Original paper

Molecular classification of diffuse gliomas

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In this study we assessed whether gliomas could be subdivided into different molecular subtypes by immunohistochemistry (IHC) reminiscent of those first described by Verhaak *et al*. in 2010 (classical, proneural, mesenchymal and neural). We also evaluated the prognostic significance of single molecular factors and searched for significant correlations between markers. In this study, we included 146 patients with glioblastomas (GBMs) and 26 with diffuse astrocytomas (DAs). The glioma samples were tested for PDGFRA, IDH1 R132H, CD44, p53, Ki-67, p21 and p27 expression. We found that gliomas could be subdivided into molecular subtypes by IHC. Fifty per cent of GBMs were of the proneural subtype, 18.5% of mesenchymal subtype and 31.5% were not otherwise classified. However, most of the DAs (92.3%) belonged to the proneural subtype. No prognostic role was found for the molecular subtypes, but predictive roles were noted. Both proneural and mesenchymal molecular subtypes showed a benefit from the addition of chemotherapy and radiotherapy; however, the mesenchymal subtype showed a greater response. Interestingly, the mesenchymal subtype did not receive any benefit from the addition of radiotherapy compared with palliative management and surgery alone. Regarding single molecular markers, only IDH1 R132H was found to have a prognostic role for GBMs. There was a trend towards better survival in tumours with lower PDGFRA expression ($p = 0.066$). In DAs, PDGFRA and Ki-67 expression had prognostic roles. The following statistically significant correlations were found in GBMs: Ki-67/p53, Ki-67/p27 and p53/PDGFRA; in DAs: p53/PDGFRA, CD44/PDGFRA, and p21/PDGFRA.

Key words: gliomas, immunohistochemistry, molecular subtypes, survival, glioblastoma.

Introduction

Glioblastoma (GBM) represents the most frequent and biologically aggressive (grade IV) type of glioma [1]. Glioblastoma has a very poor prognosis in spite of aggressive multimodal treatments. Therapy remains mostly palliative due to rapid tumour growth and recurrences that occur in almost every case. The highly invasive nature of this neoplasm prevents the possibility of complete surgical removal and contributes to widespread infiltrative disease in the surrounding brain parenchyma. Diffuse astrocytoma (DA) is a type of low-grade glioma defined as a grade II neoplasm by the World Health Organization (WHO) [1]. The proliferative potential of DAs and their rate of growth is much lower than GBMs. DA is also very infiltrative and has a tendency to progress towards a high-grade malignancy called anaplastic astrocytoma (grade III) and finally secondary GBM (grade IV). Low-grade gliomas and secondary GBMs are known to bear IDH1 mutations and IDH1 R132H is the most frequent one. IDH1 mutations

are associated with younger patients, as well as a much better prognosis in both low-grade and high-grade gliomas [2]. Extensive research is being directed towards elucidating the molecular and genetic signatures in gliomas. This can provide more data about their pathogenesis and help to improve management of gliomas, possibly allowing these tumours to be stratified into different prognostic groups. While investigation of individual gene or protein alterations can provide data on potentially important prognostic markers, new techniques like DNA microarrays allow the measurement of large numbers of genes and even provide expression profiles for complete tumour genomes simultaneously. At present, several molecular subtypes of GBM have been defined on the basis of molecular signatures. For example, Verhaak *et al*. described four glioblastoma molecular subtypes based on gene expression analysis: classical, proneural, mesenchymal and neural subtypes. These were characterized by different molecular alterations and gene expression patterns [3]. In other studies, glioblastomas were divided into different subtypes on the basis of the activity of various signal transduction pathways [4] and protein expression profiles [5, 6, 7]. The tumours possessing similar molecular signatures and expression patterns likely share common pathogenesis, reflecting similar therapy responses and prognoses. Glioma subtyping is possible and offers promise for routine practice, but molecular data must be altered to a simpler, cheaper tool for daily use. Immunohistochemistry (IHC) is an important component of tissue testing in pathology laboratories in the emerging molecular era. It is also a good surrogate for the more expensive traditional cytogenetic and molecular methods. One of the proteins of interest in many studies is platelet-derived growth factor receptor alpha (PDGFRA). The proneural subtype of GBM was characterized by PDGFRA amplification as well as p53 and IDH1 mutations [3]. The protein CD44 is the best known molecule associated with invasion in many tumours, including gliomas [8, 9]. Increased expression of CD44 was also found in the mesenchymal subtype of GBMs [3]. This indicates that CD44 is worthy of attention as a potential determinant of biological aggressiveness and could be used in molecular subtyping. Other proteins that have been reported to be specific for certain molecular glioma subtypes are c-mer proto-oncogene tyrosine kinase (MERKT), epidermal growth factor receptor (EGFR), oligodendrocyte transcription factor 2 (OLIG-2) and assorted others. The aim of our study was to investigate expression of PDGFRA, CD44, IDH1 R132H, Ki-67, p53, p21 and p27 in GBMs and DAs and to identify significant correlations of marker expression as well as to compare expression with clinical parameters such as age and gender. In addition, we evaluated whether gliomas could be subdivided into several molecular subtypes.

Material and methods

A retrospective study was carried out in compliance with laws and regulations considering principles of ethics in accordance with the Declaration of Helsinki.

In our study, archived formalin-fixed, paraplast-embedded tissues of 146 GBMs, including 5 secondary GBMs and 26 DAs were analyzed by immunohistochemistry (IHC). Anaplastic astrocytomas were excluded from the study because we evaluated the two most contrasting grades of diffuse gliomas. The cases were identified by an archive search of all consecutive patients (2009-2014) who were subjected to neurosurgical treatment by routine indications in a single university hospital. The diagnosis and grade of the gliomas were verified by 2 pathologists in accordance to the 2016 WHO classification of tumours of the CNS [1] as detailed in Table I. Immunohistochemical visualization for IDH1 R132H was used for detection of IDH-mutant glioma cases.

