Post-operative Combined Radiation and Chemotherapy with Temozolomide and Irinotecan in Patients with High-grade Astrocytic Tumors. A Phase II Study with Biomarker Evaluation

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Abstract. *Background: Clinical studies have shown that temozolomide (TMZ) and irinotecan demonstrate activity in high grade astrocytic tumors (HGAT). However, the optimal schedule of administration is unknown. Patients and Methods: In the present study, a total of 45 HGAT patients, 38 with glioblastoma multiforme (GBM) and 7 with anaplastic astrocytoma (AA), were treated with TMZ, 150 mg/m^2 on days 1-5, followed by irinotecan, 150 mg/m^2 on days 6 and 17, every 4 weeks for 6 cycles or until the occurrence of unacceptable toxicity or disease progression. Radiation therapy (60 Gy) was initiated on the first day of treatment. Results: Twenty-two patients completed six cycles of treatment. Most frequently recorded side-effects included neutropenia (37%), nausea/vomiting (66%), diarrhea (31%) and infection (44%). Five episodes of vaso-occlusive disease, all of them fatal, were observed. After a median follow-up of 49.8 months, median progression-free survival for patients with GBM was 7.7 months, while median overall survival was 12.8 months. There were six long-term survivors, three of them with GBM. Two out of the five biomarkers studied, epidermal growth factor receptor (EGFR) and vascular endothelial growth factor-C (VEGF-C), were found to be overexpressed in 74% of the tumors, however they had no predictive value for progression-free or overall survival. Conclusion: The combination of TMZ and irinotecan, as administered in this study, was accompanied by high rates of toxicity, especially myelotoxicity and infection. Further development of this regimen in the treatment of HGAT is not recommended.*

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High grade astrocytic tumors (HGAT) account for 60% of all primary brain tumors in adults and they are the third and fourth leading causes of cancer-related mortality in men between 15 and 55 years of age, and in women between the ages of 15 and 34 years, respectively (1). Approximately 17,000 new cases of primary brain tumors occur in the USA and nearly 12,000 patients die of their disease each year (2). Histologically, HGAT are separated according to grade into anaplastic astrocytomas (AA) and glioblastomas multiforme (GBM).

Surgical debulking followed by external beam radiotherapy (RT) to approximately 60-65 Gy is considered the standard treatment for HGAT. However, the role of post-radiation or post-surgery chemotherapy for these patients remains unclear. A meta-analysis of 16 published randomized trials indicated a small (10%), but statistically significant increase in 1-year survival rate with the addition of chemotherapy (3). It is, therefore, apparent that new active drugs are needed to improve the outcome of patients with HGAT.

Temozolomide (TMZ, Temodal®) is a novel oral alkylating agent that has demonstrated efficacy in the treatment of a variety of solid tumors, including primary malignant brain tumors (4-6). Because of its small molecular weight, TMZ efficiently crosses the blood brain barrier (7) and, for this reason, is considered to be a promising agent against primary brain tumors and secondary central nervous system malignancies (8). TMZ has been extensively investigated in the treatment of HGAT at first relapse with disease progression, on a nitrosourea and procarbazine-containing regimen (9). In addition, camptothecins are among the most

active agents against brain tumor xenografts (10, 11). Irinotecan (CTP-11, Campto®) has demonstrated encouraging activity in phase II studies in patients with progressive or recurrent HGAT (12, 13). Moreover, the combination of irinotecan with alkylating agents including TMZ in tumor xenografts derived from adult high-grade gliomas, showed a supra-additive effect (14, 15).

Motivated by the existing preclinical and clinical work suggesting that the combination of TMZ and irinotecan is an interesting regimen to be tested as first-line treatment for HGAT, a feasibility study in such patients was conducted. The primary end-points of the study were progression-free survival (PFS), safety and tolerability. Secondary end-points were response rate and overall survival (OS).

Patients and Methods

Patient eligibility. In order to be eligible for the study (HE 17/00), patients were required to have a histologically proven HGAT, be aged 15-75 years, have a performance status (PS)≤2 according to the Eastern Cooperative Oncology Group (ECOG) scale, life expectancy of at least 12 weeks, adequate bone marrow reserve and adequate renal and liver function.

Tumor tissue was obtained for central review and translational research studies. All histology was reviewed by one of the authors (G.K.). The protocol was approved by the Hellenic Cooperative Oncology Group (HeCOG) Protocol Review Committee and the Institutional Review Board of the AHEPA Hospital, Aristotle University of Thessaloniki, Greece. Each patient provided study-specific written informed consent before entry to the protocol.

Patients were excluded from the study: if they had a second malignancy, except for *in situ* carcinoma of the cervix or adequately excised basal cell carcinoma of the skin; and, if they had a history of atrial or ventricular arrhythmias, congestive heart failure, active infection or other serious underlying medical conditions that precluded the patient from receiving protocol treatment.

Treatment. The treatment was initiated within 2-4 weeks from the initial operation. TMZ, 150 mg/m^2, was given orally on days 1-5, and irinotecan, 150 mg/m^2 as a 90-min infusion, was administered on days 6 and 17. Each cycle was repeated every 4 weeks. Patients were treated with six cycles of chemotherapy, unless evidence of disease progression or unacceptable toxicity occurred during this period.

Dolacentron was used as an antiemetic for all patients. Corticosteroid dosing was left to the discretion of the treating physician. Low molecular weight heparin was given prophylactically during the whole treatment period especially to patients with impaired PS. Valproic acid, oral administration of 500 mg three times a day, was recommended as an anticonvulsant treatment for patients with a history of seizures. However, eventually 21 patients received valproic acid, while 8 received phenytoin and 8 oxcarbazepine. Since these drugs inhibit SN-38 conjugation and possibly increase the effect of irinotecan, myelotoxicity was monitored carefully.

