes	exon 6, splice acceptor, A -> G	aberrant splicing
11	yes	exon 6, base 509, G -> T	Ser -> Ile
12	yes	exon 6, base 512, A -> C	Gln -> Pro
13	yes	exon 6, base 517, deletion	frame shift
14	yes	exon 7, base 737, C -> T	Pro -> Leu
15	yes	exon 7, base 754, G -> T	Asp -> Tyr
16	no	exon 7, bases 760-764, deletion	frame shift
17	yes	exon 9, base 1121, deletion	frame shift

Fig. 1. Summary of *PTEN/MMAC1* mutations in glioblastomas. *A*, locations of *PTEN/MMAC1* mutations shown with respect to the amino acid sequence. Missense mutations, ●; frameshift mutations, *filled arrows*; splice junction mutations, *open arrows*. The *underlined* portion of the amino acid sequence represents the phosphatase domain (10), and the portion in *bold* represents a highly conserved tyrosine phosphatase motif (9). *B*, cDNA sequence location and consequence of *PTEN/MMAC1* mutations.

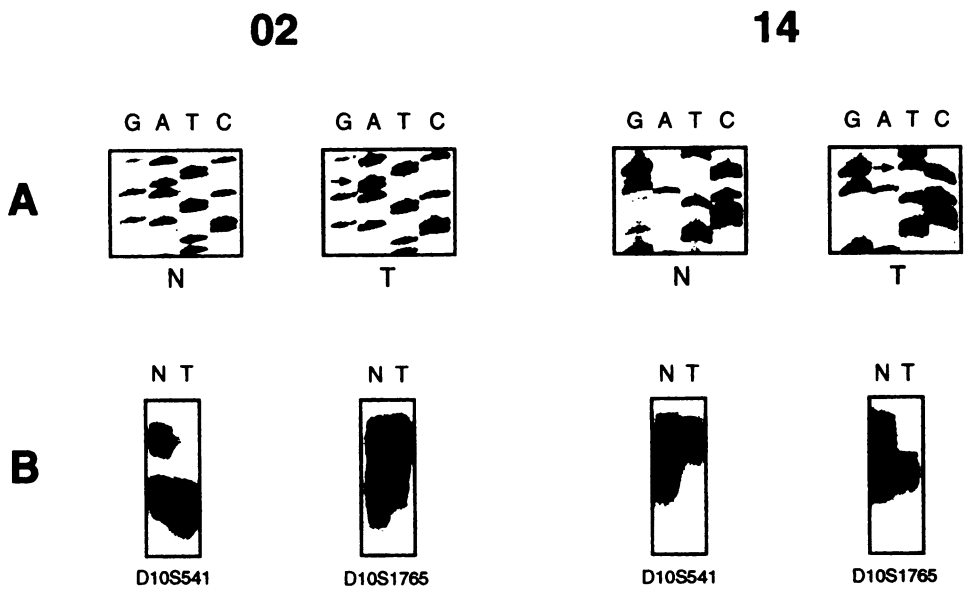

Fig. 2. Mutation and deletion of *PTEN/MMAC1*. *A*, portions of sequencing gels showing the presence of *PTEN/MMAC1* mutations (*arrows*) in tumor (*T*) but not in paired normal (*N*) DNA (*numbers* at the *top* correspond with those used in Fig. 1*B*). *B*, microsatellite analysis showing LOH in the tumor specimens for markers flanking *PTEN/MMAC1*.

(summarized in Fig. 1). Six of the mutations were located in the phosphatase domain of the protein, and three were localized to the highly conserved 12-amino acid tyrosine phosphatase core motif. All mutations were observed among the glioblastomas (grade IV); none were evident among the 10 grade III tumors.

Corresponding normal DNAs were available from 14 of the 17 tumors with *PTEN/MMAC1* mutations, and comparison of sequences for normal-tumor pairs confirmed that all mutations were tumor specific (Fig 2*A* for representative data). Microsatellite analysis with markers near to and flanking *PTEN/MMAC1* was performed on the same 14 cases to identify instances of combined LOH and mutation. Thirteen of the tumors showed LOH with one or both markers, suggesting that a wild-type *PTEN/MMAC1* allele had been deleted in nearly all of the tumors with a *PTEN/MMAC1* mutation (Fig. 2*B*). Thirty-two additional tumors with paired normal DNA samples were examined for LOH in the region of *PTEN/MMAC1*, and of these, loss was evident in 27 cases. Because there were no *PTEN/MMAC1* mutations identified among this subset of gliomas, these data suggest that 10q LOH and *PTEN/MMAC1* mutations may not be linked. Furthermore, because 40 of 46 (87%) tumors subjected to LOH analysis displayed 10q loss, these data also indicate that the majority of tumors in this series were of a clonal origin.

To address the accuracy of the DHPLC method for mutation screening, exons 1 and 5 were sequenced entirely for all 73 tumors. No sequence alterations other than the seven evident through the DHPLC analysis were identified; results that indicate that this method provides a reliable means for detecting mutations. In addition, the fact that the DHPLC screening was able to identify mutations in tumor samples with LOH indicates that this form of analysis is sensitive to the detection of heteroduplex formation even when the ratio of wild-type:mutant sequence is extremely low; this should also be the case for the converse situation (*i.e.*, high wild-type:mutant sequence ratio).

The tumor DNAs were next examined for homozygous deletions of *PTEN/MMAC1* by use of competitive PCR assays. Extent of amplification for exons 2, 6, and 7 was compared against reference segments of DNA, and this analysis identified three specimens with *PTEN/MMAC1* signal dosages that were reduced by more than 90%, relative to normal DNA, for each of the exons examined (representative results shown in Fig. 3).

