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Clinical Article

Methylation Status of the O6-Methylguanine-Deoxyribonucleic Acid Methyltransferase Gene Promoter in World Health Organization Grade III Gliomas

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Objective : We analyzed the methylation status of the O6-methylguanine-DNA methyltransferase (MGMT) gene promoter in World Health Organization (WHO) grade III gliomas in association with other molecular markers to evaluate their prevalence.
Methods : The samples of a total of 36 newly WHO grade III glioma patients including 19 anaplastic oligodendrogliomas (AO), 7 anaplastic oligoastrocytomas (AOA), and 10 anaplastic astrocytomas (AA) were analyzed. The methylation status of the MGMT gene promoter was confirmed by methylation-specific polymerase chain reaction. The 1p/19q chromosomal deletion status and EGFR amplification were assessed by Fluorescence In-Situ Hybridization. MGMT, EGFR, EGFRvIII, and p53 expression were analyzed by immunohistochemical staining.
Results : The MGMT gene promoter was methylated in 32 (88.9%) and unmethylated in 4 (11.2%). Among them, all of the AO and AOA had methylated MGMT gene promoter without exception. Significant associations between MGMT gene promoter hypermethylation and 1p/19q deletion was observed ($p = 0.003$). Other molecular markers failed to show significant associations between MGMT gene promoter statuses.
Conclusion : There was extensive epigenetic silencing of MGMT gene in high grade gliomas with oligodendroglial component. Together with frequent 1p/19q co-deletion in oligodendroglial tumors, this may add plausible explanations supporting the relative favorable prognosis in oligodendroglial tumors compared with pure astrocytic tumors.

KEY WORDS : MGMT gene promoter · Methylation · 1p/19q · Oligodendroglioma · Methylation-specific PCR.

INTRODUCTION

Anaplastic astrocytoma (AA), anaplastic oligodendroglioma (AO), and anaplastic oligoastrocytoma (AOA) are classified into the histological categories of World Health Organization (WHO) grade III gliomas, even though their classification based on the known molecular biology information remains controversial[8,9,12,17]. O6-methylguanine-DNA methyltransferase (MGMT) is an enzyme in the DNA repair process that specifically removes cytotoxic O6-alkylguanine adducts, thus mediating resistance to alkylating agents[16]. The role of this DNA repair enzyme in glioblastoma, that protects the tumor cells against alkylating and methylating chemotherapeutic agents, resulting in drug resistance is well studied[18]. MGMT methylation prevalence in glioblastoma was reported to be from 30% to 50%[2,6,15]. However, the investigation for the prevalence of MGMT gene promoter methylation status as well as their clinical implication in WHO grade III glioma is sparse. Therefore, we evaluated the methylation status of the MGMT gene promoter in WHO grade III gliomas using the methylation-specific polymerase chain reaction (MSP) method.

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MATERIALS AND METHODS

Study population

A total of 36 newly diagnosed World Health Organization (WHO) grade III glioma patients were included in this study. Histological diagnosis according to the WHO 2007 classification was assorted AO in 19 patients, AOA in 7 patients and anaplastic astrocytoma (AA) in 10 patients. The study population consisted of 18 men and 18 women ranging in age from 20 to 79 years (mean, 46.6 years). All patients underwent surgical removal or biopsy sampling of their tumor. And, the adjuvant conventional radiotherapy

and chemotherapy were performed after pathologic diagnosis in all patients.

Methylation-specific polymerase chain reaction

The methylation status of the MGMT gene promoter was confirmed by MSP. Tissue was dissected from paraffin blocks and put into polyethylene microtubes. After deparaffinization with xylene and alcohol, the blocks were dissolved in a lysis buffer solution containing proteinase K. For the MSP, purified DNA was modified by sodium bisulfite treatment using an EZ DNA methylation-Gold Kit™ (Catalog No. D5005; Zymo Research, Orange, CA, USA). The primer sequences for the MGMT were as follows : methylated forward : 5' TTT CGA CGT TCG TAG GTT TTC GC 3', methylated reverse : 5' GCA CTC TTC CGA AAA CGA AAC G 3', unmethylated forward : 5' TTT GTG TTT TGA TGT TTG TAG GTT TTT GT 3', unmethylated reverse : 5' AAC TCC ACA CTC TTC CAA AAA CAA AAC A 3'. The annealing temperature was 64 °C. The PCR products obtained were electrophoresed in 2% agarose gels and visualized under ultraviolet illumination after staining with ethidium bromide. CpGenome universal unmethylated (Catalog No. S7822; Chemicon, Temecula, CA, USA) and methylated DNA sets (Catalog No. S 7821; Chemicon) were used as negative and positive controls respectively.

