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Original Article

O^6-methylguanine methyltransferase promoter methylation status of glioblastoma cell line clonal population

Mitsuhiro Anan,[1] Rolando Fausto Del Maestro,[2] Nobuhiro Hata[1] and Minoru Fujiki[1]

Department of [1]Neurosurgery, Oita University Faculty of Medicine, Oita, Japan and [2]Neurosurgical Simulation and Artificial Intelligence Learning Centre, Montreal Neurological Institute and Hospital, McGill University, Montreal, Quebec, Canada

Glioblastoma (GBM) remains a treatment-resistant malignant brain tumor in large part because of its genetic heterogeneity and epigenetic plasticity. In this study, we investigated the epigenetic heterogeneity of GBM by evaluating the methylation status of the O^6-methylguanine methyltransferase (MGMT) promoter in individual clones of a single cell derived from GBM cell lines. The U251 and U373 GBM cell lines, from the Brain Tumour Research Centre of the Montreal Neurological Institute, were used for the experiments. To evaluate the methylation status of the MGMT promoter, pyrosequencing and methylation-specific PCR (MSP) were used. Moreover, mRNA and protein expression levels of MGMT in the individual GBM clones were evaluated. The HeLa cell line, which hyper-expresses MGMT, was used as control. A total of 12 U251 and 12 U373 clones were isolated. The methylation status of 83 of 97 CpG sites in the MGMT promoter were evaluated by pyrosequencing, and 11 methylated CpG sites and 13 unmethylated CpG sites were evaluated by MSP. The methylation status by pyrosequencing was relatively high at CpG sites 3–8, 20–35, and 7–83, in both the U251 and U373 clones. Neither MGMT mRNA nor protein was detected in any clone. These findings demonstrate tumor heterogeneity among individual clones derived from a single GBM cell. MGMT expression may be regulated, not only by methylation of the MGMT promoter but by other factors as well. Further studies are needed to clarify the mechanisms underlying the epigenetic heterogeneity and plasticity of GBM.

Correspondence: Mitsuhiro Anan, MD, PhD, Department of Neurosurgery, Oita University Faculty of Medicine, 1-1 Idaigaoka Hasama-machi Yufu, Oita, Japan. Email: annmitsu@oita-u.ac.jp

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INTRODUCTION

Glioblastoma (GBM) has been a major focus of basic and clinical research in neuro-oncology; however, because of its phenotypic and genetic diversity, effective therapies are still lacking. The first choice for multimodal treatment of GBM is the combination of conventional radiation therapy with the alkylating agent temozolomide (TMZ).[1] O^6-methylguanine methyltransferase (MGMT) is a cellular DNA repair protein that rapidly reverses alkylation, including methylation, at the O^6 position of guanine, thereby neutralizing the cytotoxic effects of alkylating agents such as TMZ.[2,3] The combined effect of radiation and TMZ on GBM patients depends on the methylation status of the MGMT promoter region.[1] Recently, intratumor heterogeneity in GBM has been reported to affect long-term survival, even in patients given treatment such as radiation therapy and chemotherapy.[4,5] Tumors are not single-cell populations but diverse populations of cells with different morphologies and functions, and the degree of methylation of the MGMT promoter varies considerably.[6,7] In this study, we evaluated the MGMT promoter methylation status of isolated single cells from GBM cell lines (U251 and U373) by pyrosequencing and methylation-specific PCR (MSP). In addition, mRNA and protein expression levels of MGMT in isolated single tumor cell clones were evaluated using reverse transcription-PCR (RT-PCR) and Western blotting.

MATERIALS AND METHODS

Isolation of single cells from glioblastoma cell lines

The GBM cell lines U251 and U373, cryopreserved in the Brain Tumour Research Centre at the Montreal

Neurological Institute, were used. U251 and U373 cells were cultured and collected in a 10-cm plate at first passage, defined as wild type (WT). Then, U251 and U373 cells were treated with 2 mM EDTA-PBS buffer, separated into single-cell clones, and plated onto 96-well plates. Individual clones were transferred and cultured in 15-cm plates until confluent.