All recurrent tumours and previously treated patients, unacceptably small samples including stereotactic biopsies, and damaged material containing large areas of necrosis or coagulation artefacts ($>$ 50% of the sample) were excluded from the study. The basic clinical data (e.g. patient's age and gender) as well as information about previous treatment or tumour recurrences were retrieved from medical records. The glioma samples were tested for PDGFRA, IDH1 R132H, CD44, p53, Ki-67, p21 and p27 expression by immunohistochemical visualization. For IHC, 3-micrometre-thick sections were cut on electrostatic glass slides (Histobond, Marienfeld, Germany). After deparaffinisation and rehydration, heat-induced antigen retrieval was performed in a microwave oven (3 $\times$ 5 min) using a basic TEG (pH 9.0) buffer (Agilent Dako, Santa Clara, United States of America), followed by blocking of endogenous peroxidase (Sigma-Aldrich Ltd., Gillingham, United Kingdom). The sections were incubated with primary antibodies (see Table II for antibody characteristics and dilution) at room temperature. Bound primary antibodies were detected by the enzyme-conjugated polymeric visualisation system EnVision (Agilent Dako), linked with horseradish peroxidase using 3,3'-diaminobenzidine (Agilent Dako) as the chromogen. Positive and negative quality controls were invariably performed and reacted appropriately. Characteristics of the primary antibodies for immunohistochemistry is shown in the Table II.

Expression of markers was evaluated by light microscopy under magnifications of 40$\times$ and 400$\times$, using an Eclipse Ci-L (Nikon, Tokyo, Japan) microscope. By intensity, the expression was evaluated

Table I. The diagnostic criteria for diffuse astrocytoma and glioblastoma by WHO 2016 classification

Entity	Criteria	Reference
Diffuse astrocytoma, grade II	Histological criteria:	Louis *et al.* 2016
	Glial tumour	
	Appropriate cellular background: mildly to moderately increased cellularity, mild to moderate nuclear atypia, fibrillary architecture, no or single[1] mitosis	
	Absence of microvascular proliferation	
	Absence of necrosis	
	Molecular parameters:	
	Presence of IDH1/2 mutations	
	IDH-mutant	
	IDH-wildtype	
Glioblastoma, grade IV	Histological criteria:	Louis *et al*. 2016
	Glial tumour	
	Appropriate cellular background: high cellularity and/ or cellular and nuclear atypia and/or brisk mitotic activity	
	At least one of the following:	
	unequivocal microvascular proliferation	
	necrosis	
	Molecular parameters:	
	Presence of IDH1/2 mutations	
	IDH-mutant	
	IDH-wild type	

[1] *in a representative tissue material. Stereotactic biopsies excluded by the study criteria*

Table II. Characteristics of the primary antibodies for immunohistochemistry

Antigen	Antibody	Clonality	Manufacturer	Dilution	Incubation time (min)	Pattern
PDGFRA	PRAH	Polyclonal	Abcam	1 : 200	60	Ct, Me
Mutant IDH1 R132H	MMAH	H09	Dianova	1 : 50	60	Ct
CD44	MMAH	DF1485	Agilent Dako, Santa Clara, USA	1 : 50	60	Me
p53	MMAH	DO-7	Agilent Dako	1 : 400	60	Nu
Ki-67	MMAH	MIB-1	Agilent Dako	1 : 100	60	Nu
p21	MMAH	SX118	Agilent Dako	1 : 25	60	Nu
p27	MMAH	SX53G8	Agilent Dako	1 : 50	60	Nu

PDGFRA – platelet-derived growth factor receptor α; MMAH – monoclonal mouse antibody against human antigen; PRAH – polyclonal rabbit antibody against human antigens; USA – United States of America; Ct – cytoplasmic; Me – membranous; Nu – nuclear

as negative vs. low vs. moderate vs. high intensity. The expression of a marker was considered positive only if the expression intensity was moderate or high. For most markers (Ki-67, p53, p21, p27, CD44, PDGFRA) the presence of nuclear, cytoplasmic or membranous staining was scored quantitatively as the fraction (%) of neoplastic cells. The expression of IDH1 R132H mutant protein was evaluated only as positive vs. negative. Regarding immunohistochemical evaluation, the cases were also classified as positive or negative. For a case to be considered positive, the fraction of positive cells had to reach a certain cut-off value. This value was based on the most frequently used level in other studies published in peer-reviewed international journals. The cut-off values for both GBMs and DAs were the following: 10% for p53, 50% for CD44 and PDGFRA, 20% for p21 and

Table III. The subtyping of gliomas by protein expression signatures

Antigen	Sybtype of glioma		
	Proneural	Mesenchymal	NOS
p53	High	Low	Low
PDGFRA	High	Low	Low
IDH1 R132H	Positive	Negative	Negative
CD44	Any	High	Low

PDGFRA – platelet-derived growth factor receptor α; NOS – not otherwise specified

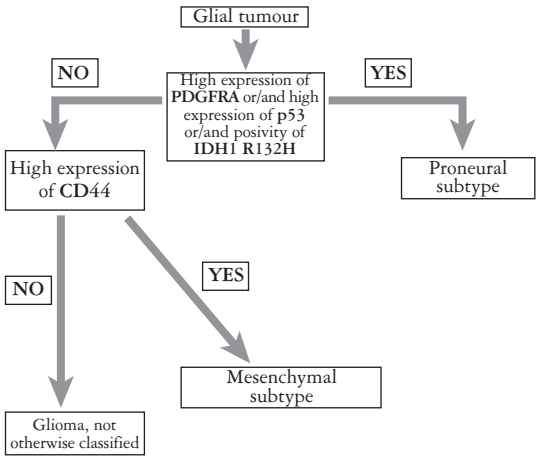

Fig. 1. Flowchart of asserting glioma cases into molecular subtypes

70% for p27. For Ki-67, we used cut-off values of 25% and 3% for GBMs and DAs respectively. The immunohistochemical profiles of p53, IDH1 R132H, PDGFRA and CD44 expression were used to determine the molecular subtype of GBM as previously described by Verhaak *et al*., 2010. Based on these protein expression signatures, three categories of GBMs were distinguished: proneural, mesenchymal and not otherwise classified, designated "Other". The proneural subtype was defined by high expression of p53 and/or high expression of PDGFRA and/or positivity of IDH1 R132H. The mesenchymal subtype was defined by high expression of CD44 and low expression of proneural markers (p53, PDGFRA and IDH1 R132H). All remaining cases which did not fit the proneural or mesenchymal subtype categories were classified as "Other". The criteria and algorithm used to distinguish three basic proposed subtypes of gliomas is shown in Table III and Fig. 1.

