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Rīga Stradiņš University

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Immunohistochemical Classification of Diffuse Gliomas

Doctoral Thesis for obtaining a doctoral degree (*Ph.D.*)

Sector – Basic Sciences of Medicine, including Pharmacology

Sub-Sector – Pathology

Riga, 2021

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Anotācija

Glioblastoma (GBM) ir viens no biežākajiem un agresīvākajiem centrālās nervus sistēmas (CNS) audzējiem, kas ir augstākās anaplāzijas pakāpes glioma (4. anaplāzijas pakāpe). Neskatoties uz standartterapiju, kas iekļauj maksimāli iespējamu audzēja rezekciju, kuru papildina ar adjuvantu ķīmijterapiju ar temozolamīdu un radioterapiju, prognoze pacientiem ar GBM ir ļoti slikta. Jaunu potenciālu molekulāru un imūnhistoķīmisku marķieru atklāšanu un pētīšana varētu palīdzēt uzlabot mūsu zināšanas par kādām kritiskām molekulārām izmaiņām, kas veicina audzēja attīstību un šādas zināšanas palielina iespēju izveidot efektīvu uz mērķa molekulu vērstu terapiju nākotnē. Jauni molekulāri un imūnhistoķīmiski prognostiski marķieri var uzlabot ārstēšanu un iespējams arī ļaut sadalīt pacientus vairākās prognostiskās grupās ar atšķirīgu ārstēšanas pieeju, kas padarītu terapiju vairāk personalizētu.

Šī pētījuma **mērķis** ir izvērtēt gliomu- GBM un difūzu astrocitomu (DA), morfoloģisko un imūnhistoķīmisko profilu, kā arī izvērtēt atsevišķus imūnhistoķīmiskus marķierus un iespēju vienkāršoti veikt gliomu subtipēšanu, izmantojot imūnhistoķīmijas (IHĶ) metodi.

Pētījums ir retrospektīvs, kurā tika analizēti formalīnā fiksēti parafīnā ieguldīti ķirurģiski rezecētu gliomu audi. Pētījumā veikta plaša morfoloģiska un imūnhistoķīmiska izvērtēšana, kur kopumā tikai vērtēti 8 imūnhistoķīmiskie parametri, iekļaujot izdzīvotības analīzi. Datu statistiskai apstrādei tika izmantota aprakstošā un analītiskā metode.

Pētījumā tika iekļauti 172 gliomu pacienti, no tiem 146 GBM un 26 DA pacienti. Pētījumā tika konstatēts, ka GBM pacientiem ir slikta prognoze ar mediāno izdzīvotību 7,9 mēneši, kas ir nedaudz zem dzīvildzes rādītājiem citās valstīs. No pētījumā izmantotajiem marķieriem, tikai izocitrātdehidrogenāzes 1 (IDH1) R132H mutācijas klātbūtne ir visnozīmīgākais prognostiskais faktors gliomām. Pacientiem ar DA, augsta trombocītu atbrīvotā augšanas faktora receptora alfa (PDGFRA) ekspresija arī ir saistīta ar labākiem izdzīvotības rādītājiem. Tika konstatēts, ka daži imūnhistoķīmiskie marķieri, tādi kā CD44, p21, PDGFRA un Ki-67 proliferācijas indekss ir parametri, kas atkarīgi no gliomu anaplāzijas pakāpes, tādējādi augstas anaplāzijas pakāpes gliomas, kā GBM, novēroja augstu CD44, Ki-67 un p21 ekspresiju. Savukārt, p27 un PDGFRA ir daudz zemāka GBM, salīdzinot ar DA. Interesanti, bet dažu marķieru ekspresijai (p27, CD44, Ki-67) bija arī dzimumatšķirības. GBMs, šūnas cikla proteīni, tādi kā p53, p21 un p27 ir arī iesaistīti dažādos mehānismos, kas regulē angioģenēzi un šūnu proliferāciju. Cieša saistība ir atklāta starp PDGFRA un p53 ekspresiju gliomās. DA gadījumos, PDGFRA novēroja negatīvu korelāciju ar CD44 un p21 kā arī mikroasinsvadu blīvumu (MVB). Pētījuma noslēgumā pierādīts, ka gliomu subtipēšana ir iespējama ar IHĶ metodi, izmantojot nelielu marķieru skaitu- PDGFRA, p53, IDH1 R132H un

CD44. Ir konstatēts arī tas, ka augsta CD44 ekspresija ir nozīmīgs GBM mezenhimāla subtipa rādītājs, kas ir saistīts ar sliktāku atbildi uz radioterapiju.

Annotation

Glioblastoma (GBM) is one of the most common and aggressive tumours of the central nervous system (CNS) and represents the highest grade (grade IV) of glioma. Despite the standard therapy with maximal possible resection, followed by radiotherapy and chemotherapy with temozolomide, the prognosis for GBM patients remains dismal. Identification and research of potential molecular and immunohistochemical markers will help to increase our awareness of critical molecular changes in tumorigenesis and increase the possibility of effective molecularly targeted therapy in future. New molecular and immunohistochemical prognostic markers can improve the treatment and even allow patients to be stratified into different prognostic groups, which will make the therapy more personalized.

The **aim** of this research was to evaluate the morphological and immunohistochemical profile of gliomas – glioblastoma (GBM) and diffuse astrocytoma (DA) – as well as to evaluate the prognostic role of single immunohistochemical markers and the possibility of glioma subtyping by immunohistochemistry (IHC).

This work was performed as a retrospective study that is based on the analysis of formalin-fixed, paraffin-embedded, surgically treated human glioma tissues. In the study, a comprehensive morphological and immunohistochemical evaluation was performed including analysis of eight immunohistochemical parameters and survival analysis by applying a wide range of descriptive and analytic statistic methods.

A total of 172 patients with gliomas were enrolled in the research work, including 146 GBMs and 26 DAs. It was found that GBM patients were characterized by poor prognosis with a median survival time of 7.9 months, which is slightly below the survival rates reported in other countries. From the selected markers, the presence of isocitrate dehydrogenase 1 (IDH1) R132H mutation was the most significant prognostic factor of better survival in gliomas. However, in patients with DAs, high platelet-derived growth factor receptor alpha (PDGFRA) expression was also associated with significantly better survival. Some immunohistochemical markers, such as CD44, p21, p27, PDGFRA and Ki-67 proliferation, are grade-specific parameters in gliomas, thus CD44, Ki-67 and p21 are significantly upregulated; however, p27 and PDGFRA are downregulated in high-grade gliomas such as GBM. Surprisingly, gender-related differences were found in the expression of some immunohistochemical markers, such as p27, CD44 and Ki-67, in gliomas. In GBMs, cell cycle proteins, such as p53, p21 and p27, are involved in a variety of mechanisms that regulate proliferation and angiogenesis. A strong relationship was also found between the expression of PDGFRA and p53 in gliomas. In DAs, PDGFRA correlated inversely with CD44, p21 and microvascular density (MVD). Finally, it was found that subtyping of gliomas is possible by IHC using a limited number of markers – PDGFRA, p53, IDH1, R132H and CD44. In addition, the expression of CD44 was shown to be

a reliable indicator of mesenchymal subtype in GBM, which has a worse response to radiotherapy.

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List of abbreviations

ALDH1A3	aldehyde dehydrogenase 1A3
CD	cluster of differentiation
CDK	cyclin-dependent kinase
CHK	checkpoint kinase
CI	confidence interval
CK	cytokeratin
CNS	central nervous system
CT	computed tomography
D2HG	D-2-hydroxyglutarate
DA	diffuse astrocytoma
DNA	deoxyribonucleic acid
EGFR	epidermal growth factor receptor
EMT	epithelial-mesenchymal transition
FISH	fluorescent in situ hybridization
G0; G1; G2	Gap 0; Gap 1; Gap 2 (phases of the cell cycle)
GBM	glioblastoma
GFAP	glial fibrillary acidic protein
HE	haematoxylin and eosin
HPF	high-power field
IDH	isocitrate dehydrogenase
IHC	immunohistochemistry
IQR	interquartile range
YKL-40	chitinase-like protein 40
MDM2	mouse double minute 2 homologue
MGMT	methylguanine-DNA methyltransferase
MRI	magnetic resonance imaging
MVD	microvascular density
NA	not applicable
NADPH, NADP	nicotinamide adenine dinucleotide phosphate
NF1	neurofibromin gene
Olig2	oligodendrocyte transcription factor 2
OS	overall survival
PCR-SSCP	polymerase chain reaction-single-strand conformation polymorphism
PDGF	platelet-derived growth factor

PDGFRA	platelet-derived growth factor receptor, alpha
PTEN	phosphatase and tensin homologue
RNA	ribonucleic acid
SD	standard deviation
TCGA	The Cancer Genome Atlas
TMA	tissue microarray
WHO	World Health Organization

Introduction

Glioblastoma (GBM) is one of the most common and aggressive tumours of the central nervous system (CNS) and represents the highest grade (grade IV) of glioma. Despite the standard therapy with maximal possible resection, followed by radiotherapy and chemotherapy with temozolomide, the prognosis for GBM patients remains dismal – with a median survival of 12–15 months (Stupp *et al.*, 2005). Total surgical resection of GBM is not possible because of the extensively infiltrative behaviour of neoplastic cells, and thus the recurrence of the tumour is inevitable (Roy *et al.*, 2015). The potential of chemotherapy and radiotherapy is also limited because of the resistance of tumour cells to standard therapy, and the haematoencephalic barrier is a major obstacle to chemotherapy. Because of the limited treatment options, it is important to identify new molecular and immunohistochemical prognostic markers that can improve the treatment and even stratify patients into different prognostic groups, which will make the therapy more personalized for individual patients. Identification and research of potential molecular and immunohistochemical markers will help in better understanding critical molecular changes in tumorigenesis and increase the possibility of effective molecularly targeted therapy in future.

There are few reliable prognostic markers in GBM: for example, O6-methylguanine-DNA methyltransferase (MGMT) promoter status and *IDH1* gene mutations are currently almost the only available clinically relevant prognostic and predictive markers (Hegi *et al.*, 2005; Kaminska *et al.*, 2019; Kim and Liau, 2012). The most common *IDH1* gene mutation, IDH1 R132H, can also be easily detected by immunohistochemistry (IHC) for IDH1 R132H mutant protein (Thota *et al.*, 2012). The critical proteins (CD44, Ki-67, p53, p21, p27) involved in the hallmarks of the cancer, such as invasion, proliferation and cell cycle regulation, have been studied extensively; however, the data are contradictory regarding the prognostic role of these markers and more researches are necessary in this area (Le Mercier *et al.*, 2012; Popova *et al.*, 2014). There are a lot of discussions regarding the prognostic role of glioma stem cell markers such as CD133 and CD44 (Bhat *et al.*, 2013; Dong *et al.*, 2019; Hermansen *et al.*, 2011; Ortensi *et al.*, 2013). A lot of research is directed towards elucidating the molecular and genetic basis of GBM. Nowadays, new high-throughput molecular techniques have emerged that allow genome sequencing, expression profiling and epigenetic analysis to be carried out. Currently, due to high-throughput genetic studies, emerging data indicate the existence of several molecular subtypes of GBM. Verhaak *et al.* found that GBMs can be divided into four molecular subtypes: classical, mesenchymal, proneural, and neural subtypes characterized by different molecular alterations and gene expression patterns (Verhaak *et al.*, 2010). Each subtype is characterized by certain genetic abnormalities: *IDH1* and *TP53* gene mutations and *PDGFRA* gene amplification in proneural

subtype and that correlated with better prognosis and younger age, however, more aggressive adjuvant treatment did not have an effect in this subtype, *EGFR* gene amplification is frequent in classical subtype, *NF1* gene deletion and expression of genes specific to mesenchymal tissues (*CD44, MET, YLK-40*) characterized mesenchymal subtype, but expression of neuron specific genes (*NELF, GABRA*) typical for neuronal subtype of GBM (Verhaak *et al.*, 2010). Other attempts at molecular subtyping of gliomas have been described and there are some overlapping of results and similarities among molecular subtypes identified across different studies (Brennan *et al.*, 2009; Liang *et al.*, 2005; Phillips *et al.*, 2006; Teo *et al.*, 2019). The unsolved issue is how to implement all these molecular data in routine clinical practice. GBM subtyping is possible and promising in routine practice but molecular data must be transformed into a more simplified and cheaper approach for daily practice and IHC might be a less expensive analogue of comprehensive and time-consuming molecular analysis. There are a few studies related to the utility of IHC in the subtyping of gliomas. For example, Le Mercier *et al*. distinguished proneural-like and classical-like GBM subtypes based on IHC for only three proteins: EGFR, PDGFRA and p53. They confirmed better survival in patients with proneural-like GBMs, whereas patients with classical-like GBMs showed a greater benefit from aggressive treatment (Le Mercier *et al.*, 2012). To evaluate the practical role of IHC in the subtyping of gliomas as well as to draw conclusions on the prognostic or predictive role of separate potential IHC markers, more studies are essential.