Evaluation of O^6-methylguanine methyltransferase promoter methylation using pyrosequencing and methylation-specific PCR

DNA from the U251 and U373 clones was purified using All Prep DNA/RNA kits (Qiagen, Toronto, ON, Canada). The methylation status of 83 CpG sites (from bp −549 to +121; transcription initiation site +1 bp) out of 97 CpG sites in the MGMT promoter region[3,8] were evaluated using pyrosequencing (PyroMark Q24, Qiagen). Eighty-three CpG sites were analyzed in eight fragments for convenience (Fig. 1). Before pyrosequencing and MSP, the collected DNA was bisulfite-converted using the EZ DNA Methylation-Gold kit (Zymo Research, Irvine, CA, USA). The HeLa cell line, which highly expresses MGMT, was used as a positive control for MGMT. At the same time, 11 methylated CpG sites and 13 unmethylated CpG sites were evaluated by MSP. The primers used for pyrosequencing and MSP are shown in Table 1.

Evaluation of O^6-methylguanine methyltransferase mRNA and protein levels

Total RNA from the U251 and U373 clones was purified using All Prep DNA/RNA kits (Qiagen), and proteins were extracted using RIPA buffer. Expression levels of MGMT mRNA were analyzed by RT-PCR. Primer used for RT-PCR are shown in Table 1. The protein extracts were used to detect MGMT by Western blotting. The HeLa cell line was used as a positive control for both RT-PCR and Western blotting. For Western blotting, a mouse monoclonal anti-MGMT antibody (clone MT3.1/MAB16200; Millipore, Temecula, CA, USA; 1:500) was used.

RESULTS

Evaluation of O^6-methylguanine methyltransferase promoter methylation using pyrosequencing and methylation-specific PCR

A total of 12 clones each from the U251 and U373 cell lines were isolated, and the methylation status of CpG sites in the MGMT promoter were evaluated by pyrosequencing and MSP. The heat map shows the percent methylation at CpG sites, ranging from 0% (yellow) to 100% (black) (Fig. 2). Clones are arranged on the vertical axis according to percent methylation, as assessed by MSP. The horizontal axis shows CpG sites 1–83 evaluated by pyrosequencing. The upper rows show WT U251 and U373. The lowest row shows the HeLa cell line as control. The percent methylation by MSP was 59% for U251 WT, 53% for U373 WT, and 0.2% for HeLa. The percent methylation by MSP was between 7.9% and 100% (median 69.2%) for U251 clones and between 42.0% and 65.4% (median 61.0%) for U373 clones. The methylation status by pyrosequencing was relatively high at CpG sites 3–8, 20–35, and 70–83 in both the U251 and U373 clones.

