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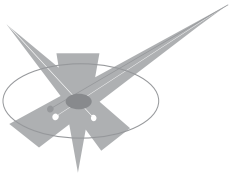

Original Paper

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Prognostic significance of proliferation rate and DNA ploidy in astrocytic gliomas treated with radiotherapy

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Summary

Aim The proliferative potential, and DNA ploidy in 50 brain tumours (15 grade I & II, and 35 grade III & IV astrocytomas) were investigated using bromodeoxyuridine (BrdUrd) incorporation and flow cytometry.

Materials/Methods Tumour samples taken from each patient during surgery were incubated *in vitro* for one hour at 37°C with bromodeoxyuridine (BrdUrd), using the high pressure oxygen method. The percentage of BrdUrd-labelled cells (BrdUrd Labelling index, BrdUrd LI), and the total DNA content were evaluated. After surgery, 21 patients received conventionally fractionated radiotherapy (RT), 11 patients received accelerated RT, and 18 patients underwent hypofractionated RT.

Results The tumours showed variability in BrdUrd LI values, which ranged from 0.3 to 15.8%. A significantly higher mean value for BrdUrd LI was shown in grades AIII & IV (3.5%), than in astrocytomas of grades AI & II (1.5%, p=0.005). A lower though not statistically significant percentage of DNA aneuploidy was observed in low-grade (40.2%) glioma than was seen in high-grade (65.7%) glioma. Univariate analysis showed that younger (≤50 years) patients (p=0.001), those with AI & II glioma (p=0.000), low tumour proliferation rate (BrdUrd LI ≤2.1%, p=0.006) and conventional or hypofractionated RT (p=0.000) had a significantly higher 5-year survival rate. Tumour ploidy had no influence on patients' survival (p=0.261). However, a Cox multivariate analysis showed that only the patients' age (>50 years), high grade tumours (AIII & IV) and accelerated RT were significantly unfavourable prognostic factors in terms of survival.

Conclusions To improve RT results, younger patients (≤50 years) with fast proliferating tumours should receive more aggressive treatment.

Key words **BrdUrd Labelling index • DNA ploidy • gliomas • radiotherapy**

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Background

The results of treatment in cases of cerebral glial tumours, in spite of continuous development and improvement in treatment protocols, are still not satisfactory. However, in the last decade, 5-year survival rates in patients with gliomas increased due to improvements in surgical techniques, radiotherapy (RT) and chemotherapy. It is suggested that further improvements will be the result of research into the molecular genetic features of brain tumours [1]. Therefore, the ability to predict the response to treatment and patients' survival, based on the biological parameters of the tumours, would be useful in clinical practice. One of the most frequently investigated factors is the proliferative potential of tumour cells, as this parameter may be used in the selection of more aggressive treatments [2] and may predict recurrence rate [3]. Many methods for the assessment of proliferative potential have been used, including: mitosis count, AgNOR count, the Ki-67 or PCNA labelling index – indicating cells in all phases of the cell cycle except the G0 phase, S phase fraction bromodeoxyuridine labelling index (BrdUrd LI) which identifies cells actively synthesising DNA and potential doubling time (Tpot), i.e. time required for a tumour cell population to double its number in the absence of cell loss. In cases of glioma, BrdUrd LI, and Tpot are considered the best methods for assessing proliferative potential and giving good correlation with clinical outcome [4–6]. The treatment of choice, in low advanced astrocytomas, is surgical resection with the preservation of neurological function. However, it has been shown that adjuvant RT increases survival time in patients with high-grade malignant gliomas [7,8]. A large number of factors can potentially influence survival rate, including the patient, the tumour and the treatment parameters.

Aim

The aim of this study was to investigate some of these factors, paying special attention to biological factors of the tumour, such as proliferation rate (BrdUrd LI) and DNA ploidy, in patients with astrocytic gliomas treated with surgery and postoperative radiotherapy (RT).

Materials and Methods

Patients

Fifty patients with primary cerebral glial tumours were included in the study. There were 26 men and 24 women. The mean age of the patients was 46.8±SD 11.8 (range 20–73) years. There were 15 patients with low-grade gliomas (AI&AII), 19 patients with AIII gliomas and 16 patients with AIV gliomas according to the Kernohan grade [9].

