

Evaluation of O6-methylguanine-DNA methyltransferase enzyme expression effect on survival of patients with Grade 4 brain astrocytoma

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Background: High-grade astrocytoma (Grade 4) or glioblastoma multiforme (GBM) are deadly brain tumors. New therapies attempt to increase lifetime and quality of life in patients with malignant astrocytoma. O6-methylguanine-DNA methyltransferase (MGMT) enzyme expression may be effective in prognosis and response to treatment of these patients. The aim of this study was assessment of MGMT enzyme expression in patients with astrocytoma Grade 4. **Materials and Methods:** In this study, 48 patients with GBM that were treated with surgery, chemotherapy and radiotherapy were investigated and followed-up for 47 months for the survival rate. Pathology blocks of patients were examined for MGMT enzyme expression using immunohistochemistry method. **Results:** The patients were 34 males and 14 females. The ages ranged from 24 to 77 years, with a mean age of 53.52 ± 13.39 years. There was no significant difference between two groups (positive and negative MGMT enzyme expression) in overall survival (median [range] 11.5 [4-30] vs. 13 [5-22], $P = 0.9$). The results of our study showed that patients although who were undergone near total surgery had higher overall survival than the group of patients who had biopsy only however, it was not significant. Patients who were treated with temozolomide (TMZ) (Temodal, Merck Canada) had significant overall median survival (14.5) more than the patients who were treated with Procarbazine (Roche, Swiss)-Lomustine (Lilly, USA)-Vincristine (Lilly, USA) regimen (8.75) ($P < 0.05$). **Conclusion:** O6-methylguanine-DNA methyltransferase enzyme expression had no effect on survival of patients with Grade 4 brain astrocytoma TMZ may increase survival rate.

Key words: Astrocytoma, chemoradiation, glioblastoma multiforme, O6-methylguanine-DNA methyltransferase, temozolomide

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INTRODUCTION

Malignant astrocytoma is also known as giant cell astrocytoma and glioblastoma multiforme (GBM) is the most lethal primary brain tumor in adults. World Health Organization classification system specifies four main groups of astrocytic neoplasms: pilocytic astrocytoma (Grade 1), diffuse low-grade astrocytoma (Grade 2), anaplastic astrocytoma (Grade 3), and glioblastoma (Grade 4).^[1]

Glioblastoma is the single most common malignant brain tumor, comprising 18.5% of all brain tumors and accounts for 54% of all gliomas in adults. The incidence rate for glioblastoma is 3.1/100,000 persons. Annual age-specific rates for glioblastoma increase from 0.36/100,000 persons at 20-34 years of age up to 14.1/100,000 persons at 75-84 years of age. The median age at diagnosis of glioblastoma is 58-63 years of age.^[2,3] There have been advances in the understanding of the molecular genetics

and of astrocytomas with Grades 3 and 4 malignancy in the past decade and it is leading to new therapeutic strategies.

Concomitant and adjuvant radiochemotherapy with the alkylating agent such as temozolomide (TMZ) has become the standard of care in patients with glioblastoma.^[4] The benefit from this treatment; however, is largely restricted to patients with tumors exhibiting promoter methylation of the O6-methylguanine-DNA methyltransferase gene (MGMT), which encodes a DNA repair protein associated with alkylator resistance.^[5]

Some studies have expressed MGMT expression in tumor tissue as a direct cause of disease and suggested that it should be replaced instead of measuring MGMT methylation. These studies also mentioned that response to TMZ is seen only in the percentage of patients with a methylated MGMT who have negative MGMT expression, not in all patients with methylated

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MGMT.^[6,7] TMZ is an alkylating agent that has excellent oral bioavailability and shows good penetration across the blood-brain barrier. MGMT gene hypermethylation is generally associated with prolonged progression-free survival and/or overall survival in patients with newly diagnosed malignant astrocytoma treated with a nitrosourea, procarbazine, or TMZ.^[8,9]

In the Phase-III European Organization for Research and Treatment of Cancer (EORTC) study of TMZ for newly diagnosed glioblastoma^[10] subset analysis of 36% of the total study group showed MGMT gene hypermethylation to be a significant independent predictor of improved survival in patients that received TMZ.^[11,12] The MGMT gene methylation status does not always correspond to the level of MGMT protein expression in individual tumors. There is conflicting evidence whether MGMT promoter hypermethylation or immunohistochemical determination of MGMT protein expression correlates better with patient outcomes.^[13,14]

However, the effect of gene expression on response to treatment is controversial and still not confirmed.^[15,16]

Now, in Iran and now TMZ is recommended to be given to all patients. Due to the high cost of alkylating drugs particularly TMZ, survey of efficacy of this drug in patients without MGMT methylation or patients with MGMT expression can be very effective in planning of treatment.

