Subtyping of gliomas of various WHO grades by the application of immunohistochemistry

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Popova S N, Bergqvist M, Dimberg A, Edqvist P-H, Ekman S, Hesselager G, Ponten F, Smits A, Sooman L & Alafuzoff I (2014) *Histopathology* **64**, 365–379

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Aims: In 2010, four subtypes (classical, proneural, mesenchymal, and neural) of glioblastoma multiforme (GBM) were defined by molecular genetic analyses. The objective of this study was to assess whether gliomas, independently of the type and grade, could be subdivided into protein-based subtypes.

Methods and results: A tissue microarray (TMA) approach was applied to incorporate tissue samples of low-grade and high-grade gliomas into five TMAs. High expression levels of epidermal growth factor receptor (EGFR), CD44, c-MER proto-oncogene tyrosine kinase (MERTK), platelet-derived growth factor receptor α, p53, oligodendrocyte transcription factor 2 (OLIG2) and isocitrate dehydrogenase 1 with the R132H mutation were assessed using immunohisto-chemistry (IHC). Glioma could be subdivided into four subtypes by IHC. The majority of the low-grade gliomas were of the proneural subtype, i.e. high p53 expression (63% of grade II). The classical subtype, with high EGFR and low p53 expression, was most common in GBMs (39%), followed by the proneural (29%) and mesenchymal (with high CD44 and MERTK expression) (29%) subtypes, a frequency that is in line with previously published data based on molecular genetics.

Conclusions: Assessment of the expression of the five proteins EGFR, CD44, MERTK, p53 and OLIG2 is sufficient for subtyping gliomas, and can be recommended for implementation in clinical practice for both low-grade and high-grade gliomas.

Keywords: glioma, immunohistochemistry, protein expression, subtyping

Introduction

Approximately 2% of all adult malignancies are brain tumours, and 80% of these are gliomas.[1] They can occur at any age, regardless of gender or ethnicity. Currently, there is no cure for glioma, and, in spite of aggressive therapy, the morbidity is high. The most aggressive type of glioma, WHO grade IV, is also referred to as glioblastoma multiforme (GBM).[2,3]

According to the cell type, gliomas are divided into astrocytic, oligodendroglial, and ependymal.[4] The neuropathological grading of gliomas is based upon the St Anne/Mayo system,[5] including the assessment of features such as (i) cellular atypia (WHO grade II),

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(ii) mitotic figures (WHO grade III), (iii) endothelial proliferation, and/or (iv) necrosis (WHO grade IV), all visualized with haematoxylin and eosin (H&E) staining. While growing, these tumours (with the exception of ependymoma) tend to diffusely infiltrate the surrounding brain tissue, as recently demonstrated for gliomas of WHO grade II/III and secondary GBM, with isocitrate dehydrogenase 1 (IDH1) bearing the R132H mutation (IDH1[R132H]).[6–8]

Accumulation of genetic alterations is involved in the initiation and progression of gliomas, leading to great genetic heterogeneity of these tumours.[9–12] In 2006, three molecular subtypes of GBM were defined on the basis of molecular profiles and the protein expression of tumour cells, i.e. proneural, proliferative, and mesenchymal.[13] Later, a number of studies were published in which GBMs were divided into different subtypes on the basis of the activity of signal transduction pathways,[9] gene expression analysis,[14] or protein expression levels[15,16] (Table 1).

One of the proteins listed, which was of interest in all of the previous reports, is epidermal growth factor receptor (EGFR), which is known to be involved in cellular processes such as proliferation, survival, angiogenesis, invasion, and metastasis. Other proteins that have been reported to be characteristic of certain GBM subtypes are the cell surface proteins CD44, c-mer proto-oncogene tyrosine kinase (MERTK), and platelet-derived growth factor receptor α (PDGFRA).[9,13–16] Furthermore, the tumour suppressor protein p53,[14–16] oligodendrocyte transcription factor 2 (OLIG2)[9,13,14,16] and IDH1 with the R132H mutation[14,16] have also been reported to be of interest.

The current strategy for diagnosis of a brain tumour incorporates assessment of the histological type (astrocytic/oligodendrocytic/ependymal) and assessment of the grade (WHO grading system). Recent observations have indicated that the molecular signature of the tumour also appears to be of significance.[13,14,17]

The objective of the current study was to attempt to identify different glioma subtypes by assessing protein expression in surgical tissue samples of adult gliomas, using immunohistochemistry (IHC) and a tissue microarray (TMA) strategy. The patients in the cohort were all inhabitants of a defined central region of Sweden.

Materials and methods

SELECTION OF MATERIAL

The general design of the study is delineated in Figure 1. The medical records at the Department of Pathology and Cytology, Uppsala University Hospital, were searched for subjects with a diagnosis of

Table 1. High expression of proteins defining previously proposed subtypes of glioblastoma multiforme (GBM)

Proteins reported to be highly expressed	Proposed subtypes				
	Philips *et al.*[13]	Brennan *et al.*[9]	Verhaak *et al.*[14]	Motomura *et al.*[16]	Le Mercier *et al.*[15]
Epidermal growth factor receptor (EGFR)	Proliferative and Mesenchymal GBM	EGFR core	Classical GBM	Mixed	Classical like
CD44	Mesenchymal GBM		Mesenchymal GBM	Astrocyticmesenchymal, Mixed	
Proto-oncogene tyrosine-protein kinase MER			Mesenchymal GBM		
Isocitrate dehydrogenase 1 mutation			Proneural GBM	Oligodendrocyte precursor type (OPT)	
OLIG2	Proneural GBM	PDGF core	Proneural GBM	Differentiated oligodendrocyte type and OPT	
P53			Proneural GBM	OPT	Proneural like
Platelet derived growth factor (PDGF) receptor α		PDGF core	Proneural GBM	OPT	Proneural like

The medical records of all patients operated for high grade glioma at Uppsala University Hospital from 1989 to 2000 and a low grade glioma from 1983 to 2000 were scrutinised. The age of the patient at operation was at least 18 years.

In total 273 subject operated for a low or a high grade glioma were identified

All diagnostic slides were retrived and reassessed and the pathoanatomical diagnosis followed the 2007 WHO criteria (Table 3).

100 cases of the low grade and 119 of the high grade tumors (219 cases) fulfilled at re-assessment the diagonostic criteria of a glioma and there was suffcient amount of material to be taken for a tissue microarray (4 case not a glioma, 50 not enough of tissue in the block). Six cases were excluded since they were not primary tumors.

