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Promoter methylation of the DNA repair gene *MGMT* in astrocytomas is frequently associated with G:C → A:T mutations of the *TP53* tumor suppressor gene

Mitsutoshi Nakamura[1], Takao Watanabe[1], Yasuhiro Yonekawa[2], Paul Kleihues[1] and Hiroko Ohgaki[1,3]

[1]International Agency for Research on Cancer, F-69372 Lyon, France and [2]Department of Neurosurgery, University Hospital Zürich, CH-8091 Zürich, Switzerland

[3]To whom correspondence should be addressed
Email: ohgaki@iarc.fr

***O*[6]-Methylguanine-DNA methyltransferase (MGMT) is a repair protein that specifically removes promutagenic alkyl groups from the *O*[6] position of guanine in DNA. Repair of *O*[6]-alkylguanine adducts by tumour cells has been implicated in drug resistance since it reduces the cytotoxicity of alkylating chemotherapeutic agents. We assessed promoter methylation of the *MGMT* gene in astrocytic brain tumours by methylation-specific PCR. *MGMT* promoter methylation was detected in 26 of 54 (48%) low-grade diffuse astrocytomas (WHO grade II) and in 12 of 16 (75%) of secondary glioblastomas (WHO grade IV) that had progressed from low-grade astrocytomas. The frequency of *MGMT* methylation was significantly lower in primary (*de novo)* glioblastomas (13 of 36, 36%, *P* = 0.0155). The majority of low-grade astrocytomas with *MGMT* methylation (24/26, 92%) contained a *TP53* mutation, whereas only 11 out of 28 (39%) cases without *MGMT* methylation carried a *TP53* mutation (*P* < 0.0001). Furthermore, G:C → A:T transition mutations at CpG sites were significantly more frequent in low-grade astrocytomas with *MGMT* methylation (15/26, 58%) than in those without (3/28, 11%, *P* = 0.0004). These results suggest that loss of *MGMT* expression as a result of promoter methylation, which frequently occurs at an early stage in the pathway leading to secondary glioblastomas, appears to be associated with increased frequency of *TP53* mutations, in particular G:C → A:T transitions.**

Introduction

Glioblastoma (WHO grade IV) is the most frequent and malignant human brain tumour. Despite progress in surgery and adjuvant therapy, glioblastoma patients still have a very poor prognosis because of incomplete resection and resistance to radio- and chemotherapy (1). Glioblastomas may develop after a short clinical history and without clinical or histopathological evidence of a less malignant precursor lesion (primary or *de novo* glioblastoma), or more slowly through progression from low-grade (WHO grade II) or anaplastic (WHO grade III) astrocytoma (secondary glioblastoma) (1,2). Recent studies have shown that these glioblastoma subtypes develop through different genetic pathways (2–7) . Primary glioblastomas occur in older patients and are characterized by *EGFR* amplification/overexpression, *PTEN* mutation, homozygous $p16^{INK4a}$ deletion, and LOH on the entire chromosome 10 (2,5–8), while secondary glioblastomas develop in younger patients and typically show frequent *TP53* mutations, LOH on chromosomes 19q and 10q (2,5,8–10), and promoter methylation of the *RB1* gene (11).

O[6]-Methylguanine-DNA methyltransferase (MGMT) is a DNA repair protein that directly and specifically removes promutagenic DNA lesions at the *O*[6] position of guanine by transferring the alkyl group to one of its own cysteine residues, without base excision (12). Intracellular levels of MGMT vary among tissues and appear to be inversely correlated with tissue-specific tumorigenesis induced by alkylating agents in rats (12,14).

MGMT activity varies significantly between tumors, over an ~300-fold range (15). Approximately 20–25% of human tumours lack detectable MGMT activity and carry the methyl repair-deficient (Mer–) phenotype (15,16). Neoplastic cell lines with the Mer– phenotype have been found to show methylation of CpG sequences in the promoter of the *MGMT* gene; methylation was not detected in MGMT-expressing cells (12,17–19). *MGMT* methylation has been found in ~40% of gliomas and colorectal carcinomas, less frequently (~25%) in non-small cell lung carcinomas, lymphomas and head and neck carcinomas, and rarely in non-glial brain tumours, breast cancer, endometrial cancer and leukemias (20).