Dose modifications. The doses of drugs were adjusted according to the complete blood count (CBC), the platelets count and liver function tests. The chemotherapy was only given when the absolute neutrophil count (ANC) was ≥1,500/μL and the platelet count ≥100,000/μL on the day of treatment.

In the case of grade II-III neutropenia and/or grade II thrombocytopenia, treatment was withheld until recovery and the dose of both drugs was reduced to 125 mg/m^2. In the case of grade IV neutropenia and/or grade III-IV thrombocytopenia, the doses of both irinotecan and TMZ were reduced to 100 mg/m^2. If the patients experienced any grade III non-hematological toxicity, treatment was withheld until recovery to grade I or less, and both drugs were given at a dose of 100 mg/m^2. If a grade IV non-hematological toxicity was recorded, then treatment was discontinued permanently and the patient was taken off the study.

Radiotherapy (RT). RT was initiated two to four weeks after surgery. A 6 MV linear accelerator was used in all cases. Patients were put on steroids and continued on the same dose until one week after the end of RT, with subsequent reduction at the discretion of the physician.

A treatment-planning CT scan, with reference marks made on the patient's head or immobilization device, was obtained. The scan slice thickness was 0.5 cm through the region that contained the target volume. The delineation of the target volume was achieved with the aid of a preoperative CT/MRI scan. The gross tumor volume (GTV) included the contrast-enhancing residual tumor and the resected tumor cavity with no margin. The planning target volume (PTV) was defined as the GTV plus a 2.5 cm margin of adjacent areas of high disease risk. The PTV was delineated on every CT slice and a 3D treatment plan was constructed. If this was not possible, a 2D plan was constructed on the slice where the maximum tumor diameter was observed.

The dose was specified at the isocenter and it was prescribed at the 90%-isodose. After the dose of 40 Gy, the target volume was reduced encompassing the GTV plus 0.5 cm. A total dose of 60 Gy with conventional fractionation (1.8-2.0 Gy daily, five days per week) was administered. All fields were simulated and verification films were taken.

Clinical and laboratory evaluation. Patients were monitored weekly by CBC and were evaluated for toxicity on day 15 of the treatment. Each patient was required to have pre- and post-surgery MRIs (at least two weeks after the initial operation). The MRI was repeated after the third and sixth cycle of chemotherapy for response assessment. All imaging material pertinent for tumor response was reviewed by one of the authors (A.K-F.). Response criteria were those proposed by Macdonald *et al.* (16).

The National Cancer Institute Common Toxicity Criteria (version 2.0) scale was used for toxicity grading.

Immunohistochemistry (IHC). Tumor samples from 28 patients (24 with GBM and 4 with AA) were immunohistochemically studied for COX-2, Epidermal Growth Factor Receptor (EGFR), Ki-67 (MIB-1), PTEN and Vascular Endothelial Growth Factor-C (VEGF-C). The source of the antibodies, the conditions of pre-treatment and staining and the visualization systems are shown in Table I. Briefly, two micron-thick paraffin sections were cut from representative tissue blocks. The tissue sections were deparaffinized by overnight incubation at 60˚C and subsequent immersion in xylenes, and rehydrated in descending ethanol baths. The slides were then treated with 0.3% hydrogen peroxide in methanol for 20 min, in

Table I. *Primary antibodies used in the study.*

Antigen	Source	Clone	Pretreatment/Time/WB	Dilution	Incubation	Visualization system
COX-2 (m, mab)	Novocastra, U.K	4H12	CB, Steamer/20 min/TBS	1:80	O/N	Super Sensitive Link-Label HRP (1)
EGFR (m, mab)	Zymed Laboratories, U.S.A	31G7	Proteinase K/10 min/TBS	1:50	O/N	LSAB (2)
Ki-67 (m, mab)	DakoCytomation, DK	MIB-1	CB, MW/21 min/TBS	1:40	O/N	Super Sensitive Link-Label HRP (1)
PTEN (m, mab)	Novocastra, U.K	28H6	CB, MW/15 min/PBS	1:150	1 h	Super Sensitive Polymer-HRP (1)
VEGF-C (r, mab)	Novocastra, U.K	Z-CVC7	CB, Steamer/20 min/TBS	1:40	O/N	Super Sensitive Link-Label HRP (1)

CB: citrate buffer; LSAB: labeled streptavidin biotin; m:mouse; mab: monoclonal antibody; MW: microwave; O/N: overnight; PBS: phosphate-buffered saline; r: rabbit; TBS: Tris-buffered saline; WB: wash buffer (1): BioGenex; (2): DakoCytomation

order to quench the endogenous peroxidase activity. Antigen retrieval was performed by heating the slides in a sodium citrate solution (pH 6.0), in a microwave oven or steamer. For EGFR antigen unmasking, the slides were treated with proteinase K (DakoCytomation) at 37˚C for 10 min. Following antigen retrieval, slides were washed in an adequate volume of buffer solution and incubated for 10 min in a Powerblock (BioGenex), in order to block nonspecific protein binding. Antibodies were diluted in antibody diluent (DakoCytomation) and applied as shown in Table I. After a 1-hour incubation with specific visualization systems and additional washings, the antigen-antibody complex was visualized using diaminobenzidine (BioGenex) as a chromogen. Slides were counterstained with Mayer's hematoxylin, dehydrated and mounted. Appropriate positive and negative control sections were stained in parallel with those of the study.