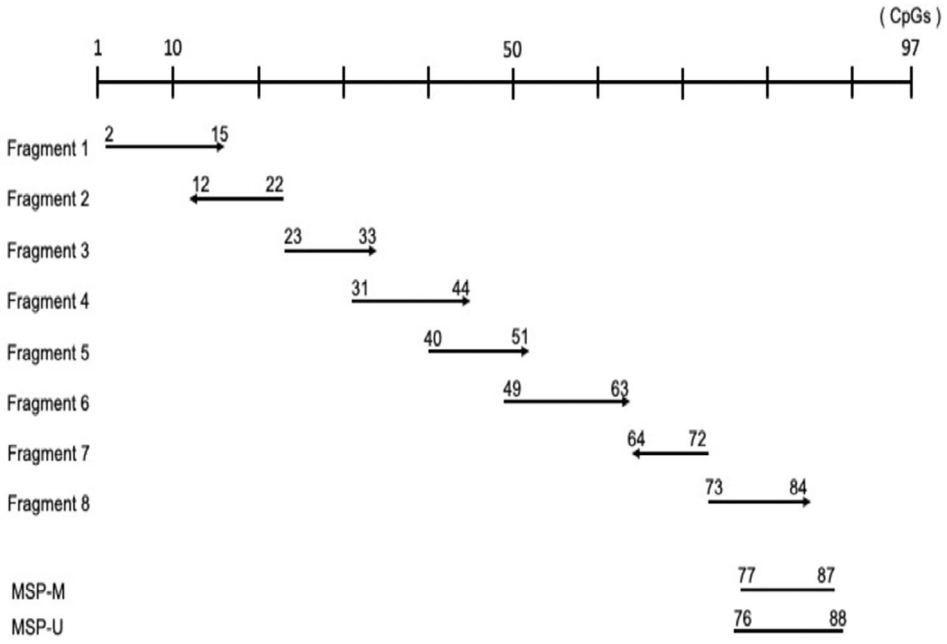

Fig 1 CpG sites in the MGMT promoter. A total of 83 CpG sites (2–84) were divided into eight fragments and analyzed by pyrosequencing. The arrow indicates the direction of detection of nucleotides by pyrosequencing. The 11 methylated CpG sites (77–87) and 13 unmethylated CpG sites (76–88 CpG sites) were analyzed by MSP. MGMT, O^6-methylguanine methyltransferase; MSP, methylation-specific PCR.

Table 1 Primers for pyrosequencing, methylation specific PCR, and RT-PCR

	Pyrosequencing		
	Forward	Reverse	Sequencing
Fragment 1	TGGGGGTTTTTGATTAGGGG	/5Biosg/CCAACTTCCCCCTAAAACTCTATACCTTAATT	TTAGGAGGGGAGAGAT
Fragment 2	/5Biosg/TGGGGGTTTTTGATTAGGGG	CCCAACTTCCCCCTAAAACTCTATACCTTAA	AAAACTCTATACCTTAATTTA
Fragment 3	GGATTATTTTTGTGATAGGAAAAGG	/5Biosg/CCTAAAACAATCTACCCATCCTC	GGTTATTTGCTAAATTAAGGTATAGAGT
Fragment 4	GGATTATTTTTGTGATAGGAAAAGG	/5Biosg/AACTATCCCAACATATCC	GGGTTAGGAGTATAGGGTAG
Fragment 5	GGATTATTTTTGTGATAGGAAAAGG	/5Biosg/AACTATCCCAACATATCC	GAGGATGAGTAGATTGT
Fragment 6	GGATTATTTTTGTGATAGGAAAAGG	/5Biosg/AACTATCCCAACATATCC	GGTAGGGAGGGAATATTT
Fragment 7	/5Biosg/AGAGTTTTAGGAGGAAGTTGGGAAG	ACCCAAACACTCACCAAAT	CTAAAAACCCCAACTATCCCAACATATC
Fragment 8	AGTTTTAGGAGGAAGTTGGGAAGG	/5Biosg/ACCCAAACACTCACCAAAT	GTTTAGGATATGTTGGGATA

	Methylation specific PCR	
	Forward	Reverse
Methylated	TTTCGACGTTCGTAGGTTTTCGC	GCACTCTTCCGAAAACGAAACG
Unmethylated	TTTGTGTTTTGATGTTTGTAGGTTTTTGT	AACTCCACACTCTTCCAAAAACAAAACA