The aim of this research was to evaluate the morphological and immunohistochemical profile of gliomas – glioblastoma (GBM) and diffuse astrocytoma (DA) – as well as to evaluate the prognostic role of single immunohistochemical markers and the possibility of glioma subtyping by IHC.

In order to achieve this aim, the following tasks were conducted:

1. Characterizing the morphological structure of gliomas;
2. Assessing angiogenesis by microvascular density (MVD) in GBMs and DAs;
3. Assessing proliferation fraction by Ki-67, as well as the expression of cell cycle regulators (p21, p27), aberrant p53 protein, PDGFRA and CD44 in GBMs and DAs;
4. Assessing the frequency of IDH1 R132H mutation by IHC in GBMs and DAs;
5. Evaluating correlations and associations between any clinical, morphological and immunohistochemical parameters in GBMs and DAs;
6. Assessing survival and factors that have an impact on it in GBMs and DAs;
7. Assessing the possibility of subtyping gliomas through the application of IHC and analysing interrelations with any clinical, morphological and immunohistochemical parameters.

Scientific hypotheses

1. Morphological and immunohistochemical characteristics have a prognostic role in GBMs.
2. Subtyping of GBM is possible through the application of immunohistochemistry.

Scientific novelty

1. In this study, several immunohistochemical markers will be evaluated regarding which contradictory and insufficient scientific evidence exists in the literature.
2. In this study, the hypothesis as to whether IHC-based classification is possible for gliomas will be tested.
3. This work is the first study in Latvia where comprehensive evaluation of GBM morphological and immunohistochemical characteristics and survival was performed.

Personal contribution

The author has conducted all stages of the study, including the preparation of the study design and the selection of IHC markers, as well as evaluating IHC results and performing scientific measurements and statistical data analysis. The author also performed the immunohistochemical visualization and took all the gross and microscopical photographs presented in this work.

Ethical concerns

This research was carried out in accordance with the Declaration of Helsinki and received approval from the Committee of Ethics of Rīga Stradiņš University, No E-9 (2), 12.09.2013.

1. Literature review

1.1. Epidemiology and risk factors

Gliomas are the most common infiltrating primary brain tumours, accounting for more than 80% of all malignant CNS tumours (Ostrom *et al.*, 2013). Malignant tumours of the brain and CNS accounted for about 256,000 new cases and 189,000 deaths globally in 2012. The highest incidence and mortality rates have been reported in more developed regions, such as Europe (age-standardized incidence rate (ASR): 5.5 per 100,.000 people), North America (ASR: 5.3 per 100,000 people) and Australia (ASR: 5.3 per 100,000 people), and the lowest in Africa (0.8 per 100,000 people) and South-Central Asia (ASR: 1.8 per 100,000 people). The incidence of these tumours has increased over the past 30 years because of improvements in diagnostic imaging techniques such as CT and MRI. The low incidence of CNS tumours in developing countries may be explained by underestimation due to the poor availability of diagnostic imaging and/or imprecise assignment of cause of death. However, true ethnic differences cannot be excluded and there is some evidence that gliomas occur more frequently in Caucasians than in people of African and Asian descent (Fan and Pezeshkpour, 1992; Kuratsu *et al.*, 2001; Ostrom *et al.*, 2018).

There were 185 recorded cases of CNS malignant tumours in Latvia in 2017, corresponding to 9.5 patients per 100,000 people (SPKC, 2017b). Thus, CNS malignancy ranks in 21st place among all malignant tumours. However, the proportion of cases histologically confirmed was lower and the age-standardized incidence rate of astrocytomas in Latvia corresponded to 3.0 patients per 100,000 males and 2.6 patients per 100,000 females (Forman *et al.*, 2012). There were 147 recorded cases of death from CNS tumours in Latvia in 2017 (SPKC, 2017a). Reported mortality and incidence rates of brain tumours were very similar in most other geographical areas (Gittleman *et al.*, 2017; Ohgaki and Kleihues, 2005a).

Glioblastoma is the most frequent and most malignant adult glioma, comprising 60–70 % of all glial tumours (Dolecek *et al.*, 2012). GBM is a relatively rare malignancy, but it is one of the most malignant human cancers and has few treatment options and a poor rate of survival. The incidence rate of GBMs is close to three to four cases per 100,000 people in many advanced countries. For example, in England the incidence of GBM was reported to be 4.6 patients per 100,000 people (Brodbelt *et al.*, 2015), and in the United States of America (USA) 3.19 patients per 100,000 people (Thakkar *et al.*, 2014).

Different studies have described the mean age of GBM diagnosis as 55 to 64 years (Bauchet *et al.*, 2010; Hilmani *et al.*, 2013; Oszvald *et al.*, 2012; Thakkar *et al.*, 2014). GBM is very uncommon in children, accounting for only 3% of all reported childhood brain tumours

(Blionas *et al.*, 2018; Ostrom *et al.*, 2013). The median ages of GBM and DA diagnosis have been reported as 64 and 45 years, respectively (Lin *et al.*, 2020; Schwartzbaum *et al.*, 2006). The median age of patients with DAs was 36 years, as reported by authors from the USA (Mayo clinic) (Schomas *et al.*, 2009).

So-called "secondary GBMs", which develop from pre-existing lower-grade gliomas, are characterized by a better prognosis and lower mean age of patients (45 years) (Ohgaki and Kleihues, 2007). In other studies, the mean age of patients with secondary GBMs was close to 40 years (Juratli *et al.*, 2013; Li *et al.*, 2015).

With regard to sex differences for gliomas, they have been reported to occur more frequently in males regardless of age or geographical location (Sun *et al.*, 2015, 2012. The male/female ratio is about 1:3 (Dobes *et al.*, 2011; Kushnir and Tzuk-Shina, 2011). However, others described a female-to-male ratio of about 1:6 (Brodbelt *et al.*, 2015; Dubrow and Darefsky, 2011).

The cause of glioma is unknown but many epidemiological studies have linked gliomas to a variety of potential risk factors, such as genetic susceptibility, environmental carcinogens, ionizing radiation, electromagnetic fields, viral infections, immunological conditions and even head injury; however, many of these associations are inconsistent and vague.

One of the few risk factors with strong evidence of an association with gliomas is ionizing radiation (Braganza *et al.*, 2012; Ostrom *et al.*, 2019). Several studies showed that therapeutic radiation is associated with an increased risk of brain tumours, and in particular, children appear to be more vulnerable to the development of radiation-induced brain tumours, including gliomas (Karlsson *et al.*, 1998; Neglia *et al.*, 1991, 2006; Ostrom *et al.*, 2019; Sadetzki *et al.*, 2005). The possible risk of brain tumour from diagnostic imaging radiation remains unclear and the data are very inconsistent. Thus, Davis *et al.* found an increased risk of gliomas in adults with cumulative exposure to three or more computer tomography scans to the head area only in those individuals with a reported positive family history of malignancy (Davis *et al.*, 2011). Again, the potential risk of brain tumours is higher in children who have received diagnostic CT scans, indicating that harmful exposures such as ionizing radiation can damage the developing brain of a child more easily. Several studies showed a significant association between CT scans and glioma in children (Mathews *et al.*, 2013; Pearce *et al.*, 2012). Studies of atomic bomb survivors in Hiroshima and Nagasaki also revealed an elevated risk of brain tumours in radiation-exposed indviduals; the risk of glioma development was non-significant but the risk appeared to be more elevated for benign brain tumours such as schwannomas (Preston *et al.*, 2007).

There have been many discussions about the possible link between cellular phones and brain tumours. Many studies have not identified any brain tumour risk in relation to long-term

mobile phone use (Deltour *et al.*, 2012; Little *et al.*, 2012; Vienne-Jumeau *et al.*, 2019). In contrast, several studies found some evidence that mobile phone use might be associated with an increased risk of malignant brain tumours (Hardell and Carlberg, 2015).

Evidence of chemically induced gliomagenesis in humans is inconclusive. N-nitroso compounds are some of the few recognized neurocarcinogens, and they are known for their ability to produce gliomas in experimental studies on rats. Repeated intravenous injections of methylnitrosourea into rats were commonly used by some of these experimental approaches to produce glioma models *in vivo* (Barth and Kaur, 2009). Human exposure to these chemical compounds is possible from both exogenous (tobacco smoke, cosmetics, dietary sources) and endogenous (formation in the gastrointestinal tract from nitrogenous precursors) sources. Many epidemiological studies that examined possible associations between human environmental exposure to N-nitroso compounds and the risk of gliomas yielded conflicting results, however the majority did not find any evidence of GBM causation (Kuan *et al.*, 2019; Mandelzweig *et al.*, 2009; Michaud *et al.*, 2009; Saneei *et al.*, 2015).

Furthermore, allergies were inversely associated with glioma risk, thereby supporting the protective role of the human immune system against gliomas. A clinical history of any form of allergy (such as asthma, eczema, hay fever) was associated with a reduced glioma risk (Brenner *et al.*, 2002; Schoemaker *et al.*, 2006; Zhang and Zhu, 2017). An inverse relationship was also found between the total immunoglobulin E level and the risk of glioma in both genders (Calboli *et al.*, 2011; Kaur *et al.*, 2019; Schwartzbaum *et al.*, 2012).

Little is known about genetic factors and susceptibility to gliomas. Only a few cases of gliomas are known to be associated with rare genetic syndromes such as Turcot syndrome (Hamilton *et al.*, 1995), Li-Fraumeni syndrome (Olivier *et al.*, 2003), melanoma-astrocytoma syndrome (Kaufman *et al.*, 1993) and neurofibromatosis type 1 and type 2 (Lobbous *et al.*, 2020; Plotkin *et al.*, 2011; Rodriguez *et al.*, 2008).

Discussions about glioma risk factors are still ongoing, and some recent studies also proposed the role of nutritional and environmental factors, such as increased body weight and vitamin deficiency, as possible risk factors (Bielecka and Markiewicz-Żukowska, 2020). Some recent studies also refer to the role that circulating pro-inflammatory factors may play in glioma pathogenesis (Feng *et al.*, 2019).

1.2. Histological characteristics and WHO classification of glial tumours: from historical perspective to current classification

Gliomas are the most common primary brain tumours that arise within brain parenchyma. The term “glioma” refers to tumours composed of cells showing a close

morphological resemblance to non-neoplastic normal glial cells such as astrocytes, oligodendrocytes and ependymal cells. Although the exact origin of these tumours remains unclear, it has been suggested that gliomas arise from glial and neural progenitor cells that are capable of differentiating into mature phenotypes (Matarredona and Pastor, 2019; Modrek *et al.*, 2014; Prager *et al.*, 2020).

Historically there have been many attempts to classify brain neoplasms and several classification schemes were developed by different authors, such as Bailey (1926), Cushing (1949), Kernohan (1949) and Ringertz (1950), among others. As classification principles developed, initially totally chaotic and fragmented knowledge about brain tumours became more organized and ordered.

The first important classification system for brain tumours was described by Bailey and Cushing in 1926 (MacKenzie, 1926). They proposed that these tumours develop from primary neuroectoderm and their system included 14 different tumour types. They also distinguished astrocytomas and oligodendrogliomas as tumours that develop from astrocytes and oligodendrocytes. Bailey and Cushing's classification was only morphology based and did not include the notion of tumour malignancy grades. Kernohan and colleagues introduced the concept of malignancy grades in 1949; they also suggested that gliomas with different microscopic appearances do not represent isolated tumour types but rather different degrees of malignancy and histological differentiation (Kernohan *et al.*, 1949). According to Kernohan *et al.*, tumours were divided into four grades based on the tumours' histologic features. In addition, Ringertz's classification system suggested that astrocytomas consisted of three grades: astrocytoma, astrocytoma with anaplastic foci and glioblastoma (Ringertz, 1950).

The main system for grading gliomas, known as the St Anne-Mayo grading system (also the Daumas-Duport grading system), was published in 1988 and this is the most popular system for grading diffuse gliomas (Daumas-Duport *et al.*, 1988). It is a four-tiered system that uses the presence or absence of four morphological criteria to assign a grade: nuclear atypia, mitoses, endothelial cell proliferation and necrosis (Daumas-Duport *et al.*, 1988). Depending on the number of the aforementioned criteria, tumours are designated as grades 1 to 4. Thus, tumours that lack any of the above-mentioned features are classified as grade 1 gliomas. Tumours with one of the above-mentioned criteria (usually nuclear atypia) are classified as grade 2 gliomas. Tumours with two of the above-mentioned criteria (usually nuclear atypia and mitoses) are designated as grade 3 gliomas. Finally, tumours with three or four of the above-mentioned features are designated as grade 4 gliomas. The current WHO grading system was based on the same criteria as the St Anne-Mayo grading system, but the WHO scheme includes some modifications of the St Anne-Mayo grading. By definition, therefore, the WHO grading system

is a modified St Anne-Mayo system. The WHO grading system also uses Roman numerals to designate tumour grades (I–IV). Thus, in the following description, Roman numerals will be used. The significant problem with the St Anne-Mayo system was the definition of grade I tumours as tumours lacking any of the four above-mentioned histological features. In reality, such gliomas without any of the above-mentioned criteria are very rare and their existence is doubtful. At least mild nuclear and cellular atypia can be found in all gliomas. Thus, in the WHO system, the grade I category was reserved only for certain circumscribed gliomas such as pilocytic astrocytomas and subependymal giant cell astrocytomas. WHO grade I gliomas are also called "non-infiltrating gliomas" and they are often surgically resectable because of clear demarcation from surrounding brain parenchyma and a lack of infiltrative growth. The term "benign glioma" is sometimes used in the literature in relation to WHO grade I gliomas, but usage of this term is disputable because the anatomic location of gliomas in brain parenchyma hinders their benign course, and in some cases of deep intracerebral localization of a WHO grade I neoplasm, total resection is not possible.

