Mgmt methylation status and glioblastoma multiforme outcome

ABSTRACT

Background: O $^{6-}$ methylguanine-methyltransferase (MGMT) promoter methylation has been associated with increased survival among patients with glioblastoma multiforme (GBM) who were treated with various alkylating agents. We examined the relationship between MGMT methylation status and clinical outcome in newly diagnosed GBM patients treated with BCNU wafers (Gliadel®).

Methods: MGMT promoter methylation in DNA from 122 newly diagnosed GBM patients treated with Gliadel was determined by a Quantitative methylation-specific polymerase chain reaction assay (QMSP) and correlated with overall survival (OS) and recurrence-free survival (RFS).

Results: The MGMT promoter was methylated in 40 (32. 7%) of 122 patients. Overall median survival was 13. 5 months (95%CI: 11. 0-14. 5) and recurrence-free survival (RFS) was 9. 4 months (95%CI: 7. 8-10. 2). After adjusting for age, KPS, extent of resection, temozolomide (TMZ) and radiation therapy (RT), newly diagnosed GBM patients with MGMT methylation who were treated with Gliadel had a 15% reduction in hazard of death compared to patients with unmethylated MGMT (Hazard ratio: 0. 85, 95%CI: 0. 56-1. 31). Patients aged over 70 with MGMT methylation and treated with Gliadel had a significantly longer median survival of 13. 5 months compared to 7. 6 months in patients with unmethylated MGMT (p= 0. 027). A similar significant difference was also found in older patients with a median recurrence-free survival of 13. 1 versus 7. 6 months (p= 0. 01) for MGMT methylated and unmethylated, respectively.

Conclusions: Methylation of the MGMT promoter in newly diagnosed GBM patients who were treated with Gliadel followed by RT and TMZ, was associated with significantly improved survival compared to the non-methylated patient population with similar treatment. For the elderly population, methylation of the MGMT promoter was associated with significantly better OS and RFS.

INTRODUCTION

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

Gliadel wafers (Eisai Inc. for Arbor Pharmaceuticals, LLC) are implanted and locally deliver Carmustine (also known as (1, 3-bis(2-chloroethyl)-1-nitrosourea (BCNU)) at the site of tumor resection, allowing for a higher concentration of local chemotherapeutic doses while minimizing systemic adverse effects [2-4]. These wafers provide a controlled- release form of local chemotherapy for approximately 3 weeks [4, 5].

Methylation of the MGMT promoter in gliomas was found to be an important predictor of the tumor responsiveness after several cytotoxic regimens [6], including BCNU treatment [7]. It was found that expression of the DNA repair protein, O^{6} – methylguanine-methyltransferase (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 forms cross-links between adjacent DNA strands. This cross-linking of double strand 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 were treated with Gliadel, we retrospectively examined the association between MGMT promoter methylation status and survival.

METHODS

Patients and Tumor Specimens

We retrospectively reviewed 185 patients with newly diagnosed GBM who received Gliadel after tumor resection, at Johns Hopkins Hospital in Baltimore, USA, between July 1997 and December 2006. Of these patients, only 122 patients had stored samples that were available for MGMT analysis. The clinical, radiological and hospital course of these patients were retrospectively reviewed. Age and gender were recorded, as well as Karnofsky performance score (KPS) at time of diagnosis, tumor location, time to recurrence and dates of death were recorded. Overall survival (OS) was calculated from the time of surgery to death, and recurrence free survival (RFS) was calculated from the time of surgery to time of recurrence or censored at the last time of follow-up. GBM was histologically confirmed in all cases. 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.

Treatment Algorithm

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

DNA Extraction

After initial patient de-identification, all original histologic slides from the GBM specimens were reviewed to reconfirm the diagnosis of GBM by a senior neuropathologist (PB). 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 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 paraffin embedded tissue after xylene deparaffinization. The microdissected tissue was digested with 1% sodium dodecyl sulfate (SDS) and 200ug/mL proteinase K (Roche, Nutley, NJ) at 48°C for 48 hours, followed by phenol/chloroform extraction and ethanol precipitation of DNA. Extracted DNA was dissolved in either LoTE (2. 5 mM EDTA, 10 mM Tris–HCl [pH 8]) or distilled water.

Bisulfite Treatment

Extracted DNA was subjected to bisulfite treatment, to convert unmethylated cytosine residues to uracil residues. Briefly, 2 µg genomic DNA from each

sample was treated with bisulfite using the EpiTect Bisulfite kit (Qiagen, Valencia, CA) according to the manufacturer’s instructions. Converted DNA was stored at -80oC.

