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ORIGINAL PAPER

Investigation of *MMAC/PTEN* Gene Mutations and Protein Expression in Low Grade Gliomas

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Abstract The *MMAC/PTEN* tumor suppressor gene has an essential biological role in the formation of glioblastomas. It is known that there are variations in genetic alterations in tumors that develop in patients with different ethnic backgrounds; thus, we aimed to evaluate the incidence of *MMAC/PTEN* mutations and protein expression among various low grade gliomas of Turkish patients. We investigated 28 low grade gliomas for mutations of the *MMAC/PTEN* gene using single strand conformational polymorphism method followed by DNA sequencing. Additionally, the level of MMAC/PTEN protein expression in the tissues of 26 tumors was assessed by immunohistochemistry. In our investigation, *MMAC/PTEN* mutations were detected in 2 of 28 tumors (7.14%). One novel sequence variant G → A transition at codon 159 was identified. This missense variation was a result of an alteration from AGG (Arginine) to AAG (Lysine). Moreover, it was observed that MMAC/PTEN protein expression was reduced to 73.08% of tumors. In conclusion, reduced MMAC/PTEN expression by genetic and/or epigenetic mechanisms in low grade gliomas might be associated with glioma tumorigenesis.

Keywords Low grade gliomas · MMAC/PTEN · SSCP · Sequencing · IHC · Turkish population

Abbreviations

SSCP	Single strand conformational polymorphism
PCR	Polymerase chain reaction
WHO	World Health Organization
IHC	Immunohistochemistry
GBM	Glioblastome multiforme
GFAP	Glial fibrillary acidic protein

Introduction

Brain tumors are the ninth most common malignancy in Turkey (representing 3.33% of all neoplasms). Their incidence is 2.34/100.000 (http://www.saglik.gov.tr/istatistikler). More than 30% of central nervous system tumors are gliomas. The World Health Organization (WHO) classification scheme divides these tumors into four grades (Kleiheus and Cavenee 2000). Grade I and II are the least malignant phenotypes. The survival of patients with gliomas is concerned with WHO tumor grade (Zhou et al. 2003). Molecular genetic analysis has added useful information regarding the characteristic pattern of aberrations for the different histological subtypes. The tumorigenesis of gliomas has been associated with several tumor suppressor genes including *MMAC/PTEN* (phosphatase, tensin homologue, deleted on chromosome10; MIM no. 601728) gene (Zhou et al. 1999). *MMAC/PTEN* encodes a major

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lipid phosphatase which signals down the phosphoinositol-3-kinase/Akt pathway and effects G1 cell cycle arrest and apoptosis (Eng 2003). Germ-line mutations in the tumor suppressor gene *MMAC/PTEN* mediate cell cycle arrest and apoptosis (Eng 2003). The alteration in the expression of *MMAC/PTEN* is correlated with the progression of gliomas (Zhou et al. 2003). Expression of MMAC/PTEN has been shown to correlate with better prognosis (Sano et al. 1999). To date, loss level of MMAC/PTEN expression has been analyzed with immunohistochemistry (IHC) analysis in low grade gliomas by two unrelated groups (Sano et al. 1999; Yakut et al. 2007). Number of cases evaluated in these two studies is very limited. So, their results on MMAC/PTEN expression in low grade gliomas have shown difference with each other. In this study, we analyzed the expression of MMAC/PTEN in larger series of low grade gliomas than two previous studies to designate the influence lacking MMAC/PTEN expression with real percentage. Furthermore, we want to determine whether sequence alterations have an effect on the loss of MMAC/PTEN protein expression in these type of gliomas in Turkish patients.