WHO grade II–IV gliomas, which are collectively called "diffuse" or "infiltrative gliomas", are less demarcated with infiltration into the surrounding brain parenchyma without a clear tumour border. Because of their highly infiltrative growth, they are not amenable to total surgical resection. Gliomas of WHO grades I and II are also called "low-grade gliomas" and gliomas of WHO grades III and IV are called "high-grade gliomas".

Thus, the WHO grade directly reflects the tumour malignancy: grade I neoplasms are the least aggressive, while grade IV gliomas are the most aggressive tumours. GBM represents the highest grade (grade IV) of glioma.

WHO grading principles for astrocytomas are summarized in Table 1.1.

Table 1.1.

WHO grading of astrocytomas

WHO grade	WHO type	Histologic criteria
I	Pilocytic astrocytoma; subependymal giant cell astrocytoma	None; certain circumscribed astrocytomas
II	Diffuse astrocytoma	One criterion: usually nuclear pleomorphism
III	Anaplastic astrocytoma	Two criteria: usually nuclear pleomorphism and mitoses
IV	Glioblastoma	Three or four criteria: nuclear pleomorphism, mitoses, vascular proliferation and/or necrosis

In 1979, the World Health Organization (WHO) published the first classification to include all known CNS tumours. The WHO classification was subsequently updated in 1993,

2000, 2007 and 2016. The latest 2016 WHO classification system, in addition to histology, uses molecular parameters to supplement the histological definition.

A part of the 2016 WHO classification of CNS tumours for the definition of diffuse astrocytoma and glioblastoma is shown in Table 1.2.

Table 1.2.

WHO classification of CNS tumours

Entity	Criteria	Reference
Diffuse astrocytoma, grade II	*Histological criteria:* Mildly to moderately increased cellularity, mild to moderate nuclear atypia, fibrillary architecture, no mitosis Absence of microvascular proliferation and necrosis *Molecular parameters:* Presence of IDH1/2 mutations IDH – mutant IDH – wild type	Louis *et al.*, 2016
Glioblastoma, grade IV	*Histological criteria:* High cellularity and/or cellular and nuclear atypia and/or brisk mitotic activity At least one of the following: 1) Unequivocal microvascular proliferation, 2) Necrosis. *Molecular parameters:* Presence of IDH1/2 mutations IDH – mutant IDH – wild type	Louis *et al.*, 2016

1.3. Pathomorphological characteristics of selected CNS tumours

1.3.1. Characteristics of DA (WHO grade II)

The anatomic location of gliomas affects the treatment decisions and prognosis of patients. The most common location for DAs is within cerebral hemispheres, with a predilection for frontal (40–51 % of cases) and temporal (10–30 % of cases) lobes (Capelle *et al.*, 2013; Duffau and Capelle, 2004; Larjavaara *et al.*, 2007, 2011; Tang *et al.*, 2017). Parietal lobes are the location for tumours in 6–23 % of cases (Duffau and Capelle, 2004; Larjavaara *et al.*, 2011; Liouta *et al.*, 2018). Another relatively frequent location for DAs is the insula, in 16–25 % of cases (Capelle *et al.*, 2013; Duffau and Capelle, 2004; Jooma *et al.*, 2019). A much lower frequency of DAs occurs in the occipital lobes (0.5–7 % of cases) (Capelle *et al.*, 2013; Duffau and Capelle, 2004; Larjavaara *et al.*, 2011; Viegas *et al.*, 2011), and unusual sites for DAs are the cerebellum and spinal cord.

DAs are more frequently localized in subcortical white matter, but due to their high infiltrative behaviour, they have a tendency to infiltrate widely subcortical deep grey matter

structures and the cerebral cortex, and through the corpus callosum they can extend to the contralateral hemisphere (Claes *et al.*, 2007; Tunthanathip *et al.*, 2017). Widespread infiltrative growth and a tendency to grow along white matter fibres can result in multifocal gliomas.

The most extreme example of infiltrative glioma growth characterized by the involvement of at least three cerebral lobes and even the entire brain, according to the 2007 WHO classification, is known as "gliomatosis cerebri" (Louis *et al.*, 2007).

Grossly, DAs are ill defined and faintly coloured, thus it is very difficult to identify DAs on gross specimens. A local mass lesion may be present in brain parenchyma but it may have vague, indistinct borders. There may be only mild effacement of white and grey matter and blurring of the grey and white matter interface. In some tumours with extensive cystic change, a focal spongy area can be seen (Louis *et al.*, 2007). In practice, gross descriptions of glioma tissue material in the pathology department are worthless and unnecessary. Essentially, the neuroimaging represents the surrogate of gross pathology for surgical glioma specimens (Perry, 2003). Magnetic resonance imaging (MRI) is now the gold standard for characterization of tumour anatomy. DAs are usually ill defined and hypointense on T1-weighted images and a hyperintense mass on T2-weighted and fluid-attenuated inversion recovery (FLAIR) MRI sequences. The majority of DAs do not show gadolinium enhancement, which tends to appear in high-grade gliomas or indicate malignant progression of previous low-grade gliomas such as DAs (Claes *et al.*, 2007; Louis *et al.*, 2007; Walker *et al.*, 2011).

Microscopically, DAs are composed of neoplastic cell proliferations that bear astrocytic differentiation and are usually interspersed among a network of non-neoplastic neuronal and glial processes. Astrocytic differentiation of cells is determined microscopically by elongated, hyperchromic, slightly irregular, atypical nuclei. The cytoplasm of cells is usually sparse and hardly observable, creating the appearance of naked nuclei (Louis *et al.*, 2007) (Figure 1.1.).

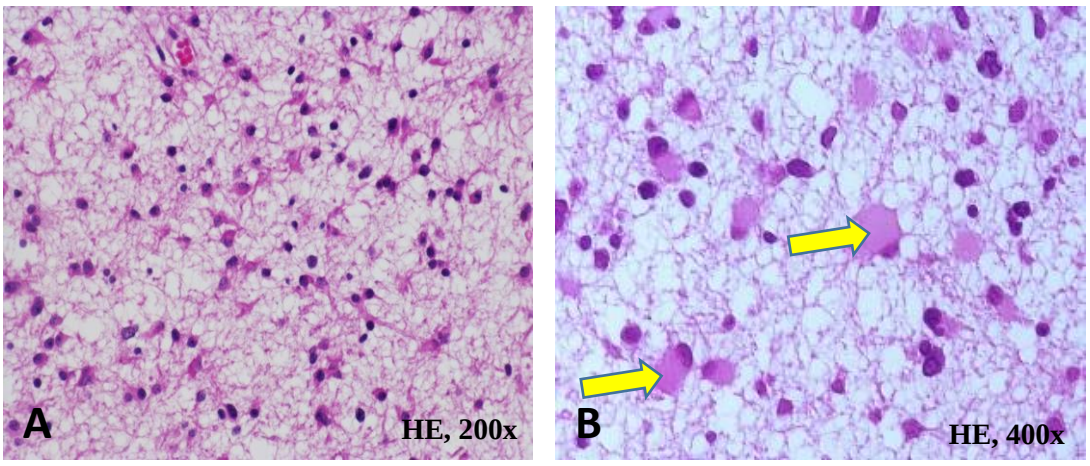


Figure 1.1. **Morphological features of diffuse astrocytoma: A, B. Moderately increased cellularity and fibrillary background of glial cell processes. Some gemistocytic astrocytes can be seen (arrows)**
Haematoxylin and eosin (HE), original magnification 200× (A) and 400×(B).
Microphotographs by A. Jakovlevs.

In comparison to normal brain, DAs show moderately increased cellularity and frequently have loose and microcystic stroma within the fibrillary background of glial cell processes. Microcysts that contain fluid with a mucinous appearance can frequently be found. Mitotic figures are usually absent in DAs (Gupta *et al.*, 2005; Louis *et al.*, 2007). Non-neoplastic, reactive astrocytes called "gemistocytes" may occasionally be identified within neoplastic tissues. Gemistocytes contain enlarged nuclei and stainable, eosinophilic cytoplasm. Some rare forms of DAs that are predominantly composed of gemistocytes are known as "gemistocytic astrocytomas" (Louis *et al.*, 2007).

Sometimes, especially in cases of small biopsies from the infiltrative edge of the tumour where cellularity is low, it could be difficult to distinguish the neoplastic cells from their non-neoplastic glial counterparts. On such occasions, ancillary immunohistochemical studies can be helpful. Currently available antibodies that recognize mutant IDH1 R132H protein can help in making a final distinction from normal glial elements (Camelo-Piragua *et al.*, 2011; Huang *et al.*, 2019; Kloosterhof *et al.*, 2011).

1.3.2. Characteristics of GBM (WHO grade IV)

In general, GBMs develop most frequently in temporal lobes – in 31% of cases – then in parietal and frontal lobes, with 24% and 23% of cases, respectively. Occipital lobes are affected in 16% of cases (Louis *et al.*, 2007). Frequently several lobes are affected by these tumours, and a combined fronto-temporal location is quite common (Louis *et al.*, 2007). Another study carried out on 645 patients with a diagnosis of GBM showed that tumours located in the frontal lobe were the most frequent – with 43% of cases – followed by the temporal lobe, with 28% of cases, and the parietal lobe, with 25% of cases (Simpson *et al.*, 1993). Larjavaara *et al.* showed similar localization patterns for GBMs – the frontal lobe accounted for 40%, the temporal lobe for 29%, the parietal lobe for 14% and the occipital lobe for 3.0% of cases – and even after accounting for tissue volume, the frequency was highest for the frontal lobe (Larjavaara *et al.*, 2007).

GBMs, as the most aggressive infiltrative glioma, have an amazing tendency to infiltrate surrounding brain far away from the main tumorous mass. Tumour infiltration often extends into the adjacent cortex and through the corpus callosum into the contralateral hemisphere. On some occasions, infiltrative spread of GBMs can lead to a bilateral symmetric lesion, usually with the involvement of both frontal lobes, known as "butterfly glioma" (Dziurzynski *et al.*, 2012). Due to the infiltrative spread of neoplastic cells, multiple distantly separated foci of glioma can occur. The incidence of such multifocal gliomas is about 8–15 % (Barnard and

Geddes, 1987; Djalilian *et al.*, 1999; Giannopoulos and Kyritsis, 2010). Some studies found an even higher rate of multifocality in gliomas – up to 24% (Di *et al.*, 2020; Syed *et al.*, 2018).

Grossly, GBM has a heterogeneous appearance showing a variable colour on cut section with greyish tumour areas and more yellowish or reddish areas representing tumour necrosis and haemorrhages. In addition, liquefied necrotic tumorous tissues can form a cystic cavity. Due to this variegated appearance, the term "glioblastoma multiforme" was introduced by Bailey and Cushing in early 1926 (Iacob and Dinca, 2009; Louis *et al.*, 2007).

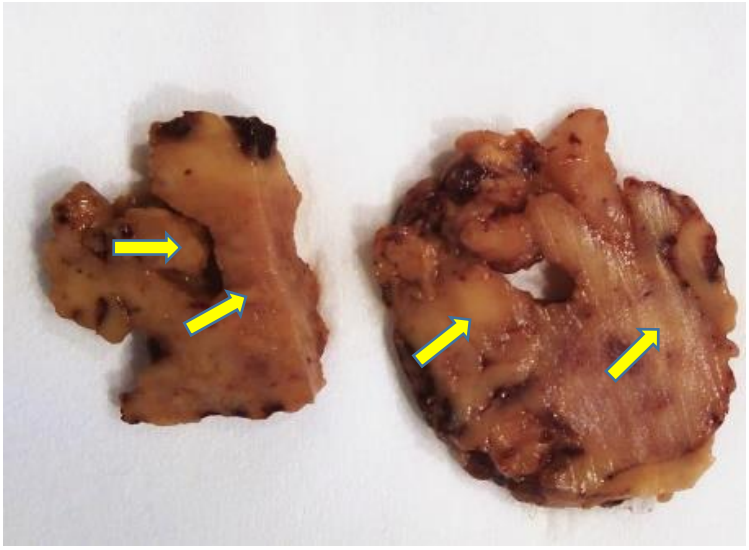


Figure 1.2. **Operation material of brain tissues with infiltrative glioma. Yellowish, poorly demarcated areas of neoplasm can be seen (arrows)**

Photo image by A. Jakovlevs.