Methylation Analysis

Bisulfite-modified DNA was used as a template for fluorescence-based real-time PCR. 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 probe; 0. 6 units platinum Taq polymerase (Invitrogen); 200 µmol/L concentrations each of dATP, dCTP, dGTP, and dTTP; and 6. 7 mmol/L $MgCl_2$. Primers and probes were designed to specifically amplify the promoter of MGMT and the promoter of a reference gene, ACTIN B; primer and probe sequences and annealing temperatures are provided inTable 1. Amplifications were carried out using the following profile: 95°C for 3 min followed by 50 cycles at 95°C for 15 s and 60°C for 1 min. Amplification reactions were carried out in 384-well plates in a 7900 sequence detector (Perkin-Elmer Applied Biosystems) and analyzed by a sequence detector system (SDS 2. 2. 1; Applied Biosystems). Each plate included patient DNA samples, positive controls (Bisulfite-converted Universal Methylated Human DNAStandards (Zymo Research) in serial dilutions 20ng to 2pg) 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 1, 000 for more efficient tabulation.

Statistical Methods

The overall survival (OS) time was defined from the date of initial diagnosis of the disease (surgery) to the time of death or censored at the time last known alive. The recurrence-free survival (RFS) was counted from the date of initial diagnosis of the disease to the time of disease recurrence or censored at the time last known alive and recurrence-free. Probabilities of OS and RFS were estimated using the Kaplan-Meier (KM) method [15] and compared using Log-rank test. Confidence intervals were calculated using the method of Brookmeyer and Crowley[14]. Cox proportional hazards model [16] was used to estimate the association between OS or RFS and MGMT methylation status, treatments and well known prognostic factors. Schoenfeld residuals were used to test the proportionality of factors in Cox proportional – hazards models. Radiation status was treated as a stratification factor in the Cox regression model. TMZ has FDA approval for newly diagnosed GBM patients aged between 18-70. Subgroup analyses were performed for patients who were aged over 70. All p values were two-sided. All analyses were performed using the Statistical Analysis System, version 9. 2. MGMT was considered as promoter methylated if the methylation ratio was higher than 8, and unmethylated if below 8.

RESULTS

Patient Population

Six hundred patients with newly diagnosed GBM underwent craniotomy between 1997 and 2006, at the Johns Hopkins Hospital. One hundred eighty five patients received Gliadel (30. 8%) after tumor resection. Methylation

specific PCR was performed in 122 of the 185 patients (66%) because 63 patients did not have sufficient paraffin embedded tumor tissue for MGMT analysis. The characteristics of the patients and type of treatments are shown inTable 2. The clinical course of forty patients who had methylation of MGMT promoter was compared to 82 patients without promoter methylation of MGMT. The similarity of distributions among patient's characteristics, and treatments between MGMT methylated and unmethylated is also shown inTable 2. There was a slightly male predominance in both groups. The median age of the MGMT methylated group was 65. 5 years compared to 60. 5 years in the non-MGMT methylated group (p= 0. 59). Most of the patients in both groups had KPS score of ≤ 80 (p= 0. 67). Most of the patients in both groups underwent gross total resection (GTR) (85% vs. 74% in the methylated and non-MGMT methylated group, respectively), (p= 0. 19).

Most of the patients in the MGMT methylated and non-MGMT methylated groups received post-operative radiation therapy (RT) (80% and 72% respectively). However, there were 31 patients (25%) without radiation treatment recorded in their medical chart. Only 33% and 29% of MGMT methylated and non-MGMT patients, respectively, were treated with TMZ due to majority of patients was treated prior to 2005 when RT+ TMZ became the stadnadrd of care for the newly diagnosed GBM patients.

Overall Survival

The Kaplan-Meier estimate of the median OS for the122 patients with newly diagnosed GBM was 13. 5 months (95% CI: 11. 0, 14. 5). Median OS for those with MGMT methylation was 13. 9 months (95%CI: 9. 5, 17. 1) compared to

12. 9 months (95%CI: 10. 9, 14. 5) (p= 0. 86) in patients non methylated. Univariate and multivariate association of survival with treatment factor, baseline prognostic factors, and MGMT methylation status are shown inTable 3. There was a 15% reduction in hazard of death (Hazard ratio: 0. 85, 95%CI: 0. 56-1. 31) for patients with MGMT methylated tumor compared to those with MGMT unmethylated tumor after adjusting for age, KPS, extent of resection, TMZ and RT. A subgroup analysis was performed among 35 patients who were 18-70 years old and treated with Gliadel, RT and TMZ (Gliadel+ Stupp's regimen) [1]. The median OS was 19. 8 months (95% CI, 14. 5, 22. 2) in this subset of patients. There was no statistically significant difference in OS among these 35 patients with MGMT promoter methylation (median OS: 20 months, 95% CI: 9. 2, 37. 0), compared to patients without MGMT promoter methylation (median OS: 18. 9 months, 95% CI: 11. 9, 22. 2), (Table 4).