Treatments

Between the years 1991 and 1997, 50 patients were treated with surgery aimed at complete removal (Department of Neurosurgery, Collegium Medicum, Jagiellonian University, Kraków, Poland) and postoperative radiotherapy with curative intent (Department of Radiation Oncology, Centre of Oncology, Kraków, Poland). Postoperative radiotherapy was given after a mean term of 54 days (23–104). Three schedules of RT were applied. Twenty-one patients received conventionally fractionated RT, with 60 Gy in 30 fractions of 2 Gy, 11 patients received accelerated RT (53 Gy in 20 fractions over 10 days; two fractions per day, and hypofractionated RT with three series each of 20 Gy in 5 fractions, separated by 4 week intervals was applied in 18 cases. Low-grade gliomas were treated with conventional fractionation only, AIII gliomas with all three treatment schedules, while AIV gliomas were treated with a twice daily or three series schedule (Table 1).

BrdUrd labelling index

The tumour specimens (about 0.5 cm^3) from each patient, were delivered fresh from the operating room shortly after excision. They were cut into small pieces and incubated *in vitro* with BrdUrd for l hour at 37°C in high oxygen pressure. After incubation, tumour fragments were fixed in 70% ethanol. The BrdUrd staining procedure and flow cytometry have been described in detail elsewhere [10]. To obtain pure nuclei from cells, tumour fragments were minced and digested at 37°C with 0.4 mg/ml pepsin (Sigma Chemicals, Poole, Dorset) in 0. l M HCl for 20 min. The suspension of nuclei was filtered through a 35 µm nylon mesh and centrifuged at 2000 rpm for 5 min. Partial DNA denaturation was achieved by resuspension of the pellet in 2 M HCl for 10 min. The nuclei suspension was then washed twice in phosphate-buffered saline (PBS). The pellet was further incubated in PBS containing 0.5% normal goat serum (Sigma Chemicals), 0.5% Tween 20 (Sigma Chemicals) and a mouse anti-BrdUrd monoclonal antibody (DAKO) for one hour. After incubation, the nuclei were washed in PBS and suspended in PBS/NGS/Tween containing goat anti-mouse IgG-FITC conjugate (DAKO) for

Table 1. Patients' characteristics and distribution of clinical and pathological features.

Kernohan Grade	Sex		Age (years) Mean (range)	Radiotherapy		
	M	F		Conventional	Accelerated	Hypofractionated
AI	5	3	39.7 (28.0–57.0)	8	0	0
AII	5	2	36.7 (20.0–47.0)	7	0	0
AIII	9	10	48.0 (27.0–73.0)	6	4	9
AIV	7	9	53.4 (29.0–69.0)*,**	0	7	9
All	26	24	46.8 (20.0–73.0)	21	11	18

Significant difference in mean age between: * AI and AIV gliomas, p=0.004; ** AII and AIV gliomas, p=0.001.

Table 2. Differences in biological parameters between AI & AII, and between AIII & AIV gliomas.

Grade	N	BrdUrd LI (%) Mean (range)	Diploid tumours (%)	Aneuploid tumours (%)
AI & AII	15	1.5 (0.4–4.6)	9 (60.0)	6 (40.0)
AIII & AIV	35	3.5 (0.3–15.8)*	12 (34.3)	23 (65.7)**
All	50	2.9 (0.3–15.8)	21 (42.0)	29 (58.0)

* Significant difference between AI & AII and AIII & AIV gliomas, p=0.005;
** Significant difference between diploid and aneuploid tumours in high-grade gliomas (AIII & AIV), p=0.040.

l hour. Finally, the nuclei suspension was washed twice in PBS and stained for total DNA using 10 µg/ml propidium iodide (PI) in PBS.

Flow-cytometric analysis

The stained material was analysed with a FACSCalibur flow cytometer (Becton Dickinson Immunocytometry Systems, Sunnyvale, CA, USA) equipped with an argon-ion laser (488 nm) and operated at 15 mW. Doublets and clumps were excluded from the analysis by gating on a bivariate distribution of the red peak vs the integral signal. Ten to 20 thousand events were collected in each histogram. BrdUrd LI was calculated as the percentage of cells which incorporated BrdUrd. In aneuploid tumours the diploid subpopulation of labelled cells was subtracted. Tumour ploidy was calculated from the DNA profile using ModFit LT software (Verify Software House, Inc. USA). Aneuploidy was assessed in cases in which the normal and neoplastic cell populations gave two separate peaks. DNA index was calculated as a ratio of the modal DNA fluorescence of abnormal to normal G1/G0 cells. Human lymphocytes were used as a reference peak.