Descriptive statistical analysis was performed and included the calculation of a 95% confidence interval (CI) by CIA software according to Altman *et al*., 2000. Survival was evaluated using Kaplan-Meier analysis and the log-rank test was used to compare the survival curves. To detect significant differences, a Mann-Whitney U-test was used. For correlation analysis, Spearman's correlation test was used. A value of $p < 0.05$ was considered to be statistically significant.

Results

Characteristics of patients with glioma

The study included 146 patients with GBM and 26 patients with DAs diagnosed during a period from 2009-2014 in a single university hospital. GBM was diagnosed in 75/146 (51.4%; 95% CI: 43.3-59.5) females and in 71/146 males (48.6%; 95% CI: 40.5-56.7). The age of the patients ranged from 34-89. The mean age ± standard deviation (SD) was 62.0 ±11.2 (95% CI: 60.2-63.8). Surgical resection was performed in every studied GBM. In addition, adjuvant therapy – including radiotherapy and chemotherapy with temozolomide – was used. The treatment type data were not available in 11 patients. In the remaining 135 GBM patients, standard treatment was given most frequently, consisting of surgery followed by adjuvant radiotherapy and chemotherapy with temozolomide; this accounted for 56/135 (41.5%; 95% CI: 33.5-49.9) of patients. Surgery was followed by radiotherapy alone in 50/135 (37.0%; 95% CI: 29.3-45.4) cases, but 29/135 (21.4%; 95% CI: 15.3-29.1) patients did not receive any adjuvant oncological treatment and only surgical resection was performed. Patients receiving adjuvant temozolomide and radiotherapy were younger compared to those who received adjuvant radiotherapy or who were treated with surgical resection alone (one-way ANOVA, $p < 0.001$). The mean age of patients receiving different types of treatment was 55.0 (95% CI: 52.5-57.5) years, 65.9 (95% CI: 62.9-68.7) years and 69.0 (95% CI: 65.3-72.7) years. DA was diagnosed in 14/26 (53.8%; 95% CI: 34.6-72.7) females and 12/26 (46.2%; 95% CI: 27.0-65.4) males. The age of patients ranged from 21 to 67 years. The mean age ± SD was 37.5 ±11.2 (95% CI: 33.0-42.0). Surgical resection was performed in all DAs. In addition, all patients received adjuvant radiotherapy. Clinical characteristics of the enrolled patients are summarized in the Table IV.

Table IV. Clinical characteristics of the enrolled patients

Parameter	Diffuse astrocytoma, grade II	Glioblastoma, grade IV
Number	26	146
IDH mutant	20	5
IDH wild-type	6	141
Age, years: mean ± SD (range)	37.5 ±11.2 (21-67)	62.0 ±11.2 (34-89)
Overall survival, months: Median (95% CI)	Could not be calculated	7.9 (6.8-9.0)
Gender: number; proportion; 95% CI		
Females	14; 53.8%; 34.6-72.7	75; 51.4%; 43.3-59.5
Males	12; 46.2%; 27.0-65.4	71; 48.6%; 40.5-56.7
Treatment: number; proportion; 95% CI		
Surgery + radiotherapy + temozolamide	56; 41.5%; 33.5-49.9	N/A
Surgery + radiotherapy	50; 37.0%; 29.3-45.4	26; 100%; 89.1-100.0
Surgery only	29; 21.4%; 15.3-29.1	N/A

SD – standard deviation; CI – confidence interval; N/A – not applicable

Immunohistochemical profile of glioma

GBM demonstrated a marked increase of Ki-67 proliferation activity compared with DAs: 44.4% (95% CI: 41.1-47.6) vs. 6.4% (95% CI: 4.7-8.0). Ki-67 proliferation indices ranged from 13-95% in GBMs and from 2-15% in DAs. Expression of aberrant p53 protein varied significantly in both groups, from absence of any immunoreactivity (0%) to strong labelling of almost all cells (99%).

There was no statistically significant difference of p53 protein expression by mean fraction of positive cells between DAs and GBMs ($p = 0.416$). By the selected cut-off, p53 expression was found in 64.3% (95% CI: 55.6-72.1) of GBMs and 75.0% (95% CI: 55.1-88.0) of DAs. Expression of p21 was significantly more frequent in GBMs than in DAs: 21.2% (95% CI: 18.7-23.6) vs. 6.9% (95% CI: 2.4-11.4). By the selected cut-off, 49.3% (95% CI: 41.3-57.4) of GBMs and only 15.0% (95% CI: 5.2-36.0) of DAs showed expression of p21. Expression of p27 was common in both GBMs and DAs. However, the mean value of p27 expression was lower in GBMs than in DAs: 69.7% (95% CI: 65.8-73.7) vs. 86.6% (95% CI: 81.6-91.7). Using the cut-off level, high p27 protein expression was found in 60.1% (95% CI: 50.9-68.7) of GBMs and 86.9% (95% CI: 67.8-95.4) of DAs. CD44 protein was expressed in a significantly greater fraction of cells in GBMs compared to DAs: 74.1% (95% CI: 69.6-78.7) vs. 13.5% (95% CI: 7.7-19.2). Strong expression of CD44 in more than 50% of neoplastic cells was found in 81.5% (95% CI: 74.4-86.9) of GBMs; however, only one (3.8%; 95% CI: 0.1-19.6) DA reached a high level of CD44 expression. Significantly increased expression of PDGFRA was observed in DAs compared to GBMs ($p < 0.001$). High PDGFRA protein expression was observed in 6.2% (95% CI: 5.0-10.6) of GBMs and 52.6% (95% CI: 30.1-75.0) of DAs, when the cut-off threshold of 50% was applied. IDH1 R132H protein expression was found in 3.4% (95% CI: 0.5-6.3) of GBMs compared with 76.9% (95% CI: 60.7-93.1) of DAs. All cases showed intense nuclear staining. Among the positive GBM cases, only one GBM morphologically showed components of lower grade glioma, thus confirming secondary GBM on morphological grounds. All IDH1 R132H positive GBMs ($n = 5$) lacked any radiological or clinical evidence of a pre-existing low-grade tumour. The mean age of patients with secondary GBMs (IDH1 R132H positive) was 50.6 (95% CI: 48.9-52.2) years compared to a mean age of patients with primary GBMs (IDH1 R132H negative) of 62.4 (95% CI: 60.7-64.0) years.