Only two out of 30 elderly patients aged above 70 years were treated with TMZ, one was MGMT methylated and another was not. Among these elderly patients, those with MGMT promoter methylation showed a significantly longer median survival of 13. 5 months (95% CI, 0. 49, 17. 1) compared to 7. 6 months (95% CI, 2. 9, 9. 4) when the MGMT promoter was non-methylated (p= 0. 027). A similar significant difference in median recurrence-free survival was also found in elderly patients where the median survival was 13. 1 versus 7. 6 months (p= 0. 01) for MGMT methylated and unmethylated, respectively.

The overall median recurrence-free survival was 9. 4 months (95%CI: 7. 8-10. 2) for all patients. There was no difference in RFS between patients 18-70 years old with and without MGMT methylation.

DISCUSSION

In this study we investigated the significance of MGMT methylation status in a series of 122 patients with newly diagnosed GBM who underwent surgical resection and implantation of Gliadel wafers. The results of our series show a reduction in hazard of death for patients who were MGMT methylated compared to non-methylated. Interestingly, this effect was much more profound in the elderly group of 35 patients who were older than 70 years old when they were diagnosed with GBM. Elderly patients who were MGMT methylated had significantly better OS, compared to non-methylated (13. 5 vs. 7. 6 months respectively, p= 0. 027).

The methylation of the MGMT promoter region leads to a reduced ability to repair DNA damage induced by alkylating chemotherapeutic agents [7]. Methylation of the MGMT promoter was found to be associated with responsiveness to alkylating chemotherapeutic agents such as temozolomide [6] and BCNU [7], and an increase in OS and progression free survival. The median survival of patients who received the combination of Gliadel, temozolomide and radiation therapy in our cohort ranged between 18. 9 to 20 months, six months greater than that for the radiation therapy and temozolomide historic cohort [1] (Figure1). For patients younger than 70 years old, the median survival of the MGMT methylated sub-group was slightly greater that MGMT non-methylated.

KPS in a known prognostic factor for patients with brain tumors [8]. Most of the patients in our study cohort had poor KPS of less than 80. Still, our results were in line with the report of Lechapt-Zalcman et al. [9] who assessed the prognostic impact of MGMT promoter methylation in patients with newly diagnosed GBM that received Gliadel in addition to radiation therapy and temozolomide. The OS of their study cohort was 17. 5 months. Patients with MGMT methylation had a significantly longer OS of 21. 7 months compared with patients without MGMT methylation who had OS of 15. 1 months.

Two recent phase III clinical trials in the elderly age of patients with malignant astrocytoma, the NOA-08 [10] and Nordic trials [11], demonstrated that temozolomide therapy alone was not inferior to radiotherapy alone, and methylation of the MGMT gene promoter was associated with a benefit from temozolomide. However, there is a concern that combination therapy of radiation therapy and temozolomide may be less active and less well tolerated in the elderly population [12]. European Organization for Research and Treatment of Cancer (EORTC)-26981/National Cancer Institute of Canada (NCIC) CE3 trial have suggested that with increasing age, the relative benefit of addition of temozolomide to radiotherapy decreases and the patients suffer from increased chemotherapy-associated side effect such as neutropenia, lymphocytopenia, thrombocytopenia , raised liver-enzyme concentrations infections and thromboembolic events. As opposed to systemic chemotherapy with its limitations, local delivery of Gliadel wafers may be promising in this subset of patients. Chaichana et al. compared 45 elderly patients who were treated

with Gliadel to 88 elderly patients who did not receive Gliadel [13]. The survival for older patients who received Gliadel was significantly longer than for patients who did not receive Gliadel (8. 7 months vs. 5. 5 months respectively, p= 0. 007). The median survival of MGMT methylated in elderly patients in the current cohort was doubled. These results may support the use of Gliadel in this sub-population.

Limitations

There are several limitations to this study. Its retrospective nature carries a potential bias. Moreover, the time period of this study ended in 2006, only one year after temozolomide became the standard of care in the treatment of GBM, thus most of the patients were not treated with the combination of temozolomide and radiation therapy. Furthermore, because this is a tertiary referral center, there is a bulk of patients who were operated in this center, but received further neuro-oncology treatments elsewhere, near their home, and therefore, their complementary oncology treatment is not available. Still, this large and unique cohort of patients with newly diagnosed GBM who were operated in one tertiary center provide novel data that may assist in optimizing and personalizing the treatment for GBM patients.