On MRI sequences, a specific finding of GBM is an area of contrast enhancement that creates a "ring-enhancing" pattern with a central area of hypointensity, representing necrosis, and a peripheral rim of hyperintensity corresponding to viable tumour tissue with a vasogenic oedema reflecting non-specific changes in blood-brain barrier permeability (Upadhyay and Waldman, 2011).

Microscopically, GBM is an anaplastic, hypercellular glioma composed of pleomorphic tumour cells of astrocytic lineage with marked nuclear and cellular atypia and a high mitotic rate. In addition to anaplastic morphology, either microvascular proliferation or necrosis is a hallmark feature of GBM diagnosis (Louis *et al.*, 2007, 2016). Necrosis is frequently found in the centre of the tumour mass in the form of a large widespread area – an ischaemic type of necrosis. This type of necrosis is not specific to GBM and is frequently found in many other malignant tumours representing ischaemic tissue death or the consequence of irradiation or chemotherapy.

Pseudopalisading necrosis is a unique and specific form of necrosis in GBMs that usually occurs in the form of scattered, serpiginous necrotic foci surrounded by a hypercellular zone of neoplastic cells. Pseudopalisading necrosis is highly characteristic of GBM and represents an event induced by severe hypoxia probably due to intravascular thrombosis and migrating neoplastic cells at the periphery of the hypoxic area (Brat *et al.*, 2004). Thrombosed blood vessels are thought to be causative of pseudopalisades and are frequently seen in GBMs, especially close to necrotic areas or in their centres (Brat *et al.*, 2004; Tehrani *et al.*, 2008).

Another important morphological feature of GBM is microvascular proliferation, which microscopically is recognized by a multilayered, mitotically active endothelium appearance. It was found that microvascular proliferation is a proliferation of a mixed population of cells composed of endothelial cells, pericytes and smooth muscle cells. This is the reason why the former term "endothelial proliferation" was replaced by "microvascular proliferation" (Louis *et al.*, 2007; Wesseling *et al.*, 1995). During the progression of vascular changes, multilayering of endothelium becomes more prominent and finally results in the formation of complex glomeruloid-type vascular structures, so called because of their close resemblance to kidney glomeruli.

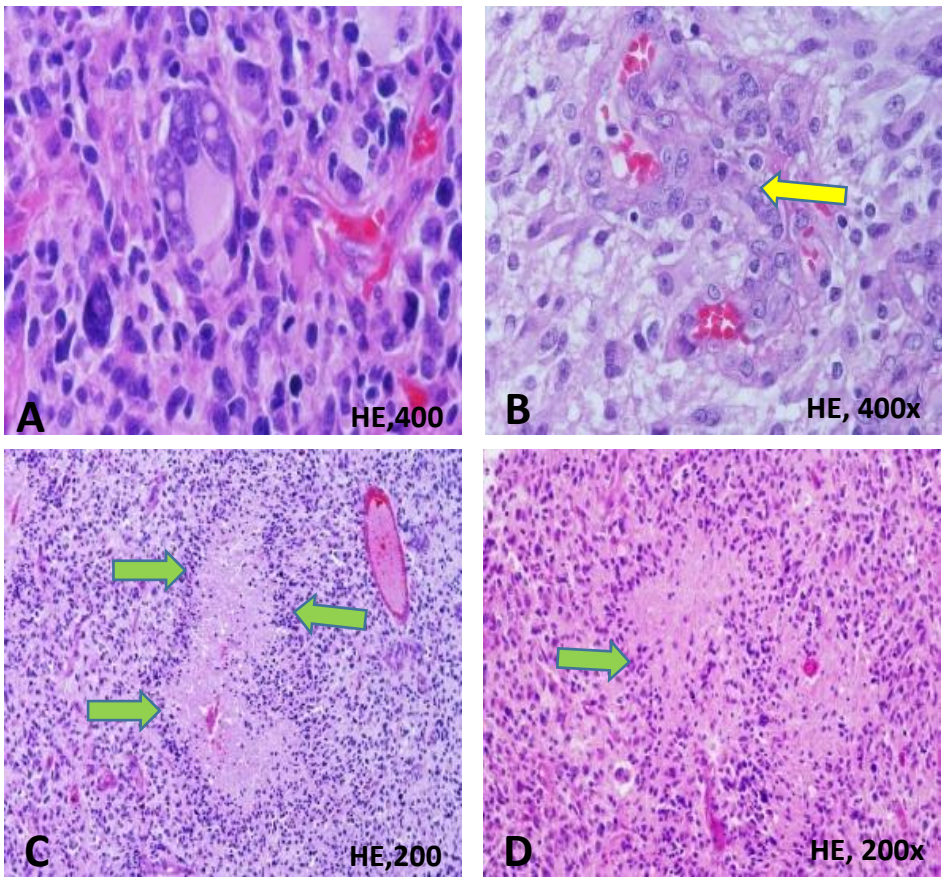


Figure 1.3. **Morphological features of glioblastoma. A: marked nuclear and cellular atypia; B: microvascular proliferation displaying multilayering of endothelium (yellow arrow); C and D: pseudopalisading necrosis. Pseudopalisades are shown by green arrows. Haematoxylin and eosin (HE), original magnification 400x (A, B) and 200x (C, D).** Microphotographs by A. Jakovlevs.

The morphology of GBM can vary from case to case and several morphological variants of glioblastoma have been widely recognized (summarized in Table 1.3.). The so-called "conventional" GBMs constitute the majority of tumours, representing about 93% of all GBMs. Less common non-conventional GBMs include gliosarcoma represented by a biphasic tumour consisting of malignant glial and mesenchymal components, a small-cell glioblastoma featuring small, round, relatively monomorphic cells with only a small amount of cytoplasm and giant-cell GBM with an extensive amount of highly pleomorphic giant cells (Louis *et al.*, 2007). Some very rare morphological subtypes, such as granular cell glioblastoma consisting of large, granular, lysosome-filled tumour cells resembling histiocytes and GBM with an embryonal component, were also recognized in literature (Louis *et al.*, 2007; Schittenhelm and Psaras, 2010; Song *et al.*, 2011; Vizcaino *et al.*, 2019).

Table 1.3.

Morphological subtypes of GBM

Histologic subtype	Frequency	Diagnostic features	Importance	References
Conventional GBM	93%	Moderate to high pleomorphism. Prominent necrosis and microvascular proliferation	The most common type of glioblastoma	(Karsy *et al.*, 2012; Louis *et al.*, 2007)
Giant-cell GBM	1–5 %	Very large, highly pleomorphic cells. Necrosis and microvascular proliferation	Not to be confused with low-grade pleomorphic xanthoastrocytoma. Slightly better prognosis	(Jin *et al.*, 2019; Kozak and Moody, 2009; Louis *et al.*, 2007; Naydenov *et al.*, 2009; Valle-Folgueral *et al.*, 2008)
Gliosarcoma	2–8 %	Biphasic morphology: malignant glial and mesenchymal components. Necrosis and microvascular proliferation	No prognostic role	(Castelli *et al.*, 2016; Kozak *et al.*, 2009; Louis *et al.*, 2007; Meis *et al.*, 1991; Morantz *et al.*, 1976)
Small-cell GBM	Variable	Monomorphic, round cells with little cytoplasm, very high proliferation rate. Necrosis and microvascular proliferation can be poorly developed. In confusing cases, detection of *EGFR* amplification, 10q and 1p/19q deletions is recommended	Anaplastic oligodendroglioma is possible differential diagnosis	(Louis *et al.*, 2007; Perry *et al.*, 2004; Takahashi *et al.*, 2014; Takeuchi *et al.*, 2016; Yadav and Madan, 2020)

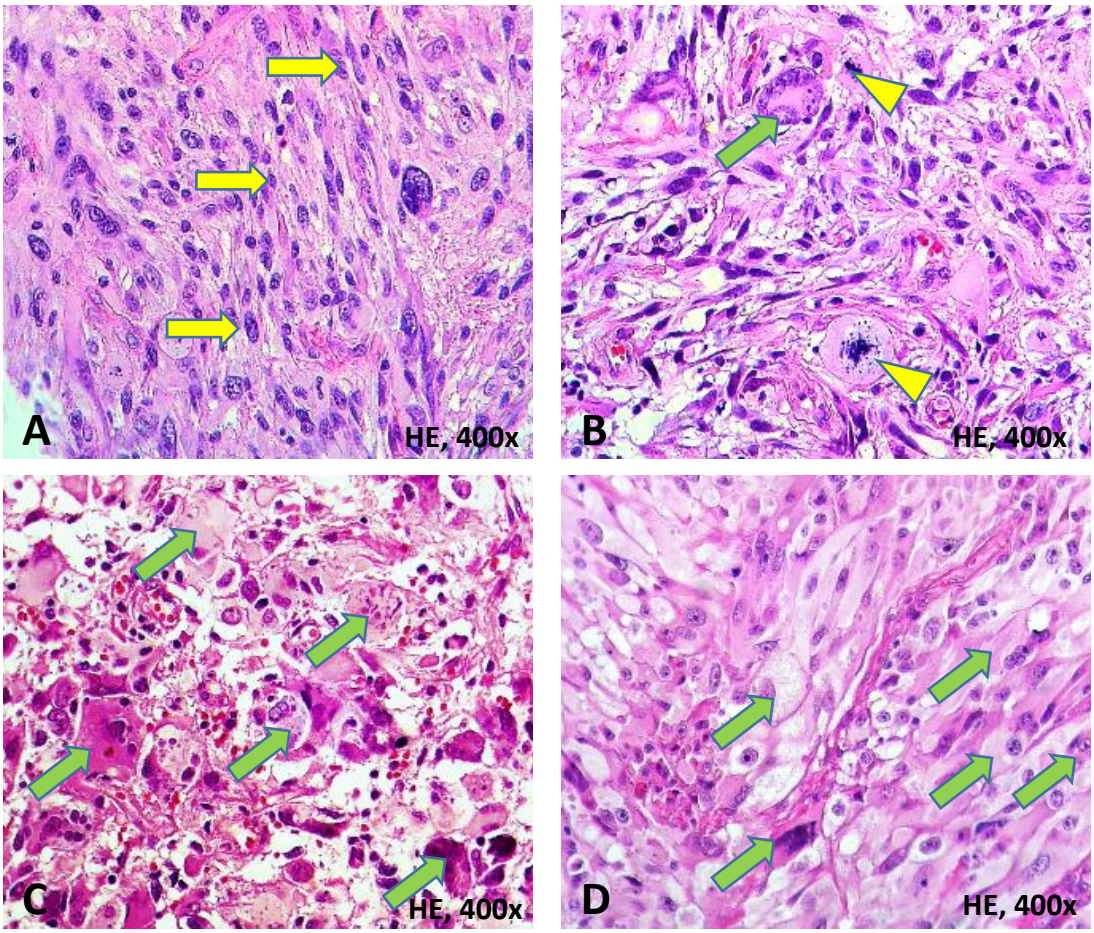


Figure 1.4. **Glioblastoma morphological subtypes. A, B: gliosarcoma. Sarcomatous component in the form of atypical spindle-shaped cells (yellow arrows). Some scattered giant cells (green arrows) and atypical mitotic figures (arrowheads) can be found. C, D: giant-cell glioblastoma composed predominantly of giant, atypical cells (green arrows). Haematoxylin and eosin (HE), original magnification**
Microphotographs by A. Jakovlevs. 400×.

1.4. Molecular subtyping of gliomas

The WHO 2007 and previous classification systems were based on morphological features of glial cells alone to subdivide glial tumours into different categories. Pathological diagnosis is "the gold standard" in determining tumour histological subtype, and the role of classic pathology is still of huge importance. However, classic morphology-based subtyping of gliomas has many limitations. First, it was found that gliomas with identical histological features might have different prognosis, clinical outcomes and responses to treatment. Second, sometimes it could be difficult to place diffuse glioma into one of the WHO entities, because they may share overlapping features on light microscopy, making final diagnosis strictly dependent on subjective interpretation of cellular and nuclear characteristics by a pathologist. Thus, subjectivity, a lack of reproducibility and an imperfect ability to predict clinical outcome limit histological subtyping

and molecular parameters are becoming more crucial for prognostic, predictive and diagnostic reasons. These problems indicate the urgent need to improve the current glioma classification, and classification based on molecular parameters will be more relevant and objective in clinical practice. In the latest WHO 2016 classification, molecular parameters were introduced in addition to previous morphology-based classification alone.

