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Clinical Study

MGMT inactivation and clinical response in newly diagnosed GBM patients treated with Gliadel ☆

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ABSTRACT

We examined the relationship between the O^6-methylguanine-methyltransferase (MGMT) methylation status and clinical outcomes in newly diagnosed glioblastoma multiforme (GBM) patients who were treated with Gliadel wafers (Eisai, Tokyo, Japan). MGMT promoter methylation has been associated with increased survival among patients with GBM who are treated with various alkylating agents. MGMT promoter methylation, in DNA from 122 of 160 newly diagnosed GBM patients treated with Gliadel, was determined by a quantitative methylation-specific polymerase chain reaction, and was correlated with overall survival (OS) and recurrence-free survival (RFS). The MGMT promoter was methylated in 40 (32.7%) of 122 patients. The median OS was 13.5 months (95% confidence interval [CI] 11.0–14.5) and RFS was 9.4 months (95% CI 7.8–10.2). After adjusting for age, Karnofsky performance score, extent of resection, temozolomide (TMZ) and radiation therapy (RT), the newly diagnosed GBM patients with MGMT methylation had a 15% reduced mortality risk, compared to patients with unmethylated MGMT (hazard ratio 0.85; 95% CI 0.56–1.31; $p = 0.46$). The patients aged over 70 years with MGMT methylation had a significantly longer median OS of 13.5 months, compared to 7.6 months in patients with unmethylated MGMT ($p = 0.027$). A significant difference was also found in older patients, with a median RFS of 13.1 *versus* 7.6 months for methylated and unmethylated MGMT groups, respectively ($p = 0.01$). Methylation of the MGMT promoter in newly diagnosed GBM patients treated with Gliadel, RT and TMZ, was associated with significantly improved OS compared to the unmethylated population. In elderly patients, methylation of the MGMT promoter was associated with significantly better OS and RFS.

1. Introduction

Glioblastoma multiforme (GBM) is the most common primary brain tumor, with a median survival of less than 2 years [1]. To date, only two alkylating agents have been shown to be consistently associated with prolonged survival: temozolomide (TMZ) and locally delivered Gliadel wafers (Eisai, Tokyo, Japan) [1–3].

Gliadel wafers are intracranially implanted and locally deliver carmustine (1,3-bis[2-chloroethyl]-1; nitrosourea [BCNU]) at the site of tumor resection, allowing for a higher concentration of local chemotherapeutic dose while minimizing systemic adverse effects [2–4]. These wafers provide a controlled release form of local chemotherapy over approximately 3 weeks [4,5].

Methylation of the O^6-methylguanine-methyltransferase (MGMT) promoter in gliomas has been found to be an important predictor of tumor responsiveness after several cytotoxic regimens [1], including BCNU treatment [6]. Expression of the DNA repair protein, MGMT, results in GBM resistance to alkylating agents. Alkylating agents cause cell death by binding to DNA, most commonly to the O^6 position of guanine, and forming cross links

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between adjacent DNA strands. This cross linking of double stranded DNA is inhibited by the cellular DNA repair protein MGMT.

In this study, through a unique analysis of 122 patients with newly diagnosed GBM who had enough tumor tissue for MGMT analysis and were treated with Gliadel, we retrospectively examined the association between the MGMT promoter methylation status and overall survival (OS) and recurrence-free survival (RFS).

2. Methods

2.1. Patients and tumor specimens

We retrospectively reviewed 160 patients with newly diagnosed GBM, who received Gliadel after their tumor resections at Johns Hopkins Hospital in Baltimore, Maryland, USA, between July 1997 and December 2006. Of these patients, only 122 had stored tumor samples that were available for MGMT analysis; 38 (24%) did not have enough tumor tissue. The clinical, radiological and hospital courses of these patients were retrospectively reviewed. Age, sex, Karnofsky performance score (KPS) at time of diagnosis, tumor location, time to recurrence and dates of death were recorded. GBM was histologically confirmed in all patients. The extent of surgical resection was determined based on a postoperative MRI performed <48 hours after surgery. Gross total resection was defined as no residual tumor enhancement on MRI, while subtotal resection was defined as residual nodular enhancement on MRI. The study was approved by the Johns Hopkins Institutional Review Board.

2.2. Treatment algorithm

Gliadel wafers were not implanted in patients after tumor resection when the tumor significantly extended into the ventricles or was multifocal.

