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

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Date of submission 30 April 2013
Accepted for publication 8 August 2013
Published online Article Accepted 13 August 2013

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

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

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 immunohistochemistry (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, oligodendrogial, 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),...
(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 (IDH1R132H).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 levels15,16 (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 p5314–16 oligodendrocyte transcription factor 2 (OLIG2)9,13,14,16 and IDH1 with the R132H mutation14,16 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.11,14,17

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

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**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 |
| CD44 | Mesenchymal GBM | Mesenchymal GBM | Astrocyticmesenchymal, Mixed |
| **Proto-oncogene tyrosine-protein kinase MER** | | | | | |
| **Isocitrate dehydrogenase 1 mutation** | Proneural GBM | PDGF core | Proneural GBM | Differentiated oligodendrocyte type and OPT |
| **OLIG2** | Proneural GBM | PDGF core | Proneural GBM | OPT | Proneural like |
| **P53** | Proneural GBM | OPT | Proneural GBM | OPT | Proneural like |
| **Platelet derived growth factor (PDGF) receptor α** | PDGF core | Proneural GBM | OPT | Proneural like | Proneural like |

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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 retrieved and retrospectively reviewed, blind to the original diagnosis. The type and grading followed the WHO criteria from 2007. 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. 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. 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.

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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

Figure 1. Flowchart summarizing the logistics of the study.
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% 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

| 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    | 1:100      |
| 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      |

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

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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).

Fifty-one 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 \(\alpha\) 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    | 1    | 52  |
| **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.
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/IDH1R132H 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

### 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 at reassessment | Astrocytoma (AC) | Oligodendroglioma (O) | Oligoastrocytoma (OAC) | Anaplastic (a) AC | aO | aOAC | GBM | Σ |
|---------------------------|------------------|---------------------|-----------------------|------------------|----|------|-----|----|
| Astrocytoma (AC)          | 34               | 7                   | 2                     | 10               | 1  | 4    |     | 76 |
| Oligodendroglioma (O)     | 7                | 7                   | 2                     | 9                |    |      |     |    |
| Oligoastrocytoma (OAC)    | 2                | 2                   | 2                     |                  |    |      |     |    |
| Anaplastic (a) AC         | 10               |                     |                       |                  |    |      |     |    |
| aO                        | 7                |                     |                       |                  |    |      |     |    |
| 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 | IDH1R132H | OLIG2 | P53 | PDGFRA |
|------------------|----|------|------|-------|-----------|-------|-----|--------|
| Astrocytoma (AC) | 43 | 30$^6$ | 24$^5$ | 8$^4$ | 56$^2$ | 50$^5$ | 31$^4$ | 34$^5$ |
| Oligodendroglioma (O) | 12 | 38$^4$ | 0$^1$ | 13$^4$ | 78$^3$ | 25$^4$ | 50$^4$ | 14$^5$ |
| Oligoastrocytoma (OAC) | 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 $^3$ | 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$^4$ | 12$^1$ |

n, Number of cases; EGFR, epidermal growth factor receptor; MERTK, proto-oncogene tyrosine-protein kinase MER; IDH1R132H, isocitrate dehydrogenase 1 mutation R132H; PDGFRA, platelet derived growth factor receptor α. Superscript indicates the number of cases not assessed.
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). 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.

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. 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.

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

| Proteins | 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 |
|          | r  | P  | r  | P  | r  | P  | r  | P  | r  | P  | r  | P  |
| 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 | NS | 0.412 ** | 0.763 * | 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.
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

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. (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

| Subtype | WHO grade | III | IV | Total |
|---------|-----------|-----|-----|-------|
| Classical | 14 | 50 ± 4 (31–71) | 11 | 54 ± 2 (34–62) | 28 | 61 ± 2 (35–77) | 53 | 56 ± 2 (31–77) |
| Pronuclear | 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.
| Subtypes \(^{14}\) | Correlated to | WHO grade | 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 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.006 |
| | Mesenchymal | IV (GBM) | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 |
| | Other | III–IV | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 |
| | \(n = 98\) | II | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 |
| 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 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.041 | 0.003 |
| | Mesenchymal | IV (GBM) | 0.003 | 0.001 | 0.000 | 0.000 | 0.022 | 0.000 | 0.000 | 0.000 | 0.045 |
| | Other | III–IV | 0.000 | 0.008 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.008 |
| | \(n = 117\) | II | 0.007 | 0.013 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.005 |
| Mesenchymal high CD44 and/or high MERTK \(n = 28\) | Classical | II–IV | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.008 |
| | Proneural | IV (GBM) | 0.003 | 0.001 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.009 |
| | Other | III–IV | 0.002 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.021 |
| | \(n = 160\) | II | 0.001 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.008 |
| Other all low \(n = 15\) | Classical | II–IV | 0.000 | 0.001 | 0.000 | 0.001 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.031 | 0.000 | 0.008 |
| | Proneural | IV (GBM) | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 |
| | Mesenchymal | III–IV | 0.037 | 0.007 | 0.000 | 0.000 | 0.001 | 0.000 | 0.002 | 0.000 | 0.000 |
| | \(n = 165\) | II | 0.007 | 0.000 | 0.001 | 0.002 | 0.000 | 0.000 | 0.000 | 0.000 | 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 \(\alpha\). Italics signify ranges between \(P = 0.05–0.005\).
assessed in relation to outcome. 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 when the original and the reassessment results are compared. 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. 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%). EGFR IHC results have been shown to reflect molecular genetic data; thus, EGFR IHC can certainly be applied. 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%). 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.

### Table 9. Molecular subtypes of glioblastoma and their definitions as described by Verhaak et al.

| Subtypes         | High expression | Gene loss           |
|------------------|-----------------|---------------------|
| Classical        | EGFR            | CDKN2A, PTEN, TP53  |
| Mesenchymal      | CD44, CHI3L1/YKL40, MERTK, MET, NFKB pathway, TNF family | NF1, PTEN |
| Proneural        | ASCL1, DCX, DLL3, IDH1R132H, NKKX2-2, OLIG2, PDGFRα, SOX, TCF3, TCF4, TP53 | |
| 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; IDH1R132H, 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; NKKX2-2, 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.
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.\textsuperscript{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.\textsuperscript{14} defined the proneural subtype of GBM as a tumour with high expression of PDGFRA and/or OLIG2 and/or p53 and/or IDH1\textsuperscript{R132H}. This subtype of GBM has previously been reported as representing approximately 20–30% of all GBMs.\textsuperscript{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%.\textsuperscript{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).\textsuperscript{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.\textsuperscript{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.\textsuperscript{28} Le Mercier et al.\textsuperscript{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.\textsuperscript{41,42}

The fourth protein assessed here, IDH1\textsuperscript{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\textsuperscript{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\textsuperscript{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.\textsuperscript{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.\textsuperscript{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.
G. Hesselager: neurosurgery. A. Smits, M. Bergqvist, and S. Ekman: selection of the cases from the medical records. I. Alafuzoff, S. N. Popova, L. Sooman, and A. Dimberg: collection of the slides from the archive and assessment of the TMAs. F. Ponten and P.-H. Edqvist: production of the tissue microarrays.

Conflicts of Interest
The authors report no conflicts of interest.

Acknowledgements
We would like to thank the research engineer Inge-Marie Olsson and laboratory technologists Inga Hansson and Ulrika Larsson for their skilful technical assistance, and Meena Strömquist for her critical reading of the manuscript. This study was supported by local ALF grants, and was approved by the local Uppsala ethical committee (Dnr 2002-330, 2005-542-31/1, and 2006/229).

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