Statistical analysis

All statistical analyses were performed using STATISTICA v.5.1 software. Descriptive statistics were used to determine the mean values of variables and standard errors of means (SE). In all statistical procedures, p values lower than 0.05 were considered significant. The statistical significance of the differences between means was assessed by use of the Mann-Witney U test (grade vs. age, LI). The probability of survival was calculated using the Kaplan-Meier method [11]. A univariate analysis was carried out using a log-rang test. Median values were used for cut-off points. All clinical and biological parameters were entered into the multivariate analysis, which was performed using a Cox proportional hazard model and a stepwise regression procedure [12].

Results

Patients with AI or AII gliomas were significantly younger than those with AIV gliomas (Table 1). Low grade (AI & II) gliomas were seen in 10 men and 5 women, while the high-grade (A III & IV) group comprised 16 men and 19 women. No difference in mean age between men and women was found (p=0.335). A significant correlation between tumour grade and patients' age (p=0.000), or tumour grade and RT schedule (p=0.000) was demonstrated.

The brain tumours showed variability in the BrdUrd LI. The BrdUrd LI ranged from 0.3 to

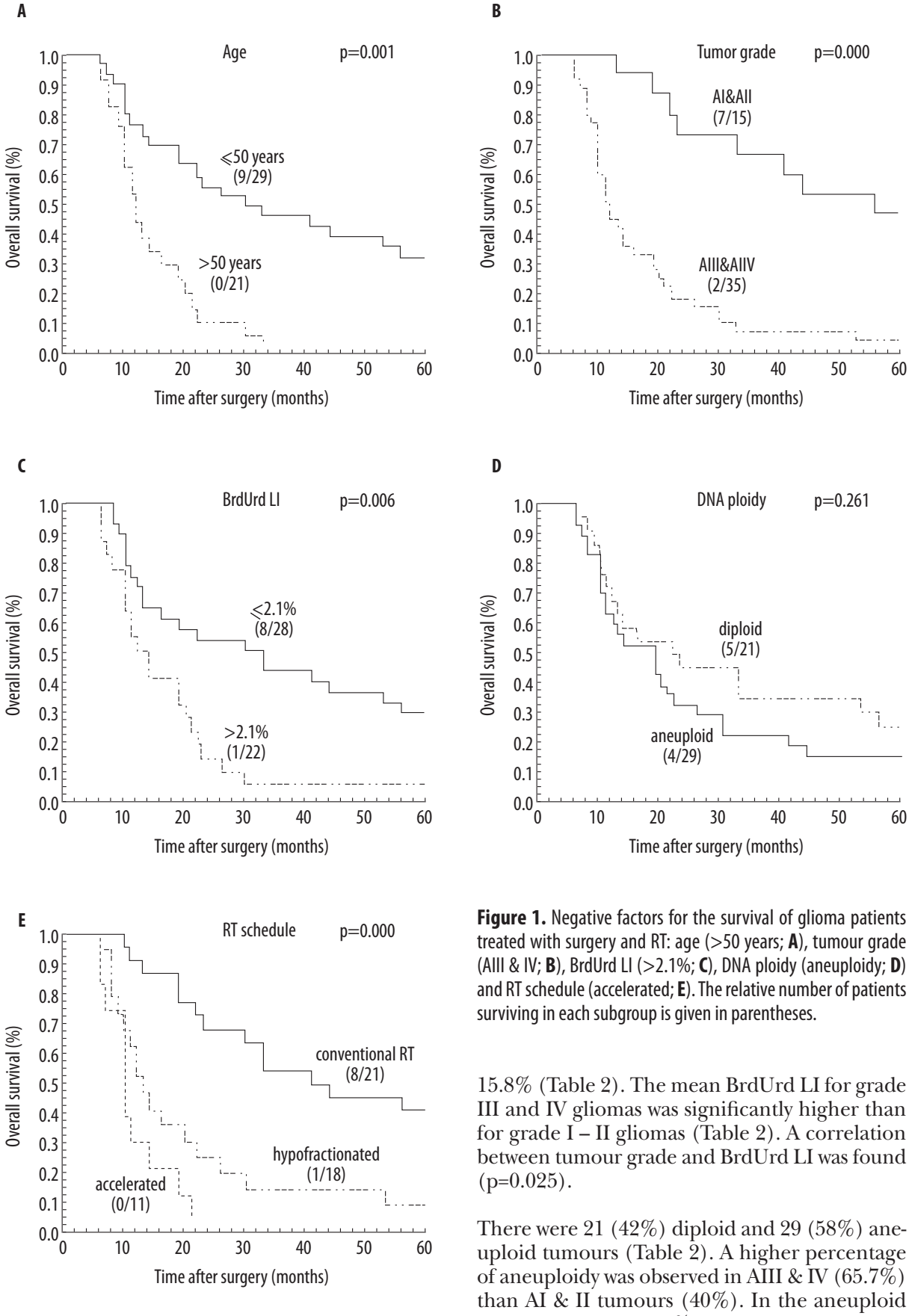

Figure 1. Negative factors for the survival of glioma patients treated with surgery and RT: age (>50 years; **A**), tumour grade (AIII & IV; **B**), BrdUrd LI (>2.1%; **C**), DNA ploidy (aneuploidy; **D**) and RT schedule (accelerated; **E**). The relative number of patients surviving in each subgroup is given in parentheses.

15.8% (Table 2). The mean BrdUrd LI for grade III and IV gliomas was significantly higher than for grade I – II gliomas (Table 2). A correlation between tumour grade and BrdUrd LI was found (p=0.025).

There were 21 (42%) diploid and 29 (58%) aneuploid tumours (Table 2). A higher percentage of aneuploidy was observed in AIII & IV (65.7%) than AI & II tumours (40%). In the aneuploid subpopulation, 18 (36%) of the 29 aneuploid tu-