	RT-PCR	
	Forward	Reverse
	GCTGATGCCGTGGAGGTCCCA	TGCAGACCACTCTGTGGCACG

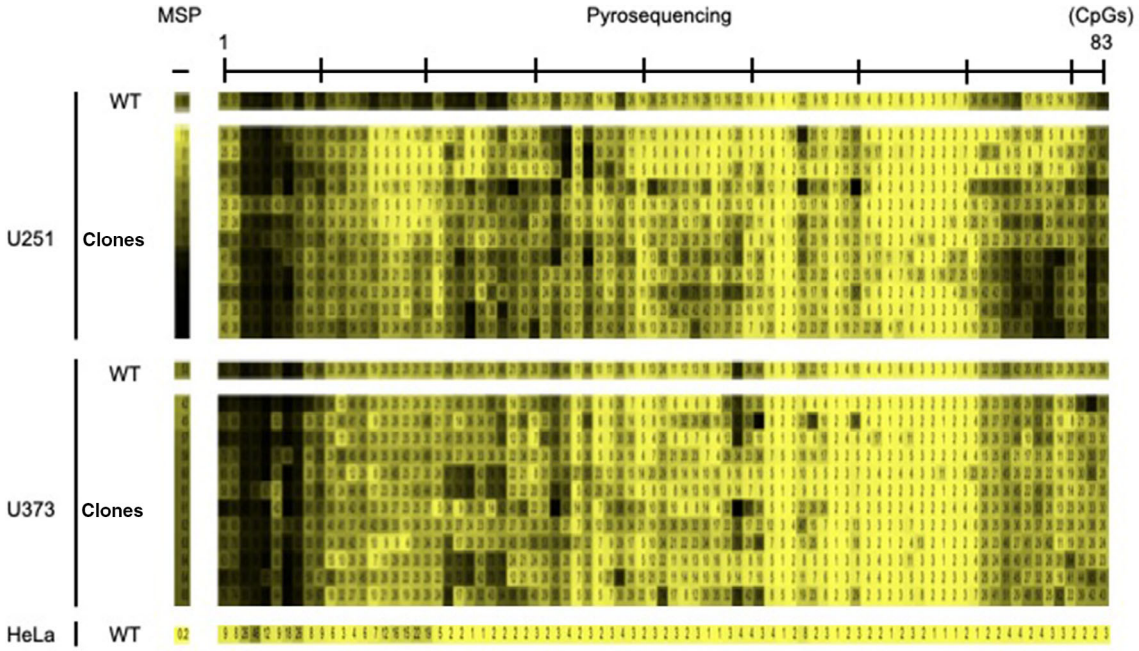

Fig 2 Heatmap of the methylation status of CpG sites in the MGMT promoter of U251, U373, and HeLa cells, respectively. U251 and U373 clones are arranged in the order of methylation status of MSP. The degree of methylation is indicated from 0% (yellow) to 100% (black).

Evaluation of O^6-methylguanine methyltransferase mRNA expression

RT-PCR was used to assess the expression of MGMT mRNA (Supplementary Figs. 1 and 2). MGMT mRNA was not expressed in the U251 WT nor the isolated U251 clones. In comparison, MGMT mRNA was detected in the U373 WT but not the U373 clones.

Assessment of O^6-methylguanine methyltransferase expression

Western blotting was used to assess the expression of MGMT protein (Supplementary Figs. 3 and 4). In U251 and U373, MGMT protein was not expressed in the WT or each clone.

DISCUSSION

Tumor tissue has been known to consist of morphologically different cell populations; more recently, it has been shown that these cell populations vary in function as well. There are two main models of tumor heterogeneity: the stochastic model and the hierarchy model. In the stochastic model, tumor cells are biologically equivalent, but their behavior is influenced by intrinsic and extrinsic factors. In the hierarchy model, only a subset of cells has the ability for tumor initiation.[9–11] Intratumor heterogeneity in GBM has been reported to reduce remission rates even in patients given treatment such as radiation therapy and chemotherapy.[4,5,12–14] Here, to investigate epigenetic heterogeneity in GBM, we isolated single-cell clones from the GBM cell lines U251 and U373 to evaluate MGMT promoter methylation. The isolated clones varied in methylation status of CpG sites in the promoter, as demonstrated by MSP and pyrosequencing. Although MSP is a simple and frequently used procedure, it can only show the methylation status of around 10 CpG sites. In comparison, with the pyrosequencing technique we used, it was possible to evaluate methylation at each of the 83 CpG sites. Moreover, even the same clone showed completely different methylation percentages among individual CpG sites. This finding is interesting and suggests that even individual cell lines are composed of diverse cell populations.