Two representative core samples were taken from the paraffin blocks and incorporated into five tissue microarray block

In 180 cases representative tumor tissue was seen in the produced tissue microarray (Table 4).

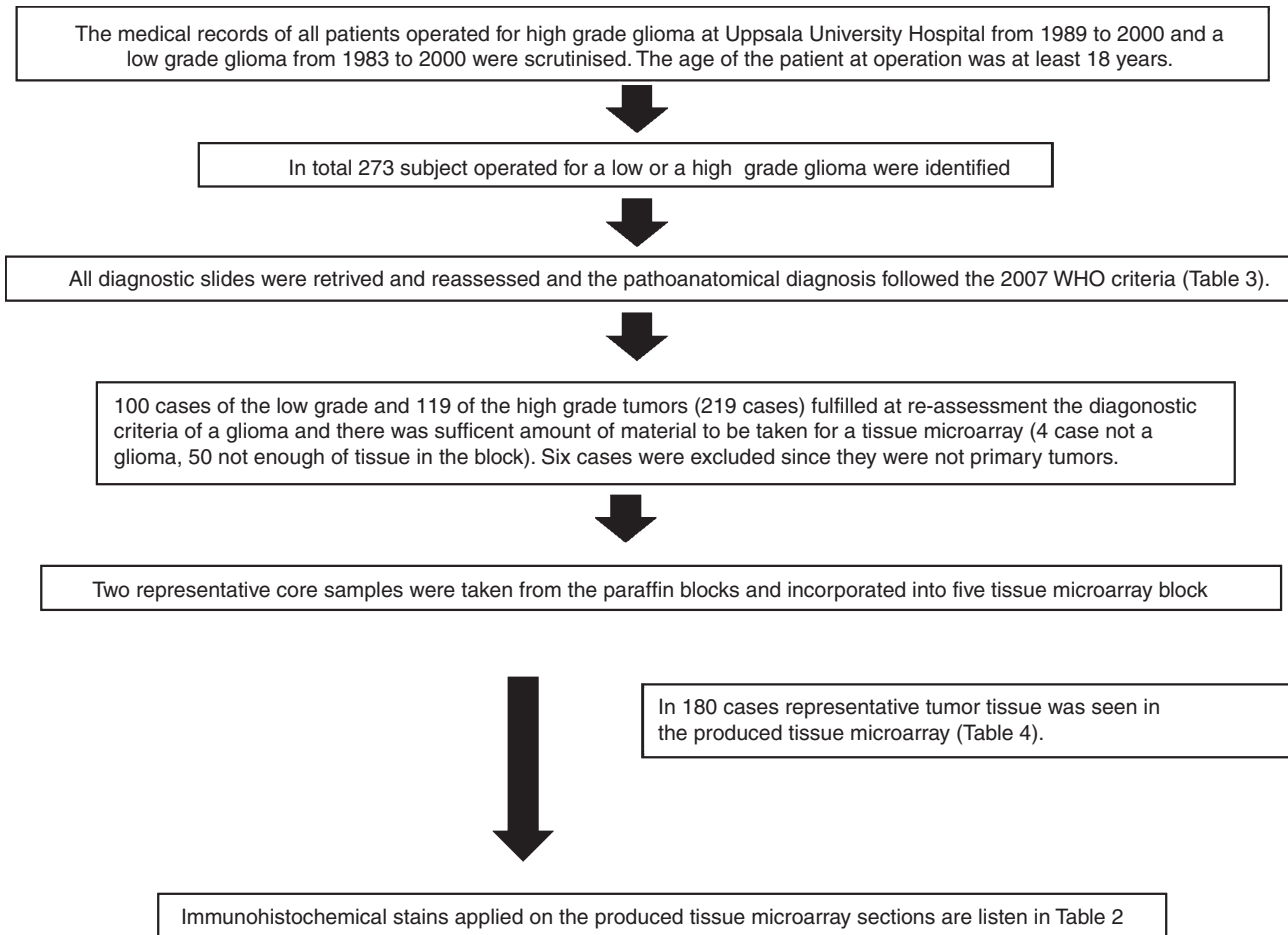Immunohistochemical stains applied on the produced tissue microarray sections are listen in Table 2

Figure 1. Flowchart summarizing the logistics of the study.

low-grade or high-grade glioma of astrocytic or oligodendrocytic phenotype who underwent an operation between 1983 and 2000 (low grade glioma) or between 1989 and 2000 (high grade glioma). In total, 273 subjects were identified. All archived slides were retrieved and retrospectively reviewed, blind to the original diagnosis. The type and grading followed the WHO criteria from 2007.[4] The material included small biopsies, and subtotal or presumably total resections. The patients were variably treated, i.e. no treatment, radiotherapy only, chemotherapy only, or a combination of radiotherapy and chemotherapy.

TISSUE MICROARRAY CONSTRUCTION

In each case, two representative tumour regions were identified and marked on the original H&E-stained slides. TMAs were prepared as previously described.[18] In total, five blocks with core samples were produced. The recipient blocks were cut into consecutive 4-μm-thick sections, and placed on SuperFrost Plus slides (Gerhard Menzel GmbH, Braunschweig, Germany); thereafter, the first section of each block was stained with H&E to assess the representativeness of the core, and this was followed by typing and grading of the tumour according to the WHO 2007 recommendations.

IMMUNOHISTOCHEMISTRY

The antibodies, dilutions and pretreatments used are shown in Table 2. Subsequently, serial paraffin wax-embedded sections were rehydrated; endogenous peroxidase activity was quenched with 3% hydrogen peroxide for 10 min, and antigen retrieval was performed. Unspecific binding sites were blocked with Background Sniper (Biocare Medical, Concord, CA, USA) for 10 min. Thereafter, sections were incubated with primary antibodies overnight at 4°C. The Power-Vision detection system (Immunologic, Duiven, the

Table 2. Antibodies listed in alphabetical order, including dilution and pre-treatments applied

