Differential Expression of MMAC/PTEN in Glioblastoma Multiforme: Relationship to Localization and Prognosis

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Abstract

MMAC/PTEN, a tumor suppressor gene located on chromosome 10q, has recently been shown to act as a phosphatidylinositol 3,4,5-trisphosphate phosphatase and to modulate cell growth and apoptosis. Somatic mutations of MMAC/PTEN have been reported in a number of human cancers, especially in glioblastoma multiforme (GBM), although the number of identified mutations (~10–35%) is significantly lower than the frequency of LOH affecting the MMAC/PTEN locus in the specimens (~75–95%). To further investigate the possible alterations that may affect MMAC/PTEN, we examined the expression of the gene by reverse transcription-PCR in a series of gliomas. A significant difference (P < 0.001) was observed between the expression of MMAC/PTEN in GBMs versus lower grades of gliomas, thus mimicking the difference in allelic deletion associated with the locus in these tumors. Furthermore, Kaplan-Meier survival plots, adjusted for age and tumor grade, showed a significantly better prognosis for patients whose tumors expressed high levels of MMAC/PTEN. Additionally, immunostaining of GBMs revealed little or no MMAC/PTEN expression in about two-thirds of the tumors, whereas the other approximately one-third of tumors had significantly higher levels of expression. However, in about two-thirds of the high-expressing specimens, a heterogeneous pattern of expression was observed, indicating that certain cells within the tumor failed to express MMAC/PTEN. The combination of these results suggest that, in addition to molecular alterations affecting the gene, altered expression of MMAC/PTEN may play a significant role in the progression of GBM and patient outcome.

Introduction

GBM is the most common and malignant primary neoplasm of the brain. Approximately 10,000 people are diagnosed with GBMs every year in the United States (1). Yet, even with advances in diagnostic procedures, surgical techniques, radiation therapy, and chemotherapy, the prognosis for patients with GBM remains dismal, with the majority of patients succumbing to the disease within a year after diagnosis (1).

A number of studies have been directed at investigating the molecular pathogenesis of GBM (2). The most frequent genetic alterations associated with the generation of GBM are deletions involving either large segments or an entire copy of chromosome 10, along with amplification of epidermal growth factor receptor gene on chromosome 7. Karyotypic analyses revealed that about two-thirds of patients with GBMs have lost an entire chromosome 10, whereas approximately one-third have an increase in the copy number of chromosome 7. Molecular analyses have indicated between 80 to >95% of GBMs have LOH associated with chromosome 10 (2, 3). However, LOH of different regions of chromosome is associated with various grades of tumor (4). Together, these studies suggest that at least two and possibly three or more tumor suppressor genes reside on chromosome 10 and that at least one locus, MMAC/PTEN, is associated with the progression of GBMs (4). Several candidate tumor suppressor genes have been cloned on chromosome 10q due to the identification of homozygous deletions at loci for MMAC/PTEN at 10q23.3 and DMBT1 at 10q26 (5–7). MMAC/PTEN has been the focus of a number of studies, because it is the first tumor suppressor gene to have homology to a protein tyrosine phosphatase and actually has activity of a dual-specificity phosphatase (8). Although, recently MMAC/PTEN has been shown to have activity against phosphatidylinositol phosphates, suggesting that MMAC/PTEN may exert its biochemical effects as a mediator of the phosphatidylinositol (PI3') kinase pathway (9).

Meanwhile, evidence has been rapidly accumulating confirming the classification of MMAC/PTEN as a tumor suppressor gene. Expression of MMAC/PTEN in cancer cells devoid of a functional gene product has been shown to inhibit cellular growth and the tumorigenic capabilities of cells (10). Furthermore, somatic mutations of MMAC/PTEN have been observed in a number of different cancers and cell lines including malignant melanomas, endometrial and prostate carcinomas, and brain tumors (7, 8). Germ-line mutations have also been found in several hereditary cancer predisposition syndromes including Cowden’s syndrome, Bannayan-Zonana syndrome, and juvenile polypsis syndrome (11, 12). One potential caveat, however, is that although a significant number of mutations have been identified, the mutations occur in only 5–40% of the various tumor specimens, with allelic deletions affecting the locus. For GBMs, about 75–95% of the specimens exhibit LOH at MMAC/PTEN, but only about 10–30% of the samples have been shown to have mutations affecting the gene (13). In the present study, we analyzed the expression of MMAC/PTEN using a semiquantitative RT-PCR assay and immunohistochemical analyses to determine whether altered expression of MMAC/PTEN may also contribute to the oncogenesis of GBM.