The MGMT protein functions to repair DNA damage caused by alkylating agents, and therefore, the methylation status of the MGMT promoter impacts the effectiveness of radio/chemotherapy and clinical outcome.[2] The methylation status of the promoter has been shown to affect MGMT expression. A study found positive MGMT expression, as detected by immunohistochemistry, in 29.5% of 44 patients with MGMT promoter methylation, as detected by MSP, and negative expression in 44.8% of 29 patients with unmethylated promoters, in a series of 73 patients.[15] The investigators found that MGMT protein expression was highly heterogeneous, underscoring the utility of combining MRS with immunohistochemical staining to evaluate epigenetic regulation in tumors.

Another study demonstrated that 73% of gliomas with methylated MGMT promoters had substantial numbers of MGMT-positive tumor cells,[16] and vice versa. In the present study, MGMT mRNA was not detected in U251 cells but was expressed in the U373 WT (Supplementary Figs. 1 and 2). This suggests that the processes required for protein expression, such as transcription and translation, are not only regulated by promoter methylation, but by other factors as well. In general, the methylation of DNA alters its topology and binding to histone proteins, thereby inhibiting the binding of transcription factors and resulting in transcriptional repression. This form of epigenetic regulation has been shown to play a key role in the pathogenesis of a growing number of human diseases.[17–19] In the current study, the methylation status of CpG sites varied greatly among the 83 CpG sites tested by pyrosequencing. For example, CpG sites 60–80, often examined in previous reports by MSP,[2,20] 3–8 and 20–35 were hypermethylated. In contrast, sites 40–70 were hypomethylated (Fig. 2). In future studies, by focusing on the transcription factors that specifically bind to these regions, it may be possible to elucidate the mechanisms regulating MGMT expression.

A limitation of this study is the absence of detectable MGMT mRNA and protein expression. We did not use a method to alter the methylation of CpG sites in the MGMT promoter in each clone. If the methylation of each clone can be reduced to the same level as in HeLa cells, which strongly express MGMT, they should start expressing the enzyme. Another strategy to overcome this limitation is to evaluate methylation of CpG sites in the MGMT promoter in clones of MGMT hyper-expressing cell lines, such as HeLa and Daoy (medulloblastoma cell line). If HeLa or Daoy clones that do not express MGMT appear, we can identify the CpG sites in the MGMT promoter that are strongly involved in regulating MGMT expression. Another limitation of this study is that neither function nor intratumor heterogeneity was examined.

In conclusion, to investigate epigenetic heterogeneity in tumors, including tumor cell lines, pyrosequencing of isolated clones is useful for evaluating in detail the methylation status of CpG sites in regions such as the MGMT promoter. This approach, using currently available methods such as pyrosequencing and MSP, as well as newer analysis methods, may help advance the precision treatment of GBM and other cancers.

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DISCLOSURE

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

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SUPPORTING INFORMATION

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Supplementary Fig S1. Results of RT-PCR for MGMT mRNA (upper) and beta-actin mRNA (lower) in U251 cell line clones. Percentage of MGMT promoter methylation by MSP for each clone is shown (%). Arrow indicates the MGMT band. Three clones were not used for pyrosequencing assay (asterisk). M, DNA ladder marker; N, negative control; WT, wild type.

Supplementary Fig S2. Results of RT-PCR for mRNAs for MGMT (upper) and beta-actin (lower) in U373 cell line clones. Percentage of MGMT promoter methylation by MSP for each clone is shown (%). Arrow indicates the MGMT band. M, DNA ladder marker; N, negative control; WT, wild type.

Supplementary Fig S3. Western blot analysis for MGMT (upper) and alpha-tubulin (lower) in U251 cell line clones. Percentage of MGMT promoter methylation by MSP for each clone is shown (%). Arrow indicates the MGMT band, and the arrowhead indicates alpha-tubulin. Three clones were not subjected to pyrosequencing assay (asterisk). M, DNA ladder marker; WT, wild type.

Supplementary Fig S4. Western blot analysis for MGMT (upper) and alpha-tubulin (lower) in U373 cell line clones. Percentage of MGMT promoter methylation by MSP for each clone is shown (%). Arrow indicates the MGMT band, and the arrowhead indicates alpha-tubulin. M, DNA ladder marker; WT, wild type.