Table 3. Univariate analysis for 50 glioma patients treated with surgery and radiotherapy.

Parameter	N	Median survival (months)	(log-rank test) p value
Age			
≤50 years	29	28	
>50 years	21	11	0.001
Grade			
AI & AII	15	50	
AIII & AIV	35	11	0.000
BrdUrd LI			
≤2.1%	28	30	
>2.1%	22	12	0.006
Ploidy			
diploid	21	19	
aneuploid	29	16	0.261
RT			
Conventional	21	37	
Accelerated	11	10	
Hypofractionated	18	12	0.000

mours were hyperdiploid (DNA index 1.2–1.8), 9 (18%) were tetraploid (DNA index 1.9–2.1) and 2 (4%) were hypertetraploid.

Nine (18%) patients survived the 5-year period. Kaplan-Meier survival curves were plotted according to patients' age, tumour grade, BrdUrd LI, tumour ploidy and RT schedule. Younger patients (≤50 years) survived significantly longer than older patients (>50 years; p=0.001, Figure 1A). Median survival time for the first group was 28 months and for the second 11 months (Table 3). A higher survival percentage was observed in the grades I & II group than in the grades III & IV group (p=0.000, Figure 1B). The median survival time for the first group was 50 months, while for the grade III & IV groups it was 11 months (Table 3).

The univariate analysis showed that patients with slowly proliferating tumours (BrdUrd LI ≤2.1%) survived significantly longer (p=0.006) than those with faster proliferating tumours (BrdUrd LI >2.1%) (Figure 1C). Median survival time for the first subgroup was 30 months and for the second group 12 months (Table 3). There was no significant difference in patients survival with respect to tumour ploidy (p=0.249, Figure 1D).

It was shown in the univariate analysis that patients receiving conventional RT treatment survived significantly longer than those treated with hypo- and accelerated RT (p=0.000, Figure 1E). When, instead of schedule RT, a total RT dose of <56 Gy >was used (not shown), significantly better survival (p=0.005) was observed in those patients who received RT doses greater than 56 Gy.

Table 4. Multivariate Cox analysis for 50 patients (final model).