Materials and Methods

Brain Tumor Specimens. All specimens were obtained from patients who underwent therapeutic procedures at The University of Texas M. D. Anderson Cancer Center. Sections of all samples were histologically evaluated by board-certified neuropathologists and classified according to the modified Ringeretz grading system. In 15 cases, surrounding tissue, verified as normal brain, was also obtained at the time of surgery. Specimens were snap-frozen in liquid nitrogen and stored at -80°C. Purity of tumor was estimated in H&E staining and stored at 80°C. Purity of tumor was estimated in H&E staining and contained >70% neoplastic cells. For immunohistochemical study, samples...
were either obtained from frozen sections or samples fixed in 10% formalin and embedded in paraffin.

**RT-PCR Assay of MMAC/PTEN Expression.** mRNAs were isolated from 10 frozen sections of each tumor using the Micro-fast track isolation kit and converted to cDNA with the use of random primers and reverse transcriptase (Invitrogen, San Diego, CA). The relative quality and quantity of mRNA/cDNA were assessed by examining the expression of enolase, and then the relative expression of MMAC/PTEN was depicted as a ratio of MMAC/PTEN to enolase. PCR primer pairs for MMAC/PTEN were designed to amplify a region from 117 to 741 bp (F1-CAGAAAGACTTGAAGGCTAT and B1-AACGGCTGAGGGAACTC), which also contained a NsiI restriction site in the MMAC/PTEN pseudo-gene (13), but not in the 10q gene. The primers designed to amplify α-enolase comprised a region from 77 to 532 bp of the coding region of the gene (EF1-TGGCAGGATGACTTCAGA and EB1-AGTGCGCTGAAGTTCACC). The PCR mixture contained 100 ng of cDNA as template in 20 mM of Tris-HCl (pH 8.0), 50 mM of KCl, 1.5 mM MgCl2, 0.2 mM of each deoxynucleotide triphosphate, 1 µl of each primer, and 1 unit of Taq polymerase (Roche Molecular System, Branchburg, NJ). PCR was carried out as follows. An initial denaturation of 3 min at 94°C was followed by 40 cycles of 45 s at 94°C, 45 s at 54°C, and 1 min at 72°C, followed by 10 min of final elongation at 72°C. The PCR products were treated with NsiI and then separated on a 2% agarose gel. Images of the gel were captured by a Gel-Doc 1000 system and analyzed using Molecular Analyst software (version 2.1, Bio-Rad, Hercules, CA). Semiquantitative expression of MMAC/PTEN was determined as a ratio of MMAC/PTEN to enolase expression.

![Fig. 1. Illustration of relative MMAC/PTEN RT-PCR expression.](image)
Immunohistochemical Study of MMAC/PTEN Expression. Rabbit antiserum was generated against a glutathione S-transferase fusion protein corresponding to amino acids 202–305 of the MMAC/PTEN gene product. Frozen sections were fixed in acetone or paraffin-embedded sections were deparaffinized and then incubated in goat serum for 30 min prior to application of protein A purified IgG anti-MMAC/PTEN (1:1000), followed by incubation overnight at 4°C. The primary antibody was visualized using the Vectastain ABC Elite kit (Vector Laboratories, Burlingame, CA), followed by counterstaining with Mayer’s hematoxylin. Incubation of the glutathione S-transferase-MMAC/PTEN fusion protein with the antiserum resulted in an inhibition of specific binding to the protein product on immunoblots and tissue sections. Western blotting of cell extracts expressing MMAC/PTEN with the antiserum revealed only a single band of Mr ~49,000.

Prognostic Data. Prognostic data of each patient were collected from The Brain Tumor Center Database at University of Texas M. D. Anderson Cancer Center. Survival times were calculated from the date of surgery. All of the patients were basically treated with similar regimens, which included surgery followed by radiation therapy and/or chemotherapy. The correlation between MMAC/PTEN expression ratio and survival data of each patient was analyzed using GraphPad Prism 2.01 software (GraphPad Software; www.Graphpad.com) and Kaplan-Meier survival curves were generated with Cox-Mantel log-rank test. SAS software (SAS Institute, Cary, NC) was also applied to calculate Cox proportional hazards regression analysis.