Therefore, the main aim of the current study was assessment of the effect of MGMT enzyme expression on prognosis and survival of patients with high-grade malignant brain astrocytoma. The secondary objective of the current study was the evaluation of the effects of some important prognostic factors including history of convulsion, method of surgery, method of chemotherapy, MGMT enzyme expression in immunohistochemistry (IHC) testing, sex in the presence of other ones on survival rate of studied sample.

MATERIALS AND METHODS

Study design and participants

A total of 52 patients with Grade 4 malignant astrocytoma that referred to radiation therapy and oncology clinic of Sayed Al-Shohada Hospital Isfahan University of medical Sciences during 2008-2012 were investigated (Research Project Number 189070).

Inclusion criteria were:

1. Patients with astrocytoma with Grade 4 malignancy who had been confirmed by a pathologist,
2. Provided sample of tumor tissue, by surgery or open biopsy,

3. All of the therapeutic methods (surgery, radiotherapy and chemotherapy),
4. Karnofsky performance status (KPS) scale index ≥ 70 in the begging of study,
5. Age > 18 years,
6. No contraindications for chemotherapy,
7. Inappropriate pathologic samples, also patients with history of chemotherapy within 5 years ago and history of radiation therapy of brain were not included.

Exclusion criteria were:

1. Not finishing radiotherapy period at least for 6 weeks,
2. Recurrence of disease after previous treatment,
3. Using antiangiogenic drugs like bevacizumab,
4. Current pregnancy, lactation or not using a reliable contraceptive method,
5. History of coagulation disorder or recurrent thrombosis experience,
6. History of heart failure or myocardial infarction in the past 6 months,
7. History of hormonotherapy,
8. Astrocytoma treatment with another method,
9. Lack of consent of patient or dead patient's legal executor for using pathologic samples,
10. Death due to any reason except astrocytoma.

Sampling was done from November of 2008 to March 2012. It last for 48 months that these patients were observed. All the patients were informed about the survival rate of the applied methods and side-effects of current standard treatments (radiotherapy and chemotherapy), in advance and patients chose different therapy methods, by their desire and possibility of performing treatment. Hence, in choosing therapy methods no interference had been occurred.

Procedures

A total of 52 patients were included. 1 patient was withdrawn during radiation therapy and 3 patients because of serious health problems received palliative treatment instead of chemotherapy. 4 patients were excluded and finally the study was performed on 48 patients. All the patients had undergone surgical procedures included open biopsy or near total surgery. Sampling was done among astrocytoma with Grade 4 malignancy patients that were cured by applying all of three therapy methods (surgery, radiotherapy, and chemotherapy) and adjuvant therapies.

Based on adjuvant therapy, patients were divided into two groups:

1. Radiotherapy + chemotherapy with TMZ (Temodal, Merck Canada)
2. Radiotherapy + chemotherapy with Procarbazine (Roche, Swiss)-Lomustine (Lilly, USA)-Vincristine (Lilly, USA) (PCV) regimen.

Pathological blocks and slides were taken sample was evaluated by IHC methods.

Furthermore, patients in two treatment groups were divided into two categories:

1. Positive MGMT expression and
2. Negative MGMT expression.

All patients were visited during and after the treatment at regular intervals and the overall survival was evaluated at least in 2 times (6 months and 1-year after start of treatment) and results of the mean overall survival of 6 months and 1-year were obtained in two subgroups.