The objective of the present study was to compare the frequency of *MGMT* methylation in primary glioblastomas with that in low-grade astrocytomas and secondary glioblastomas derived from them. We further correlated *MGMT* methylation with the frequency and pattern of *TP53* mutations.

Materials and methods

Tumor samples and DNA extraction

The surgical specimens of brain tumours were obtained from patients treated between 1979 and 1997 in the Department of Neurosurgery, University Hospital of Zürich, Switzerland. Tumors were fixed in buffered formalin and embedded in paraffin. Pathological diagnosis was made according to the WHO classification (21).

Thirty-six patients with primary glioblastoma (20 males, 16 females, mean age 56.8 ± 11.2 years, range 34–73 years) had a preoperative clinical history of <3 months (mean, 1.5 months) and a histological diagnosis of glioblastoma at the first biopsy, without any evidence of a less malignant precursor lesion. Sixteen patients with secondary glioblastoma (eight males, eight females, mean age 39.7 ± 8.8 years, range 26–53 years) had at least two biopsies, with clinical and histological evidence of progression from low-grade diffuse astrocytoma (WHO grade II). Low-grade diffuse astrocytomas (15 cases) from the same patients with secondary glioblastomas and 39 additional low-grade diffuse astrocytomas (31 males, 23 females, mean age 36.3 ± 10.5 years, range 22–62 years) were also examined. DNA was extracted from typical tumor areas that were manually microdissected from paraffin sections as described previously (22).

Peritumoral normal brain tissue was available in sections of 11 low-grade astrocytomas and was also carefully microdissected manually and analysed.

Methylation-specific PCR for MGMT methylation

DNA methylation in the CpG islands of the *MGMT* gene was determined by methylation-specific PCR (23). Sodium bisulfite modification was performed

Abbreviations: BCNU, 1,3-bis-(2-chloroethyl)-1-nitrosourea; CCNU, 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea; MGMT, *O*[6]-Methylguanine-DNA methyltransferase.

Table I. Correlation between *MGMT* promoter methylation and *TP53* mutations in astrocytic brain tumours

Tumor	Methylation status	All cases	Percent of cases with *TP53* mutation		
			G:C → A:T at CpG	G:C → A:T not at CpG	All *TP53* mutations
Low-grade astrocytoma	Methylated	26/54 (48%)	15/26 (58%)*	3/26 (12%)	24/26 (92%)**
	Unmethylated	28/54 (52%)	3/28 (11%)	1/28 (4%)	11/28 (39%)
Secondary glioblastoma	Methylated	12/16 (75%)	6/12 (50%)	0/12 (0%)	11/12 (92%)***
	Unmethylated	4/16 (25%)	1/4 (25%)	0/4 (0%)	1/4 (25%)
Primary glioblastoma	Methylated	13/36 (36%)	0/13 (0%)	0/13 (0%)	1/13 (8%)
	Unmethylated	23/36 (64%)	2/23 (9%)	0/23 (0%)	5/23 (22%)

Significantly different between methylated and unmethylated cases (*$P = 0.0004$, **$P < 0.0001$, ***$P < 0.03$).

using the CpGenome™ DNA Modification Kit (Intergen, Oxford, UK) as described previously (24). Control methylated DNA (Intergen) and unmethylated DNA (normal blood) were treated with bisulfite by the same method. Primer sequences for the methylated and unmethylated reactions have been reported by Esteller *et al.* (20,25). Sense primers were located at the 3′ end of exon 1 and antisense primers were at the 5′ end of intron 1, the region where the presence of an enhancer element has been reported (17). PCR was carried out in a 10 µl volume containing PCR buffer (20 mM Tris pH 8.4, 50 mM KCl), 2 mM $MgCl_2$, dNTPs (250 mM each), primers (0.5 mM each), 0.5 U PLATINUM® *Taq* DNA polymerase (Gibco-BRL, Cergy Pontoise, France) and ~40 ng bisulfite-modified DNA. Amplification was carried out in a Robocycler (Stratagene) with initial denaturing at 95°C for 5 min followed by 35 cycles of denaturing at 95°C for 50 s, annealing for 50 s at 59°C and extension for 50 s at 72°C, and then a final extension for 2 min at 72°C. Amplified products were electrophoresed on a 3% agarose gel, and were visualized by ethidium bromide. For each PCR reaction, we included methylated and unmethylated DNA, and normal blood DNA without bisulfite modification, as positive and negative controls.