Antibody	Source	Code	Clone	Epitope	Pretreatment	Dilution
CD44 cell-surface glycoprotein	Santa Cruz	sc-7297	Polyclonal	Full-length	Citrate Buffer, pH 6.0, MW 3 × 5 ^{min}	1:100
Proto-oncogene tyrosine-protein kinase MER	Novus Biologicals	NB 110-57199	Y323	N-terminus	Citrate Buffer, pH 6.0, MW 3 × 5 ^{min}	1:50
Epidermal growth factor receptor	Invitrogen	28-0005	31G7	Extracellular domain	Proteinase K	1:100
Glial fibrillary acidic protein	DakoCytomation	Z0334	Polyclonal	Full-length	0.03% protease XXIV	1:500
Isocitrate dehydrogenase 1	Dianova	DIA H09	H09	aa125-137	Tris-EDTA, pH 9.0, MW 3 × 5 ^{min}	1:500
Ki67	DakoCytomation	M7240	MIB-1	345 and 395 Kd	Citrate Buffer, pH 6.0, MW 3 × 5 ^{min}	1:100
Microtubule-Associated protein 2	Sigma-Aldrich	M4403	HM-2	Full-length	Citrate Buffer, pH 6.0, MW 3 × 5 ^{min}	1:500
Oligodendrocyte transcription factor 2	Abnova	H00010215-M03	3C9	Full-length	Citrate Buffer, pH 6.0, MW 3 × 5 ^{min}	1:100
Platelet derived growth factor receptor α	Santa Cruz	sc-338	Polyclonal	C-terminus	Tris-EDTA, pH 9.0, MW 3 × 5 ^{min}	1:100
Tumour protein 53	DakoCytomation	M7001	DO-7	Full-length	Citrate Buffer, pH 6.0, MW 3 × 5 ^{min}	1:50

Netherlands) was used with the Romulin AEC or DAB chromogen kit (Biocare Medical).

LIGHT MICROSCOPY

A case was included when at least 50% of the total area of at least one of the core samples remained on the slide, and if the core was representative regarding the original slide, i.e. tumour present.

All IHC stains were assessed at magnifications of ×40 to ×400. The staining pattern of neoplastic cells with glial fibrillary acidic protein (GFAP) and microtubule-associated protein 2 (MAP2) was used to confirm the phenotype of the tumour cells, i.e. astrocytic, oligodendrocytic, or mixed (Figure 2). Nuclear staining (Ki67 and p53) was scored on a scale from 0 to 3, as follows: 0, absence of labelled nuclei in the core; 1, up to 10 positive nuclei; 2, 10–30 positive nuclei; and 3, >30 positive nuclei. Scores of 2 and 3 were considered to indicate high expression of the protein. In sections stained for OLIG2, PDGFRA, CD44, MERTK, EGFR and IDH1^{R132H}, the presence of nuclear, membrane or cytoplasmic staining was scored on a scale from 0 to 3, as follows: 0, absence

of immunoreactivity (IR); 1, up to 10% of cells immunoreactive; 2, 10–50% of cells immunoreactive; and 3, >50% of cells immunoreactive. A score of 3 was considered to indicate high expression of these proteins. To confirm the reliability of the assessment of IHC stains, sections were reassessed several times by at least two evaluators blinded to the original results, and a consensus score was ascribed to each tumour sample.

Photographs were taken with an Olympus BX46 microscope equipped with an Olympus DP72 digital camera, and images acquired using Cell Acquisition software (Olympus Optical, Tokyo, Japan). IBM SPSS Statistics 20 (IBM Corporation, Armonk, NY, USA) was used for statistical analysis. For significant differences, the non-parametric Kruskal–Wallis rank test and Spearman's correlation tests were used.

Results

Two hundred and nineteen cases were reassessed. The male/female ratio was 1.5, and the age at operation ranged from 19 to 80 years (mean ± standard error of the mean, 48 ± 1 years). The agreement
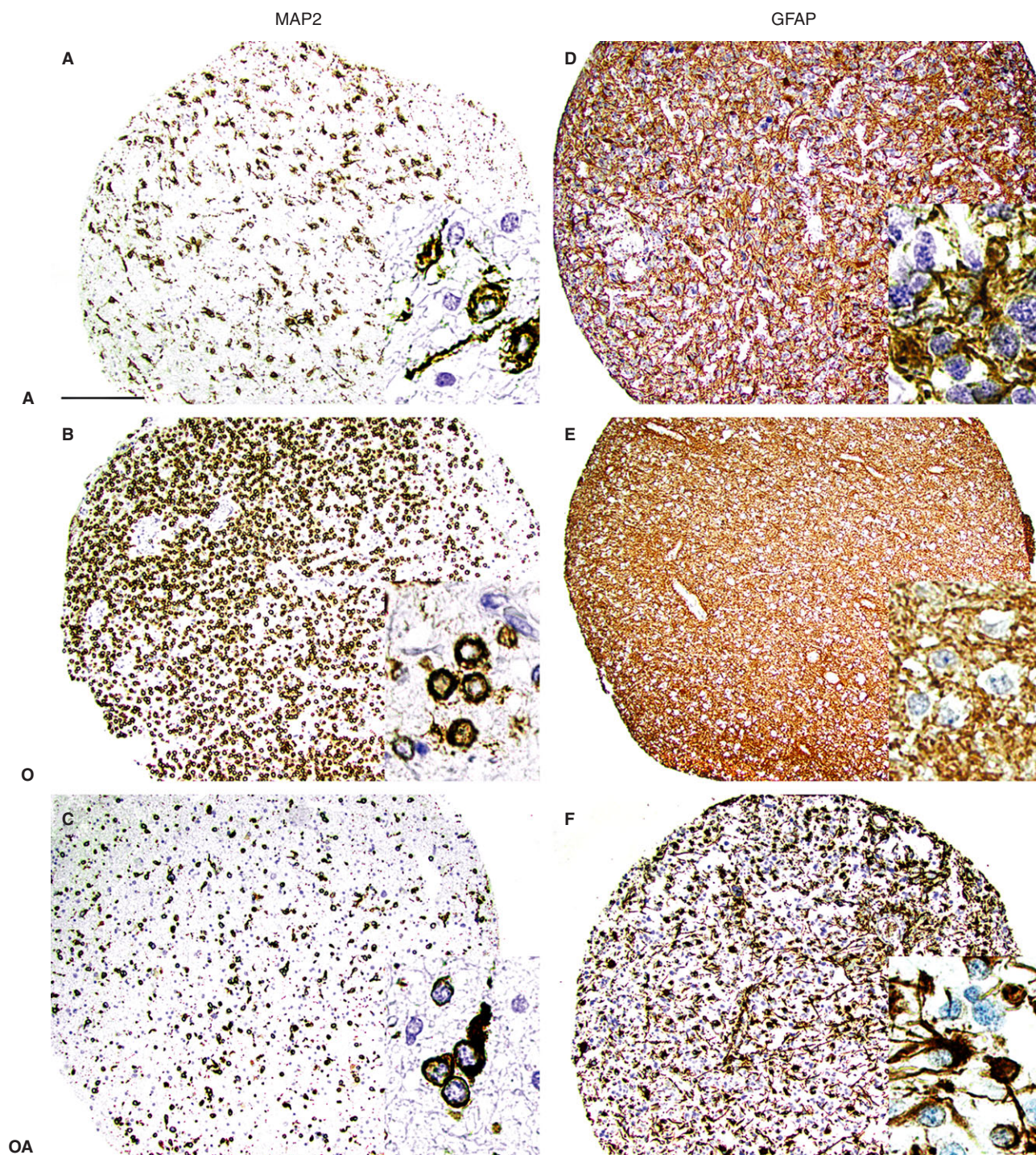
MAP2   GFAP