Immunohistochemistry protocol

The steps of the IHC protocol we used were as follows:

1. Tissues were fixed and paraffinized,
2. Paraffin embedded blocks were cut in 3 μ sections,
3. Deparaffinizing and rehydrating the section were done,
4. Microwave was done for 15 min,
5. Endogen hydrogen peroxidase enzymes were blocked with H₂O₂ 3% solution,
6. Protein blocking was done for 10 min,
7. Samples were placed in MGMT antibody (MGMT antibody [SPM287] prediluted [ab54306], ABCAM PLC Company, London, UK) for 12 h,
8. Polymer envision was done for 30 min,
9. Diaminbenzidine procedure was done for 5 min, 10. Background staining with hematoxylin was done, 11. Montization was done, and
12. Stained samples viewed by a pathologist under light microscope.

A washing step with phosphate buffered saline buffer was done once between the above steps and the final result was announced by the pathologist as positive or negative IHC.^[17,18]

Karnofsky performance status scale index

The KPS scale index allows patients to be classified as to their functional impairment. This can be used to compare effectiveness of different therapies and to assess the prognosis in individual patients. The lower the karnofsky score, the worse the survival for most serious illnesses.^[19,20]

Treatment protocol

At the beginning of the study, treatment with TMZ (temodal and merck sharp) was recommended to all patients. If the patient accepted the treatment, radiation therapy was done after surgery with 45 cGy dose to the tumor bulk plus calculated edema volume with magnetic resonance imaging (MRI) in T2 phase plus 3 cm of tumor margin. Patient's treatment was done with 1.8 cGy radiation dose daily and then radiation of tumor bulk with 1 cm margin was done up to 45 cGy doses. Simultaneously, patients received

TMZ with daily dose of 75 mg/m² of body surface. After completion of chemoradiation, chemotherapy with TMZ was done with dose of 150 mg/m² for 5 days every 28 days for six courses.^[4]

In the patients group that not agree with treatment with TMZ (Temodal, Merck Canada), radiation therapy was done after surgery with 50 cGy dose to the tumor bulk plus calculated edema volume with MRI in T2 phase plus 3 cm of tumor margin. Patient's treatment was done with 1.8 cGy radiation dose daily and then radiation of tumor bulk with 1 cm margin was done up to 45 cGy doses. Then the chemotherapy regimen with PCV (lumostine 130 mg/m² on day one and procarbazine 60 mg/m² daily on days 8-21 and vincristine 2 mg on days 8 and 29 in periods of 56 days for six courses) was performed.^[4]

Statistical analysis

The quantitative data are presented as mean \pm standard deviation or median (range) while qualitative ones as number (percent). Kaplan–Meir with log-rank test was used for comparing the crude survival rate between studied groups and proportional Cox regression was used for investigating the effects of predictor variables on survival rate when adjusting the effects of confounding factors. Statistical independent *t*-test, Chi-square or Fisher exact tests were used for comparing the variables between studied groups. All analyses were done using Statistical Package for Social Sciences version 20 (SPSS Inc., Chicago, IL, USA) and *P* < 0.05 were considered as significant.

RESULTS

Forty-eight patients included 34 males (71%) and 14 females (29%) entered into the study. Their ages ranged from 24 to 77 years, with a mean age of 53.52 (\pm 13.39) years. Twenty-four patients (50.0%) received PCV regimen and 24 patients (50.0%) received TMZ regimen patient baseline characteristics are shown in Table 1. Table 2 contains the results of comparing the factor affecting survival rate in two groups of patients categorized base on MGMT enzyme expression based on IHC test.

After follow-up of patients the survival time of patients based on surgery methods, type of chemotherapy and IHC were compared. The results are presented in Table 3 and Figures 1-5. There was no significant difference in overall survival rate between the two groups of positive and negative MGMT enzyme expression with IHC (*P* = 0.94). Overall survival was also not significantly different in the two gender groups (*P*=0.91). Although the group of patients who were undergone near total surgery had overall survival rate more than those who were undergone biopsy; however, the difference was not statistically significant (*P* = 0.37). Patients who received standard chemotherapy and chemoradiation with TMZ had

Table 1: Patient baseline characteristics

Characteristics	Mean (%)
Age (±SD) (year)	53.52±13.39
KPS before radiation therapy (0-100)	86.67±6.61
History of convulsion	
Positive	28 (58.3)
Negative	20 (41.7)
Method of surgery	
Biopsy	29 (60.4)
Near total	19 (39.6)
MGMT enzyme expression in IHC testing	
Positive	12 (25)
Negative	36 (75)
Time of surgery (min)	88.79±1.031
Mean (±SD) of survival (month)	12.26±6.068
Status of patients' survival	
Survive	3 (6.3)
Dead	45 (93.8)