For the evaluation of the assay results, the products from the controls were examined first. The MGMT gene promoter fragments in the controls should be observed at 80 and 92 base pairs in the methylated DNA-methylated primer and unmethylated DNA-unmethylated primer combinations respectively. The methylated DNA-unmethylated primer and unmethylated DNA-methylated primer controls should not show any bands. If the control results were acceptable, patient samples were evaluated for the presence of amplification with the methylated and unmethylated primers. The results were interpreted as positive if MGMT gene promoter methylation was detected as a fragment of 80 base pairs observed on the gel, and negative if MGMT gene promoter methylation was not detected with the methylated primers.

Fluorescence *in-situ* hybridization

The 1p/19q chromosomal deletion status and epidermal growth factor receptor (EGFR) amplification were assessed by rluorescence *in-situ* hybridization (FISH) on paraffin sections obtained during surgery. The procedure and materials are followed as previously described[7].

Immunohistochemistry

MGMT, EGFR, epidermal growth factor receptor variant III (EGFRvIII), and p53 expression were analyzed by immunohistochemical staining. Immunohistochemical staining was done using the conventional labeled streptavidin-biotin-peroxidase method (LSAB Kit, DAKO, Glostrup, Denmark) according to the manufacturer's protocol. The used antibodies are as follows; MGMT (Neomarkers, Fermont, CA, USA), EGFR (Zymed, San Francisco, CA, USA), EGFRvIII (DAKO, Glostrup, Denmark), and p53 (DAKO, Glostrup, Denmark). Immunohistochemistry results were semiquantitatively graded as < 5% (negative), 5-10% (1+), or >10% (2+), based on the percentage of tumor cells showing immunoreactivity. We considered more than 1+ as positive for corresponding markers.

Statistical analysis

For analyzing associations between markers, the chi-square test was used for parametric comparisons. Statistical significance was accepted at probability values of less than 0.05. These statistical analyses were performed with the aid of SPSS software (version 12.0; SPSS Inc., Chicago, IL, USA).

RESULTS

Methylation status of the MGMT gene promoter and MGMT expression

Of the 36 patients whose samples were analyzed, the MGMT gene promoter was methylated in 32 (88.9%) and unmethylated in 4 (11.2%). The results according to the histological classification is summarized in Table 1. Interestingly, all the high grade gliomas with oligodendroglial component (AO or AOA) had methylated MGMT gene promoter. Comparison with the immunohistochemistry result revealed that 81.5% of the analyzable samples showed matched results which include methylated MGMT gene promoter and negative MGMT gene promoter expression or vice versa.

Table 1. The prevalence of methylation status of MGMT gene promoter according to the histological classification. Results are from the methylation-specific polymerase chain reaction method. Numeric is number of patients

Histological diagnosis	Total	MGMT gene promoter status	
		methylated	unmethylated
Anaplastic oligodendroglioma	19	19	0
Anaplastic oligoastrocytoma	7	7	0
Anaplastic astrocytoma	10	6	4

MGMT : O6-methylguanine-DNA methyltransferase

Relationships between MGMT gene promoter status and other molecular markers

Associations between the MGMT gene promoter methylation status and other molecular markers such as 1p/19q deletion, EGFR amplification, EGFR expression, EGFRvIII expression and p53 expression are summarized in Table 2. Of 19 patients with AOs, 1p/19q co-deletion was observed in 18 patients. And, 3 of 7 patients with AOA revealed intact 1p/19q while the others had deletion in either 1p or 19q. All patients with AA had intact 1p/19q chromosome except for 2 patients who showed deletion in 1p and in 19q, respectively. Significant associations between MGMT gene promoter hypermethylation and 1p/19q deletion was observed (p = 0.003) which implies strong evidence of MGMT gene promoter hypermethylation in high grade oligodendroglial tumors. EGFR amplification was not observed in any of the present series of samples and other molecular markers failed to show significant associations between MGMT gene promoter statuses.