Materials and Methods

Patients

A total of 28 patients who were scheduled for surgical treatment, fulfilling all inclusion criteria as assessed by conventional histopathological examination, were evaluated prospectively. Approved form the Institutional Review Board was obtained, and written informed consent of the patients was provided. Clinical and histopathological

Table 1 Characterizations of patients

Case no	Age (years)	Sex	Histo-pathology	Grade	GFAP	KI 67 (‰)	Tumor location	*MMAC/PTEN* mutation	IHC analysis
1	37	F	Mix oligoastrocytoma	II	+	10	Right temporal	–	2
2	53	M	Oligodendroglioma	II	–	30	Left temporal	–	2
3	26	F	Ependimoma	II	+	15	Right lateral ventricle	159 G → A	3
4	18	M	Pilocytic astrocytoma	II	NS	20	Cerebellum	–	2
5	61	M	Oligodendroglioma	II	NS	High	Right temporal	–	1
6	24	M	Astrocytoma	II	NS	2	Right frontotemporal	–	3
7	33	M	Astrocytoma	II	NS	138	Left occipital	159 G → A	1
8	42	M	Oligodendroglioma	II	–	25	Left temporal	–	3
9	23	M	Astrocytoma	II	+	2	Left frontal	–	1
10	25	F	Oligodendroglioma	II	+	20	Right parietooccipital	–	2
11	43	F	Oligodendroglioma	II	–	135	Left temporal	–	1
12	17	M	Astrocytoma	II	NS	2	Cerebellar vermis	–	1
13	19	F	Oligodendroglioma	II	–	8	Temporobasal	–	3
14	40	M	Mix oligoastrocytoma	II	+	140	Left frontal	–	2
15	42	F	Astrocytoma	II	+	5	Right frontal	–	1
16	19	M	Ependimoma	II	+	680	Right oxipital	–	2
17	46	M	Oligodendroglioma	II	NS	2	Right temporal	–	1
18	73	M	Oligodendroglioma	II	+	1	Left temporal	–	3
19	29	M	Astrocytoma	II	+	27	Left frontal	–	2
20	15	M	Astrocytoma	II	+	1–2	Right temporal	–	NM
21	16	F	Astrocytoma	II	+	2	Left occipital	–	1
22	55	M	Oligodendroglioma	II	NS	15	Left talamus	–	2
23	64	F	Gliosarcoma	II	+	2	Left parietal	–	2
24	64	M	Oligodendroglioma	II	–	300	Right frontal	–	2
25	42	F	Oligodendroglioma	II	+	60	Left frontal	–	3
26	42	M	Oligodendroglioma	II	–	27	Left frontal	–	2
27	46	F	Oligodendroglioma	II	–	10	Right frontal	–	NM
28	31	M	Oligodendroglioma	II	–	2–5	Left parietal	–	3

The expression rate of MMAC/PTEN protein; *1* highly reduced, *2* reduced, *3* expression, *nm* not materialized, *NS* non stained

features of the tumors and oncological outcome were also evaluated with regard to the presence of *MMAC/PTEN* mutations in the tumors. The expression of glial fibrillary acidic protein (GFAP) was also determined. The patients were followed up for at least 24 months or until death (Table 1).

Mutation Detection

Genomic DNA was extracted using standard methods from each tissue sample taken from the tumors at the time of surgery (Tunca et al. 2007). Exons 1–9 of *MMAC/PTEN* gene were subjected to polymerase chain reaction (PCR) analysis as previously described (Tunca et al. 2007). The high-quality amplified products were assessed with single strand conformational polymorphism (SSCP) analysis (Tunca et al. 2007). Samples that showed one or two bands separated from the wild-type bands were identified as SSCP positive. All the samples that contained aberrant migration patterns during gel electrophoresis were subjected to the SSCP analysis procedure at least twice to rule out contamination.

Sequencing Analysis

All samples with different SSCP bands were sequenced using the BigDye Terminator Chemistry (Applied Biosystems, Foster City, CA) and the *MMAC/PTEN* primers which was used PCR and was analyzed using an automated ABI Prism 310 Genetic Analyzer (Applied Biosystems). Results of sequencing analysis were compared with wild-type samples and normal sequencing of *MMAC/PTEN* gene (MIM no. 601728, Gen Bank accession no. U93051).