KPS = Karnofsky performance status; IHC = Immunohistochemistry; MGMT = O6-methylguanine-DNA methyltransferase; SD = Standard deviation

Table 2: Comparison of survival factors in patients with positive and negative MGMT enzyme expression based on IHC tests

Variable	IHC positive (%)	IHC negative (%)	P value
Overall survival (month)	12.78±5.29	12.14±6.29	0.940
Survive patients	0 (0)	3 (7.7)	0.587
Chemotherapy regime			
PCV	2 (22.2)	22 (56.4)	0.064
Temozolomide	7 (77.7)	17 (43.6)	
Age (±SD) (year)	57.88±15.59	52.5±12.84	0.28
KPS before radiation therapy (0-100)	80 (80-90)	90 (80-100)	0.096
History of convulsion	4 (44.4)	16 (41)	0.85
Method of surgery			
Biopsy	6 (66.7)	23 (59)	0.67
Near total	3 (33.3)	16 (41)	
Time of surgery (min)	88.67±0.71	88.82±1.09	0.69

KPS = Karnofsky performance status; IHC = Immunohistochemistry; MGMT = O6-methylguanine-DNA methyltransferase; SD = Standard deviation; PCV = Procarbazine (Roche, Swiss)-Lomustine (Lilly, USA)-Vincristine (Lilly, USA)

significantly higher overall survival rate compared to those treated with radiotherapy alone and chemotherapy with PCV regime ($P = 0.047$) also, there was no significant difference in overall survival rate between the two groups of with and without history of convulsion ($P = 0.56$) [Table 3].

DISCUSSION

As mentioned, the aim of this study was to determine the effect of MGMT enzyme expression on prognosis and survival of patients with high-grade malignant brain astrocytoma followed by chemotherapy.

Temozolomide is a new oral alkylating and methylating agent that has demonstrated good tolerance and promising