TP53 mutations

TP53 mutations in all glioblastomas and most low-grade diffuse astrocytomas except for nine cases were reported previously (5,8,9). For these nine cases, pre-screening for mutations by PCR–SSCP analysis was carried out in exons 5–8 of the *TP53* gene. Sequencing primers used were as follows: 5′-TCT GTC TCC TTC CTC TTC CTA C-3′ (sense) and 5′-AAC CAG CCC TGT CGT CTC TCC A-3′ (antisense) for exon 5; 5′-CTG GGG CTG GAG AGA CGA CA-3′ (sense) and 5′-GCC ACT GAC AAC CAC CCT TA-3′ (antisense) for exon 6; 5′-TGC CAC AGG TCT CCC CAA GG-3′ (sense) and 5′-GGG TCA GAG GCA AGC AGA GG-3′ (antisense) for exon 7; 5′-TCC TTA CTG CCT CTT GCT TC-3′ (sense) and 5′-TCT CCT CCA CCG CTT CTT GT-3′ (antisense) for exon 8. Samples which showed mobility shifts in SSCP analysis were further analyzed by DNA sequencing. After PCR amplification with the same set of primers, PCR products were sequenced on a Genetic Analyzer (ABI PRISM™ 310, Perkin-Elmer Biosystems) using ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kits (Perkin-Elmer Applied Biosystems).

Results

Frequency of MGMT methylation

MGMT promoter methylation was detected in 26 of 54 (48%) low-grade astrocytomas and in 12 of 16 (75%) secondary glioblastomas that had progressed from low-grade astrocytomas (Table I, Figure 1). In five out of the 12 cases of secondary glioblastomas with MGMT methylation, the preceding low-grade astrocytoma in the same patient also showed methylation, whereas in the remaining seven cases, promoter methylation was only observed after progression to glioblastoma. The frequency of MGMT methylation was significantly lower in primary (*de novo*) glioblastomas (13 of 36, 36%, $P = 0.0155$, Table I) than in secondary glioblastomas.

We assessed 11 samples of normal brain tissue (five of which were adjacent to low-grade astrocytomas with *MGMT* methylation), but none showed *MGMT* methylation.

Correlation between MGMT methylation and TP53 mutations

The majority of low-grade astrocytomas with *MGMT* methylation (24/26, 92%) contained a *TP53* mutation, whereas only

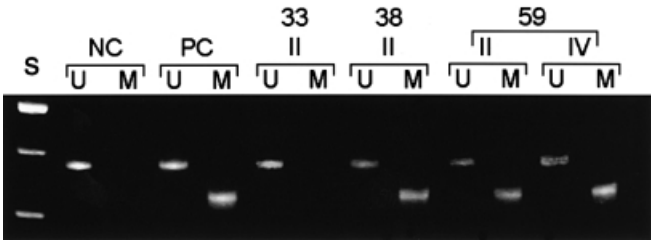

Fig. 1. Methylation-specific PCR of *MGMT* promoter in low-grade astrocytomas (II) and a glioblastoma (IV). In low-grade astrocytoma from patient 33, only unmethylated base is present, while in a tumour from patient 38, both methylated and unmethylated bases are observed. In patient 59 with two biopsies for low-grade astrocytoma and glioblastoma, methylated bases are detected already in low-grade astrocytoma. S, molecular size marker; U, PCR product amplified by unmethylated-specific primers. M, PCR product amplified by methylated-specific primers. PC, positive control for unmethylated and methylated DNA; NC, negative control (DNA from normal blood sample).

11 out of 28 (39%) low-grade astrocytomas without *MGMT* methylation contained a *TP53* mutation (Table I, $P < 0.0001$). G:C → A:T transition mutations at CpG sites were significantly more frequent in low-grade astrocytomas with *MGMT* methylation (15/26, 58%) than in those without *MGMT* methylation (3/28, 11%: $P < 0.0004$, Table I). In secondary glioblastomas, *TP53* mutations were significantly more frequent in cases with *MGMT* methylation (92%) than in those without (25%; $P = 0.027$). In primary glioblastomas, the difference was not significant, probably because this glioblastoma subtype rarely contains *TP53* mutations.