Immunohistochemical characteristics of glioma patients are summarized in the Table IV.

Results of the immunohistochemical visualization results showing high and low level of expression of all studied markers in gliomas are illustrated in Fig. 2.

Associations and correlations between the studied immunohistochemical variables

We found very weak positive correlations between p53 and the proliferation fraction by Ki-67 ($r_s = 0.196$; $p = 0.027$) and PDGFRA ($r_s = 0.181$; $p = 0.043$) in GBMs. Ki-67 also showed a trend towards a very weak negative correlation with p27 ($r_s = 0.199$; $p = 0.055$).

In DAs, there was a significant moderate, positive correlation between PDGFRA and p53 ($r_s = 0.544$; $p = 0.013$). In contrast, the correlation between PDGFRA and CD44 was negative, but also of moderate strength ($r_s = –0.592$; $p = 0.006$). There was

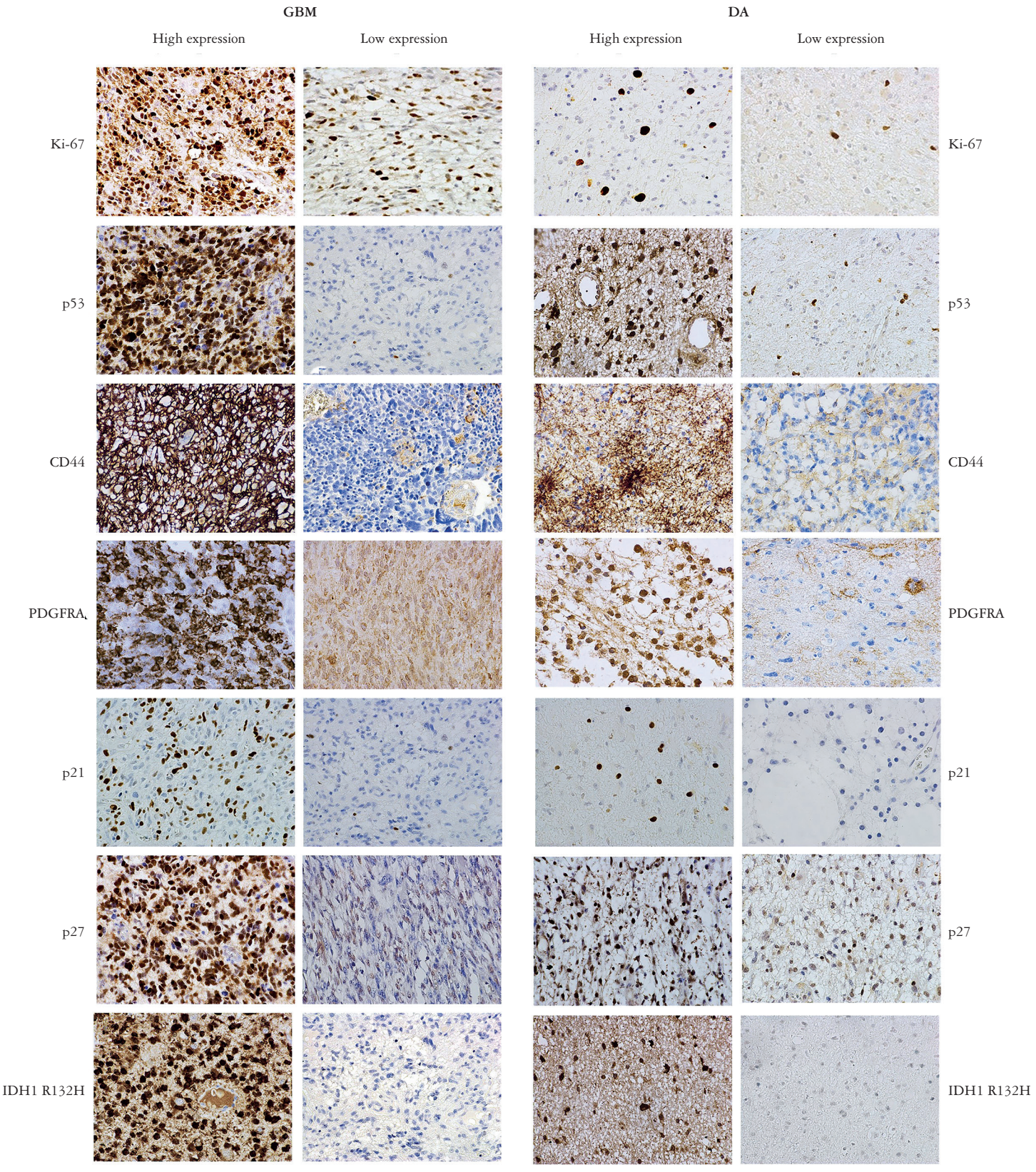

Fig. 2. Immunohistochemical detection of Ki-67, p53, CD44, PDGFRA, p21, p27, IDH1 R132H in GBM and DA. A staining intensity with high and low expression levels is shown at original magnification of 200× for all immunohistochemical markers