Figure 2. Staining results obtained with antibodies against microtubule-associated protein 2 (MAP2) and glial fibrillary acidic protein (GFAP). A, astrocytic differentiation; O, oligodendrocytic differentiation; OA, oligoastrocytic differentiation. Note the difference at both low and high (insert) magnification regarding the compartmentalization of the labelling (cell membrane versus cytoplasm) and the shape of the cell, depending on the glioma subtype (astrocytic versus oligodendrocytic) (A–C). Note the absence of GFAP labelling of the cell membrane in pure oligodendrocytic tumours (insert in E), in contrast to astrocytic or mixed tumours (D,F). Bar: 200 μm.

rate for reassessment of original slides and following the WHO 2007 criteria regarding low-grade glioma was 77% ($n = 128$), and that regarding high-grade glioma was 98% ($n = 91$) (Table 3). Regarding grade, the highest agreement rate was noted for grade IV tumours (82%), and the poorest for grade III tumours (9%). A significant proportion of original grade III tumours were reassessed as being grade IV tumours, i.e. GBM. Regarding subtype, the highest agreement rate was for astrocytomas (76%), and the poorest was for oligoastrocytomas (7%).

Core samples from the 219 cases were included in the TMA. Most samples were surgical (67%), and the cores were taken from viable representative tumour areas. When the produced TMA sections were examined, in 39 cases there was insufficient material for assessment. Comparing results from reassessment of the TMA cores and the whole sections, agreement regarding the grade of the tumour was 100% (Table 4), whereas the highest agreement regarding subtype was for astrocytomas (87%).

Representative photomicrographs of the staining results from application of the antibodies listed in Table 2 are presented in Figures 2 and 3, and the results are summarized in Table 5. Only those cases that showed high protein expression are included in Table 5. The group of anaplastic oligodendrogliomas contained only two cases, and these were therefore excluded from the subsequent analyses. In 15 cases (8% of all tumours), two of which were GBMs (3% of GBMs), none of the proteins assessed was present to a high extent.

The number of EGFR-positive cells varied significantly, from a few scattered strongly immunoreactive cells per core to labelling of almost all cells (Figure 3A,B). Strong EGFR IR was noted in all glioma subtypes, independently of the WHO grade (Table 5). Forty-five per cent of low-grade gliomas, 57% of high-grade gliomas and 58% of GBMs showed high EGFR IR.

Sixteen per cent of low-grade gliomas, 37% of high-grade gliomas and 43% of GBMs showed high CD44 IR (Figure 3C,D; Table 5), whereas strong CD44 IR was not seen in oligodendrogliomas, independently of grade.

Ten per cent of low-grade gliomas, 16% of high-grade gliomas and 15% of GBMs showed high MERTK IR (Figure 3E,F; Table 5).

Both astrocytic and oligodendrocytic tumours were labelled using IDH1^{R132H} antibody (Figure 3G,H; Table 5). Sixty-seven per cent of grade II/III tumours and 11% of GBMs showed high IDH1^{R132H} IR.

Fifty-one per cent of low-grade gliomas, 70% of high-grade gliomas and 75% of GBMs showed OLIG2-immunoreactive nuclei (Table 5).

Forty-five per cent of low-grade gliomas, 44% of high-grade gliomas and 41% of GBMs showed p53-immunoreactive nuclei (Table 5). The tumours of mixed phenotype (oligoastrocytic tumours) were most frequently p53-immunoreactive (69%), whereas only 33% of astrocytic tumours were p53-immunoreactive.

Platelet-derived growth factor receptor α antibody primarily labelled scattered cells in a tumour (Figure 3I,J). Twenty-three per cent of low-grade gliomas,

Table 3. Agreement rate between original diagnosis and the diagnosis given upon reassessment of original slides, following current WHO criteria

	Low-grade glioma			High-grade glioma			
	AC	O	OAC	aAC	aO	GBM	Σ
Original diagnosis							
Astrocytoma (AC)	**36**	5		8	2	1	128
Oligodendroglioma (O)	8	**19**	3		5		
Oligoastrocytoma (OAC)	17	7	**3**	5	2	7	
Anaplastic (a) AC				**4**	3	34	91
aOAC						4	
Glioblastoma (GBM)	2				6	**36**	
Glioma not defined					2		
Σ		100			119		219

100% agreement given in bold.

Table 4. Agreement rate between the diagnosis given upon reassessment of the whole original section and at the assessment of the tissue microarray (TMA) cores

	Diagnosis assessing TMA core							
	Low-grade glioma			High-grade glioma				
	AC	O	OAC	aAC	aO	aOAC	GBM	Σ
Diagnosis at reassessment								
Astrocytoma (AC)	**34**	3	10					76
Oligodendroglioma (O)	7	**7**	9					
Oligoastrocytoma (OAC)	2	2	**2**					
Anaplastic(a) AC				**10**	1	4		104
aO				7	**1**	8		
aOAC				1				
Glioblastoma (GBM)							**72**	
Σ		76			104			180

100% agreement given in bold.