Correlation between MGMT methylation and age of patients

There was no significant difference in age of patients with low-grade astrocytomas between those with and without *MGMT* methylation. The patients with glioblastomas with *MGMT* methylation were significantly younger (47 ± 13 years) than those without *MGMT* methylation (55 ± 13 years, $P = 0.0267$).

Discussion

The present study shows that *MGMT* promoter methylation is frequent (75%) in secondary glioblastomas. Low-grade astrocytomas, the less malignant precursor lesions, also showed *MGMT* methylation in 48% of cases. Methylation-specific PCR only indicates whether or not specific cytosine residues in the MGMT promoter are methylated, but does not directly reveal loss of gene expression. However, methylation identified with the primers used in this study has been shown to be associated with loss of MGMT protein expression in a variety of human tumours including gliomas, in contrast to retention of protein expression in the majority of tumours lacking

methylation (20). Thus, our results strongly suggest that loss of expression of the *MGMT* gene by promoter methylation is frequent in the pathway leading to secondary glioblastomas.

We have also shown that *MGMT* methylation is often associated with a *TP53* mutation in secondary glioblastomas and low-grade astrocytomas. Except for two cases, all low-grade astrocytomas with *MGMT* methylation contained a *TP53* mutation, the majority of which were G:C → A:T transition mutations (18/26, 69%) and of these, most were at CpG sites (15/18, 83%). In contrast, only 11 out of 28 (39%) low-grade astrocytomas lacking *MGMT* methylation contained a *TP53* mutation; in these cases, G:C → A:T mutations were also less frequent (14%). For comparison, the frequency of G:C → A:T *TP53* mutations in 749 human brain tumours listed in the IARC *TP53* database is 52%, of which 66% are located at CpG sites (www.iarc.fr/p53/index.html); this frequency is lower than that of our cases with *MGMT* methylation.

A similar correlation has been reported for colon carcinomas (25), for which a strong association was found between *MGMT* methylation and the presence of G → A mutations in the K-*ras* gene: 71% (36/51) of the tumours with a G → A mutation had *MGMT* methylation, whereas only 32% (12/37) of those with other K-*ras* mutations (not involving G → A transitions) and 35% (55/156) of the tumours without K-*ras* mutations had *MGMT* methylation (25). These and our results suggest that epigenetic silencing of MGMT by promoter methylation may lead to preferential occurrence of G:C → A:T transitions in transformation-associated genes in human neoplasms. It is of interest that spontaneous G:C → A:T transitions are detected more frequently in the adenine phosphoribosyl transferase gene of Chinese hamster ovary cells lacking MGMT activity (28%) than in those expressing MGMT (5%) (26).

The aetiology of human brain tumours is still largely unknown. With the exception of the very rare causation by therapeutic irradiation (22), epidemiological studies have failed to unequivocally identify environmental carcinogens that operate in the evolution of gliomas. Several endogenous pathways may lead to G:C → A:T transitions at CpG sites, which are the most frequently observed *TP53* mutations in human brain tumours (27). The best-characterized underlying mechanism is the deamination to thymine of 5-methylcytosine that is clustered at CpG sites. This is considered to occur spontaneously or to be factor-mediated, e.g. through the action of oxygen radicals or by nitric oxide produced by nitric oxide synthase in conditions of chronic inflammation (28). The possibility exists that the loss of MGMT-mediated repair due to methylation causes accumulation of G:C → A:T mutations resulting from O^6-alkylG → T mispairing. However, analyses of tumour samples do not allow unequivocal distinction between the two major underlying mechanisms, i.e. C → T transitions in the transcribed strand of the gene resulting from either cytosine mutation (following deamination) or from mutation of the paired guanine on the complementary strand due to O^6-alkylguanine mispairing with T.

It has been shown that O^6-methylation of the guanine moiety at CpG islands is not efficiently repaired by MGMT if normal 5-methylcytosine is present in the *TP53* sequence (27). This raises the possibility that *TP53* mutations at CpG sites are not a result of deamination of 5-methylcytosine alone. They may, in addition, result from endogenous or exogenous factors that produce DNA adducts at the O^6 position of guanine. A great variety of adducts at this position have been shown to be substrates for repair by MGMT (12). Such adducts typically result from exposure to *N*-nitrosamides and related alkylating agents that cause brain tumours in rats (29) but there is no evidence that these carcinogens are involved in the aetiology of human brain tumours.