Predicting Deleterious Amino Acid Substitutions

SIFT web-based program was used to calculates the probabilities of having an amino acid at a specific position which was relative to the most frequent amino acid at that position (http://blocks.fhcrc.org/sift/SIFT.html).

Protein Expression

The level of MMAC/PTEN protein expression has been determined by IHC. Formalin-fixed paraffin-embedded tissues were available from 26 patients. IHC staining, using mouse monoclonal antiserum to human MMAC/PTEN (clone17.A, Thermo Scientific), was performed as described before (Guler et al. 2005). Briefly, after antigen retrieval in 1 M sodium citrate (pH 6.0) in pressure cooker for 3 min, primary antibody was applied at 1/25 dilution for 30 min, and detection was conducted with strepavidin–biotin complex using the UltraTek HRP Anti-Polyvalent Lab Pack (ScyTek Laboratories, USA).

One section from each tumor was stained and evaluated for extent and intensity of staining. Because of the heterogeneous staining of some tumors, both staining intensity and extent of staining in neoplastic cells were taken into account in expression level scoring. Intensity was graded as strong or reduced expression. Extent of staining was classified as the fraction of stained neoplastic cells: >25, 25–50, and >50%. Cases with strong expression in more than half of the neoplastic cells were scored as high expression; cases with reduced staining intensity but in more than 50% of tumor cells or strong staining in 25–50% of neoplastic cells were scored as reduced expression; cases with reduced expression in 50% of neoplastic cells or strong staining in <25% of neoplastic cells were scored as highly reduced expression. The vascular endothelial cells in each section was used as internal positive control of staining, an endometrial adenocarcinoma section with adjacent normal endometrium was used as external positive control, and a negative control was also included.

Results

We investigated sequence alterations of the *MMAC/PTEN* gene and protein expression in low grade gliomas. The age range of the patients was 15–73 years (mean age ± SEM, 37.32 ± 3.11 years). In total, there were 18 male and 10 female patients.

The presence of *MMAC/PTEN* mutations was detected in the tumors of two patients (2/28; 7.14%) by SSCP. Mutation was clustered in exon 5. As a result of sequencing

