Wspolczesna Onkol 2011; 15 (4): 198–202

DOI: 10.5114/wo.2011.24313

Aim of the study: To evaluate the results of survival of glioblastoma patients treated in Warmia and Mazury Oncology Centre in the years 2003-2008.

Material and methods: Eighty-two patients (45 males; 37 females) with newly diagnosed histologically confirmed glioblastoma multiforme were treated. Thirty-eight patients were treated radically; this group includes 25 patients who had radiochemotherapy with temozolomide, and 13 patients treated only with radical radiotherapy. Forty-four patients had palliative radiotherapy. The tumour tissues of 23 patients were investigated for MGMT gene promoter methylation in the Department of Molecular Biology, Memorial Cancer Centre, Institute of Oncology, Warsaw.

Results: The median overall survival (OS) of all patients was 10.5 months. The median OS for patients who were treated radically was 16.8 months and for patients who were treated palliatively it was 8.9 months. The median OS for patients treated with radiotherapy plus temozolomide was 27.4 months (for patients with methylated promoter of *MGMT* gene, 28 months; with unmethylated promoter, 26.5 months). One patient died of pulmonary and cerebral aspergillosis during radiochemotherapy. Leukoencephalopathy and cognitive disturbances were diagnosed in one 70-year-old patient after radiotherapy with temozolomide.

Conclusions: The OS for patients treated with radiotherapy and temozolomide was longer than in the group treated only with radiotherapy. Differences were not observed in the median OS for patients with methylated and unmethylated promoter of *MGMT* gene, treated with radiotherapy plus temozolomide. The median OS for patients who had only radical radiotherapy is similar to the median OS for patients who had palliative radiotherapy.

Key words: glioblastoma multiforme, radiochemotherapy, temozolomide, *MGMT* gene promoter methylation.

Treatment results, including clinical prognostic factors and *MGMT* gene promoter methylation, in patients with glioblastoma multiforme in Warmia and Mazury Oncology Centre

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Primary malignant brain tumours constitute 2-3% of newly diagnosed cancers in the Polish population. According to the Department of Epidemiology and Cancer Prevention, Institute of Oncology in Warsaw, 2600-2800 newly diagnosed malignant primary brain tumours were registered in the years 2004-2006. The greatest morbidity is observed in adults between 45 and 79 years of age, with the majority of episodes and mortality in males [1]. In Warmia and Mazury, 70-90 newly diagnosed malignant brain tumours occurred annually between 2004 and 2006 [1]. Glioblastoma is the most frequent primary malignant brain tumour in adults. Previous standard therapy included surgical resection, followed by radiotherapy. Results of the therapy are not satisfactory because median survival was 4.3-17 months, depending on age, performance, neurological and mental status, and extent of the surgery [2, 3]. A recent trial by EORTC26981/NCIC CE3 demonstrated that the addition of temozolomide to the standard postoperative radiotherapy improved median survival from 12.1 months to 14.6 months and the 2-year overall survival (OS) from 10 to 26%, and the 5-year overall survival (OS) from 1.9 to 9.8% [4, 5]. Therefore, the combined treatment was introduced in many countries as a treatment option for newly diagnosed glioblastoma. After analysis of patient subgroups, the greatest survival benefit was observed in patients treated with temozolomide and radiotherapy, whose tumours contained methylated *MGMT* (O^6-methylguanine-DNA methyltransferase) gene promoter and in patients with recursive partitioning analysis (RPA) classes III and IV [3, 5, 6]. R. Stupp *et al.* demonstrated a slight benefit in patients aged 60-70 and in RPA class V, too [5]. Treatment results in patients with unmethylated *MGMT* gene promoter are an issue of discussions and further analysis [5, 7]. The aim of the present study was to assess treatment of patients with glioblastoma multiforme in Warmia and Mazury Oncology Centre in the years 2003-2008, taking into consideration clinical prognostic factors and *MGMT* gene promoter methylation.

Material and methods

Patients

Between February 2003 and September 2008, 247 patients with primary malignant brain tumours were registered in the Radiotherapy Department. Eighty-two patients had a histologically confirmed glioblastoma (45 males, 37 females). Overall survival time was assessed from the surgery procedure to death or to January 2010 (follow-up 0-88 months; median follow-up 11 months) (Table 1).