Parameter	RR (relative risk)	p value
Age		
≤50 years	1.0	
>50 years	2.2	0.038
Grade		
AI & AII	1.0	
AIII & AIV	2.9	0.022
Radiotherapy		
Conventional	1.0	
+ Hypofractionated		
Accelerated	2.6	0.018

All the clinical data were entered into the Cox multivariate analysis (patients' age, tumour grade, schedule of RT) along with biological prognostic factors (BrdUrd LI and DNA ploidy). The analysis revealed that there were only three unfavourable prognostic factors affecting the survival rate of the patients: age >50 years (p=0.038), high tumour grade (A III & IV) (p=0.022) and an accelerated schedule of RT (p=0.018, Table 4). As the univariate and multivariate analyses revealed that age was a strong prognostic factor, we performed separate multivariate analyses for younger (≤50 years) and older patients (>50 years). It appeared that, for younger patients, significant positive prognostic factors with respect to survival were: conventional or hypofractionated RT (p=0.033) and BrdUrd LI (<2.1%). However for older patients, none of the analyzed parameters had an impact on survival.

Discussion

This study was conducted in order to determine whether BrdUrd LI or DNA ploidy can identify patients with poorer outcomes, who might benefit from more aggressive treatments. The BrdUrd LI method revealed a variability in proliferative potentials for both low and high-grade gliomas which may confirm that this group of tumours represents heterogeneous diseases of different biological behaviors. Some authors studying cell kinetics in gliomas have also used BrdUrd LI to identify differences in proliferation rates between

and within groups of brain tumours of different histology [5,6,13–15]. In our study, the BrdUrd LI for the examined tumours ranged between 0.3 and 15.8% and high grade gliomas showed higher proliferation rates. These values fall within ranges given by other authors for these groups of tumours [13,14]. Correlation of tumour cell kinetic parameters with post-treatment follow-up showed that patients with tumours of lower proliferative potential (BrdUrd LI ≤2.1%) survived significantly longer than those with tumours of higher proliferative potential (BrdUrd LI >2.1%). Several studies on BrdUrd LI in brain tumours have shown the significance of that parameter in patients' survival [4,16] and recurrence rate [3], indicating a greater probability of survival in patients whose tumours had LI's below 1–3%. Also some other studies have revealed a highly significant correlation between low S-phase fraction <3% [17], low Ki-67 clone MIB-1 LI [2,18] and longer patients' survival. Our investigation showed that the proliferation rate of the tumour correlated with its grade. BrdUrd LI appears to reflect the aggressiveness of the tumour, as high-grade gliomas had significantly higher proliferation rates. Therefore, this parameter seems to be as important in selecting the treatment as the histopathological diagnosis and therefore should be considered an important factor in determining the prognosis of individual patients with brain tumours.

The examined tumours showed a relatively low rate of aneuploidy (58%). A lower percentage of aneuploidy was recorded for low-grade (40.0%) tumours than for high-grade gliomas (65.7%), though this difference was not statistically significant. Our data fell within ranges described by other authors (57.3–89.0%) in the case of high-grade tumours [10,19]. Tetraploid and hypertetraploid tumours were more frequent (24%) in AIII & IV than in low-grade tumours (6.9%), and only the high-grade subgroup displayed hypertetraploidy which might be indicative of a higher degree of malignancy in these tumours. Nevertheless, DNA ploidy was not predictive in terms of survival. In the literature, opinions on the assessment of DNA ploidy as a prognostic factor in brain tumours are inconclusive. Some authors considered diploidy to be a prognostic factor [19], others did not observe any significant association between DNA ploidy and survival time [4,17,20].

In our series, the age of patients appeared to be a significant prognostic factor. As in other studies [2,4,21,22], younger patients (≤50 years) survived significantly better than older patients. It is known that brain tumours in elderly patients seem to have an intrinsic resistance to cancer treatment, though there is no clear explanation for this fact. The brain may have a unique role in the process of aging; differentiated post-mitotic neurons are not replaced by cell division and little turnover of DNA occurs in glial cells. Moreover, cell aging in the brain may be associated with a decrease in oxygen uptake [23], which may be related to lower levels of cytotoxic free radicals under irradiation. Alternatively, the difference may lie with scavenging enzymes that neutralize or inactivate nucleophilic molecules. Rosenblum et al. [24] have shown that patients' age inversely correlates with *in vitro* cell kill from biopsies, and that patients with sensitive cells were significantly younger than those with resistant cells. Older people may have accumulated genetic damage throughout their lives, which is linked with exposure to exogenous mutagens and a possible decrease in various host functions, such as DNA repair and other defence mechanisms [25].