Table V. Immunohistochemical profile of glioma

Variable	Diffuse astrocytoma	Glioblastoma
Ki-67		
Mean of Ki-67 expressing cells ±SD (%); 95% CI	6.4% ±3.9; 4.7-8.0	44.4% ±18.5; 41.1-47.6
Median of Ki-67 expressing cells (%); IQR	5.5; 6	41.0; 24
Range of Ki-67 expressing cells (%)	2-15	13-95
No. of high Ki-67 expression status; %; 95% CI (cut-off 25% and 3% for GBMs and DAs respectively)	16; 66.7; 46.7-82.0	111; 88.1; 81.2-92.6
p53		
Mean of p53 expressing cells ±SD (%); 95% CI	43.4 ±31.7; 30.0-56.8	35.3 ±37.6; 28.7-42.0
Median of p53 expressing cells (%); IQR	52.0; 63	15.0; 71
Range of p53 expressing cells (%)	0-95	0-99
No. of high p52 expression status; %; 95% CI (cut-off 10%)	18; 75; 55.1-88	81; 64.3; 55.6-72.1
CD44		
Mean of CD44 expressing cells ±SD (%); 95% CI	13.5 ±14.3; 7.7-19.2	74.1 ±27.8; 69.6-78.7
Median of CD44 expressing cells (%); IQR	8.5; 15	86.5; 36
Range of CD44 expressing cells (%)	1-50	5-100
No. of high CD44 expression status; %; 95% CI (cut-off 50%)	1; 3.8; 0.1-19.6	119; 81.5; 74.4-86.9
p21		
Mean of p21 expressing cells ±SD (%); 95% CI	6.9 ±9.5; 2.4-11.4	21.2 ±15.0; 18.7-23.6
Median of p21 expressing cells (%); IQR	2.5; 7	19.0; 19
Range of p21 expressing cells (%)	0-32	1-68
No. of high p21 expression status; %; 95% CI (cut-off 20%)	3; 15.0; 5.2-36.0	72; 49.3; 41.3-57.4
p27		
Mean of p27 expressing cells ±SD (%); 95% CI	86.6 ±11.6; 81.6-91.7	69.7 ±21.2; 65.8-73.7
Median of p27 expressing cells (%); IQR	92; 17	74; 31
Range of p27 expressing cells (%)	62-97	2-98
No. of high p27 expression status; %; 95% CI (cut-off 70%)	20; 86.9; 67.8-95.4	68; 60.1; 50.9-68.7
PDGFRA		
Mean of PDGFRA expressing cells ±SD (%); 95% CI	42.3 ±35.5; 25.7-59.0	7.9 ±17.3; 5.0-10.7
Median of PDGFRA expressing cells (%); IQR	42.0; 68	1.0; 4
Range of PDGFRA expressing cells (%)	1-95	0-90
No. of high PDGFRA expression status; %; 95% CI (cut-off 50%)	10; 52.6; 30.1-75.0	9; 6.2; 2.3-10.1
IDH1 R132H		
No. of positive IDH1 R132H expression status; %; 95% CI	20; 76.9; 60.7-93.1	5; 3.4; 0.5-6.3
No. of negative IDH1 R132H expression status; %; 95% CI	6; 23.1; 6.9-39.3	141; 96.6; 93.7-99.5

SD – standard deviation; IQR – interquartile range; CI – confidence interval; No. – number; PDGFRA – platelet-derived growth factor receptor α; GBM – glioblastoma; DA – diffuse astrocytoma; CD – cluster of differentiation

Table VI. Correlations between IHC variables in GBMs and DAs by r_s and p values

IHC markers	GBM		DA	
	R_S	P value	R_S	P value
Ki-67/p53	**0.196**	**0.027**	0.339	0.106
Ki-67/CD44	–0.162	0.070	–0.130	0.544
Ki-67/PDGFRA	0.098	0.274	–0.002	0.992
Ki-67/p21	–0.060	0.503	0.146	0.551
Ki-67/p27	**–0.199**	**0.055**	0.276	0.226
p53/CD44	–0.073	0.414	–0.382	0.066
p53/PDGFRA	**0.181**	**0.043**	**0.544**	**0.013**
p53/p21	0.019	0.829	–0.20	0.282
p53/p27	0.037	0.725	0.149	0.518
CD44/PDGFRA	–0.141	0.090	**–0.592**	**0.006**
CD44/p21	0.037	0.659	0.170	0.474
CD44/p27	–0.144	0.128	0.302	0.162
PDGFRA/p21	–0.152	0.067	**–0.603**	**0.008**
PDGFRA/p27	–0.056	0.555	0.149	0.555
p21/p27	0.119	0.211	–0.290	0.229

CD – cluster of differentiation; PDGFRA – platelet derived growth factor receptor α; r_s – Spearman's rank correlation coefficient; GBM – glioblastoma; DA – diffuse astrocytoma; IHC – immunohistochemistry

a strong negative correlation between PDGFRA and p21 in DAs ($r_s = –0.603$; $p = 0.008$). The full results for the correlations between IHC markers are summarized in Table VI where statistically significant correlations ($p < 0.05$) are indicated in bold.

The Mann-Whitney U test was used to assess differences between the IDH1 R132H positive and negative groups of GBMs (primary vs. secondary) and DAs. There was a trend towards a younger age in secondary GBMs ($p = 0.060$). There was also a significantly higher level of p53 expression in secondary GBMs ($p < 0.001$). No significant differences were found for IDH1 R132H status in DAs.