Scoring system for interpretation of immunohistochemical markers. The evaluation of the immunostains was done by two independent observers, one neuropathologist (G.K.) and one general pathologist (M.B.), blinded as to the patient's clinical characteristics and survival data.

COX-2: For COX-2 the percentage of positive tumor cells was determined semiquantitatively, by assessing the whole stained section. Results were graded on a scale of 0 to 4, based on the percentage of specific tumor cell staining, as described by Joki *et al.* (17): grade 0, no staining reaction or <5% of the tumor cells stained positive; grade 1, ≥5% to <25% of the tumor cells; grade 2, ≥25% to <50% of the tumor cells; grade 3, ≥50% to <75% of the tumor cells; grade 4, ≥75% of the tumor cells. The intensity of immunostaining was determined as: 0, absence of staining; 1+, weak staining; and 2+, strong staining. An immunoreactive score was calculated by multiplying the grade derived from the percentage of positive cells by the intensity of staining, as proposed by Krajevska *et al.*(18, 19). For heterologous staining patterns, each component was scored independently and the results were summed, as previously described (17).

EGFR: EGFR immunostaining was graded as either absent or present in single tumor cells, as previously described (20, 21). Extent of staining was scored as 0 (0-5%), 1 (>5%-50%), 2 (>50%-90%) and 3 (>90%), according to the percentage of immunoreactive tumor cells (22). Only tumors included in the latter two groups were considered as having EGFR overexpression (22).

Ki-67 (MIB-1): Ki-67 immunostaining was scored counting 500 neoplastic cells in 10 representative fields (hot points), using a high power (x40) objective with a grid screen. All stained cells were recorded as positive, independently of the staining intensity. The percentage of positively stained cells, termed the proliferation index, was calculated and recorded.

PTEN: PTEN immunohistochemical expression was evaluated according to a previously established rank scale of 0 to 2 (23, 24). Vascular endothelial cells were used as a control marker of staining intensity. PTEN immunohistochemical expression in neoplastic cells was graded as: 2, if their staining intensity was equal or higher to that of endothelial cells; 1, if their staining intensity was reduced compared to that of endothelial cells; and 0, if staining intensity was undetectable in the tumor cells. Tumors with PTEN scoring of 0 or 1 were considered PTEN deficient. The percentage of neoplastic cells was assessed by counting 100 cells in four different x40 microscope fields. PTEN immunoreactivity in less than 5% of the tumor cells was regarded as negative.

VEGF-C: The VEGF-C staining pattern was assessed semi-quantitatively, taking into account the percentage of positive tumor cells (at least 500 tumor cells were counted), which was termed the labeling index (LI). LI was then used to classify tumor cells into four grades: grade 0, LI <5%; grade 1, LI ≥5%-≤25%; grade 2, LI >25%-≤50%; and grade 3 LI >50% (25). The intensity of immunoreactivity was graded on a relative 3-tier rank scale as: grade 1, slight or not detectable staining; grade 2, moderate staining; and grade 3, intense staining. The immunoreactive score for VEGF-C was calculated by multiplying the LI grade by the intensity grade. Only tumors with an immunoreactive score ≥4 were considered as having VEGF-C overexpression.

Statistical analysis. The primary objective of the study was to evaluate the 6-month PFS rate. According to Fleming's single-stage design, assuming the expected 6-month PFS rate to be at least 70% and the minimum acceptable rate 50%, a total of 37 patients would provide 80% power to test the hypothesis, with a two-sided a=5%.

Survival was estimated from the date of surgery to the date of the last follow-up or until the patient's death. PFS was deemed as the time between surgery and progression, documented clinically and/or radiologically. Patients, who died from the disease without having a documented progression, or from any cause during the chemotherapy period or within a month from its completion, were considered as events for the estimation of PFS. Time to treatment failure (TTF) was calculated from surgery to the date of treatment discontinuation for any reason, or disease progression, or death from any cause during chemotherapy (or within a month from its completion).

Univariate Cox regression analysis was used to assess the relationship between time to event end-points and immunohisto-chemical markers.

Table II. *Selected patient and tumor characteristics.*

	GBM		AA	
No.	38		7	
Age				
Median	58.5		47	
Range	30-75.5		39-59	
<58	17 (45%)		6 (86%)	
≥58	21 (55%)		1 (14%)	
	N	%	N	%
Gender				
Men	26	68	6	86
Women	12	32	1	14
Performance status				
0	13	34	1	14
1	13	34	5	71
2	11	29	1	14
3	1	3	-	-
Hemisphere				
Right	24	63	3	43
Left	14	37	3	43
Bilateral	-	-	1	14
Tumor location				
Frontal	5	13	2	29
Temporal	13	34	3	43
Parietal	20	53	1	14
Occipital	7	18	1	14
Type of operation				
Gross total resection	17	45	5	71
Subtotal resection	9	24	2	29
Biopsy	12	32	-	-

Values are rounded up.
GBM: glioblastoma multiforme, AA: anaplastic astrocytoma.

Table III. *Selected treatment characteristics (n=45).*

Number of cycles delivered	194
Number of cycles per patient [n (%)]	
1	5 (11)
2	3 (7)
3	11 (24)
4	2 (4)
5	2 (4)
6	22 (49)
Number of cycles at full dose	162
Number of cycles with delay	22
Median interval between cycles (days)	28
Number of cycles at full dose for TMZ[a]	183
Number of cycles at full dose for irinotecan	164
DI of TMZ delivered	
Median (mg/m^2/day)	163
Range	37-188
RDI of TMZ	
Median	0.87
Range	0.2-1.0
DI of irinotecan delivered	
Median (mg/m^2/day)	68
Range	41.5-76.9
RDI of irinotecan	
Median	0.91
Range	0.5-1.0

[a]≥90% of the dose defined in the protocol.
TMZ: temozolomide, DI: dose intensity, RDI: relative dose intensity.