Malignant gliomas are among the most devastating cancers, commonly producing profound and progressive disability, and leading to death in most cases. The infiltrating nature of high-grade gliomas makes complete resection virtually impossible. Thus, standard treatment generally consists of cytoreductive surgery followed by radiotherapy. Adjuvant chemotherapy is not widely accepted because of the low sensitivity of gliomas to traditional anti-neoplastic agents, poor penetration of most drugs across the blood-brain barrier, and the significant systemic toxicity associated with current agents. However, a novel alkylating agent – temozolomide looks promising. Radiotherapy of low grade gliomas is a controversial issue. Subgroups of patients with low-grade gliomas and poor prognostic factors are more likely to benefit from immediate postoperative RT, whereas patients with low-risk disease, particularly oligodendrogliomas, can reasonably be left for observation only. However, one cannot exclude the possibility that delayed RT may result in a worsening of long-term clinical outcome [26]. In the opinion of Marimanoff and Stup [27], postoperative RT should not be administered routinely to patients with low-grade gliomas. If RT is deemed necessary, as is the case in progressive or inoperable disease causing neurological symptoms, a total dose of ≤50 Gy, with a fractional dose of ≤1.8 to 2 Gy, should be administered using modern, highly conformal techniques. Among patients who

undergo biopsy only, and not total resection, a greater radiation dose can significantly improve survival, with a threshold at approximately 54 Gy [26]. Our data may confirm the above, because in our series patients receiving total doses greater than 56 Gy (cut off point) survived significantly better than those receiving dose <56 Gy. Other studies have shown that radiation doses ≥60 Gy confer a survival advantage in high grade gliomas [8], although another study [7] has also shown that even dose escalation, with three dimensional conformal radiation to 90 Gy, cannot prevent local failures in high-grade gliomas. Moreover, the current data provide no arguments for the use of accelerated RT, as found by Nieder [28] in meta analysis. On the other hand, a meta-analysis performed by Steward [29] has demonstrated a small but consistent improvement in the survival of approximately 5–10% at 1 and 2 years, in patients with high-grade gliomas who received adjuvant chemotherapy in addition to RT.

Among patients who underwent surgical resection of their tumours and who were treated with RT, survival is most strongly linked to the tumour grade, which largely agrees with other studies. Patients with grade III or IV tumours had a significantly worse prognosis than those with grade I or II tumours. In the univariate analysis, BrdUrd LI also appeared to be a significant prognostic factor in terms of survival, however this parameter was dependent on tumour grade, and this was probably why it lost its significance in multivariate analysis. Patients receiving conventional RT survived significantly better than those treated with hypo- and accelerated RT. However, it should be stressed that the former method was applied mostly to low-grade gliomas. Therefore, it cannot be excluded, that tumour grade, and not the RT schedule, affects survival more significantly.

In the Cox analysis, where all biological and clinical parameters were considered, age, high tumour grade and RT schedule appeared to be the only factors influencing survival. This revealed again, that in the case of such tumour types, the histological grade is the strongest biological factor influencing the prognosis. When we performed separate analyses for 2 patient subgroups differing in age, the proliferation rate and conventional or hypofractionated RT became important prognostic factors for younger patients (<50 years) while none of the analyzed parameters was important for elderly patients. The dependencies obtained may confirm the findings of Fisher [21] and Rodrigues Pereira et al. [18] who analyzed proliferation rates based on Ki-67 LI and revealed that it is a useful predictor, although dependent on other prognostic factors, particularly age. Therefore, although multivariate analysis is the most useful tool to evaluate prognostic factors and to construct appropriate statistical models, it is important to keep in mind that these models are sensitive to the variables chosen, definitions of those variables, and statistical methodology.

Nevertheless, the assessment of all unfavourable prognostic factors in the glioma patients subjected to surgery and postoperative RT, may give grounds for applying proper RT schedules. Treatment with radiation or chemotherapy should not be introduced until malignant transformation or faster growth indicators are found.

Conclusions

1.For astrocytic gliomas treated with surgery and radiotherapy (RT) the most significant prognostic factors are: young age (≤50 years), low-grade (AI & AII), and – possibly – the RT schedule.

2.To improve RT results, younger patients (≤50 years) with faster proliferating tumours (BrdUrd LI >2.1%) should receive more aggressive treatment.

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