Survival and prognostic markers of GBM patients

The survival data were available for 135 patients, all of which have been included in the survival analysis. At the end of the study, 2/135 (1.5%; 95% CI: 0-5.2) patients were alive, but 133/135 (98.5%; 95% CI: 94.8-99.6) had died during the observation period. The overall median survival time was 7.9 months (95% CI: 6.8-9.0). One-year, two-year and three-year survival rates for patients with GBM were 36.3%, 9.6% and 1.5%, respectively. There was a statistically significant difference in overall survival (OS) regarding the patient's age (log-rank, $p < 0.001$); median OS times for patients ≤ 65 and > 65 years old were 11.7 (95% CI: 8.1-15.3) and 5 (95% CI: 3.2-6.8) months, respectively. This indicates younger age at diagnosis is associated with significantly longer survival time in GBM patients. Significant difference in median OS was observed in GBM patients by type of treatment (log-rank, $p < 0.001$). Tumours treated with the current standard of care – surgery followed with radiotherapy and chemotherapy with temozolomide – had a median OS of 12.1 (95% CI: 11.2-13.0) months, vs. surgery plus radiotherapy: 7.5 (95% CI: 5.4-9.6) months, vs. surgery only: 2.9 months (95% CI: 1.4-4.4). In GBMs, a statistically significant survival difference was also found in patients regarding IDH1 R132H mutant protein expression (log rank, $p = 0.040$). Patients with secondary GBMs had a median OS of 18.3 (95% CI: 18.0-18.5) months vs. 7.7 (95% CI: 6.3-9.0) months in patients with primary GBMs. A trend towards a difference in OS was found in patients with GBM by PDGFRA expression (log rank, $p = 0.066$). The median OS of patients with high PDGFRA expression was 6.4 (95% CI: 2.8-9.9) months vs. 8.3 (95% CI: 6.4-10.1) months in patients with low PDGFRA expression. Regarding other immunohistochemical markers, no significant survival differences were found.

Survival and prognostic markers of DA patients

The survival data were available for 25 patients, which have been included in the survival analysis. At the end of the study, 14/25 (56.0%; 95% CI: 37.0-73.3) patients were alive, but 11/25 (44.0%; 95% CI: 26.6-62.9) died during the observation period. Due to the small study group and small

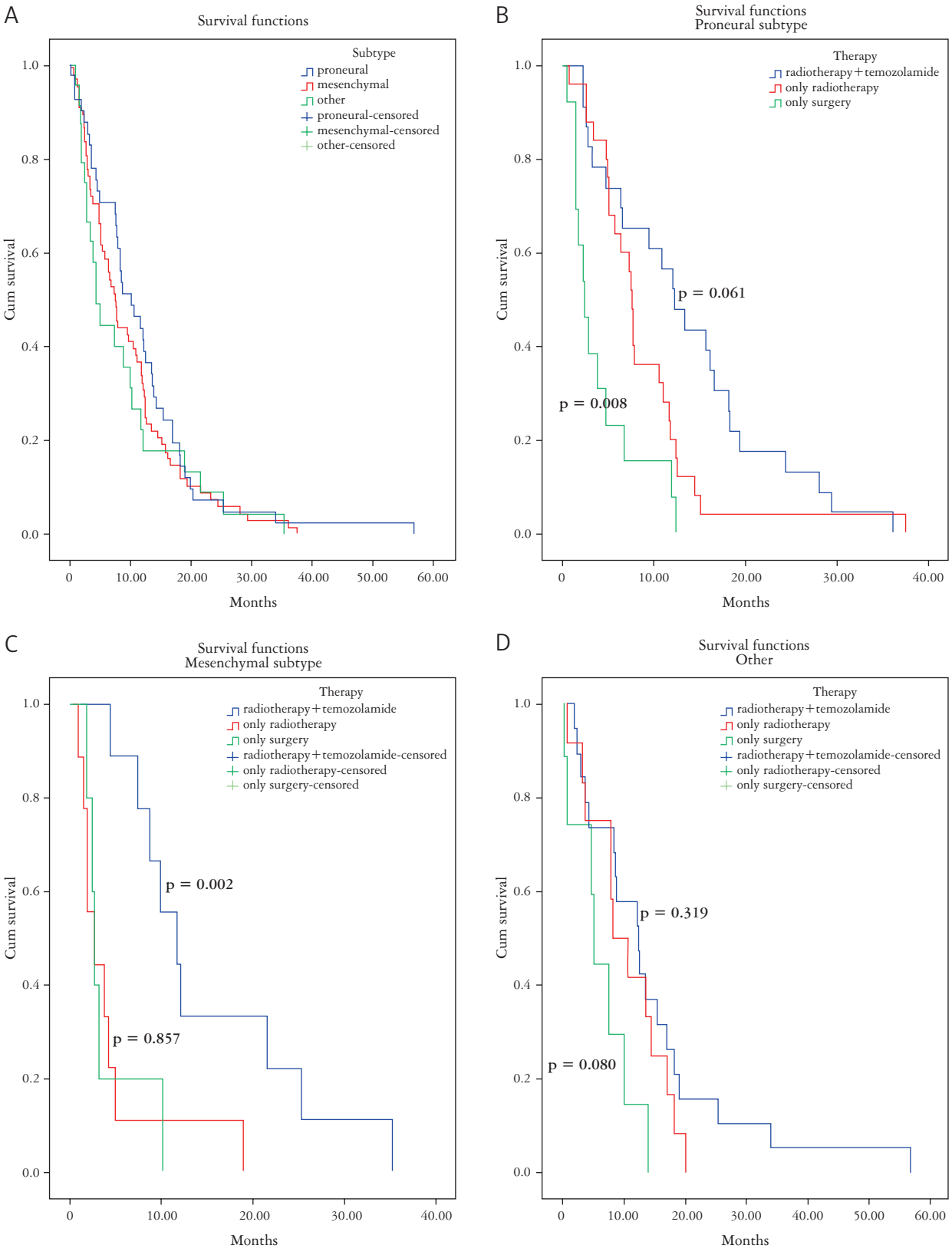

Fig. 3. Kaplan-Meier survival curves by glioblastoma (GBM) subtype and treatment type. In general, there are no differences in overall survival between molecular subtypes of GBMs, however, Kaplan-Meier analysis has showed that survival is different by distinct treatment type. Thus, in proneural and mesenchymal GBMs addition of temozolomide improved survival, however, mesenchymal subtype appears to be resistant to addition of radiotherapy. A) Survival by GBM molecular subtype. B) Survival by proneural subtype and treatment type. C) Survival by mesenchymal subtype and treatment type. D) Survival by other subtype and treatment type