Results

Patient population. From January 2001 until October 2003, 45 patients (38 with GBM and 7 with AA) entered the study. Selected patient and tumor characteristics are shown in Table II. Most of the patients presented with a PS of 0-1.

Compliance to treatment and toxicity. Twenty-two patients (17 out of 38 with GBM and 5 out of 7 with AA) completed six cycles of treatment, while 23 patients discontinued treatment. Selected treatment characteristics are shown in Table III.

Nine patients died during the treatment period: three patients died from acute massive pulmonary embolism, one of them at the end of the first cycle and the other two in the period between the two irinotecan infusions of the third cycle; two patients died from acute myocardial infarction after the first and third cycle, respectively; a 63-year old woman was admitted to the hospital after the third cycle with febrile neutropenia and pneumonia and, despite the appropriate treatment, she succumbed three days later from sepsis; finally, three patients died from tumor progression. Other reasons for treatment discontinuation were tumor progression (10 patients), withdrawal of consent (1), moving to another hospital (1) and long hospitalization for *Pneumocystis carinii* pneumonia (PCP) (2). The latter patients, even though they were taken off the study, continued treatment with TMZ monotherapy.

The incidence of the various toxicities among the 45 registered patients is depicted in Table IV. Twenty-five patients (56%) exhibited myelosuppression. Twenty-eight episodes of infection were recorded in 20 patients. Among them, three patients had fungal infections and six viral (one of them developed herpes zoster in the face and was treated successfully with Famciclovir). Two patients were diagnosed with PCP, as previously stated. They were both treated successfully with trimethoprime-sulfomethoxazole. In total, 17 patients were hospitalized, three because of febrile neutropenia. Microorganisms isolated from blood, sputum and urine were: *Streptococcus* in two cases, *Staphylococcus aureus* in one, *Escherichia coli* in two and *Pneumocystis carinii* in two. Seventeen patients were treated with

Table IV. *Incidence, N (%), of various toxicities (N=45).*

	Grade				
	1	2	3	4	5
Anemia	5 (11)	4 (9)	1 (2)	0	0
Neutropenia	9 (20)	2 (4)	6 (13)	0	0
Leukopenia	9 (20)	7 (16)	6 (13)	0	0
Thrombocytopenia	4 (9)	3 (7)	3 (7)	0	0
Nausea and Vomiting	20 (44)	10 (22)	0	0	0
Diarrhea	8 (18)	5 (11)	1 (2)	0	0
Constipation	9 (20)	3 (7)	0	0	0
Anorexia	5 (11)	1 (2)	0	0	0
Alopecia[a]	1 (2)	5 (11)	8 (18)	0	0
Allergic reactions	1 (2)	3 (7)	0	0	0
Gastritis	3 (7)	2 (4)	0	0	0
Stomatitis	0	2 (4)	0	0	0
Infection	3 (7)	2 (4)	14 (31)	0	1 (2)
Fatigue	3 (7)	7 (16)	0	0	0
Dizziness	2 (4)	0	0	0	0
Athralgias	0	1 (2)	0	0	0

[a]Outside the irradiation field.

antibiotics. Additionally, G-CSF administration was required in five patients at some time during the treatment period and erythropoietin in six. Finally, three patients were transfused with red blood cells.

Response to treatment and survival. Best response rates are shown in Table V. Eleven patients, all with GBM, were not evaluated for the response to treatment: seven died before having an MRI, which was planned, according to the protocol, after the third cycle, one moved to another hospital after the second cycle, one demonstrated early deterioration of his clinical condition, one had a gross total resection and for one patient there were no MRI films available for review. Median duration of PR and SD was 12.4 months (range: 3.28-61.21 + months) and the corresponding times for AA were 7.57, 15.28, 32.39, 40.72, 49.80 and 61.18, respectively.

After a median follow-up of 49.8 months (range, 1.38–61.21+), 35 out of the 38 patients with GBM (92%) have progressed, and died. Among the 7 patients with AA, four patients have progressed so far and three of them have died. In patients with GBM, median PFS was 7.7 months [range: 1.38–61.21+, 95% confidence interval (CI): 1.22–14.19], median TTF was 4.19 months (range: 0.72–61.21+, 95% CI: 2.27–6.13), while median survival was 12.79 months (range: 1.38-61.21+, 95% CI: 10.37–15.20, Figure 1).

Tumor biomarker expression analysis. Individual data of the IHC assessed protein expression in 28 patients are given in Table VI.

Table V. *Best response data.*

	GBM (N=38)		AA (N=7)
	N(%)	95% CI	N
PR	3 (8%)	1.7-21.4	1
SD	14 (37%)	21.8-54	5
PD	10 (26%)	13.4-43.1	1
NE	11 (29%)	15.4-45.9	0

Values were rounded up. GBM: Glioblastoma multiforme, AA: Anaplastic astrocytoma, CI: confidence interval, PR: partial response, SD: stable disease, PD: progressive disease, NE: non-evaluable.