For a long time, two GBM subtypes were known, reflecting different pathogenetic pathways: primary GBM, which develops *de novo*, and secondary GBM, which results from progression from lower-grade glial neoplasm (Kleihues and Ohgaki, 1999). Later molecular investigations have shown so many molecular distinctions between these two subtypes that even different diseases were suspected on the basis of their distinct genetic and clinical profiles (Kleihues and Ohgaki, 1999; Ohgaki and Kleihues, 2007). However, these two subtypes are indistinguishable by classical histopathological examination. A lot of research is directed towards elucidating the molecular and genetic basis of GBM. While investigations of individual gene or protein alterations can provide data on potentially important prognostic markers, new techniques like DNA microarrays allow large numbers of genes and even the expression profile of all tumour genome to be measured simultaneously.

Tumours that possess similar molecular signatures and expression patterns likely share common pathogenesis, reflecting similar therapy responses and prognosis. So tumours can be stratified according to their common molecular signatures in different molecular subtypes among tumours that appear histologically indistinguishable but could carry different clinical outcomes. Five molecular subtypes of breast cancer represent one such successful example where molecular subtyping has now been incorporated into the routine clinical practice. Extensive molecular analysis is highly expensive and time-consuming, so its everyday application is limited. Immunohistochemical tumour profiling is an alternative variant to transfer molecular data into routine practice.

The Cancer Genome Atlas (TCGA) is the most comprehensive cancer research project to date; it began in 2005 and is still in progress. The main aim of TCGA is to understand the molecular basis of human cancer and improve our ability to diagnose and treat malignancy. So far, the TCGA project has produced a huge amount of data, such as whole genomic sequence, expression and epigenetic analysis of more than 30 different types of cancer from over 11,000 patients (Wang *et al.*, 2016c). GBM was the first type of cancer to be studied by TCGA. The data generated by TCGA are publicly available and have been widely used by the scientific community, independent researchers and teams worldwide in their studies. To date, several high-throughput genetic studies in GBM patients have been published and several molecular subtypes have been described.

Verhaak *et al.* in their study used TCGA data and found that GBMs can be divided into four molecular subtypes: classical, mesenchymal, proneural and neural subtypes characterized by different molecular alterations and gene expression patterns (Table 1.4.). The response to treatment also differed according to the molecular subtype. Different response to treatment in these subtypes was not associated with *MGMT* gene methylation status, thus GBM subtype seems to be an independent therapy response predictor. Interestingly, these subtypes also expressed genes associated with certain types of brain cells (Verhaak *et al.*, 2010).

Table 1.4.

Characteristics of the molecular subtypes of glioblastoma as described by Verhaak *et al.*

Subtype	Molecular changes	Clinical correlations	Cell type genetic signature
Proneural subtype	*IDH1*, *TP53* and *PDGFRA* mutations	Better prognosis Younger age Resembles primary GBM No benefit from combined treatment	Oligodendrocytic signature
Classical subtype	*EGFR* amplification Frequent expression of mutant EGFRvIII protein	The maximal benefit from treatment	Astrocytic signature
Neural subtype	Expression of genes that are mainly expressed in normal neurons: *NELF*, *GABRA* and others	Little potential benefit	Astrocytic and oligodendroglial signatures; also normal neuron signature
Mesen-chymal subtype	*NF1* mutations Expression of proteins that are specific for mesenchymal tissues: YKL-40 and MET	Responds to treatment	Cultured astroglial cell and mesenchymal signature

Another large study carried out by Phillips *et al*. classified GBMs into three subtypes: proneural, proliferative and mesenchymal (Phillips *et al.*, 2006). Proneural and mesenchymal subtypes showed a close resemblance to corresponding TCGA subtypes. In contrast, the proliferative subtype was defined by overexpression of markers associated with proliferation. The proliferative subtype was characterized by a significantly higher amount of Ki-67 positive tumour cells than proneural and mesenchymal GBMs. However, mesenchymal GBMs showed a higher Ki-67 index in vasculature than other subtypes, in line with the higher expression of angiogenesis markers such as VEGF (Phillips *et al.*, 2006).

GBM subtyping was also described in some smaller studies prior to the two mentioned above (Liang *et al.*, 2005; Mischel *et al.*, 2003; Nutt *et al.*, 2003). Liang *et al*. separated GBMs into two groups by expression of oligodendroglial markers: OLIG1 and OLIG2 (Liang *et al.*, 2005). Mischel *et al*. divided GBMs into EGFR-overexpressing and EGFR-non-expressing. These two subtypes showed different gene expression patterns (Mischel *et al.*, 2003).

Nutt *et al.* showed that gene expression profiling correlated with survival better than histological classification (Nutt *et al.*, 2003). GBM subtyping is possible and promising in routine practice but molecular data must be transformed into a more simplified and cheaper approach for daily practice. There are few studies concerning this issue. Le Mercier *et al.* distinguished proneural-like and classical-like GBM subtypes based on IHC for only three proteins: EGFR, PDGFRA and p53. They confirmed better survival in patients with proneural-like GBMs, whereas patients with classical-like GBMs showed greater benefit from aggressive treatment. The authors suggested that markers such as NF1, YKL-40 and MET could be used to distinguish mesenchymal-like GBMs (Le Mercier *et al.*, 2012).

Popova *et al.* performed large IHC-based subtyping of gliomas. In their study, they used tissue microarrays prepared from 273 patients with various WHO grade gliomas. All slides were stained with 10 markers, namely CD44, MER, EGFR, GFAP, IDH1 R132H, Ki-67, MAP2, OLIG2, PDGFRA and p53. Most of these markers were selected based on molecular signatures of GBM described by Verhaak *et al.* Researchers were able to subdivide all gliomas into three molecular subtypes just by IHC profiling. Thus, they found that the majority of the low-grade gliomas (63% of grade II) belong to the proneural subtype (high p53 expression). However, the classical subtype with high EGFR and low p53 expression was the most common in GBMs (39%), followed by proneural (29%) and mesenchymal (high CD44 and MERTK) (29%) subtypes (Popova *et al.*, 2014).

A recent study by Orzan *et al.* also provided data indicating that a restricted panel of several immunohistochemical markers can be successfully used in transcriptional profiling of GBM molecular subtypes (proneural, mesenchymal, classical) with high accuracy and with a prognostic role (Orzan and Pagani, 2020). Major studies together with proposed glioma subtypes are summarized in Table 1.5.

The extensive tumour heterogeneity remains a challenging issue in molecular classification of GBM. Several molecular subtypes of GBM can coexist in one tumour, hypothetically due to different subpopulations of tumour cells with distinct responses to therapy (Bergmann *et al.*, 2020; Bernstock *et al.*, 2019; Nicholas *et al.*, 2011).

In one study it was also shown that significant intratumoural heterogeneity in GBMs was associated with worse prognosis, so molecular subtype heterogeneous tumours showed shorter OS compared with tumours with shared homogeneous molecular signatures (Liesche-Starnecker *et al.*, 2020).

Table 1.5.

Proposed molecular subtypes of glioma by different authors

Author	Method	Types	Defining criteria/properties	Significance
Le Mercier *et al.*, 2012	IHC (100 GBM)	Classical-like	EGFR+ p53- PDFRA-	Radiotherapy alone not effective T+R: effective
		Proneural-like	p53+ PDFRA+	Longer overall survival Radiotherapy alone effective
Verhaak *et al.*, 2010	Data by The Cancer Genome Atlas, GBM	Classical	*EGFR* amplification and increased EGFR protein expression. Lack of *TP53*, *NF1*, *PDGRFA*, *IDH1* mutations. *CDKN2A* (*p16INK4A* and *p14ARF*)	Greatest benefit from aggressive treatment
		Proneural	*PDGFRA* amplification or mutation, or *IDH1* mutation *TP53* mutations frequent cell cycle/proliferation signature	Secondary GBMs Younger patients Trend toward longer survival No benefit from aggressive therapy
		Neural	Neuronal genetic markers	Possible treatment benefit
		Mesenchymal	*NF1* deletion or mutation Higher activity of *CD44* and *MERTK* More marked inflammation and necrosis	Benefit from aggressive treatment: decreased lethality
Brennan *et al.*, 2009	Proteomic	3 subtypes	EGFR activation PDGFR activation NF1 loss	
Popova *et al.*, 2014	IHC, TMA, 273 high and low grade	Classical	EGFR high p53 low	
		Proneural	p53 high, OLIG2 high	
		Mesenchymal	CD44 high MERTK high	
Phillips *et al.*, 2006	Gene expression profiling	Proneural	OLIG2	
		Proliferative	EGFR	
		Mesenchymal	EGFR CD44	
Brown *et al.*, 2015	Gene expression profiles 483 patients	Proneural	CD133	Cell cycle genes activated
		Mesenchymal	CD44	Invasion- and migration-related genes
Conroy *et al.*, 2014	IHC, FISH, TMA 123 patients with GBM	Proneural	IDH1 R132H	
		Classical	EGFR	
		Mesenchymal	CD44, VIM, YKL40 (High expression ≥ 2)	

Table 1.5 (end)

Author	Method	Types	Defining criteria/properties	Significance
Motomura *et al.*, 2012	79 patients with GBM. IHC	Differentiated oligodendrocyte	Olig2 + p53 - p16 -	
		Oligodendrocyte precursor	PDGFRA + p16 + p53 + nestin - CD44 -	More favourable outcome
		Astrocytic Mesenchymal	CD44 + PDPN + p16 - p53 -	Worse prognosis
		Mixed type	Various positivity of p16, EGFR, p53, CD44, Hes-1, Olig2, EGFR	Longest median OS

* Abbreviations in the table: DA, diffuse astrocytoma; GBM, glioblastoma; IHC, immunohistochemistry; OS, overall survival; T, temozolomide; R, radiotherapy.

1.5. Immunophenotyping of gliomas. The general features, characteristics and prognostic role of selected immunohistochemical markers

Immunohistochemistry (IHC) is an important component of pathology laboratory testing in the emerging molecular era and is a good surrogate of much more expensive traditional cytogenetic and molecular methods.

The **Ki-67 antigen** was first described by a group of German scientists in 1983 (Gerdes *et al.*, 1983). Ki-67 is an important nuclear non-histone protein that is strongly related to cellular proliferation. Ki-67 was discovered more than 30 years ago, but the exact function of this nuclear protein is still unknown. The recent data suggest that Ki-67 is associated with early steps of ribosomal RNA transcription (Bullwinkel *et al.*, 2006). Rahmanzadeh *et al*. showed that downregulation of Ki-67 causes inhibition of ribosomal RNA synthesis (Rahmanzadeh *et al.*, 2007).

Ki-67 antigen expression is variable during the different cell cycle phases. Ki-67 is found in highest concentration during the late Gap 1 (G1) and early S phases, but it is also present at detectable levels during Gap 2 (G2) as well as mitosis (M) (Scholzen and Gerdes, 2000; Seigneurin and Guillaud, 1991). Ki-67 is not expressed in cells in the Gap 0 (G0) phase or when resting (Scholzen and Gerdes, 2000). This fact makes it an excellent marker for determining proliferating cell fraction, which is defined as the percentage of tumour cells with nuclear staining. The fraction of proliferating tumour cells usually correlates with prognosis, thus Ki-67 is an excellent prognostic marker as well. MIB-1 is highly sensitive and the most frequently used antibody for assessing the Ki-67 labelling index (Lindboe and Torp, 2002).

Because Ki-67 is a marker of proliferation it directly reflects the biological potential of tumours and increasing values of Ki-67 strongly correlate with a higher grade of glioma. However, due to a possible overlap of Ki-67 proliferation indices between different groups of gliomas, Ki-67 cannot be used alone but rather in addition to established WHO histological grading criteria. It can be especially useful in cases with doubtful morphology: for instance, if histology indicates low-grade glioma, but some other features indicate a higher-grade lesion (Johannessen and Torp, 2006).

As regards the diagnostic value of Ki-67, most studies show statistically significant differences in Ki-67 index between high-grade (III–IV) and low-grade (II) astrocytomas. The difference is most significant between DA and AA, as well as DA and GBM, but not between AA and GBM (Duregon *et al.*, 2016; Hsu *et al.*, 1997; Neder *et al.*, 2004; Rodriguez-Pereira *et al.*, 2000; Thotakura *et al.*, 2014). The expression range of Ki-67 in DAs was variable in many studies: 0–18.8 % of neoplastic cells with a median count of cells reaching 5.2% (Hilton *et al.*, 1998; Thotakura *et al.*, 2014; Torp and Alsaker, 2002; Tove *et al.*, 2012).

In regard to GBM, Ki-67 LI is usually elevated with mean values ranging from 12% to 36%. There are a number of studies regarding the prognostic role of Ki-67 in both DAs and GBMs, and the most relevant studies regarding Ki-67's prognostic role are summarized in Table 1.6.