Treatment

Patients received radical radiotherapy (some with concomitant and adjuvant temozolomide) or palliative radiotherapy. Twenty-five patients (age 23-70 years; median age 47 years) received radical three-dimensional radiotherapy (54-62 Gy; dose per fraction 1.8-2 Gy) plus concomitant oral temozolomide at a daily dose of 75 mg/m^2. After a 4-week break, the patients received up to 6 cycles of adjuvant oral temozolomide (150-200 mg/m^2 for 5 days and every 28 days). Thirteen patients (age 24-61 years; median age 54 years) received radical three-dimensional radiotherapy alone. Ten patients received radiotherapy in two phases. Forty patients (age 37-78 years; median age 63 years) received palliative three-dimensional radiotherapy with a dose of 30 Gy and a second phase with a dose of 21 Gy in daily fractions of 3 Gy.

Four patients (age 47-69 years; median age 55.5 years) had two-dimensional palliative radiotherapy with a dose of 20 Gy in daily fractions of 4 Gy on the whole brain. The radiotherapy was delivered using linear accelerators with a nominal energy of 6 MV. The decision about the kind of treatment for a particular patient was made based on the patient's age, performance status, neurological status, extent of surgery, results of MRI and CT, co-existent diseases and possibility of obtaining a refund for the temozolomide treatment. The patients were retrospectively classified according to the RPA classification (by the extent of surgery, interview and physical examination, including neurological examination, and nurses' reports). The tumour tissues of patients treated with temozolomide were investigated for *MGMT* gene promoter methylation in the Department of Molecular Biology, Memorial Cancer Centre, Institute of Oncology in Warsaw. The overall survival time was assessed from the surgery procedure to the patient's death or to January 2010, when information about deaths was confirmed by the Centre for Document Personalization, Minister of Interior and Administration. It is not possible to assess progression-free survival because patients did not have a homogeneous follow-up schedule. The patients who had palliative radiotherapy often did not come to follow-up visits. STATISTICA Version 6 was used to perform statistical analyses.

MGMT promoter methylation analysis

The *MGMT* promoter methylation was determined by methylation-specific PCR (MSP) as described previously by Esteller *et al.* DNA was extracted from formalin-fixed, paraffin-embedded tissue using the Sherlock AX kit (A&A Biotechnology) according to the manufacturer's recommendations. DNA quality was assessed spectrophotometrically as well as by control multiplex PCR as proposed in the BIOMED-2 study. Bisulfite conversion of 1 µg of the DNA was performed using the Epitect kit (Qiagen) according to the manufacturer's instructions. PCR reaction in a volume of 15 µl contained 1 × PCR buffer, 1.5 mM $MgCl_2$, 0.25 mM dNTPs, 10 pmol of each primer, 0.5 u FastStart DNA polymerase (Roche) and 1 µl of the converted DNA. The sequences of primers are shown in Table 2. PCR reaction conditions are shown in Table 3. PCR products were electrophoresed in 8% polyacrylamide gel and visualized in UV light after ethidium bromide staining. All the positive results of the PCR reaction specific for the methylated gene variant, indicating the methylated *MGMT* promoter, were confirmed by direct DNA sequencing. DNA sequencing was performed using BigDye Sequencing Kit V.3.1 (Applied Biosystems) and an AbiPrism 3100 Sequencing Analyzer (Applied Biosystems) according to the manufacturer's recommendations. DNA isolated from peripheral blood of healthy donors was used as a negative control and the positive methylated control was the same DNA treated *in vitro* with the DNA methyltransferase SssI (New England Biolabs), according to the manufacturer's recommendation.

Results

MGMT promoter gene assessment

In 22 evaluated tumours, 12 (46%) had detectable *MGMT* gene promoter methylation (Table 1, Fig. 1). For 3 patients, paraffin-embedded tumour tissues were not available.