Relationships between EGFR amplification by FISH and expression of EGFR or EGFRvIII on immunohistochemical staining

EGFR amplification was assessed by FISH in 32 available samples and the results were negative for all cases. Of 32 samples, immunohistochemical staining was done in 30 with EGFR and 17 with EGFRvIII. Positive immumoreactivity was observed with EGFR in 14 (46.7%) samples and with EGFRvIII in 13 (76.5%).

Table 2. The relations between MGMT gene promoter status and other molecular markers. Numeric is number of patients

Variable	MGMT gene promoter status		p-value
	Methylated (n = 32)	Unmethylated (n = 4)	
1p/19q deletion (n = 36)			
Deletion (either)	24	0	0.003
No deletion (both)	8	4	
EGFR† amplification (n = 32)			
Yes	0	0	NA§
No	28	4	
EGFR† expression (n = 33)			
Positive	13	3	0.233
Negative	16	1	
EGFRvIII† expression (n = 17)			
Positive	11	2	0.659
Negative	3	1	
p53 expression (n = 32)			
Positive	9	1	0.773
Negative	19	3	

MGMT: O6-methylguanine-DNA methyltransferase, EGFR: Epidermal growth factor receptor
EGFRvIII: Epidermal growth factor receptor variant III, NA: not available

DISCUSSION

The present study clearly showed that high grade oligodendroglial tumors are always hypermethylated in the MGMT gene promoter. Only a few studies reported the status of MGMT gene promoter methylation in gliomas other than glioblastoma. Möllemann et al.[13] reported that MGMT hypermethylation and low or absent expression are frequent in oligodendroglial tumors that 46 of 52 tumors (88%) showed MGMT gene promoter hypermethylation. Among their 23 AOs and 11 AOAs, the MGMT gene promoter was not methylated in only 1 and 2 samples respectively and those samples showed no 1p/19q co-deletion[13]. Dong et al.[3] reported that the prevalence on MGMT gene promoter hypermethylation detected by MSP analysis was 60% of 43 oligodendroglial tumors including 67% of 15 AO/AOAs. Their data showed significant association between hypermethylation of MGMT gene promoter and 1p/19q co-deletion[3]. Alonso et al.[1] also reported that as much as 80% of 41 oligodendroglial tumor samples were hypermethylated in their MGMT gene promoter and among them, 85% of 13 AOs were hypermethylated. Contrast to the high frequency of methylation rate of MGMT gene promoter in AO/AOAs, that of AA was found to be relatively low that less than 50% were methylated according to the previous studies[4,5]. Taken together with the previous and the present study, transcriptional silencing of the MGMT gene by hypermethylation in oligodendroglial tumor especially in high grade may contribute to the tumor's sensitivity to chemotherapy. Nutt et al.[14] showed that the low activity of MGMT is sufficient to account for increased sensitivity of oligodendrocytic cells to chemotherapeutic drugs.

There are evidences that the MGMT gene promoter methylation is associated with tumor progression in oligodendroglial tumors. The presence of hypermethylation of MGMT in WHO grade II astrocytoma/oligodendroglioma is only 31%[5]. Lavon et al.[11] performed a longitudinal assessment of epigenetic aberrant MGMT gene promoter methylation with 46 paired early and progressive oligodendrogliomas from 23 patients, and correlated them with tumor phenotype in a series of progressive oligodendroglial tumors. They demonstrated that the MGMT gene promoter methylation is more pronounced at

tumor progression, particularly in tumors with an intact 1p in oligodendroglial tumors and it was postulated that the MGMT promoter methylation is a late event in progressive oligodendrogliomas[11]. It is accepted that the oligodendroglial tumor has a tendency to be methylated in various gene promoters including MGMT and this may be associated with the initiation and/or progression of oligodendroglial tumors[10]. However, whether MGMT gene methylation is an actual etiologic event for oligodendroglial tumors or only a prognostic factor needs further investigation[10].

CONCLUSION

Our study for MGMT gene promoter methylation status in 36 WHO grade III gliomas revealed extensive epigenetic silencing of MGMT gene in high grade gliomas with oligodendroglial component. Together with frequent 1p/19q co-deletion in oligodendroglial tumors, this may add plausible explanations supporting the relative favorable prognosis in oligodendroglial tumors compared with pure astrocytic tumors.

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