Results

The expression of MMAC/PTEN was evaluated in 135 brain specimens by RT-PCR and compared with the expression of enolase, a constitutively but low expressed gene product. Of the 135 samples, 78 were diagnosed as GBMs, 42 were diagnosed as lower grade gliomas, which included anaplastic astrocytomas and oligodendrogliomas. Fifteen specimens were from surrounding tissue, adjacent to the tumor, and histologically verified as normal brain. Of the 120 glioma specimens, 36 of 42 (84%) were known to have LOH affecting the MMAC/PTEN loci. About 9% of the GBMs with LOH (3 of 34) were known to have mutations affecting MMAC/PTEN (13), including frameshift and nonsense mutations resulting in a truncated protein product and one missense mutation. Semiquantitative RT-PCR analysis showed that the relative expression of MMAC/PTEN in GBMs was significantly reduced compared to the expression in the normal tissues and lower grade non-GBMs (Fig. 1a). The relative mean expression of MMAC/PTEN to enolase in GBMs was 0.30 ± 0.16 compared with 0.44 ± 0.18 for lower grade non-GBMs. Histologically diagnosed normal brain tissues revealed a relative expression of 0.52 ± 0.16. As depicted in Fig. 1b, there was a heterogeneous relative expression of MMAC/PTEN in the various tissues. However, overall the relative RT-PCR expression of MMAC/PTEN in GBMs was significantly reduced compared with that observed in lower grade gliomas and normal brain tissues (P > 0.001). Mutations in MMAC/PTEN did not appear to significantly alter the reduced expression because two specimens with the observed mutations exhibited a moderately reduced level of expression (0.298 and 0.233), and the missense mutation had a significantly reduced relative expression level (0.075).

The expression of MMAC/PTEN in 42 cases of GBMs and 8 cases of lower grade glioma was also evaluated by immunohistochemical analysis. Representative cases illustrating expression of MMAC/PTEN are shown in Fig. 2. Vascular endothelial cells and neurons were immunoreactive with anti-MMAC/PTEN antibody and served as an internal positive control for this study (Fig. 2A). Interestingly, nuclear or perinuclear staining was observed in a number of cells including neurons and endothelial cells, although MMAC/PTEN has been reported to have cytoplasmic localization (10). About two-thirds of GBM cases, 29 of 42 (69%), revealed little or no staining of the majority of tumor cells (Fig. 2B). The other approximate one-third of
GBM cases (13 of 42; 31%) revealed strongly positive staining with anti-MMAC/PTEN for at least part of the specimen (Fig. 2, C and D). Two distinct patterns of positive staining, however, were observed in this group. In one pattern, representing 5–10% of the GBMs (3 of 42), revealed that all of the tumor cells were stained for MMAC/PTEN (Fig. 2C). In the other pattern, differential staining of the tumor was revealed that all of the tumor cells were stained for MMAC/PTEN this group. In one pattern, representing 5–10% of the GBMs (3 of 42),

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MMAC/PTEN expression as determined by RT-PCR are shown. The

fourth quartile of patients survived significantly longer than the other three quartiles of patients. b, the survival of all evaluable glioma patients when divided in high (approximately normal level of expression) and low expression of MMAC/PTEN. The differences in the survival curves were statistically significant (log-rank test, hazard ratio, 3.3; 95% CI, 1.6–4.3; P = 0.0003). Similar statistically significant results were obtained if only GBM patients were analyzed.

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Of the 120 tumor cases, 89 were gliomas that were eligible to be included in a survival analysis. For this set of patients, GBM was the diagnosis for 61 cases (~70%), and lower grade gliomas in the rest. Four quartiles were initially created to analyze the potential association between the variables because about one-quarter to one-third of the specimens appeared to express MMAC/PTEN. Specimens from patients with high expression of MMAC/PTEN represented by a fourth quartile exhibited a significant increase in survival when compared with the other three quartiles. No significant differences in survival among patients the other three quartiles were observed. From this preliminary analysis, Kaplan-Meier survival curves were generated and revealed that patients with a low level of relative MMAC/PTEN expression assessed by RT-PCR (<0.46) had significantly shorter survival than patients with a high level (>0.46) of relative expression (hazard ratio, 3.3; 95% CI, 1.6–4.3; P = 0.0003; Fig. 3). Multivariate analysis showed that, after adjustment for age and histological grade, relative MMAC/PTEN expression was still associated with patient survival time (hazard ratio, 2.4, 95% CI, 1.4–4.6; P = 0.02). Therefore, even within the various grades of tumor, patients with either GBM or lower grade gliomas, which had relatively high expression of MMAC/PTEN, exhibited a better prognosis than those with low level of expression of the gene product. However, the number of cases available for the lower grade tumors was limited and needs additional verification.