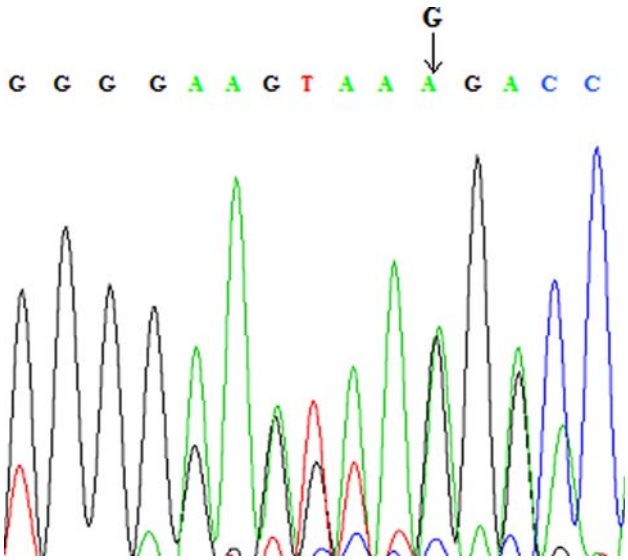

Fig. 1 Novel sequence alterations in *MMAC/PTEN* gene; 159 G→A

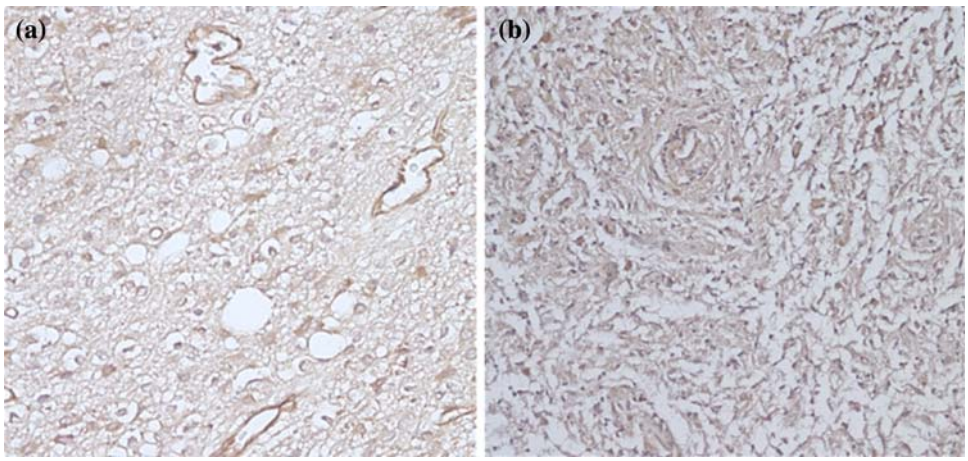

Fig. 2 **a** Reduced of MMAC/PTEN expression; **b** highly reduced of MMAC/PTEN expression

analysis for SSCP-positive samples, one novel gene alteration was identified. It was G → A transition at codon 159. This missense variation was a result of an caused to alteration from AGG (Arginine) to AAG (Lysine; Table 1; Fig. 1).

Predicting Deleterious Amino Acid Substitutions

As a result of analysis by using SIFT program, we observed that mutation from AGG (Arginine) to AAG (Lysine) at codon 159 was not tolerated.

Protein Expression

In IHC analysis, only seven (26.92%) tumors showed strong MMAC/PTEN protein expression. In 11 tumors (42.31%), there was reduced MMAC/PTEN expression, and in eight (30.77%) tumors highly reduced MMAC/PTEN expression was detected (Fig. 2). Totally, reduced MMAC/PTEN expression was observed in 73.08% of all tumors. MMAC/PTEN protein expression was assessed by IHC for two tumors exhibiting *MMAC/PTEN* mutation; the level of MMAC/PTEN protein expression was highly reduced in only one.

Discussion

Molecular studies indicate that the variation in expression of MMAC/PTEN which has important roles in the regulation of cell proliferation, apoptosis, and tumor invasion is related to the progression of gliomas (Zhou et al. 2003; Ohgaki et al. 2004). *MMAC/PTEN* gene alterations are important in glioma formation (Tunca et al. 2007) and patient prognosis; thus, that it may be intimately involved in the generation of the malignant nature of the glioblastoma multiforme (GBM) (Sano et al. 1999). In addition, it was determined that MMAC/PTEN is also an important prognostic variable in astrocytomas and may be helpful in their routine pathologic evaluation (Kelley et al. 2005). Expression levels of MMAC/PTEN protein can be evaluated by IHC methods. As a matter of fact, in clinical practice, it was shown that evaluation of MMAC/PTEN is accomplished by examining diffuse gliomas for chromosomal imbalances using comparative genomic hybridization and IHC (Yakut et al. 2007). It was also observed that the loss of expression of MMAC/PTEN by IHC may predict a subset of patients with reduced survival (Sano et al. 1999). In our study, 73.08% of low grade gliomas showed reduced levels of MMAC/PTEN protein expression by IHC analysis. This result demonstrates that the loss of MMAC/PTEN protein is significant for low grade glioma development as for other tumor types.