In DAs, Ki-67 correlated with prognosis in most studies with different cut-off levels determined by authors in their studies (McKeever *et al.*, 1998; Schiffer *et al.*, 1997; Torp and Alsaker, 2002). However, in some other studies, Ki-67 did not have an impact on survival (Hilton *et al.*, 1998). In terms of Ki-67 in GBM, the results are more inconclusive, with some studies showing a prognostic role (Ho *et al.*, 2003; Jin *et al.*, 2011; Liang *et al.*, 2020) and others not finding any significant association with prognosis (Moskowitz *et al.*, 2006; Tsidulko *et al.*, 2017; Yang *et al.*, 2013).

Table 1.6.

Ki-67 prognostic role in DAs and GBMs

Authors	**Number of patients with gliomas**	**Main results/ cut-off point**	**Conclusions**
(Schiffer *et al.*, 1997)	DA – 50 patients	Ki-67 (MIB-1) Cut-off point: 8.0	Ki-67 index > 8% was associated with worse survival
(McKeever *et al.*, 1998)	DA – 50 patients	Ki-67 (MIB-1) Cut-off point: 2.0%	Ki-67 index > 2.0% is associated with worse prognosis in DA
(Hilton *et al.*, 1998)	DA – 96 patients (58 of whom received post-operative therapy)	Ki-67 (MIB-1) mean: 1.15 Ki-67 (MIB-1) median: 0.49	Ki-67 index did not correlate with survival in either irradiated or non-irradiated patients with DA

Table 1.6 (end)

Authors	Number of patients with gliomas	Main results/ cut-off point	Conclusions
(Torp and Alsaker, 2002)	DA – 22 patients	Ki-67 (MIB-1) range: 0.1– 9.5 % Ki-67 (median): 2.7% Cut-off point: 2.7%	Ki-67 was associated with worse prognosis in DA
(Tove *et al.*, 2012)	DA – 109 patients	Ki-67 (MIB-1) mean and median: 5.2% and 4.5%; Range: 0.1–16 %; Cut-off point: 4.45%	Ki-67 index did not correlate with prognosis in DA
(Ho *et al.*, 2003)	GBM – 249 patients	Ki-67 (MIB-1) mean: 36%, median: 34%; Cut-off point: 35%	Ki-67 predicts long-term survival in GBM
(Moskowitz *et al.*, 2006)	GBM – 116 patients	Ki-67 (MIB-1) mean: 12.5%; Range 0–76 %; Cut-off point: 12.5% The median OS was 14 months	Ki-67 proliferation rate does not predict patient survival as independent variable; Ki-67 proliferation index did not have an impact on survival rates in GBM
(Jin *et al.*, 2011)	GBM – 156 patients	Ki-67 (MIB-1) Cut-off point: 25%	Ki-67 is an independent prognostic factor in GBM
(Wakimoto *et al.*, 1996)	DA – 19 patients; GBM – 28 patients	Ki-67 (MIB-1) mean: DA – 1.2% and GBM – 12%	Ki-67 index is an independent prognostic factor for patients with all grades of gliomas
(Di *et al.*, 1997)	DA – 29 patients; GBM – 24 patients	Ki-67 (MIB-1) mean: DA – 1.2%; GBM – 12%	Ki-67 index is an independent prognostic factor for patients with all grades of gliomas
(Torp, 2002)	DA – 22 patients; GBM – 9 patients	Ki-67 (MIB-1) median: DA – 2.7%; GBM – 12.1% Cut-off point: 5.2%	Ki-67 index is an independent prognostic factor for patients with all grades of gliomas
(Neder *et al.*, 2004)	DA – 10 patients GBM – 25 patients	Ki-67 (MIB-1) mean: DA – 2.3%; GBM – 12.2% Cut-off point: 2.7%	Ki-67 index is an independent prognostic factor in DAs, but not in GBMs
(Yang *et al.*, 2013)	DA – 464 patients GBM – 200 patients	Ki-67 (MIB-1) Cut-off point: 10%	High Ki-67 expression was associated with worse prognosis in DAs but not in GBMs
(Alkhaibary *et al.*, 2019)	GBM – 44 patients	Ki-67 (MIB-1) Cut-off point: 27%	Ki-67 index did not correlate with prognosis in GBM patients
(Wong *et al.*, 2019)	GBM – 77 patients	Ki-67 (MIB-1) Cut-off point: 22%	High Ki-67 expression was associated with worse prognosis in GBM patients

* Abbreviations in the table: DA, diffuse astrocytoma; GBM, glioblastoma; IHC, immunohistochemistry; OS, overall survival.

* Table was created from PubMed database search results, summarizing main studies for Ki-67 prognostic role in gliomas. Information about methods, Ki-67 status and used cut-offs is also summarized in table.

p53 is a nuclear protein encoded by the *TP53* gene and is the most common mutated gene with about 50% of human cancers having a loss of p53 function (Soussi and Wiman, 2007). p53 protein is usually present in cells at low levels under normal conditions, but the accumulation of p53 is a direct result of DNA damage, hypoxia or oncogene activation. p53 produces a variety of cellular responses but the main role of p53 is maintenance of genome integrity by induction of apoptosis and cell cycle arrest if significant DNA damage occurs. Thus, the p53 protein has been called the "guardian of the genome" (Lane, 1992). p53 exerts its function by transcriptional regulation; it is able to activate or suppress the expression of a variety of genes and hundreds of p53 target genes have been identified (Fischer *et al.*, 2014; Wei *et al.*, 2006).

As p53 is an important cell cycle regulator, its function must be strictly controlled to allow normal cellular functioning. Several mechanisms are involved in the regulation of p53 transcription, protein stability, subcellular localization and DNA binding activity. These mechanisms take control of p53 activity under normal circumstances while resulting in rapid activation of p53 as the response to cellular stress (Lessel *et al.*, 2017; Woods and Vousden, 2001). In normally functioning cells, p53 cellular concentration is kept at low levels; different mechanisms are involved in this regulation, including regulation of the stability of the p53 protein. One of the most important regulators of p53 is the MDM2 protein, which can both inhibit p53 activity and promote intracellular degradation of p53 through the proteasome-dependent pathway (Ryan *et al.*, 2001). Wild-type p53 protein has a very short half-life period; p53 undergoes rapid intracellular degradation, which prevents accumulation of p53, thus its level in normal cells is low.

The MDM2 protein is as an E3 ligase, which can attach ubiquitin residues to the p53 protein, allowing it to be degraded by proteasomes (Woods and Vousden, 2001).

The expression of MDM2 is regulated by p53 in the form of a positive autoregulatory feedback loop between these two proteins. Under cellular stress conditions, MDM2 function is inhibited, which prevents p53 degradation and leads to accumulation of the p53 protein (Ryan *et al.*, 2001). For instance, DNA damage induces expression of checkpoint kinase 1 (Chk1) and checkpoint kinase 2 (Chk2), which have been shown to inhibit interaction between p53 protein and MDM2 (Chehab *et al.*, 2000).

p53 protein function is realized within nucleus, thus different nuclear transport mechanisms exist. It was shown that nuclear import of p53 is dependent on its interaction with microtubules, dynein and g-actin (Saha *et al.*, 2016; Vousden and Woude, 2000).

In addition, the ability of p53 to bind DNA is also under control and can be achieved by modification of p53 molecule, including phosphorylation and acetylation (Gu and Roeder, 1997; Meek, 1999; Yogosawa and Yoshida, 2018).

Finally, p53 activation results in cell cycle arrest or apoptosis that can be mediated by downstream effects of p53 by activation, suppression of p53 target genes and involvement of complex secondary messenger signalling pathways. For instance, the p53 protein can increase the expression of cyclin-dependent kinase inhibitor p21 (WAF1/CIP1), which is an important regulator of G1/S transition (Gartel, 2009).

Due to numerous cellular processes influenced by the p53 protein, detection of p53 in tumour tissues seems to be a promising prognostic and predictive test. IHC is a reliable method for assessing p53 protein expression in tissues in routine practice. Mutations in the TP53 gene lead to overexpression of mutant p53 protein, which is more stable than wild-type p53 and thus accumulates in the nuclei, creating an ideal target for IHC detection. The most sensitive method of assessment of TP53 gene mutations is gene sequencing; however, this is expensive and time-consuming, and is thus limited in pathology practice. Many studies show good correlation between the presence of TP53 mutation and overexpression of p53 protein detected by IHC, therefore IHC can be used as a surrogate for mutational analysis (Hall and Lane, 1994; Newcomb *et al.*, 1998; Simmons *et al.*, 2001; Wang *et al.*, 2014; Yemelyanova *et al.*, 2011). The limitation of IHC is the inability to detect nonsense mutations in the TP53 gene resulting in the loss of p53 expression due to the formation of a stop codon (Simmons *et al.*, 2001). However, TP53 missense mutations that result in accumulation of mutant p53 are more common (Muller and Vousden, 2014).

There is no consensus about the prognostic role of TP53 mutations and p53 protein overexpression in gliomas. The data about the p53 protein are contradictory in both low-grade and high-grade gliomas. Different cut-off points for p53 positive value were used in different studies. The cut-off values used by various investigators ranged from 5% to 50%; however, 5% and 10% cut-offs for positive values were observed more frequently. Many studies failed to identify any prognostic role of p53 overexpression or TP53 gene status in GBMs (Gross *et al.*, 2005; Houillier *et al.*, 2006; Newcomb *et al.*, 1998; Simmons *et al.*, 2001; Takano *et al.*, 2012). However, some authors have found that p53 overexpression predicts better prognosis in GBMs (Fischer *et al.*, 2018; Ohgaki *et al.*, 2004; Schmidt *et al.*, 2002) . There are also contrasting data indicating that the presence of TP53 mutation was a predictor of poor prognosis in GBMs (Wang *et al.*, 2014). Ruano *et al.* found that p53 had a prognostic role only in co-expression with EGFR but not alone, and p53 expression in EGFR expressing GBM was associated with worse outcome (Ruano *et al.*, 2009).

As regards DAs, several researchers did not find any prognostic role of p53 overexpression or *TP53* gene status (Gillet *et al.*, 2014; Hilton *et al.*, 2002; Peraud *et al.*, 2002; Takano *et al.*, 2012). Stander *et al.* found that only positive TP53 mutation status and not p53 protein overexpression was a predictor of worse survival in DAs (Stander *et al.*, 2004). However, Okamoto *et al.* found that the mutated *TP53* gene correlates with poor prognosis in DAs only in univariate analysis. Multivariate analysis found no association between TP53 status and survival of patients with low-grade diffuse glioma, suggesting that after age adjustment, TP53 mutations are not a significant predictive factor (Okamoto *et al.*, 2004). Pardo *et al.* identified p53 positive status as a poor prognosticator in both DAs and GBMs (Pardo *et al.*, 2004).

Interestingly, mutations in the *TP53* gene were found more frequently in the so-called proneural molecular subtype of GBM in 54% of cases (Verhaak *et al.*, 2010).

The structure of the most relevant studies along with patients' group size, methods, p53, *TP53* gene status and major conclusions in brief are presented in Table 1.7.

Table 1.7.

p53 prognostic role in DAs and GBMs

Authors	Patients	Methods and p53 status	Conclusions about p53
(Newcomb *et al.*, 1998)	GBM – 95 patients	IHC for p53; TP53 gene mutations were detected by PCR-SSCP analysis; Four-point scale for p53; Cut-off point for p53: > 5%; p53 positive in 54% of GBMs	p53 overexpression did not correlate with prognosis and age; TP53 gene mutation strongly correlates with p53 immunoreactivity
(Simmons *et al.*, 2001)	GBM – 233 patients	IHC for EGFR, MIB-1 and p53; TP53 gene mutations were detected by PCR-SSCP analysis. Four-point scale for p53 (0–3) Cut-off point for p53: > 5% p53 positive in 42% of GBMs	p53 is not associated with survival TP53 gene mutation strongly correlates with p53 immunoreactivity
(Schmidt *et al.*, 2002)	GBM – 97 patients	TP53 gene mutations were detected by PCR-SSCP analysis; TP53 mutations in 22% of GBMs	Presence of TP53 mutation predicts better prognosis
(Ohgaki *et al.*, 2004)	GBM – 715 patients (677 with primary and 38 with secondary GBMs	TP53 gene mutations were detected by PCR-SSCP analysis; TP53 mutations in 31% of primary and 65% of secondary GBMs.	Presence of TP53 mutation predicts better prognosis (univariate analysis); however, after age adjustment TP53 was not associated with survival
(Gross *et al.*, 2005)	Primary GBM – 31 patients	IHC for p53 and p21; mean for p53 – 7.9%, median – 4.3%, range 0–28 %	p53 is not associated with survival