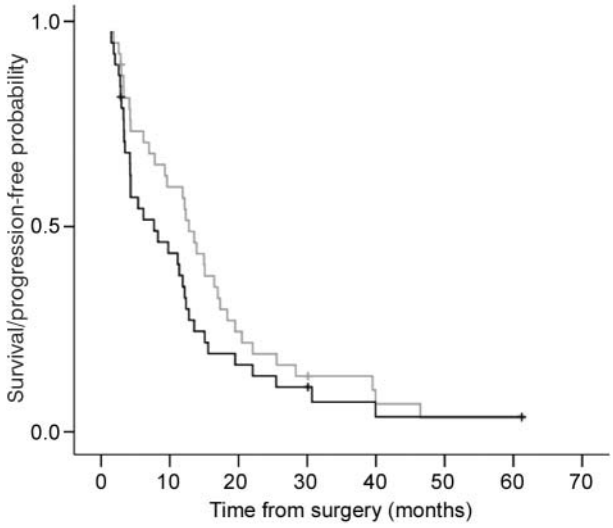

Figure 1. *Progression-free survival (——) and overall survival (——) of patients with GBM.*

COX-2: COX-2 protein expression was detected by IHC in all tissue samples tested (Figure 2a). The staining pattern was cytoplasmic, whereas granular staining was also detected in a small number of tumor cells. GBM tumors showed a large range of distribution and intensity of staining (range from 1 to 7 as expressed by immunoreactive score) whereas, all of the AA tumors tested showed high immunoreactive scores (≥7 in 4 out of 4 cases).

EGFR: EGFR was immunohistochemically detected in the cytoplasm and membrane of neoplastic cells in 24 out of 28 cases (86%) (Figure 2b). However, EGFR overexpression was noticed in 18 out of 24 (75%) of GBM and in 2 out of 4 of AA patients.

Ki-67 (MIB-1): The proliferation index estimated by the expression of Ki-67 ranged from 5% to 80% for the entire study group. Particularly, in the AA tumors the expression of Ki-67 ranged from 5% to 12% of tumor cells, while in the

Table VI. *Characteristics, protein expression and survival of a cohort of 28 patients with HGAT.*

Study number	Histology	Gender	Age (years)	Survival (months)	PFS	TTF	MIB-1 (%)	COX-2 grade x INT	VEGF-C LI grade x INT	EGFR expression	PTEN % of cells*
170009	GBM	M	66	15.1	15.1	15.1	20	2	1	1	80
170018	GBM	F	70	15.0	11.1	11.1	13	3	1	2	60
170006	GBM	M	58	61.2+	61.2+	61.2+	38	1	4	2	40
170013	GBM	M	56	2.6	2.6	2.1	32	6	4	0	80
170026	GBM	M	55	12.3	3.5	3.5	8	1	3	3	60
170027	GBM	M	47	9.6	2.8	2.8	20	1	4	3	90
170005	GBM	M	73	13.5	13.5	0.7	38	2	1	2	30
170029	GBM	M	61	13.9	4.3	4.1	18	2	6	0	>95
170022	GBM	F	53	39.3	39.3	39.3	10	5	6	1	>95
170032	GBM	M	54	19.5	19.5	4.3	45	1	6	2	90
170036	GBM	M	65	6.2	6.2	3.5	75	6	4	3	50
170008	GBM	F	51	3.3	3.3	3.1	18	3	3	3	70
170046	GBM	F	30	30.1+	30.1+	30.1+	18	6	4	3	90
170017	GBM	M	59	1.8	1.8	1.3	18	1	6	3	70
170040	GBM	M	50	11.9	11.9	11.9	20	4	9	2	80
170043	GBM	F	63	2.95	2.95	2.85	8	6	9	2	>95
170001	GBM	M	76	4.3	4.3	3.9	5	7	4	2	>95
170021	GBM	F	59	4.2	4.2	4.1	25	7	9	2	90
170014	GBM	F	52	2.9	2.9	2.9	6	7	9	2	80
170042	GBM	F	35	17.3	12.4	12.4	67	6	6	2	>95
170044	GBM	M	67	7.8	2.0	2.0	80	7	6	1	>95
170020	GBM	M	64	4.1	1.5	1.3	23	6	9	3	50
170016	GBM	M	41	16.5	9.8	9.8	15	6	2	3	60
170019	GBM	M	60	22.1	22.1	22.1	30	1	ND	0	>95
170003	AA	M	41	61.2+	61.2+	61.2+	12	8	9	2	60
170028	AA	M	47	49.8+	49.8+	49.8+	9	7	1	1	70
170045	AA	M	46	32.4+	32.4+	32.4+	5	8	9	0	>95
170024	AA	M	58	9.0	2.75	2.2	8	7	6	2	>95

HGAT: High grade astrocytic tumors, GBM: Glioblastoma multiforme, AA: Anaplastic astrocytoma, PFS: progression-free survival, TTF: Time to treatment failure, LI: Labeling index, INT: Intensity, ND: Not done.
*All cases were graded as 2.

GBM tumors the mitotic activity was higher, as expected, ranging from 5% to 80%. Seven out of the 24 GBM tumors showed a Ki-67 proliferation index of >30%.

PTEN: Staining for PTEN demonstrated nuclear positivity in all cases (Figure 2c). The immunoreaction was equal or increased compared to that of endothelial cells in all 28 tumors. The percentage of stained tumor cells ranged from 30% to over 95%.

VEGF-C: VEGF-C overexpression (immunoreactive score ≥4) was detected in 20 out of 27 cases (74%) (Figure 2d), more specifically in 17 out of 23 (74%) of the GBM tumors and in 3 out of 4 of the AA tumors. The staining pattern was cytoplasmic. Eleven out of the 23 GBM tumors (48%) and 3 out of the 4 AA tumors showed an immunoreactive score of ≥6.