All tumors were examined for *EGFR* amplification by Southern blot analysis, and this analysis revealed >5× increases in *EGFR* gene dosage for 31 of 63 glioblastomas (49%; data not shown); only 1 of the 10 grade III tumors showed amplification of this gene. Among the 20 glioblastomas having either homozygous deletion or mutation of *PTEN/MMAC1*, there were 8 instances of *EGFR* amplification (40%); 23 of the remaining 43 grade IV tumors (53%) had amplification of this gene (Table 1). These data indicate that no statistically significant difference exists concerning the incidence of *PTEN/MMAC1* alterations in tumors with or without *EGFR* amplification ($P = 0.31$, χ^2 test).

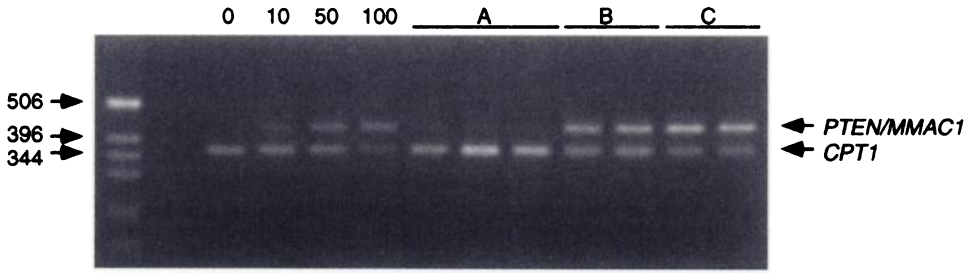

Fig. 3. *PTEN/MMAC1* homozygous deletions. Tumor DNAs were subjected to 30 cycles of amplification in the presence of primer pairs specific for exon 7 of *PTEN/MMAC1* and for a portion of the *CPT1* gene. Reaction products were resolved in a 2.0% agarose gel, stained with ethidium bromide, and photographed using a digital photodocumentation system. Results for samples containing 0, 10, 50, and 100% of normal DNA and 100, 90, 50, and 0% SW1088 DNA (a glioblastoma cell line lacking *PTEN/MMAC1* DNA), respectively, are shown to the *left*. Densitometric analysis of the results for the tumor DNAs in group A indicated homozygous deletion of *PTEN/MMAC1*. Examples indicating hemizygous (*B*) or no (*C*) deletion of *PTEN/MMAC1* are also shown. Marker sizes (in bp) are shown to the *left*.

Table 1 *PTEN and EGFR alterations in glioblastomas*

	PTEN/MMAC1^{m}* or *PTEN/MMAC1*$^{-}$†	*PTEN/MMAC1*^{wt}#
↑ *EGFR*@	8 (40%)	23 (53%)
EGFR$_n$¶	12 (60%)	20 (47%)

* *m*, mutant.
† −, homozygous deletion.
# *wt*, wild type.
@ ↑, amplification.
¶ n, no amplification.

Discussion

From a cancer genetics perspective, the data presented here support a tumor suppressor function for the *PTEN/MMAC1* gene. The observation of *PTEN/MMAC1* mutations in 17 of 63 glioblastomas agrees with the frequency of mutation previously reported (9) in this type of tumor.

In addition to subtle sequence alterations of *PTEN/MMAC1*, data presented in two studies indicate that homozygous deletions of *PTEN/MMAC1* can be detected in approximately 25% of glioblastoma cell lines (9, 10). Because of this, we felt it important to address the occurrence of such deletions in the panel of tumors examined here. Competitive PCR assays revealed three tumor DNAs that are likely to have lost both copies of *PTEN/MMAC1*, indicating a frequency of homozygous deletion of only about 5% in primary glioblastomas. This figure probably represents an underestimate of the incidence of homozygous deletions due to the normal tissue component and genetic heterogeneity of the tumor cells within the specimens from which DNAs were prepared. Additional tumors in this series of glioblastomas may have subpopulations of *PTEN/MMAC1* null cells that are present in proportions that cannot be detected by the assay we have used. The data presented have been interpreted conservatively, and only the DNAs showing less than 10% normalized *PTEN/MMAC1* dosage for all three exons tested were considered to have homozygous deletions.

Direct demonstration of a growth-suppressive effect for the *PTEN/MMAC1* protein has yet to be accomplished. However, the tyrosine phosphatase activity of its associated protein has been confirmed (11), and this activity has promoted speculation regarding its possible opposition to the signaling cascade of RTKs (9). In addition to the consideration of enzymatic activities, support for a biochemical link between *PTEN/MMAC1* and RTK function stems from the observation that *EGFR* amplification is largely restricted to tumors having LOH on chromosome 10, thereby suggesting that inactivation of a tumor suppressor gene on this chromosome must precede amplification of the RTK-encoding oncogene (18, 19). However, a plausible alternative hypothesis would consider the inactivation of *PTEN/MMAC1* and amplification of *EGFR* in the same tumor as redundant and therefore unlikely to occur together. This type of relationship has been shown for the *p53* and *MDM2* genes (20, 21) as well as for the *p16*^{INK4}*/CDKN2*, *RB*, and *CDK4* genes (22, 23). Neither of these possibilities seems to be favored by the data shown here, because there is no apparent association between the status of the *PTEN/MMAC1* and *EGFR* genes in these tumors.

Regardless of the biochemical pathway(s) that involve *PTEN/MMAC1*, data from the cumulative mutational analysis of this gene support the likelihood that its inactivation plays a significant role in glial tumor development. As concerns stage of development, our preliminary analysis of a small number of anaplastic (grade III) gliomas suggest, as did previous chromosome 10 LOH studies (2–8), that this inactivation is strongly associated with the high-grade malignancy characteristic of glioblastoma.

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