Authors	Patients	Methods and p53 status	Conclusions about p53
(Houillier *et al.*, 2006)	GBM – 220 patients	IHC for p53; Cut-off point for p53: > 50%; p53 expressed in 37% of cases	p53 is not associated with survival
(Ruano *et al.*, 2009)	GBM – 194 patients	FISH for EGFR, MDM2, CDK4 Direct gene sequencing for TP53 gene mutations IHC for EGFR, p21, CDK6 and p53. Cut-off point for p53: not described Strong p53 immunopositivity in 11.2% of GBMs	p53 is not an independent prognostic factor. p53 expression was significantly associated with poorer survival in the EGFR+ GBM
(Wang *et al.*, 2014)	GBM – 68 patients	TP53 gene were detected by PCR-SSCP analysis; IHC for p53; Cut-off point for p53: > 10%; TP53 mutation and p53 overexpression were identified in 35% and 41% of cases	TP53 gene mutation strongly correlates with p53 immunoreactivity Presence of TP53 gene mutation predicts worse overall survival
(Hilton *et al.*, 2002)	DA – 71 patients	IHC for p53; Cut-off point for p53 positive value: > 10%. Median of p53 positive cells – 10%, range 0–77 %	p53 is not associated with survival
(Peraud *et al.*, 2002)	DA – 159 patients	PCR-SSCP analysis for TP53 IHC for p53; Cut-off point: not mentioned; TP53 mutation in 49%; p53 overexpression by IHC in 47%	TP53 gene status is not associated with survival
(Stander *et al.*, 2004)	DA – 159 patients	TP53 gene were detected by PCR-SSCP analysis; IHC for p53; TP53 mutation in 46% of DA	positive TP53 mutation status (but not p53 overexpression) was found to be an independent unfavourable predictor of survival
(Pardo *et al.*, 2004)	DA – 29 patients; Grade III, IV astrocytomas – 45 patients	IHC for p53; Cut-off point for p53: >25%; High p53 expression: DA-13%; grade III, IV astrocytomas- 71%	High expression of p53 correlated with poor prognosis in both DA and grade III, IV astrocytomas
(Takano *et al.*, 2012)	DA – 42 patients; GBM – 53 patients (41 primary, 11 secondary GBMs)	IHC for MIB-1, mutant IDH1, VEGF and p53 protein; Cut-off point for p53: > 10%; p53 positive in 30% of DAs; 40% and 30% of primary and secondary GBMs	p53 overexpression did not correlate with overall survival in both multivariate and univariate analysis for DAs and GBMs No correlation between IDH1 and p53 status
(Gillet *et al.*, 2014)	61 Grade II gliomas (8 DA, 21 oligoastrocytomas, 32 oligodendrogliomas)	IHC for MIB-1, p16, p53; Cut-off point for p53: > 10%; p53 overexpression in 75% of DA and 34% of oligodendrogliomas	p53 is not associated with survival in grade II gliomas p53 expression is associated with astrocytic differentiation

* Abbreviations in the table: DA, diffuse astrocytoma; GBM, glioblastoma; IHC, immunohistochemistry; PCR-SSCP, polymerase chain reaction-single-strand conformation polymorphism; IDH, isocitrate dehydrogenase

* Table was created from PubMed database search results, summarizing main studies for p53 prognostic role in gliomas. Information about methods, p53 status and used cut-offs is also summarized in table.

In regard to the characteristics of patients and tumours, no correlation was found between the gender and age of patients (Ali and Jalal, 2013; Lin *et al.*, 2015). Stark et al. analysed age-related expression of several markers such as p53, Mdm2, EGFR and MSH2 in GBMs and they found that p53 protein expression was much more frequent in patients of a younger age (Stark *et al.*, 2003). This may be associated with the higher rate of TP53 gene mutations in secondary GBMs, which tends to occur in younger patients (Ohgaki and Kleihues, 2007; Olafson *et al.*, 2020).

Interestingly, p53 did not show any correlation with histopathological grade in a number of studies (Ali and Jalal, 2013; Hu *et al.*, 2013; Nayak *et al.*, 2004).

Some studies have also proposed p53 as a potential molecular target in the treatment of gliomas (Forte *et al.*, 2019; Manfredi, 2020).

p21 and p27 are both cell cycle inhibitors that can bind to cyclin-dependent kinase (CDK) complexes and induce cell cycle arrest (Coqueret, 2003).

The CDK inhibitor **p21** is a small protein composed of only 165 amino acids that belongs to the CIP/Kip family of CDK inhibitors (Karimian *et al.*, 2016). p21 is also known under the names $p21^{Waf1/Cip1}$ and p21/CDKN1A (Abbas and Dutta, 2009).

p21 is able to cause cell cycle arrest in G1/S and G2/M checkpoints by inhibiting CDK4,6/cyclin D and CDK2/cyclin E complexes (Karimian *et al.*, 2016).

Furthermore, p21 is also involved in other important processes such as apoptosis, DNA replication and cellular motility (Abbas and Dutta, 2009; Gartel and Tyner, 2002; Stivala *et al.*, 2012). Specific functions of p21 depend on its subcellular localization. For example, p21 in the nucleus acts as a negative cell cycle regulator, but in cytoplasm, it can inhibit apoptosis and promote cell motility (Cazzalini *et al.*, 2010; Cmielova and Rezacova, 2011).

p21 protein expression can be induced by both p53-dependent and p53-independent mechanisms. As mentioned before, DNA damage causes accumulation of the genome guardian protein p53, which can arrest the cell cycle. Thus, the cell cycle inhibitory effect of p53 is realized indirectly through induction of p21 expression by p53 (Benson *et al.*, 2014; Eckner, 2012).

p21 protein also has crucial functions during mitosis and is important in the termination of mitosis. It has been shown that loss of p21 causes overduplications of centrioles and aberrant centrosome numbers that lead to chromosomal instability (Duensing *et al.*, 2006). Loss of p21 has also been shown to cause prolonged mitosis and mitotic defects (Kreis *et al.*, 2015).

p21 is also involved in the control of stem cell self-renewal and maintenance of their quiescent state. Several studies indicate that p21 can limit the proliferation of basal

keratinocytes, hematopoietic stem cells and neural stem cells (Cheng *et al.*, 2000; Kippin *et al.*, 2005; Pechnick *et al.*, 2008; Topley *et al.*, 1999).

For example, the loss of p21 inhibitory effect on the neural stem cell niche may cause exhaustion of the stem cell population due to rapid expansion and proliferation of stem cells and terminal differentiation of multipotent neural stem cells into mature astrocytes (Marques-Torrejon *et al.*, 2013; Porlan *et al.*, 2013). The influence of p21 on stem cells is thought to be indirect through the regulation of expression of several pluripotency factor genes, such as SOX2 (Marques-Torrejon *et al.*, 2013).

A large number of studies have shown that p21 may have a dual role, and together with its tumour suppressor effect it may also act as an oncoprotein because of its antiapoptotic properties (De la Cueva *et al.*, 2006; Gartel, 2006; Parveen *et al.*, 2016). In some other studies it was shown that p21 may also induce apoptosis through p53-dependent and p53-independent pathways (Gartel, 2005; Kreis *et al.*, 2019).

Several lines of evidence suggest that the cytoplasmic location of p21 may promote cell motility (Kreis *et al.*, 2019; Lee and Helfman, 2004). High levels of cytoplasmic p21 in tumour cells enhance their motility, thus contributing to increased invasion and metastasis formation (Abbas and Dutta, 2009; Lee and Helfman, 2004).

p21 plays multiple roles in cell cycle regulation and can show contrasting effects, being at the same time a tumour suppressor and an oncoprotein. There are many additional roles of p21 that are poorly understood, and further studies are essential to better understand this protein that plays a dual role in oncogenesis.

Expression of p21 has been extensively studied in many human malignancies; however, there are a limited number of studies regarding p21 in gliomas and its prognostic relevance.

In one study by Kirla *et al.*, the expression of p21 and p27 proteins was evaluated by IHC in 77 patients with malignant astrocytomas, including 25 AAs and 52 GBMs. These tumours were grouped in three categories: < 30% positive cells, 30–50 % positive cells and > 50% positive cells. The mean expression of p21 in GBM was found to be 19.8%. No association between the expression of p21 and survival was found in patients with high-grade gliomas. However, p27 in this study was found to be a powerful predictor of survival in high-grade gliomas ($p = 0.007$) (Kirla *et al.*, 2003).

In one recent study published in 2016 by Trabelsi *el al.*, molecular and immunohistochemical profiling of 110 different gliomas (including 60 GBMs) were carried out together with survival analysis. In this study, p21 was found to have no effect on overall survival. In contrast, an association between free relapse survival and p21 status was identified.

Only tumours with more than 20% of immunopositivity were considered positive for statistical analysis (Trabelsi *et al.*, 2016).

Zolota *et al.* analysed the expression of several cell cycle markers (p21, p27, p14 and p16) in biopsies from 67 patients with astrocytomas, including 16 low-grade and 51 high-grade tumours. Specimens containing more than 10% of stained cells were considered to be p21 positive. Expression of p21 was found to be more frequent in high-grade than low-grade gliomas: 41.1% versus 12.5% ($p = 0.031$). No associations were found between p21 status and survival (Zolota *et al.*, 2008).

However, in a different study, p21 expression was found to be an important indicator of shorter disease-free survival. Nevertheless, gliomas expressing both p21 and p53 proteins showed an association with short overall survival (Korkolopoulou *et al.*, 1998).

In has also been suggested by others that upregulation of p21 in gliomas may lead to increased cell cycle progression and play a part in maintaining a hyperproliferative state in those cells (Besson and Yong, 2000; Morris-Hanon *et al.*, 2017).

p27 is another cell cycle inhibitor and belongs to the same CIP/Kip family of CDK inhibitors as p21 protein (Moller, 2000). In contrast to other tumour suppressors, p27 is rarely mutated in human cancer but it is frequently deregulated, showing reduced levels or being dislocated (Slingerland and Pagano, 2000). Levels of p27 are high in normal and quiescent cells but decline in a proliferative state. For example, normal proliferating basal cells of epidermis and colorectal crypt cells showed decreased p27 as compared with terminally differentiated less proliferative cells (Jordan *et al.*, 1998).

Progressive loss of p27 has been described in different types of neoplasia, especially during progression from benign preneoplastic conditions to invasive cancers (Catzavelos *et al.*, 1997; Korkolopoulou *et al.*, 2002b).

The activity and binding capacity of p27 is regulated by many post-translation modifications of the protein structure, such as phosphorylation and ubiquitination (Abbastabar *et al.*, 2018; Lee and Kim, 2009). p27 is strictly regulated with phosphorylation by different oncogenic tyrosine kinases on multiple binding sites, producing different responses such as inhibiting the activity of CDK or leading to intracellular degradation of p27 and cellular proliferation (Gesbert *et al.*, 2000; Grimmler *et al.*, 2007; Jakel *et al.*, 2011).

In addition to the cell cycle regulatory potential of p27, its cytoplasmic localization may influence the structure of actin cytoskeleton and promote cell motility (Besson *et al.*, 2004). Some malignant metastatic cancers may show an increased amount of cytoplasmic p27, supporting its oncogenic role in that localization (Denicourt *et al.*, 2007; Li *et al.*, 2006; Wang *et al.*, 2008).

In scientific literature, there are many discussions about the role of the p27 protein and its prognostic significance. To the best of our knowledge, loss of expression of nuclear p27 has correlated with poor prognosis in many human cancers (Chetty, 2003; Juuti *et al.*, 2003; Kenney *et al.*, 2013; Kim *et al.*, 2014; Shamma *et al.*, 2000; Zhang and Sun, 2001). Interestingly, several studies have shown that cytoplasmic location of p27 is associated with poor prognosis in different cancers (Chen *et al.*, 2011; Currier *et al.*, 2019; Duncan *et al.*, 2010; Kruck *et al.*, 2012).

In regard to p27 expression in gliomas, many controversies exist and there are few studies that describe the prognostic role of p27 in gliomas.

He *et al.* evaluated 160 high-grade gliomas and 32 low-grade gliomas, including 132 GBMs and 19 DAs. Using 5% as a cut-off, p27 protein was observed in the nucleus in 52 cases (27.1%) of glioma, and cytoplasmic expression was found in 126 cases (65.6%) of all gliomas. In GBMs, cytoplasmic p27 expression was found in 81.8%. Only cytoplasmic location of p27 was found to be associated with poorer prognosis ($p = 0.007$) in GBMs (He *et al.*, 2012).

In another study by Yang *et al*., 96 gliomas were evaluated using the IHC approach, including 45 GBMs and 16 DAs. High cytoplasmic and nuclear staining were identified in 77.7% and 46.6% of GBMs, as well as in 25.0% and 81.2% of DAs, respectively. The cut-off value for high p27 expression was 70%, and low expression was considered if less than 70% of the cells were stained. Thus, expression of p27 and its nuclear or cytoplasmic location were dependent on the WHO grade of gliomas. Therefore, cytoplasmic location of p27 is more frequent in high-grade gliomas and was to be associated with worse prognosis in gliomas ($p = 0.009$), while increased nuclear p27 expression showed some tendency towards better prognosis ($p = 0.06$). (Yang *et al.*, 2011).