None of the aforementioned immunohistochemical markers had any predictive value for OS, PFS or TTF ($p>0.05$ in all cases).

Discussion

The prognosis usually associated with the diagnosis and local treatment of HGAT continuously prompts investigators towards the evaluation of more effective and well-tolerated adjuvant treatment regimens. In the present study our experience with post-operative administration of TMZ and irinotecan in such patients is reported. To our knowledge this is one of the first studies reporting on the efficacy of this combined modality treatment in patients with newly diagnosed HGAT.

TMZ has proven efficacy when given to patients with newly diagnosed GBM. In the pivotal study by Stupp *et al.* (26) in patients with GBM, it has been clearly demonstrated that concomitant administration of RT and TMZ, followed by adjuvant TMZ for six months, had improved survival of patients with GBM compared to which had been achieved by RT alone. Median OS of patients treated with combined

treatment had been 14.6 months, significantly longer than the 12.1 months that had been reported with RT monotherapy. These results were in accordance with those from a Greek randomized phase II trial (27). The latter trial had made use of an intensified adjuvant treatment, administering TMZ 150 mg/m^2 for five days, after RT every 2 weeks. The schedule of TMZ administration and duration of chemotherapy are two issues still under debate. In a new ongoing randomized trial, we are currently exploring the impact of adjuvant TMZ treatment prolongation from six to twelve months on the survival of patients with HGAT.

According to our protocol patients with AA were also eligible to enter the present study. It is well established that AA patients have a more protracted natural history than patients with GBM. However, it is reasonable that patients with AA should be treated with combined chemotherapy and RT as well, since a meta-analysis of 12 randomized trials comparing RT with or without chemotherapy had suggested that chemotherapy appeared to have a similar survival benefit for patients with AA and GBM (28, 29).

It is unclear why the response rate achieved in our study with the combination of TMZ and irinotecan appears to be inferior to that reported with TMZ monotherapy. Patient selection, differences in drug scheduling and dosing, or simply strictly applied response criteria may have been responsible for this discrepancy. The schedule of administration of irinotecan immediately after the completion of the 5-day course of TMZ was selected because, at the time when this protocol was designed, there were no clinical data on the concomitant use of TMZ and irinotecan with RT. Furthermore, the administration of only two doses of irinotecan per cycle was chosen, because frequent admissions to the hospital are tiresome for these patients, who usually have low PS and difficulty in complying with tedious treatment schedules. Notably, median survival in other studies with TMZ monotherapy has been similar to that reported in our patients. However, comparisons between different phase II studies are dangerous, since they may lead to erroneous conclusions.

Chemotherapy in our study was accompanied by considerable toxicity. The most commonly recorded side-effects were myelotoxicity, nausea/vomiting and diarrhea, mostly due to the addition of irinotecan. The high incidence of myelotoxicity could be attributed to the concomitant administration of anticonvulsants, since, to some extent, anticonvulsants interfere with the metabolism of irinotecan and alter its toxic profile. Pharmacokinetic studies have shown that concomitant administration of certain anticonvulsants with irinotecan produces reduced exposure to the active irinotecan metabolite SN-38 (12). Therefore, the use of anticonvulsants should be limited to patients with a documented history of seizures.

It is worthwhile mentioning, that patients with GBM are at risk of developing two other life-threatening medical conditions, *i.e.,* infections and vaso-occlusive disease. Twenty-eight episodes of infection, most of them of mild to moderate severity, were recorded. Of note, there were three cases of pneumonia, one of them fatal, in an older patient who was admitted to the hospital because of febrile neutropenia. The microorganisms isolated in the other two cases of pneumonia were *Pneumocystis carinnii*. The high incidence of infections among patients with HGAT could probably be attributed to the impaired physical condition of these patients and to the prolonged use of corticosteroids. Immuno-compromized patients are more susceptible to the development of opportunistic infections, such as PCP, and, therefore, are candidates for some form of chemoprophylaxis. Stupp *et al.* (26) observed two cases of PCP and they subsequently decided to introduce prophylactic pentamidine inhalations to their patients during the concomitant treatment phase. No additional opportunistic infections occurred following the introduction of this therapeutic policy. Nevertheless, the possibility that the high rate of infections observed in the present study was due to the combined TMZ/irinotecan chemotherapy regimen cannot be excluded.

Moreover, despite the fact that almost all of our patients received prophylactic low-molecular weight heparin, six patients developed vaso-occlusive disease, four of them fatal. It has been reported that thromboembolic events occur frequently in patients with HGAT and contribute to the high mortality associated with this disease. Biochemical factors, such as access to the systemic circulation of tissue factor (TF), the receptor of factor VII/VIIa and a key player in the initial phase of coagulation (30), VEGF release by the glioma cells (31), increased circulating levels of plasminogen activator inhibitor-1 (PAI-1) (32), or clinical factors, such as immobility, surgery, or administration of chemotherapy (33, 34), may all predispose these patients to thromboembolism. The role of prophylactic use of anticoagulants is currently under intensive clinical testing.

In the era of molecular oncology an increasing number of translational research studies aim to identify molecular markers with prognostic or treatment-specific predictive value. In the present study, the expression of five such markers, which have been implicated in the malignant process of human gliomas was evaluated immunohisto-chemically. However, our results should be interpreted cautiously, given the relatively small sample size and the retrospective nature of the study.