number of death cases, statistical calculations are insufficient and overall median survival time could not be calculated. Within the first year following surgical operation, all patients were alive (25/25). Two years after the initial operation, 3/25 (12%; 95% CI: 4.2-29.9) patients had died, but 22/25 (88%; 95% CI: 70.0-95.8) were alive. Three years after the initial operation, 5/25 (20%; 95% CI: 8.8-39.1) patients had died, but 20/25 (80%; 95% CI: 60.9-91.1) were alive. In DAs, statistically significant differences in survival were found in patients regarding PDGFRA expression (log rank, $p = 0.017$). Interestingly, there were no significant survival differences regarding Ki-67 expression using the selected cut-off of 3%. However, when we tried a higher cut-off value of 5.5%, which was based on the median Ki-67 expression rate in DAs, a statistically significant difference in survival was found (log rank, $p = 0.037$).

Molecular subtypes of gliomas

The majority of GBM cases were of the proneural subtype: 73/146 (50.0%; 95% CI: 42.0-58.0) cases, followed by "Other" (not otherwise classified) for 46/146 (31.5%; 95% CI: 24.5-39.4) patients, and the mesenchymal subtype in 27/146 (18.5%; 95% CI: 13.0-25.6) patients. There were no associations between the molecular subtypes of the GBMs and any of the clinical or other immunohistochemical parameters (Ki-67, p21, p27). There were no differences in OS between molecular subtypes of GBMs (log rank, $p = 0.424$) (Fig. 3A). Furthermore, the level response to therapy was evaluated on survival in the different GBM subtypes. As shown in Fig. 3B, in the proneural subtype there was a trend suggesting the addition of temozolomide improved OS compared with radiotherapy alone ($p = 0.061$). However, a visual trend towards a difference between the Kaplan-Maier curves was more readily apparent. Radiotherapy also improved OS in the proneural subtype compared with surgery alone ($p = 0.008$). In the mesenchymal subtype, the addition of temozolomide significantly improved OS compared with radiotherapy alone ($p = 0.002$). However, addition of radiotherapy did not improve OS of the patients with the mesenchymal subtype compared with surgery alone ($p = 0.857$) (Fig. 3C). Thus, addition of radiotherapy did not provide any benefit compared to surgery alone in patients with the mesenchymal subtype. In "Other" GBMs (not otherwise classified), there was only a statistically significant difference in OS between the GBMs threated with adjuvant chemotherapy and radiotherapy compared with solely surgically treated ($p = 0.031$). There were no statistically significant differences between the other groups: temozolomide + radiotherapy vs. radiotherapy alone ($p = 0.319$) and radiotherapy vs. surgery only ($p = 0.080$) (Fig. 3D).

Most of the DAs, 24/26 (92.3%; 95% CI: 75.8-97.9) patients, belong to the proneural subtype; the remaining 2/26 (7.6%; 95% CI: 2.1-24.1%) DAs were not otherwise classified ("Other").

Discussion

Diffuse gliomas do not rank among the tumours with the highest incidence; nevertheless, they rank among the most aggressive human malignancies with limited treatment options. Thus, better understanding of specific signaling pathways and molecular alterations determining the biological features of gliomas – e.g. invasion, proliferation, resistance to current therapy – can provide hope for improving specific management, specifically, development of personalized therapy as well as targeted management.

In the last decade, several studies have been performed to identify subtypes of gliomas based on molecular and proteomic signatures [2, 3, 7, 10, 11]. For example, Verhaak *et al.* described four glioblastoma molecular subtypes based on gene expression analysis: classical, proneural, mesenchymal and neural [3]. Existence of proneural and mesenchymal GBM molecular subtypes was also suggested by other large-scale molecular study carried out by Phillips *et al.* [12]. The latest 2016 WHO classification of CNS tumours is a huge step forward as it significantly improves classification of gliomas by incorporating molecular data in a glioma diagnostics. However, discussions about previously described proneural and mesenchymal subtypes are still ongoing [13, 14, 15]. From all these subtypes, especially, mesenchymal subtype is one of the most consistent subtypes described in the literature [14, 15, 16]. Identification of mesenchymal signature in glioma tissues brings certain clinical interest because it is associated with worse prognosis [3, 17].

However, molecular techniques are time consuming and expensive, and thus some molecular data must be replaced with cheaper and simpler tools such as IHC. To date, several authors have shown successful attempts of molecular glioma subtyping by the means of IHC [5, 6, 18, 19].

In the current study, we used IHC to assess the expression of some of the proteins that have been reported to be significant in subtyping (PDGFRA, IDH1 R132H, p53, CD44) or determining basic biological features of malignant tumours such as invasion, proliferation and cell cycle regulation (Ki-67, p21, p27).

In this study, no survival differences were found between molecular GBM subtypes. With regard to clinical outcome, the mesenchymal subtype is described as a subtype with an unfavourable prognosis;

this is in contrast to the better prognosis by which the proneural subtype is characterized [3, 12, 14, 20]. However, we found a predictive role for these molecular subtypes: a response to adjuvant therapy was found to be different for proneural vs. mesenchymal subtypes. Thus, both molecular subtypes showed a benefit from addition of chemotherapy to radiotherapy; however, in the mesenchymal subtype, the beneficial effect of adjuvant chemotherapy was more prominent compared to radiotherapy alone. Interestingly, the mesenchymal subtype did not show any beneficial effect from the addition of radiotherapy compared with palliative management and surgery alone ($p = 0.857$), but in the proneural subtype addition of radiotherapy was beneficial ($p = 0.008$). This finding may indicate a possible radioresistance of the mesenchymal subtype of GBM. In contrast, Verhaak *et al*. reported that more intensive treatment significantly reduced mortality in the mesenchymal subtype of GBM. However, in the study by Verhaak *et al*. the effect of radiotherapy alone was not reported; all patients with less intensive and more intensive therapy received combined concurrent or non-concurrent chemo-radiotherapy or chemotherapy of varying intensities [3]. Supporting our finding, Brown *et al*. reported radioresistancy in the mesenchymal subtype while preserving chemo-sensitivity of the mesenchymal subtype [21]. Several authors have also reported that the mesenchymal subtype is associated with a stem cell phenotype, enriched with the presumed stem cell marker CD44 [22]. However, glioma mesenchymal stem cells are characterized by extensive radioresistency [23, 24, 25], which may explain the failure of radiotherapeutic effectiveness in the mesenchymal subtype of GBMs in this study.