Toxicity

During concomitant radiochemotherapy, leukopenia, neutropenia and thrombocytopenia grade 3 and 4 as per CTCAE (Common Terminology Criteria for Adverse Events) occurred in 2 of 25 patients. One of these patients died of pulmonary and cerebral aspergillosis, which was confirmed in autopsy. Grade 3, as per CTCAE, leukoencephalopathy and severe deterioration of the mental status were observed in one 70-year-old patient after radiotherapy with temozolomide. Seven patients discontinued treatment after the phase of concomitant radiochemotherapy. The remaining patients received 1 to 6 cycles (median 4 cycles). None of the patients developed severe adverse events during adjuvant chemotherapy with temozolomide. Thirteen patients who were treated with radical radiotherapy alone did not have toxicity grade 3 and 4 as per CTCAE. One patient had steroid-dependent diabetes. Eighteen of 40 patients received two phases of the planned treatment: 30 Gy and 21 Gy in daily fractions of 3 Gy. Twenty-two patients discontinued radiotherapy because of deterioration of their performance status. Four patients who were treated with palliative two-dimensional radiotherapy had good early treatment tolerance.

Overall survival

The analysis included 82 patients (45 males; 37 females). At the time of the analysis (January 2010), 15 patients were alive. The follow-up lasted for a period of 2.7 months up to

Table 1. Characteristics of patient groups

	Radical treatment (n = 38)	Palliative treatment (n = 440)	Test χ^2
WHO			
0-1	36	16	$p < 0.001$
2	2	16	
3	0	12	
RPA			
III	15	0	$p = 0.001$
IV	21	7	
V	2	23	
VI	0	14	
Extent of surgery			
Radical resection	22	18	$p = 0.125$
Subtotal surgery or biopsy	16	26	
Age			
≤ 50	16	6	$p = 0.004$
> 50	22	38	

Table 2. Primer sequences

Primer	Sequence 5' → 3'	PCR product
mgmt mf	TTTCGACGTTCGTAGGTTTTCGC	81 bp
mgmt mr	GCACTCTTCCGAAAACGAAACG	
mgmt uf	TTTGTGTTTTGATGTTTGTAGGTTTTTGT	93 bp
mgmt ur	AACTCCACACTCTTCCAAAAACAAAACA	

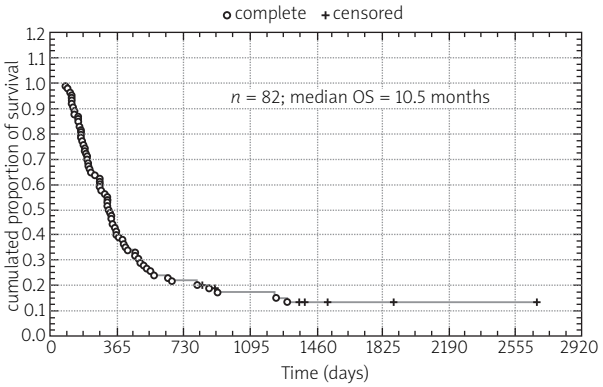


Fig. 2. The overall survival (OS) of patients who were treated in Warmia-Mazury Oncology Center

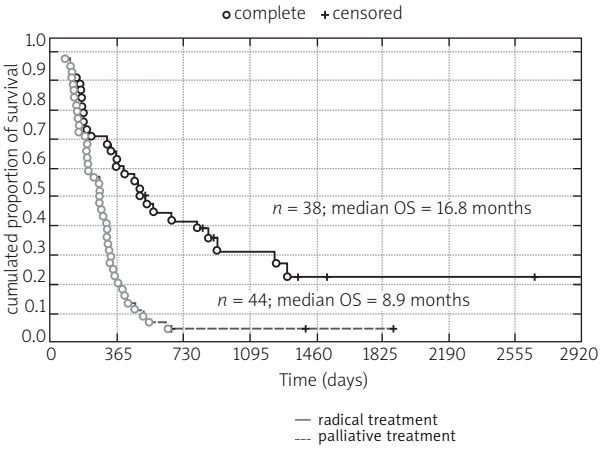
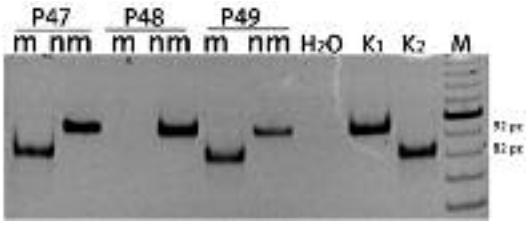


Fig. 3. The overall survival (OS) of patients with glioblastoma multiform depending on treatment methods



m – methylated DNA specific PCR product; nm – unmethylated DNA specific PCR product; H_2O – no template control; K_1 – unmethylated control DNA; K_2 – methylated control DNA