COX-2 expression has been previously detected in human gliomas (17, 35). In our study, there was considerable heterogeneity in COX-2 expression,

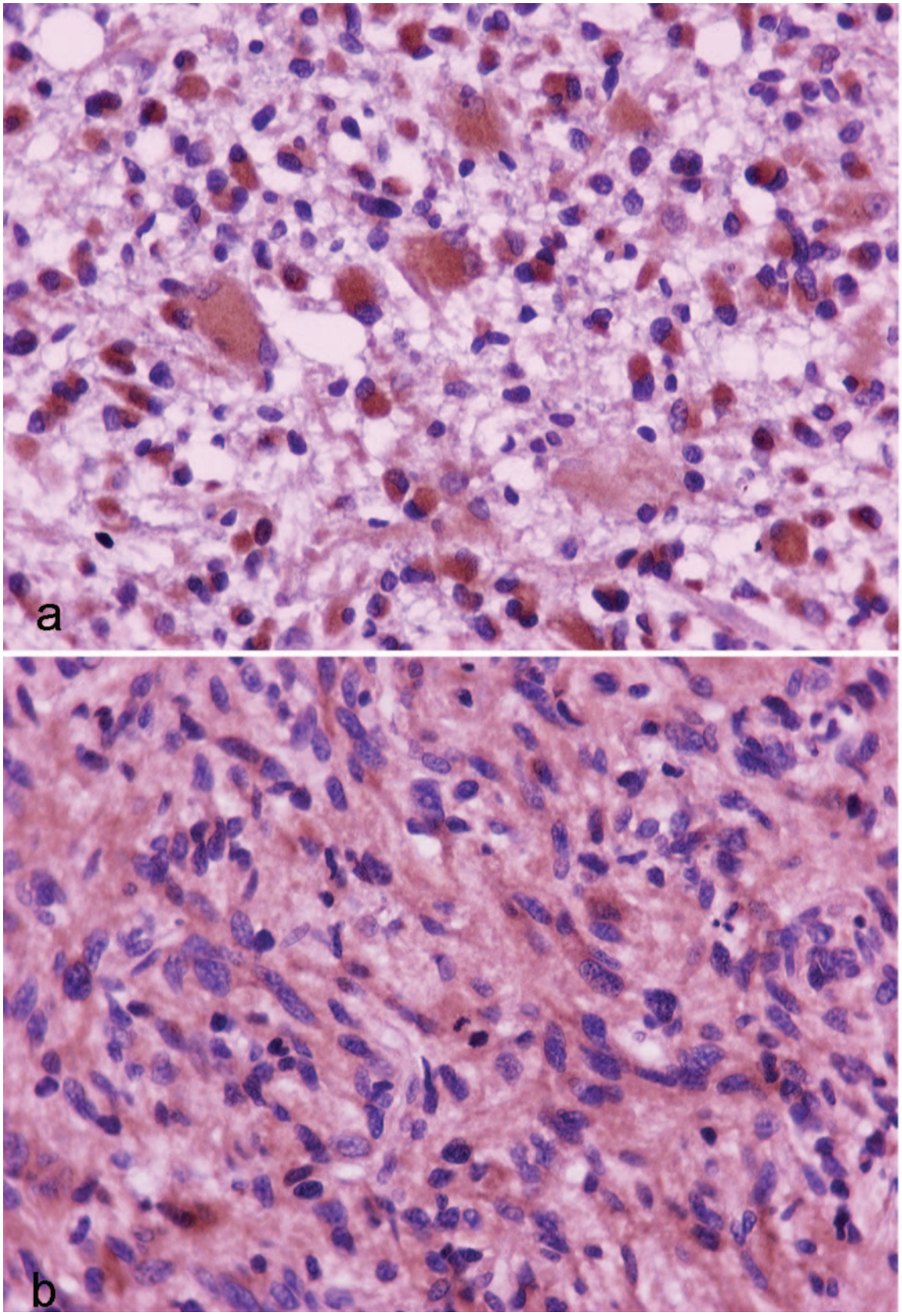

Figure 2. *continued*

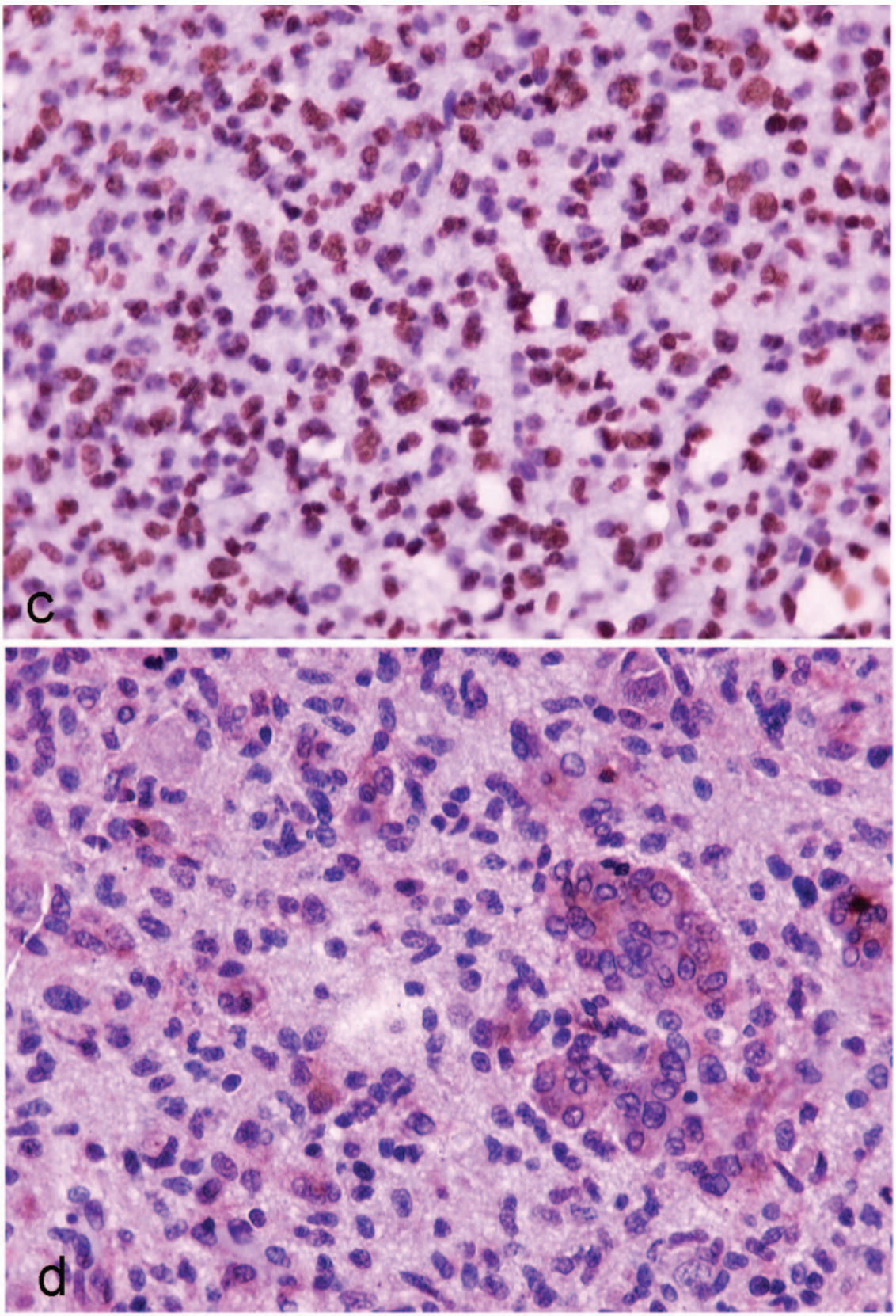

Figure 2. *Immunochistochemical expression of (a) COX-2, (b) EGFR, (c) PTEN and (d) VEGF-C in the tumors of patients with HGAT (x 40).*

especially in patients with GBM. These findings are in line with those reported by other investigators in gliomas (17) and colon cancer (36). EGFR is frequently overexpressed in human gliomas (20). In the present study, EGFR overexpression was seen in 18 out of the 24 (75%) GBM patients, which was similar to that reported by Bredel *et al.*(22), whereas EGFR overexpression was seen in 2 out of the 4 AA patients. Our results are similar to previous observations that EGFR is overexpressed in 40%-50% of GBM tumors but only in 10% of AA tumors, suggesting that EGFR overexpression is a late event in glial tumor development (37, 38).

PTEN is a tumor suppressor gene, implicated to play a role in the progression of many human cancers (39). Loss of PTEN expression has been reported in human gliomas (36, 40) and in other carcinomas (24, 41). Although PTEN does not have a nuclear localization signal, PTEN protein has been immunohistochemically detected in the cytoplasm and cell membrane, as well as in the nucleus. In the present study, PTEN nuclear positivity in, virtually, all of our tumor samples was observed. Accordingly, no reduction in immuno-histochemical expression of PTEN was detected, whereas other investigators using different PTEN antibodies successfully detected reduced PTEN immunoreactivity in up to 50% of GBM tumor samples (42). This may be due to the specificity of the monoclonal antibody used in our study, which recognizes an epitope located approximately 200 aminoacids from the C-terminus region of the PTEN protein.