Table 5. Percentages of cases with high protein expression while applying immunohistochemistry

Diagnosis in TMA	n	EGFR	CD44	MERTK	IDH1[R132H]	OLIG2	P53	PDGFRA
Astrocytoma (AC) II	43	30[10]	24[5]	8[4]	56[2]	50[5]	31[4]	34[5]
Oligodendroglioma (O) II	12	38[4]	0[3]	13[4]	78[3]	25[4]	50[4]	14[5]
Oligoastrocytoma (OAC) II	21	76[4]	10[1]	15[1]	95[1]	65[1]	70[1]	5[1]
Low-grade gliomas Σ	76	45[18]	16[9]	10[9]	70[6]	51[10]	45[9]	23[11]
AC III	18	50[2]	33	6	61	67	39	44
O III	2	100[1]	0[1]	0[1]	100[1]	0[1]	0[1]	0[1]
OAC III	12	58	9[1]	33	58	50	67	8
Glioblastoma multiforme	72	58[3]	43	15	11[1]	75[3]	41	4
High-grade gliomas Σ	104	57[6]	37[2]	16[1]	26[2]	70[4]	44[1]	12[1]

n, Number of cases; EGFR, epidermal growth factor receptor; MERTK, proto-oncogene tyrosine-protein kinase MER; IDH1[R132H], isocitrate dehydrogenase 1 mutation R132H; PDGFRA, platelet derived growth factor receptor α. Superscript indicates the number of cases not assessed.

12% of high-grade gliomas and 4% of GBMs showed PDGFRA-immunoreactive cells (Table 5). In 38% of astrocytic tumours, PDGFRA-immunoreactive cells were seen, as compared with 6% of oligoastrocytomas.

In order to assess the patterns of association, a correlation test was carried out. Only correlations that were high ($r \geq 0.4$) and significant ($P < 0.05$) are shown in Table 6. The highest and most significant positive correlation was noted for EGFR/MERTK in grade II oligodendrogliomas, and the most significant negative correlation was noted for MERTK/IDH1[R132H] in grade III astrocytomas.

An algorithm was used for subtyping the glioma samples on the basis of their protein expression (Figure 4; Table 7). The number of tumours with a pattern reminiscent of the classical and mesenchymal
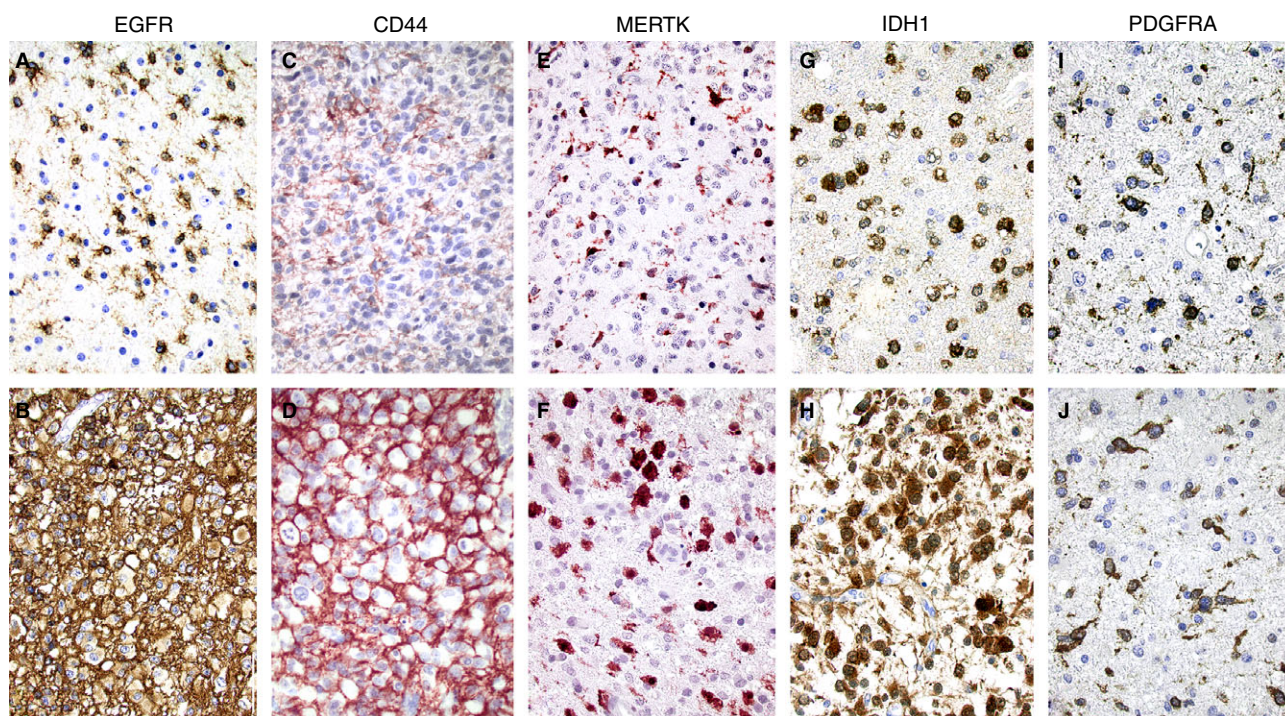
| EGFR | CD44 | MERTK | IDH1 | PDGFRA |

Figure 3. Immunohistochemical detection of epidermal growth factor receptor (EGFR) and c-MER tyrosine kinase (MERTK) in anaplastic oligodendroglioma grade III, CD44 in glioblastoma multiforme grade IV, isocitrate dehydrogenase 1 with R132H mutation (IDH1) in oligodendroglioma grade II, and platelet-derived growth factor receptor α (PDGFRA) in astrocytoma grade II. A staining pattern with moderate immunoreactivity is shown in the upper panel (**A,C,E,G,I**), and a staining pattern with strong labelling is shown in the lower panel (**B,D,F,H,J**). Bar: 50 μm.

molecular subtypes increased with increasing WHO grade. In contrast, 63% of WHO grade II tumours showed a pattern reminiscent of the proneural molecular subtype, and 80% of the tumours that did not show overexpression of one of the analysed proteins were also of WHO grade II.

In order to detect significant differences in protein expression between groups, the non-parametric Kruskal–Wallis rank test was applied. Detailed results are given in Table 8. EGFR, p53 and CD44 IR frequently showed significant differences between subtypes and WHO grades. No significant differences were noted for PDGFRA, and no highly significant differences (<0.005) were noted for microtubule-associated protein 2 (MAP2).

Discussion

In 2006, it was proposed that malignant glial tumours such as GBM should be subtyped on the basis of their protein constituents. Thereafter, in 2010, subtyping of GBM into four defined molecular subgroups (classical, mesenchymal, proneural, and neural) was proposed (Table 9).[14]

Here, applying the IHC technique, we assessed the expression of some of the proteins that have been reported to be of significance for the subtyping of GBMs.[9,13,16] According to our results, different protein-based 'subtypes' can indeed be identified by the application of IHC, independently of the grade or the histological phenotype of the tumour. Our findings indicate that assessment by means of a selective IHC panel should probably be included as part of routine practice in neuropathological diagnostics.