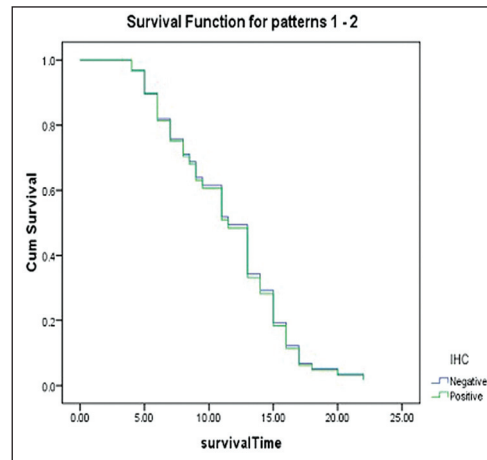


Figure 1: Survival rate in different levels of immunohistochemistry

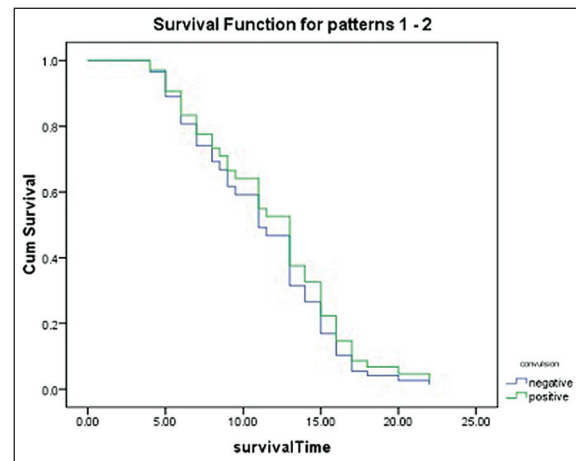


Figure 2: Survival rate in different levels of convulsion

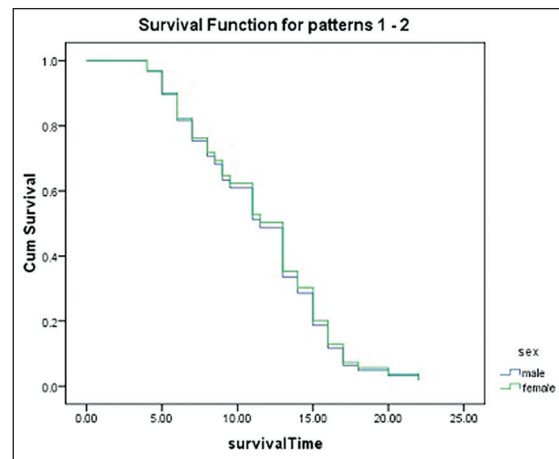


Figure 3: Survival rate in different levels of gender

activity in astrocytic tumors. TMZ exerts cytotoxicity mainly by methylating DNA at the O6 position of guanine. This leads to futile cycling of the DNA mismatch repair pathway and eventual double-strand DNA breaks and induction of apoptosis.^[22,23]

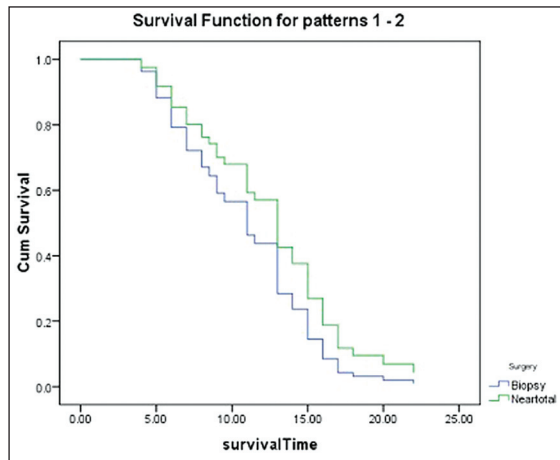


Figure 4: Survival rate in different levels of surgery

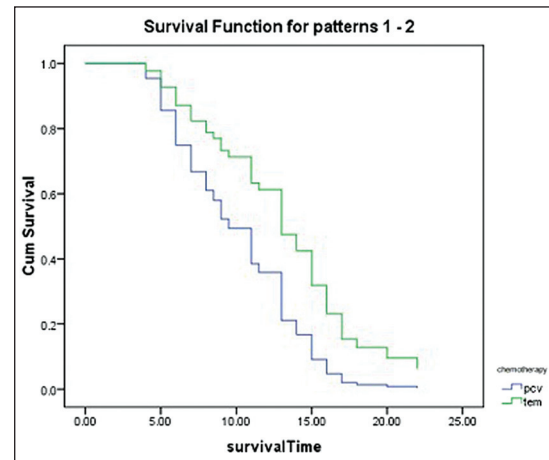


Figure 5: Survival rate in different levels of chemotherapy

Table 3: The results of Cox regression on the effects of predictors of survival rate in the study samples

Status	Median (range)	HR (95% CI for HR)*	P value
History of convulsion			
Negative	10 (4-30)	1	0.56
Positive	13 (4-27)	1.18 (0.56-2.52)	
Method of surgery			
Biopsy	9.5 (4-27)	1	0.37
Near total	14 (4-30)	1.47 (0.63-3.45)	
MGMT enzyme expression in IHC testing			
Negative	11.5 (4-30)	1	0.94
Positive	13 (5-22)	0.97 (0.44-2.14)	
Method of chemotherapy			
PCV	8.75 (4-20)	1	0.047
Temozolomide	14.5 (6-30)	2.08 (1.01-4.32)	
Sex			
Male	12.25 (4-30)	1	0.91
Female	11.25 (4-27)	1.05 (0.46-2.27)	

SD = Standard deviation; KPS = Karnofsky performance status; IHC = Immunohistochemistry; MGMT = O6-methylguanine-DNA methyltransferase; HR = Hazard ratio; 95% CI = 95% confidence interval; Resulted from Cox proportional regression; PCV = Procarbazine (Roche, Swiss)-Lomustine (Lilly, USA)-Vincristine (Lilly, USA)

In a large study of 162 patients with recurrent anaplastic astrocytoma treated in first or second line with TMZ, an objective response rate of 35% was achieved.^[24] Furthermore, stabilized and responding patients showed improved quality of life.^[25]

In this study, patients that treated with TMZ regimen had two advantages more than those treated with PCV regimen because temozolamide group had received the first-line alkylating agent and also received chemoradiation while in the patients that treated with PCV regime radiotherapy was prescribed alone.