Regarding DAs, the significance of subtyping in low-grade gliomas should be further evaluated. The low-grade gliomas are enriched primarily with markers for the proneural subtype [26, 27]. In addition, high frequency of *IDH1* mutations in low-grade gliomas account for the majority of the proneural subtype [28]. Because CD44 expression was very low in DAs, a mesenchymal designation might only be of importance in GBMs but not in DAs. Presence of the *IDH* gene mutations is one of the known prognostic factors for a relatively favourable prognosis in patients affected with high-grade gliomas [2, 29, 30, 31]. IDH gene mutation identify secondary GBMs with much better prognosis [32]. Median overall survival rates of mutated and non-mutated *IDH1* patients with GBMs are 3.8 and 1.1 years, respectively [2]. Presence of IDH mutation in glioma so greatly determines prognosis of the patient that testing for IDH mutation now is also included in diagnostic criteria's of gliomas in the 2016 WHO classification of tumours of the CNS [1]. Screening for the IDH gene mutations is also possible by the means of IHC. Antibodies specific for the products of IDH1 R132H mutation may be of sufficient value of screening tool to replace more formal mutational analysis [33, 34].

In this study, patients with secondary GBMs (IDH-mutant) had significantly better prognoses than those with primary GBMs (IDH-wild-type): median OS was 18.3 months vs. 7.7 months ($p = 0.040$). Regarding DAs, patients with IDH1 R132H negative and IDH1 R132H positive tumours did not show any statistically significant survival differences ($p = 0.336$) in this study. Also, an association between IDH1 and p53 was also found in GBMs ($p = 0.001$); the finding that these two abnormalities frequently coexist is supported by other authors [32]. Interestingly, another single molecular marker, PDGFRA, showed a trend ($p = 0.066$) toward better prognosis in GBMs and was significantly ($p = 0.017$) associated with a better prognosis in DAs. The clinical impact of PDGFRA in gliomas has been debated in several studies but there is no consensus yet. Increased expression of PDGFRA has been reported in proneural GBMs that have better prognosis, as described by several researchers [3, 12]. However, other authors reported no association between PDGFRA expression and prognosis in glioma patients [35]. In the current study, Ki-67 proliferation fraction was found to be a useful indicator for a worse prognosis in DAs; however, prognostic significance was found by using a higher cut-off value of 5.5% instead of the 3% cut-off chosen initially. Although Ki-67 proliferation fraction is not included in the latest WHO classification system, assessment of Ki-67 may be useful for identification of increased proliferative activity in DAs with otherwise typical low-grade gliomas. Such cases of DAs might need more careful follow-up and have a worse prognosis [36]. In this study, Ki-67 proliferation fraction correlated with p53 protein expression ($r_s = 0.196$; $p = 0.027$), indicating oncogenic properties of p53 upregulate proliferation in neoplastic cells. Also, another cell cycle inhibitor, p27, had a trend towards an inverse correlation with Ki-67. Thus, loss of p27 and upregulation of p53 may be indicators for more proliferative features in GBMs. Regarding p53 correlation with PDGFRA in both GBMs and DAs, it seems to be a reasonable association, because both *TP53* and *PDGFRA* gene mutations are more frequently associated with the proneural subtype of GBMs as described by Verhaak *et al*. [3]. In DAs, CD44 expression has a negative correlation with PDGFRA ($r_s = -0.592$; $p = 0.006$). In addition, Conray *et al*. showed that high scores of CD44 were rarely found in gliomas with high PDGFRA expression [37]. Cautiously considering that PDGFRA pathway activation in different classifications has been considered to be a marker for proneural/proneural-like glioblastoma but CD44 expression is point-

ing towards the mesenchymal subtype, a negative association seems to be more reasonable. Interestingly, PDGFRA also had a negative correlation with p21 ($r_s = –0.603$; $p = 0.008$) in DAs.

In addition to mesenchymal and proneural GBM subtypes, Verhaak *et al*. described classical and neural subtypes. The neural subtype is relatively poorly characterised and defined by the expression of genes such as *NELF* and *GABR*, which are mainly expressed in normal neurons. However, the classical subtype is characterised by *EGFR* gene amplification and frequent expression of the EGFRvIII protein. Thus, it would be necessary in future studies to include other IHC markers such as EGFR, NELF and GABR, as well as other mesenchymal markers, such as YKL-40, MERTK and CD44. We believe GBMs which remained unclassified in this study, called "Other" could be classified by using additional markers.

In conclusion, our results indicate an existence of two mutually exclusive molecular signatures in gliomas reminiscent to those proneural and mesenchymal subtypes. In our study, assessment of the expression of only four proteins (p53, CD44, PDGFRA and IDH1) by IHC was able to define these subtypes of gliomas. This assessment is cheaper and less time consuming than other molecular approaches and is easily applicable in routine practice. Because of simple reproducibility, our findings have huge practical interest and the panel of these four markers can be used for simple molecular subtyping which is crucial for prediction of treatment response in glioma patients. In addition, detection of IDH1 R132H mutation by IHC is simple and cheap prognostic test for glioma patients.

The authors declare no conflict of interest.

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