The molecular signature was not compared with the IHC profile in this study, owing to the lack of frozen tissue. Nevertheless, we believe that it was worth carrying out the study in order to assess the usability of archived paraffin-embedded material for the assessment of protein expression with IHC. Moreover, the frequency of cases with a certain protein subtype in our study was well in line with the frequency obtained in assessment of subtypes using molecular techniques.[14]

We did not include any data regarding the clinical outcome, owing to the heterogeneity of the included cases. The operations were performed within a timeframe of 17 years, and included material obtained using various surgical techniques (whole or partial

Table 6. Correlation between protein expressions in different types of glioma

	Spearman's rank order of correlation													
	Astrocytoma (A) grade II		A III		A Σ		Oligodendroglioma grade II		Oligoastrocytoma (OA) grade II		OA III	OA Σ	Glioblastoma multiforme
Proteins	r	P	r	P	r	P	r	P	R	P	r	r	r
EGFR/CD44	NS		NS		NS		0.699	*	NS		NS	NS	NS
EGFR/MERTK	NS		NS		NS		0.883	**	NS		NS	NS	NS
EGFR/IDH1^{R132H}	NS		NS		NS		NS		NS		NS	NS	NS
EGFR/OLIG2	NS		NS		NS		0.702	*	NS		NS	NS	NS
EGFR/P53	NS		NS		NS		0.654	*	NS		NS	NS	NS
EGFR/PDGFRA	NS		NS		NS		0.787	*	NS		NS	NS	NS
CD44/MERTK	NS		NS		0.412	**	0.763	*	0.457	*	NS	NS	NS
CD44/OLIG2	NS		NS		NS		0.730	*	NS		NS	NS	NS
CD44/P53	0.450	**	NS		NS		NS		NS		NS	NS	NS
CD44/PDGFRA	0.417	**	NS		NS		0.651	*	NS		NS	NS	NS
MERTK/IDH1^{R132H}	NS		−0.593	**	NS		NS		NS		NS	NS	NS
MERTK/OLIG2	NS		NS		NS		0.801	**	NS		NS	NS	NS
MERTK/PDGFRA	0.425	**	NS		NS		0.701	*	NS		NS	NS	NS
IDH1/OLIG2	NS		NS		NS		0.594	*	NS		NS	NS	NS
OLIG2/PDGFRA	NS		NS		NS		0.629	*	NS		NS	NS	NS
P53/OLIG2	0.446	**	NS		0.400	**	0.890	**	0.501	*	NS	NS	NS
P53/PDGFRA	0.502	**	0.477	*	0.492	**	0.684	*	NS		NS	NS	NS

NS, Non significant; EGFR, epidermal growth factor receptor; MERTK, proto-oncogene tyrosine-protein kinase MER; IDH1 ^{R132H}, isocitrate dehydrogenase 1 mutation R132H; PDGFRA, platelet derived growth factor receptor α.
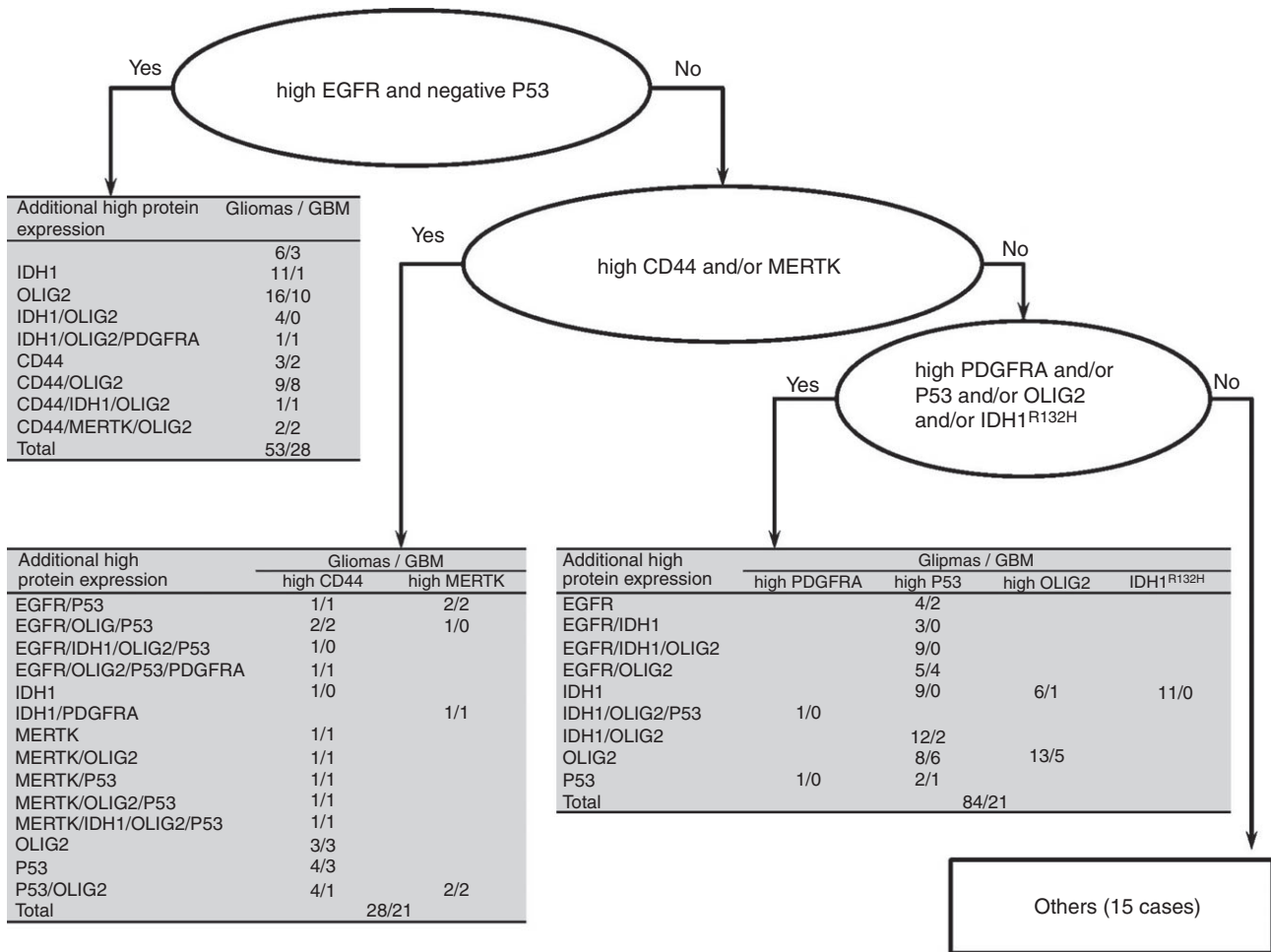
*$P < 0.01$.