Both genetic and epigenetic mechanisms have a role in loss of protein expression. So, in this study, we want to determine whether sequence alterations have an effect on the loss of MMAC/PTEN protein expression in low grade glioma. Furthermore, the frequencies and types of mutations may vary in tumors of patients in the different populations (Fukushima et al. 2006). As a matter of fact, in our previous study, we determined eight novel mutations in Turkish GBM patients (Tunca et al. 2007). These results support this hypothesis. For this aim, we evaluated 28 patients with low grade gliomas and found a novel mutation in 7.14% of the patients (2/28). Therefore, it is shown that genetic mechanisms also have an effect on the loss of MMAC/PTEN protein expression in low grade gliomas.

In gliomas, *MMAC/PTEN* mutations are most often detected in GBM which is the most frequent and malignant human brain tumors and only occasionally in less malignant gliomas, this situation indicates that *MMAC/PTEN* alterations appear as a late event also in tumorigenesis of gliomas (Zhou et al. 1999; Daumas-Duport et al. 1997; Wang et al. 1997; Maier et al. 1998; Duerr et al. 1998). In our previously study on GBM, *MMAC/PTEN* mutations were detected in 15 of 62 tumors (24.19%) (Tunca et al.

2007). The findings of that study and results of this study on low grade gliomas indicate that the effects and frequencies of *MMAC/PTEN* mutations on glioma oncogenesis in Turkish population are similar to other populations in literature (Tunca et al. 2007).

We determined one novel alteration localized at codon 159 in exon 5 of *MMAC/PTEN* gene. This codon localized in the N-terminal phosphatase domain which has an important biological role in lipid and protein phosphatase activity of MMAC/PTEN protein. This domain is defined by the first 185 amino acids (exon 1–6) of *MMAC/PTEN* gene (Eng 2003). Specially, exon 5 encodes amino acids which are items of CKAGKGR phosphatase catalytic core motive of N-terminal phosphatase domain of MMAC/PTEN. In the literature, ~30% of all germ-line and somatic mutations have been determined in this catalytic core. In vitro studies showed that *MMAC/PTEN* mutations occurred in the catalytic core motive caused loss of lipid phosphatase activity (Eng 2003). Our missense mutation was a result of the cause to variation of amino acid configuration in N-terminal phosphatase domain. Despite the fact that the core motive have an essential role in phosphatase activity (Furnari et al. 1997), to carry out this activity, it needs the whole part of N-terminal domain (Eng 2003). In addition, we used web-based tool SIFT to calculate the probabilities of having an amino acid at a specific position relative to the most frequent amino acid at that position. A cutoff for these probabilities is used to classify the mutations as tolerated and nontolerated (Mathe et al. 2006). As a result of this analysis, it was determined that mutation from AGG (Arginine) to AAG (Lysine) at codon 159 was not tolerated. This situation is shown that this mutation may have an important role in the function of MMAC/PTEN protein and glioma tumorigenesis. Furthermore, novel of this alteration supports the suggestion that there is variability in mutations underlying the gliomas of patients in different populations (Fukushima et al. 2006). Therefore, this mutation may be specific for Turkish population. In addition, our findings in glioma patients revealed 7.14% of the *MMAC/PTEN* gene mutation and added new knowledge about *MMAC/PTEN* gene mutation types and frequencies.

In this study, we reported decreased expression of MMAC/PTEN in a series of 28 low grade gliomas. We found only one *MMAC/PTEN* mutation in this group; nevertheless, we observed a reduced or absent expression of MMAC/PTEN for the vast majority of tumors. Similar to the results described herein, decreased expression of MMAC/PTEN was reported in prostate cancer xenographs and their several cell lines (Whang et al. 1998). Furthermore, they found that the treatment of one xenograph with 5-azodeoxcytidine restored the expression of MMAC/PTEN, suggesting that methylation of a promoter may affect MMAC/PTEN expression. We also suggest that epigenetic mechanisms may affect MMAC/PTEN protein expression.

In conclusion, this study reveals new and important information regarding reduced level of MMAC/PTEN protein expression by genetic and/or epigenetic mechanisms in glioma formation. We believe that these types of studies will contribute to clarify the biological mechanisms of glioma tumorigenesis.

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