If *MGMT* methylation is directly associated with preferential occurrence of *TP53* mutations, one would expect that normal tissue surrounding tumours might also show *MGMT* methylation. Lack of MGMT activity (Mer– status) has been found in ~60% of histologically normal brain samples adjacent to a primary brain tumour (15,16,30). The incidence of Mer– status in non-neoplastic cerebral tissue from brain tumour patients was age-dependent, increasing from 21% in children (<19 years) to 75% in adults over 50 (30). In contrast, the Mer– status was found in only 12% of normal brain specimens from patients operated for conditions other than primary brain tumours, and was not age-dependent (30). In the present study, we assessed 11 normal brain tissue samples (five of which were from an area adjacent to a low-grade astrocytoma with *MGMT* methylation), but no single case showed *MGMT* methylation.

One possibility would be that *MGMT* methylation occurs after the development of low-grade astrocytomas but *before* acquisition of a *TP53* mutation. *TP53* mutations constitute an early event in the pathway leading to secondary glioblastomas; >65% of low-grade astrocytomas contain *TP53* mutations (5,9,31) and this fraction does not increase during malignant progression. In a clonal assay, it was shown that in low-grade astrocytomas, the fraction of mutated cells is low (8–21%) and increases during progression, reaching values of >95% in glioblastomas (32), suggesting that *TP53* mutation is first present in a small fraction of tumour cells which, because of their growth advantage, then gradually expand during progression (32,33). However, it cannot be excluded that the lower fraction of mutated cells in low-grade astrocytomas is a result of contamination with non-neoplastic reactive astrocytes.

As the present results are based on DNA from biopsies rather than cultured cells, identification of *MGMT* methylation and *TP53* mutation does not necessarily imply that both events occurred in the same tumour cell. In fact, methylation was more frequent in secondary glioblastomas than in low-grade astrocytomas from which they were derived.

The activity of MGMT can be modified in several ways. O^6-benzylguanine efficiently inactivates the MGMT protein, thereby increasing the chemotherapeutic effectiveness of methylating and chloroethylating agents *in vitro* and in human tumour xenograft models (34). Other factors that may affect levels and activity of MGMT include polymorphisms in the *MGMT* gene, exposure to formaldehyde, presence of metal ions and the extent of DNA depurination (12,35).

In an analysis of 152 adult gliomas, MGMT activity was inversely correlated with the age of patients (16). In the present study, the mean age of patients with low-grade astrocytoma with *MGMT* methylation was similar to that of patients with tumours lacking methylation. The glioblastoma patients with *MGMT* methylation were younger than those without *MGMT* methylation, but this is likely to reflect the younger age of patients with secondary glioblastoma compared to primary glioblastoma (5).

O^6-Alkylguanine adducts are produced by cytostatic monomethylating agents (procarbazine, temozolomide, dacarbazine), bifunctional chloroethylnitrosoureas that are used for the

treatment of gliomas including 1,3-bis-(2-chloroethyl)-1-nitrosourea (BCNU) and 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea (CCNU), and related cancer therapeutic drugs (36). Brain tumours expressing low levels of MGMT have been found to be more sensitive to chemotherapy, and the therapeutic efficacy was enhanced by depletion of MGMT by O^6-benzylguanine *in vitro* and in patients (12,34,37). Patients with malignant gliomas expressing high levels of MGMT had a shorter time to treatment failure and death following radiation plus adjuvant chemotherapy with BCNU (38). Furthermore, the frequency of the Mer– phenotype among tumours recurring after surgery, radiation and alkylating agent-based chemotherapy was 7-fold lower than in tumours treated with surgery alone and 6-fold lower than in tumours recurring after surgery and radiation (15). Recently, Esteller *et al.* (39) assessed *MGMT* promoter methylation in anaplastic astrocytomas and glioblastomas that were subsequently treated with BCNU, and showed that *MGMT* promoter methylation was significantly associated with longer overall survival and time till progression.

The present study shows that *MGMT* methylation is significantly more frequent in secondary glioblastomas that progressed from low-grade astrocytomas than in primary glioblastomas (75 versus 36%). It is still a matter of controversy whether the prognosis of patients with secondary glioblastoma is better than (40) or similar to (41) that of patients with primary (*de novo*) glioblastomas. It thus remains to be shown whether susceptibility to chemotherapy differs between primary and secondary glioblastomas and whether this correlates with *MGMT* methylation.

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