At follow-up, the group that had received temozolamide regime had a higher overall survival. Patients who were

treated with TMZ had overall survival more than patients who were treated with PCV regimen ($P = 0.003$).

In study of van den Bent *et al.* investigated TMZ as first-line chemotherapy in recurrent oligodendroglial tumors and mixed oligoastrocytomas after surgery and radiation therapy. TMZ provides an excellent response rate with good tolerability in chemotherapy-naïve patients with recurrent oligodendroglioma.^[26]

The results of our study show that patients who were undergone near total surgery had higher mean survival and overall survival than the group of patients who had biopsy only ($P < 0.05$). In our study, type of surgery due to a decrease in the number of tumor cells was clearly affected on the overall survival. Hence, it can be brought the numbers of malignant cells to $<10^{-1}$ cells (final D_{10}) with a smaller number of D_0 (dose of radiation that remains only 37% of malignant cells after irradiation).

Most retrospective studies published over the past two decades have shown a survival advantage for malignant astrocytoma patients who underwent a “total” or “subtotal” resection versus a biopsy or “minor” resection.^[27,28] In other similar studies, the extent of resection was not a predictor of survival independent of tumor histology, patient age, and performance status.^[29] In some studies of glioblastoma resection of $>75\%$ of enhancing tumor was associated with improved survival.^[30] In a large study of glioblastoma patients, resection of at least 78% of enhancing tumor was an independent predictor of better overall survival.^[31] Another study showed no survival benefit unless $>97\%$ of the tumor was resected.^[32] Study of McGirt *et al.* have shown that glioblastoma patients with any residual enhancing tumor at all have shorter survival than those who undergo gross total resection.^[33]

Tumor size was not evaluated in our study due to the lack of access to patients’ records and tumor size was not

mentioned in the report of the radiologist. But the history, physical examination, treatment, tumor histology, and follow-up after treatment of patients were available.

However, a recently-completed Phase-II clinical trial with brain tumor patients yielded mixed outcomes; while there was some improved therapeutic activity when TMZ was given to patients with TMZ-resistant anaplastic glioma, there seemed to be no significant restoration of TMZ sensitivity in patients with TMZ-resistant GBM.^[34]

An international Phase-III study by the EORTC randomized nearly 600 patients with newly diagnosed glioblastoma to either receive radiation therapy without any chemotherapy, or to receive daily TMZ (75 mg/m²) given concurrently with radiation therapy followed by six cycles of monthly TMZ (200 mg/m²/day for 5 days) after radiation therapy. Patients who received TMZ had a significant prolongation of survival (median survival 14.6 months vs. 12.1 months for the radiation therapy-only group), a 37% relative reduction in the risk of death, significantly prolonged median time to tumor progression (6.9 months vs. 5.0 months), and a higher 2-year survival rate (26.5% vs. 10.4%).^[10]

In study of Rivera *et al.*, MGMT promoter hypermethylation was associated with longer progression-free survival and overall survival in glioblastoma patients who received no chemotherapy until the time of first tumor recurrence.^[35]

Results of our study showed there was no significant difference between two groups of patients with positive and negative MGMT enzyme expression in overall survival ($P = 0.202$).

On the other hand, a study by National Cancer Institute of Canada Clinical Trials Group showed the presence of MGMT protein in brain tumors predicts poor response to TMZ and these patients receive little benefit from chemotherapy with TMZ.^[11]

CONCLUSION

We found that TMZ was effective in both groups of patients with negative and positive MGMT enzyme expression and it seems to evaluating of the expression of MGMT enzyme before treatment not be necessary routinely. According to the results of our study, TMZ can be used as the first choice for chemoradiation and chemotherapy of patients with malignant astrocytoma after near total surgery with minimal side-effects.

Given the degree of benefit and the lower toxicity of TMZ relative to nitrosourea-based regimens, TMZ regimen recommended to be standard therapy for patients with newly diagnosed glioblastoma.

Author contribution

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