2.3. DNA extraction

After the initial patient de-identification, all original histologic slides from the GBM specimens were reviewed by a senior neuropathologist (PB) to reconfirm the diagnosis of GBM. A representative block with tumor was retrieved for DNA extraction. Histologic slides from the formalin fixed, paraffin embedded tissue were obtained, one representative slide was stained with hematoxylin and eosin (H&E) and the tumor was marked by the senior neuropathologist (PB). An additional five correlating unstained 10 micron slides were also obtained. The tumor cells in the unstained slides were microdissected according to the marked H&E stained reference slide. DNA was extracted from the paraffin embedded tissue after xylene deparafinization. The microdissected tissue was digested with 1% sodium dodecyl sulfate and 200 μg/mL proteinase K (Hoffmann-La Roche, Basel, Switzerland) at 48°C for 48 hours, followed by phenol/chloroform extraction and ethanol precipitation of the DNA. The extracted DNA was dissolved in either LoTE (2.5 mM ethylenediaminetetraacetic acid, 10 mM tris-hydrochloric acid [pH 8]) or distilled water.

2.4. Bisulfite treatment

Extracted DNA was subjected to bisulfite treatment to convert the unmethylated cytosine residues to uracil residues. Briefly, 2 μg of genomic DNA from each sample was treated with bisulfite using the EpiTect Bisulfite kit (Qiagen, Germantown, MD, USA) according to the manufacturer's instructions. The converted DNA was stored at −80°C.

2.5. Methylation analyses

The methylation analyses were performed using quantitative methylation-specific PCR. The bisulfite modified DNA was used as a template for fluorescence-based real time polymerase chain reactions (PCR). The amplification reactions were carried out in triplicate in a final volume of 20 μL that contained 3 μL bisulfite-modified DNA, 600 nmol/L concentrations of forward and reverse primers, 200 nmol/L of probe, 0.6 units of platinum Taq polymerase (Invitrogen, Carlsbad, CA, USA), 200 μmol/L concentrations each of deoxyadenosine triphosphate, deoxycytidine triphosphate, deoxyguanosine triphosphate, and deoxythymidine triphosphate, and 6.7 mmol/L of magnesium chloride. The primers and probes were designed to specifically amplify the promoter of MGMT and the promoter of a reference gene, ACTIN B. The primer and probe sequences and annealing temperatures are provided in Table 1. The amplifications were carried out using the following profile: 95°C for 3 min, 50 cycles at 95°C for 15 s, 60°C for 1 min. The amplification reactions were carried out in 384-well plates in a 7900 sequence detector (PerkinElmer, Waltham, MA, USA) and analyzed by a sequence detector system (Applied Biosystems, Foster City, CA, USA). Each plate included the patient DNA samples, positive controls (Bisulfite-converted universal methylated human DNA standards [Zymo Research, Irvine, CA, USA] in serial dilutions of 20 ng to 2 pg), and molecular grade water was used as a non-template control. The β-actin gene was used to normalize and act as an internal loading control. The methylation ratio was the ratio of values for the gene-specific PCR products to those of the ACTIN B, and then multiplied by 1000 for more efficient tabulation.

2.6. Statistical analyses

The baseline patient and disease characteristics were summarised using descriptive statistics for all 160 patients. The OS time was defined from the date of initial diagnosis of the disease (surgery) to the time of death, or it was censored at the time the patient was last known to be alive. The RFS was counted from the date of the initial diagnosis to the time of disease recurrence, or it was censored at the time the patient was last known to be alive and recurrence-free. The probabilities of OS and RFS were estimated using the Kaplan–Meier method [7] and compared using the log-rank test. Confidence intervals (CI) were calculated using the method of Brookmeyer and Crowley. The Cox proportional hazards model was used to estimate the association between OS or RFS and MGMT methylation status, treatments and well known prognostic factors among the 122 patients who had enough tissue

Table 1
Primer and probe sequences

Gene	Forward primer	Probe	Reverse primer
MGMT	CGA ATA TAC TAA AAC AAC CCG CG (1029–1051)	AAT CCT CGC GAT ACG CAC CGT TTA CG (1084–1109)	GTA TTT TTT CGG GAG CGA GGC (1130–1150)
ACTIN B	TGG TGA TGG AGG AGG TTT AGT AAG T (390–414)	ACC ACC ACC CAA CAC ACA ATA ACA AAC ACA (432–461)	AAC CAA TAA AAC CTA CTC CTC CCT TAA (496–522)

MGMT = O^6-methylguanine-methyltransferase.