Discussion

In this study, we examined 135 surgical specimens of brain for the expression of MMAC/PTEN by RT-PCR and observed that the expression of MMAC/PTEN was significantly suppressed in GBMs when compared with the lower grade gliomas. This observation was initially demonstrated by RT-PCR and supported by immunohistochemical analysis. Use of a cutoff point (<0.46), which reflects the level of MMAC/PTEN expression in normal brain tissue and differential survival, would imply that ~80% of the GBMs and 40% of the lower grade gliomas would be considered as having low expression. The RT-PCR and immunostaining analyses also showed that normal cells within the tumor and some tumor cells express MMAC/PTEN, and thus a considerable heterogeneity of expression levels is found. However, survival analyses showed that patients with tumors that express increased or normal levels of MMAC/PTEN have a better prognosis. Additionally, the number of tumors with low expression closely paralleled the frequency for lack of immunostaining of MMAC/PTEN seen in about 70% of the GBMs and 25% of the lower grade gliomas. Furthermore, for the ~30% of GBMs specimens that exhibited strong immunostaining for MMAC/PTEN, about two-thirds of those cases had regions that failed to stain. This would suggest that the majority of GBMs (>85%) have tumor cells that do not express MMAC/PTEN, a percentage that closely parallels the observed frequency of allelic deletion affecting the MMAC/PTEN locus. The relative frequency of tumors with low expression as assessed by this analysis would agree with our recent results that demonstrated a significantly poorer prognosis for glioma patients who have allelic deletion of MMAC/PTEN (~75%) than for those with an intact locus, irrespective of tumor grade (4). The combination of these results imply that the alteration of MMAC/PTEN is important in glioma patient prognosis; thus, that it may be intimately involved in the generation of the malignant nature of GBMs.

Similar to the results described herein, decreased expression of MMAC/PTEN was reported previously in a series of 10 prostate cancer xenografts and their several cell lines. Whang et al. (14) found only one mutation of MMAC/PTEN in 10 prostate xenografts examined; nevertheless, they observed a reduced or absent expression of MMAC/PTEN for the vast majority of tumors. Furthermore, they found that the treatment of one xenograph with 5-azodeoxycytidine restored the expression of MMAC/PTEN, suggesting that methylation of a promoter may affect MMAC/PTEN expression.

The lack of observed MMAC/PTEN expression may be accounted for by several different mechanisms. One possibility is altered methylation of the transcriptional regulatory region of the gene. This is known to occur for several tumor suppressor genes (14, 15), although direct evidence for this has been lacking for MMAC/PTEN. Another possibility is that the MMAC/PTEN gene may be targeted for homozy-
gous deletion. In support of this possibility, we have shown previously that the frequency of homozygous deletion in cancer cell lines is significantly higher than in tumor specimens, suggesting that homozygous deletions may be underrecognized in some tumor specimens (13). However, due to the large number of tumors with decreased MMAC/PTEN expression, this possibility would likely account for only a fraction of the cases. A third possibility arises from a study of Li et al. (16), who described the identification of TEP1 (MMAC/PTEN) based on its similarity to dual specificity phosphatases. In their study, they also observed that TEP1 (MMAC/PTEN) expression was regulated in part by transforming growth factor β. Because transforming growth factor β and its altered signaling pathway has been implicated in a number of human cancers, the possible relationship between these two interesting pathways needs to be further examined.

Yet, regardless of the mechanism(s) involved in down-regulation of MMAC/PTEN or tumor suppressor genes in general, the lack of expression appears to serve as an excellent means to achieving loss of function.

In this study, we also observed specific nuclear along with cytoplasmic localization of MMAC/PTEN in both normal and neoplastic cells of the tissue sections. Although this represents a novel observation with potentially interesting consequences, clearly more definitive studies need to be performed to confirm this result, because differences in antisera and technical procedures may affect this observation.

MMAC/PTEN is altered in a number of human cancers, including malignant melanomas, breast, prostate, and uterine carcinomas, and especially in GBMs (5, 6, 15, 17–20). With the exception of endometrial carcinoma, alterations to MMAC/PTEN have been observed to primarily occur in the malignant or high-grade forms of primary brain tumors as well as in malignant melanomas, meningiomas, and prostate carcinomas (5, 17–20). This would implicate MMAC/PTEN in tumor progression in most cancers examined. Although additional studies are clearly needed to address the potential roles of MMAC/PTEN in tumor progression, our present study also showed that patients with tumors expressing low levels of MMAC/PTEN had a significantly poorer prognosis than those with tumors expressing higher levels. This correlation was found to be significant even within the different grades of glioma. The relationship between MMAC/PTEN alterations and patient prognosis gives rise to a number of interesting possibilities worthy of further study.

References

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