VEGF-C is a member of a family of growth factors involved in the regulation of angiogenesis. In brain tumors, VEGF-C mRNA expression detected by PCR has been reported in medulloblastomas, astrocytomas (43, 44) and glioblastoma cell lines (45). It was believed that activation of VEGFR-3 by its ligand VEGF-C was restricted predominantly to the endothelial cell lining in lymphatic vessels (46, 47). However, it has been recently reported that activation of this receptor may also enhance cancer cell mobility and invasiveness and contribute to the promotion of tumor cell metastasis (48). The role of VEGF-C expression in the biology of gliomas has been poorly investigated. In a study by Jenny *et al.* (49), it was found that at least 50% of the cells expressing VEGF-C were monocytes or macrophages, as opposed to tumor cells. Whether VEGF-C is implicated in the inflammatory process or in tumor angiogenesis in GBM is still unknown.

In our study increased VEGF-C expression (immuno-reactive score ≥4) was detected in tumor tissues from 17 out of the 23 GBM (74%) and in three out of the four AA patients. However, specific correlations between VEGF-C expression and the other immunohistochemical markers could not be identified.

Several clinical studies have reported different findings regarding the prognostic or predictive significance of molecular markers. Small sample size in most of the published series, or other confounding factors, such as patient selection, extension of surgery, post-operative treatment, or methodological differences may account for the conflicting data. This may also be the case in our study.

In conclusion, the present study has shown that post-operative administration of combined modality treatment with RT and chemotherapy with TMZ and irinotecan was feasible in patients with HGAT, but was accompanied by high rates of toxicity. Neutropenia, nausea/vomiting and diarrhea were the most common side effects. Of note, the major health problem for these patients remains the occurrence of vaso-occlusive disease and opportunistic infections. Physicians dealing with the treatment of these patients should take these medical conditions into serious consideration and adopt aggressive preventive therapeutic policies. It does not appear from the present study that irinotecan has a synergistic or even an additive effect with TMZ in the treatment of patients with HGAT. Further development of this regimen, at least as used in the present study is not recommended.

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