**$P < 0.001$.

Figure 4. Algorithm for grouping of the gliomas on the basis of their protein expression, reminiscent of the molecular subtypes defined by Verhaak et al.,[14] (classical = high EGFR and negative p53; mesenchymal = high CD44 and/or MERTK; proneural = high PDGFRA and/or p53 and/or OLIG2 and/or IDH1) and a summary of the high protein expression observed in 180 gliomas.

Table 7. Low and high-grade gliomas and the subtypes defined by protein expression

	WHO grade							
	II		III		IV		Total	
Subtype[14]	n	Age, years (range)	n	Age, years (range)	n	Age, years (range)	n	Age, years (range)
Classical	14	50 ± 4 (31–71)	11	54 ± 2 (34–62)	28	61 ± 2 (35–77)	53	56 ± 2 (31–77)
Proneural	48	37 ± 1 (19–63)	15	48 ± 4 (33–77)	21	55 ± 4 (23–79)	84	43 ± 2 (19–79)
Mesenchymal	2	43 ± 0.5 (42–43)	5	42 ± 4 (32–54)	21	59 ± 3 (22–80)	28	55 ± 3 (22–80)
Other	12	44 ± 4 (25–70)	1	48	2	45 ± 19 (26–64)	15	45 ± 3 (25–70)

Age – mean ± SE.

resections of tumour or diagnostic biopsy); the consequent treatment comprised no treatment, radiotherapy only, different protocols of chemotherapy, or both radiotherapy and chemotherapy. It has previously been reported, regarding meningiomas, that the above issues are of significance when protein expression is

Table 8. Significant correlation of protein expression by means of Kruskall–Wallis rank test in different glioma subtypes and glioma WHO grades

Subtypes[14]	Correlated to	WHO grade	Significant correlations Kruskal–Wallis rank test Proteins assessed									
			EGFR	IDH1^R132H	OLIG2	P53	PDGFRA	CD44	MERTK	GFAP	Ki67	MAP2
Classical high EGFR and negative P53 n = 53	Proneural	II–IV	0.000			0.000					0.006	
	Mesenchymal	IV (GBM)	0.000			0.000						
	Other	III–IV	0.000			0.000						
	n = 98	II	0.000			0.001						
Proneural high IDH1^R132H and/or high OLIG2 and/or high P53 and/or high PDGFRA and/or n = 84	Classical	II–IV	0.000	0.000		0.000		0.000	0.001	*0.041*	0.003	
	Mesenchymal	IV (GBM)	0.003			0.001		0.000	*0.022*		*0.045*	
	Other	III–IV	0.000	*0.008*		0.000		0.000	*0.008*			
	n = 117	II	*0.007*	*0.013*	0.000	0.000				0.005		
Mesenchymal high CD44 and/or high MERTK n = 28	Classical	II–IV				0.000		0.000	0.000	0.008		
	Proneural	IV (GBM)	0.003			0.001		0.000	0.000			*0.009*
	Other	III–IV	0.002			0.000		0.000	0.000			*0.021*
	n = 160	II						0.001			0.008	
Other all low n = 15	Classical	II–IV	0.000	0.001	0.000	0.001		*0.031*		0.000	0.008	
	Proneural	IV (GBM)										
	Mesenchymal	III–IV			*0.037*							
	n = 165	II	*0.007*	0.000	0.001	0.002				0.000		

EGFR, Epidermal growth factor receptor; MERTK, proto-oncogene tyrosine-protein kinase MER; IDH1^R132H, isocitrate dehydrogenase 1 mutation R132H; PDGFRA, platelet derived growth factor receptor α.
Italics signify ranges between $P = 0.05–0.005$.

Table 9. Molecular subtypes of glioblastoma and their definitions as described by Verhaak *et al.*[14]

Subtypes	High expression	Gene loss
Classical	EGFR	CDKN2A, PTEN, TP53
Mesenchymal	*CD44, CHI3L1/YKL40, MERTK, MET, NFκB* pathway, *TNF* family	NF1, PTEN
Proneural	*ASCL1, DCX, DLL3,* IDH1[R132H], *NKX2-2,OLIG2, PDGFRA, SOX, TCF3, TCF4, TP53* mutation	
Neural	*GABRA1, NEFL, SLC12A5, SYT1*	

ASCL1, Achaete-scute homolog 1; CHI3L1/YKL40, chitinase 3-like protein 1; DCX, doublecortin; DLL3, delta-like 3; EGFR, epidermal growth factor receptor; GABRA1, gamma-aminobutyric acid receptor subunit alpha-1; IDH1[R132H], isocitrate dehydrogenase 1 mutation R132H; MET, c-Met proto-oncogene; MERTK, proto-oncogene tyrosine-protein kinase MER; NEFL, neurofilament light polypeptide; NFκB, nuclear factor kappa-light-chain-enhancer of activated B cells; NKX2-2, homeobox protein Nkx-2.2; OLIG2, oligodendrocyte transcription factor 2; PDGFRα, platelet-derived growth factor receptor alpha; SLC12A5, potassium-chlorid transporter member 5; SYT1, synaptotagmin 1; TCF, transcription factor; TNF, tumour necrosis factor; TP53, tumour protein 53.

assessed in relation to outcome.[19] Thus, to assess reliable tumour characteristics (morphology, protein expression, and molecular genetics) in the future, prospective studies should be undertaken. The patients should be asked to provide consent for specific sampling of the tissue in addition to diagnostic material (fresh tissue for cell cultures, and fresh frozen tissue), collection of medical data including information regarding the surgical technique (biopsy, partial resection, and total resection), radiological findings at different stages, and treatment strategy. An autopsy should also be carried out, if possible, to assess the spread of a certain type of tumour. The constructed database should later be completed with information regarding survival. Previous reports have indicated that there is a certain level of discrepancy in diagnoses of tumours when the original and the reassessment results are compared.[20–23] Both down-grading and up-grading of tumours have been reported. In line with previous reports, we also noted a certain level of discrepancy (85% agreement rate) when the archival material included in this study was reassessed. The primary reason for the discrepancy observed is probably the evolution of diagnostic criteria over time, as previously reported.[24] Our findings, however, emphasize that, when studies are undertaken on archived material sampled from one or several centres, a reassessment of the diagnosis should be considered as an obligatory requirement to secure high quality and reliability of the work.