Fig. 1. Analysis of MGMT promoter methylation with the use of methylation specific PCR (MSP) of three examplary tissue samples

98.3 months. The median overall survival (OS) for patients who were treated in Warmia and Mazury Oncology Centre was 10.5 months (Fig. 2). Median OS was 16.8 months for patients treated with radical intent and 8.9 months in patients treated with palliative intent (Fig. 3). The median OS for 25 patients in the chemoradiotherapy group was 27.4 months; for 13 patients in the radical radiotherapy group it was 10.2 months (Fig. 4). The median OS for patients with methylated promoter of *MGMT* gene was 28 months and for patients with unmethylated promoter of *MGMT* gene was 26.5 months (Fig. 5).

The patients who were treated radically were younger, with a better performance status, and they were classified with lower RPA class. The differences were statistically significant (Table 3). No significant difference in the extent of the surgery was found between the groups of patients treated radically and palliatively. In the univariate analysis, it was found that performance status as per WHO, RPA class and age are factors that influence the median OS significantly (Table 4).

Discussion

The treatment results in patients with glioblastoma are unsatisfactory. Hence, new treatment options are sought. The addition of concomitant and adjuvant chemotherapy with temozolomide improves the overall survival (OS) and the progression-free survival. *MGMT* gene promoter methylation status proved to be a strong prognostic and predic-

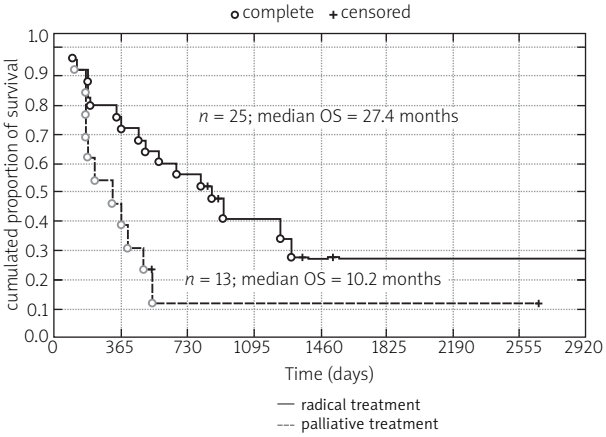


Fig. 4. The overall survival (OS) of patients with glioblastoma multiform depending on the method of radical treatment

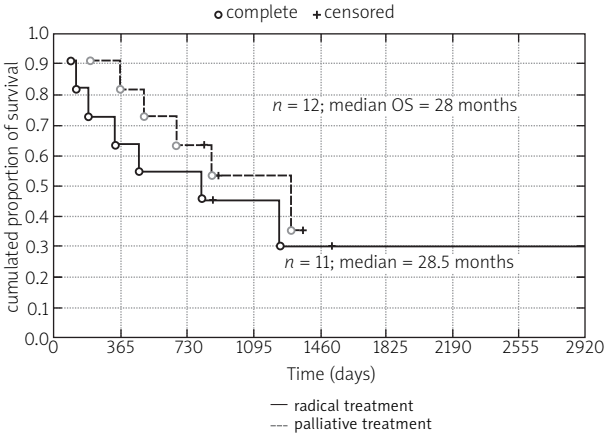


Fig. 5. The overall survival (OS) of patients who were treated with radiotherapy and temozolomide in relation to MGMT promoter methylation status

Table 3. PCR reaction conditions

PCR reaction	Cycling conditions
Methylated variant-specific	94°C 3 min, 38 cycles (94°C 30 s, 62°C 30 s, 72°C 30 s), 72°C 5 min
Unmethylated variant-specific	94°C 3 min, 38 cycles (94°C 30 s, 59°C 30 s, 72°C 30 s), 72°C 5 min

tive factor for survival in patients treated with radiotherapy and temozolomide [1, 6, 7]. We analysed the patients' overall survival depending on the *MGMT* gene promoter methylation status and clinical prognostic factors, such as the performance status, age, and RPA class. In our analysis, the median overall survival for patients treated in Warmia and Mazury Centre is 10.5 months; it is shorter than overall survival in the study of M. Weller *et al.* (12.5 months) [7]. M. Weller *et al.* demonstrated results of treatment in patients with glioblastoma registered in the German Glioma Network. The authors showed that the patients who had radiochemotherapy with temozolomide were younger and had a better performance status than patients treated according to another treatment pattern. In our oncology centre, we found a similar tendency. This is confirmed by the analysis of patient groups with radical and palliative treatment (Table 3). Median overall survival for patients treated with temozolomide in Warmia and Mazury Centre was 27.5 months; it is longer than median overall survival in the EORTC26981/NCIC CE.3 trial and in the German Glioma Network [7]. No significant differences were found in median overall survival for patients with methylated (28 months) and unmethylated promoter of *MGMT* gene (26.5 months), treated with radiochemotherapy. However, one must bear in mind the methodological limitations of our analysis of the assessed group of patients due to the low number of patients and the retrospective character of the study. The *MGMT* gene promoter methylation status is a strong prognostic factor for increase in OS for patients treated with temozolomide, according to the studies of M. Weller *et al.* [7], M. Hegi *et al.* [6] and R. Stupp *et al.* [5]. A five-year analysis of results of the EORTC26981/NCIC CE.3 trial also showed that the survival of patients, irrespective of the methylation status of the *MGMT* gene promoter, treated with the combined therapy was significantly longer than that of patients treated with radiotherapy alone [5]. In the earlier and five-year analysis of the aforementioned study, it was shown that the patient's survival depends on the RPA class, which includes age, performance status and extent of the surgery. Analysis of data of patients of the Warmia and Mazury Centre did not reveal a difference between OS for patients with methylated and unmethylated *MGMT* gene promoter, treated with temozolomide. An interesting finding is the small difference between median OS for patients treated with radical radiotherapy alone and palliative radiotherapy (10.2 months vs. 8.9 months) despite the fact that patients qualified for palliative treat-

Table 4. Univariate analysis according to clinical prognostic factors

Parameter	Number of patients	Median overall survival (OS) (months)	*p*
Patients	82	10.5	
WHO			
0-1	53	12.3	0.0034
2	17	8.0	
3	12	8.0	
RPA			
III	15	29.3	0.0001
IV	28	13.1	
V	25	8.9	
VI	14	6.9	
Extent of surgery			
Radical resection	40	13.3	0.0137
Subtotal surgery or biopsy	42	8.9	
Age			
≤ 50	22	17.5	0.0051
> 50	60	10	

ment had worse clinical prognostic factors and a poor performance status. This is probably due to the small number of patients who had radical radiotherapy alone, because in our oncology centre we qualified patients for combined treatment if there was a possibility of obtaining individual authorisation for temozolomide treatment from the National Health Fund. A controversial issue is combined treatment of patients aged over 65 years. In the Warmia and Mazury Oncology Centre, one 70-year-old patient was treated. Shortly after the concomitant radiochemotherapy, this patient developed leukoencephalopathy grade 3 as per CTCAE and serious cognitive disturbances. The patient did not receive adjuvant chemotherapy. A. E. Sijben *et al.* [8] and A. A. Brandes *et al.* [9] demonstrated a positive influence of radiochemotherapy with temozolomide on OS of patients over 65 years old. A. A. Brandes *et al.* reported a high percentage of two-and three-year OS for elderly patients with a confirmed methylation of the *MGMT* gene promoter [9]. Grade 3 leukoencephalopathy and grade 3 mental deterioration were demonstrated in a patient after the combined therapy [9], like in one elderly patient treated in our department. R. Stupp *et al.* [5] demonstrated a survival benefit after the combined modality treatment in patients aged over 60 years. However, these subgroup analyses on limited patient data lack statistical power [5]. Treatment reports on the treatment of elderly patients over 65 years old were presented at the ASTRO 2010 conference. A tendency for hypofractionated radiotherapy (40 Gy/15 fr) and concomitant and adjuvant treatment with temozolomide [10, 11] or salvage chemotherapy was observed [12].

Conclusions

Concomitant radiochemotherapy with temozolomide should be routine clinical practice for all patients with a good performance status.

The outcomes of treatment only with radiotherapy in patients with glioblastoma are worse and similar to outcomes of palliative radiotherapy.

Elderly patients (over 65 years old) should be selected for the combined treatment very carefully. The assessment of *MGMT* gene promoter methylation status can be helpful in controversial cases.

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