During the last few years, the number of studies applying TMAs in tumour research has increased dramatically. There are several benefits of using the TMA method, specifically: there is the possibility of including a large number of cases; each sample is handled methodologically in a similar way; the limited area to be assessed ensures a certain level of assessment uniformity; and, finally, assessment of thousands of cases is economically feasible. Thus, today, archival tumour tissues are often used for TMA construction. The use of the TMA technique in this study was successful; only 17% of the originally included cases were lost during processing, the tissue in the core samples was considered to be representative of the tumour in the whole section, and there was 100% agreement regarding the WHO grade (whole section versus TMA core).

Our aim was to assess whether gliomas could be subdivided into IHC types on the basis of their protein expression. Fifty-eight per cent of GBMs in this study were EGFR-immunoreactive, a finding that is within the previously reported range (40–73%).[15,25–28] EGFR IHC results have been shown to reflect molecular genetic data; thus, EGFR IHC can certainly be applied.[29–31] In our sample, 29% of all gliomas and 39% of all GBMs showed an IHC pattern (high EGFR expression; low p53 expression) reminiscent of the classical type. The frequency for GBM in our study is somewhat higher than previously reported (27% and 35%).[15] This discrepancy in percentages might be attributable to selection bias. The expression of EGFR was easily demonstrated by means of IHC, and could therefore be routinely assessed. On the basis of our results, this approach should probably be applied in cases not only of high-grade glioma, but also of low-grade glioma. The significance of this subtyping in low-grade gliomas should be further investigated. It is noteworthy that the enrichment of proteins of significance for the proneural subtype of glioma has previously been described for WHO grade II gliomas.[17]

In this study, 16% of all gliomas and 29% of GBMs showed an IHC pattern reminiscent of the mesenchymal molecular subtype. The frequency for GBM in this study is the same as previously reported.[14] It is notable that only a few low-grade gliomas showed this IHC profile, indicating that expression of these proteins might be of significance only in cases of GBM. Verhaak *et al.*[14] defined the proneural subtype of GBM as a tumour with high expression of PDGFRA and/or OLIG2 and/or p53 and/or IDH1[R132H]. This subtype of GBM has previously been reported as representing approximately 20–30% of all GBMs.[14] Interestingly, when IHC was applied, 29% of GBMs showed an IHC pattern reminiscent of the proneural molecular subtype.

In 44% of all gliomas and in 42% of GBMs, high expression of p53 was seen. In primary GBMs, the frequency of p53 positivity has been reported to range from 21 to 53%.[15,32–35] This wide range is probably attributable to both sample selection and the methodology used. In line with p53, the reported frequency of OLIG2 expression in gliomas varies from low to 100%, independently of the detection techniques used. The major pitfall in the reported studies seems to be the low number of cases included (usually <20 GBMs).[36–40] Here, we assessed OLIG2 expression in as many as 180 gliomas (72 GBMs), and high OLIG2 IR was noted in 51% of low-grade and 70% of high-grade tumours; our results for low-grade gliomas are in line with previous reports indicating an enrichment of proneural gene expression in WHO grade II gliomas with oligodendroglial differentiation.[17]

In this study, PDGFRA was expressed by 16% of all gliomas and by 12% of all high-grade gliomas (WHO grade III and GBM), a frequency in line with previously published data.[28] Le Mercier *et al.*[15] reported high PDGFRA expression in as many as 53% of all GBMs, as compared with 4% in our study. In these two studies, different antibody and assessment strategies were applied; thus, these results are not comparable. With regard to assessment strategies, Le Mercier *et al.* used computerized quantitation of labelled cells in a section, whereas we assessed the IR in TMA cores manually. Our findings are theoretically in line with previous reports indicating that *PDGFRA* amplification is not seen as overexpression of PDGFRA protein.[41,42]

The fourth protein assessed here, IDH1[R132H], was present in 44% of all gliomas and in 11% of all GBMs. Thus, as expected, most of our GBMs were negative for IDH1[R132H], indicating that they were primary GBMs.

On the basis of our results, PDGFRA IHC is not reliable, whereas assessment of both p53 IHC and OLIG2 IHC seems to be of interest. Assessment of IDH1[R132H] should probably always be included, in order to differentiate between a primary and a secondary GBM, to support the differentiation between WHO grade III and grade IV gliomas, and to visualize infiltrating neoplastic cells in the preserved brain tissue.[7]

No significant correlations were detected between patterns of protein expression and GBM. Most of the correlations noted were for the oligodendroglial WHO grade II tumours. There were only 12 cases; thus, the influence of selection bias cannot be excluded. It is noteworthy, however, that the expected positive correlations were seen between CD44/MERTK and the mesenchymal subtype, and between several proteins and the proneural subtype (Table 6). Thus, our findings imply that protein expression might be of interest in the low-grade gliomas, whereas it has no informative value in the high-grade tumours.

On the basis of our results, including analysis using the Kruskal–Wallis rank test, the proteins whose expression was most significant were EGFR, p53, CD44, and MERTK. EGFR was by far the most significant for identifying a protein subtype of glioma. The molecular subtypes have so far been implemented for GBM; however, here we noted that the assessment of protein expression was successfully applied for both high-grade and low-grade tumours. The significance of expression of these proteins regarding the fate of the tumour can only be assessed in prospective studies, i.e. comparison of protein expression in original and recurring tumours.

Our results suggest that expression of EGFR, p53, CD44, MERTK and OLIG2 can indeed define protein subtypes of glioma that are reminiscent of the previously defined molecular subtypes (classical, mesenchymal, and proneural). Assessment of expression of these five proteins by means of IHC is more economical and less time-consuming than the molecular approach. Furthermore, assessment of chromosome 1p19q codeletion, O(6)-methylguanine-DNA methyltransferase (MGMT) promoter methylation and serine/threonine-protein kinase B-Raf (BRAF) are certainly recommended for defining a glioma.[43] Thus, current glioma diagnostics should probably include not only the WHO classification, but also the assessment of the above-mentioned proteins in order to stratify patients for specific treatment strategies.

Author contributions

I. Alafuzoff and S. N. Popova: study design, reassessment of all slides, and writing of the manuscript.

Conflicts of Interest

The authors report no conflicts of interest.

Acknowledgements

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