A group of Japanese researchers evaluated the expression of p21, EGFR and p27 in 59 high-grade gliomas. Low expression of p21 and p27 was observed in 84.8% and 45.8% of tumour samples. In this study, low expression of p27 was associated with poor prognosis (Nabika *et al.*, 2010).

Piva *et al.* reported decreased p27 expression in high-grade gliomas and attributed these reduced levels of p27 to increased degradation in a proteasome-dependent pathway (Piva *et al.*, 1999). Many other studies supported the role of proteosomal degradation of p27 as a major mechanism that leads to loss of p27 in many human cancers (Loda *et al.*, 1997; Lu and Hunter, 2010; Mishra *et al.*, 2009). However, p27 protein concentration can also be regulated at the level of transcription and translation (Hengst and Reed, 1996; Servant *et al.*, 2000).

Interestingly, p27 as a potential therapeutic target in gliomas has also been discussed. For example, Chen *et al.* stated that transfection of native, functional p27 genes into human astrocytoma cells diminished the malignant potential of infected cells (Chen *et al.*, 1996).

However, Park *et al*. described the possible application of p27 gene therapy in gliomas (Park *et al.*, 2004).

CD44 is a transmembrane glycoprotein that serves as a major surface hyaluronic acid receptor and is involved in cell matrix adhesion, cell migration and various cellular signalling pathways (Dzwonek and Wilczynski, 2015; Naor *et al.*, 1997). The extracellular domain of CD44 molecule can bind different ligands such as hyaluronan, proteoglycans, cytokines and growth factors. However, the intracellular portion of CD44 molecule is involved in different signal transduction pathways (Dzwonek and Wilczynski, 2015). In addition, CD44 is one of the most structurally variable surface molecules with many CD44 variants or isoforms (CD44v) that exist due to extensive alternative splicing. The most frequent form of CD44 is standard CD44 (CD44s), which is expressed ubiquitously, and expression of splice variants is strictly limited to certain types of normal or neoplastic cells (Afify *et al.*, 2007). In addition to different isoforms, the CD44 molecule can undergo extensive post-translational modification, contributing to significant structural diversity of CD44 (Naor *et al.*, 2002).

One of the principal functions of CD44 is cellular adhesion mediated via binding with hyaluronan. The binding capacity with hyaluronan is different for distinct isoforms of CD44. In addition, CD44 can be involved in degradation of hyaluronan (Sneath and Mangham, 1998).

In terms of the nervous system, CD44 has been identified as a marker of neural stem cells as well as astrocyte and oligodendrocyte precursors (Liu *et al.*, 2004; Naruse *et al.*, 2013). The CD44 molecule may play a role in developing brain and in differentiating neural stem cells into different mature phenotypes. For example, it was shown that elevated CD44 expression in glial precursor increases differentiation toward astrocytic lineage but inhibits differentiation into oligodendrocytes (Liu *et al.*, 2004).

The significance of CD44 in neoplastic processes is disputable. Many studies have shown that high expression of CD44 and its isoforms may be associated with poor prognosis in different cancers (Chai *et al.*, 2014; Gunthert *et al.*, 1995; Yamamichi *et al.*, 1998). In addition, there are contradictory observations that unregulated CD44 can even be associated with favourable prognosis or does not have any prognostic role in malignant processes (Naor *et al.*, 2002).

Although CD44 is well known as a molecule involved in adhesion and thus implicated in the invasiveness of cancer, it is also known as a tumour stem cell marker for different malignancies, including GBM (Al-Hajj *et al.*, 2003; Bradshaw *et al.*, 2016; Dalerba *et al.*, 2007; Jijiwa *et al.*, 2011; Sahlberg *et al.*, 2014; Zoller, 2011).

GBMs enriched with stem cell signature (CD44+/CD133+) were found to be more sensitive to chemotherapy with temozolomide (Brown *et al.*, 2015).

A unique CD44 expression pattern associated with possible localization of stem cells was identified by Katz *et al.*, who stated that CD44 is expressed at much higher levels in the perivascular area, which is recognized as a possible stem cell niche (Katz *et al.*, 2012).

Interestingly, CD44 was described as a marker of mesenchymal differentiation and the so-called "mesenchymal molecular subtype" of GBM is characterized by high transcription levels of several mesenchymal markers, such as YLK-40, MET and also CD44 (Brown *et al.*, 2015; Koev *et al.*, 2014; Phillips *et al.*, 2006; Verhaak *et al.*, 2010).

In respect of CD44 expression in gliomas, it is more prominent in high-grade gliomas than in low-grade gliomas and normal brain (Kuppner *et al.*, 1992; Ranuncolo *et al.*, 2002; Yoshida *et al.*, 2012). Ranuncolo *et al.* found high expression of CD44 (more than 70% of tumour cells) in 59% of GBMs and only in 9.5% of low-grade gliomas (Ranuncolo *et al.*, 2002).

However, others found no association between the grade of glioma and CD44 expression (Ylagan and Quinn, 1997). The expression of CD44 is more intense and predominant in glioma cells, but not in normal brain tissues (Yoshida *et al.*, 2012).

In terms of the prognostic role of CD44 in gliomas, more evidence suggests that high CD44 is associated with worse prognosis in gliomas (Anido *et al.*, 2010; Dong *et al.*, 2019; Motomura *et al.*, 2012; Pietras *et al.*, 2014; Ranuncolo *et al.*, 2002; Si *et al.*, 2020; Xu *et al.*, 2010).

Other researchers found that the CD44 expression pattern correlated with poor prognosis only in GBMs with a proneural gene expression signature (Pietras *et al.*, 2014). Some recent studies have also identified CD44 as a possible molecular target for the treatment of GBM by CD44 inhibitors (Chen *et al.*, 2019; Wang *et al.*, 2020).

Thus, CD44 seems to be an important prognostic indicator and new studies are important to evaluate its biological role. In several recent studies, CD44 is also discussed as a potential therapeutic target in gliomas (Xu *et al.*, 2020).

Platelet-derived growth factors **(PDGFs)** are a family of growth factors involved in numerous biological functions, such as cell growth, proliferation and angiogenesis. Five main isoforms exist: PDGF-A, PDGF-B, PDGF-C, PDGF-D and AB heterodimer. All PDGFs bind with two tyrosine kinase receptors called "PDGF-α" (**PDGFRA**) and "β-receptors" (PDGFRB), inducing kinase activation and complex downstream signalling, including Ras-MAPK, PI3K and STAT pathways (Katz *et al.*, 2007; Ostman and Heldin, 2007). PDGFRs are linked to the Ras-MAPK pathway via effector proteins such as Shc and Grb2, which bind the activated PDGFR tail and then activate Ras leading to downstream activation of the MAPK cascade. MAPK then activates gene expression, producing numerous cellular responses such as cell growth, division, differentiation and migration (Dunn *et al.*, 2005; Seger and Krebs, 1995) .

In normal conditions, PDGF isoforms are involved in a wide range of functions in many different types of tissue. PDGFs have a huge role during embryogenesis. Thus, PRGFRA signalling is crucial in the development of the facial skeleton, testes, kidneys, lungs, hair follicles and glial cells (Andrae *et al.*, 2008; Soriano, 1997). Interestingly, PDGFRA signalling is very important for the development of mature oligodendrocytes from oligodendrocyte precursors and neural stem cells. PDGFRA signalling directs the differentiation of stem cells toward oligodendrocytes and not any other lineage (Zheng *et al.*, 2018; Zhu *et al.*, 2014).

PDGFR mutations in tumours are relatively rare events, however in some malignancies mutations such as amplification, deletion, translocation or fusion may occur and may play a role in the pathogenesis of some neoplasms. For example, in the skin tumour dermatofibrosarcoma protuberans a specific translocation between gene encoding collagen 1A1 and the PDGFB gene occurs, which leads to the formation of specific fusion protein (Nakamura *et al.*, 2015). About 5–7 % of gastrointestinal stromal tumours (GISTs) are caused by mutational activation of PDGFRA-associated overexpression of this receptor (Lasota and Miettinen, 2006; Rossi *et al.*, 2005). PDGF receptor gene rearrangements are found in certain leukaemias (Toffalini and Demoulin, 2010). PDGFRA signalling is important in high-grade gliomas, especially GBM. PDGFRA gene amplifications resulting in overexpression of receptors were found in 5–10 % of GBMs (Fleming *et al.*, 1992; Puputti *et al.*, 2006).

In large gene profiling studies, PDGFRA amplifications were found in 15% of all gliomas, and the proneural GBM subtype is enriched with PDGFRA signature; this also has better prognosis (Phillips *et al.*, 2006; Verhaak *et al.*, 2010).

About 11% of GBMs were found to have PDGFRA gene amplification and thus this is the second-most common receptor tyrosine kinase gene amplified in GBMs after EGFR (Ozawa *et al.*, 2010).

PDGFRA expression was more frequently found in low-grade gliomas than in high-grade gliomas in many studies (Chen *et al.*, 2013; Popova *et al.*, 2014).

In many studies, the IHC approach was used to detect PDGFRA expression; however, there are many discussions about the regulation of PDGFRA expression. In addressing the causes of PDGFRA, an interesting study was published on expression in gliomas by Chen *et al.* where they used microarray gene analysis and FISH to find correlations. They found no association between the presence of PDGFRA gene mutation and PDGFRA expression ($p > 0.,05$). This was unusual in contrast to the very strong association between EGFR gain and EGFR expression in the same glioma group. Thus, the authors hypothesized that PDGFRA expression in gliomas is not dependent on genetical abnormalities, but is niche factor dependent. To test this hypothesis, the authors used glioma cell *in vitro* cultures enriched with

different growth factors. PDGFRA expression was shown to be strongly dependent on the presence of fibroblast growth factor 2 (FGF2) in culture medium (Chen *et al.*, 2013).

Another study supported the fact that PDGFRA expression did not correlate with genetic findings such as PDGFRA gene amplification (Szerlip *et al.*, 2012). Thus, IHC detection of PDGFR expression is an independent test, which did not indicate the presence of PDGFRA gene mutation and is possibly associated with microenvironment in gliomas.

With regard to PDGFRA and patients' clinical data, no correlation was found between PDGFRA expression and patient age or gender (Martinho *et al.*, 2009).

The clinical impact of PDGFRA in gliomas is debated in several studies but there is no consensus. As discussed before, increased expression of PDGFRA has been reported in proneural GBMs that have better prognosis (Ko *et al.*, 2020; Phillips *et al.*, 2006; Verhaak *et al.*, 2010). However, other authors reported no correlation between PDGFRA expression and prognosis in glioma patients (Martinho *et al.*, 2009).

Some recent studies have also shown promising results that inhibition of PDGFRA signalling is beneficial in the treatment of GBM (Miklja *et al.*, 2020; Sang *et al.*, 2019; Song *et al.*, 2018).

Isocitrate dehydrogenases (IDHs) are important metabolic enzymes that catalyse oxidative decarboxylation of isocitrate into a-ketoglutarate. $NADP^+$ is used as a co-factor, being converted into NADPH during reaction (Guo *et al.*, 2011). There are three different IDH1 enzymes in human cells: IDH1, IDH2 and IDH3. All of these enzymes catalyse the same reaction but each of the enzymes has some different and unique features. There is different subcellular localization of IDH enzymes, thus IDH1 localizes in cytosol and peroxisomes, while the activity of IDH2 and IDH3 is limited to mitochondria (Krell *et al.*, 2011). However, specific mutations have been discovered only in IDH1 and IDH2 genes, and not in IDH3.

IDH enzymes play many important roles in cells, including the regulation of glutamine metabolism, lipogenesis, regulation of cellular redox status and energy metabolism. NADPH, which is produced in IDH catalysed reaction, protects against oxidative stress and decreases lipid peroxidation and oxidative DNA damage (Lee *et al.*, 2002).

IDH1 gene mutations were identified for the first time in glioblastoma by large-scale genome sequencing studies in 2008 (Parsons *et al.*, 2008). It was also noticed that almost all IDH1 mutated GBMs were secondary GBMs but IDH1 mutations were very rare in primary GBMs (Parsons *et al.*, 2008). Later, Yan et al. examined a large number of glioma patients and identified that IDH1 mutations occur very frequently (> 80%) in diffuse and anaplastic astrocytomas (WHO grades II and III) as well as oligodendrogliomas, and IDH1 mutation was shown to be associated with a favourable outcome (Yan *et al.*, 2009b). In recent years, it was

found that IDH mutations are not unique to gliomas but can be identified in other tumours, such as cholangiocarcinomas, melanomas, prostate cancer, chondrogenic tumours and acute myeloid leukaemia (Amary *et al.*, 2011; Borger *et al.*, 2012; Kang *et al.*, 2009; Lopez *et al.*, 2010; Paschka *et al.*, 2010). The presence of IDH mutations in different tumours indicates the possible important role of IDHs in carcinogenesis rather than just a silent insignificant finding. Many researchers have initiated a series of studies to identify the mechanism by which IDH enzymes cause malignant transformation. IDH mutations have some interesting and unique features. First, IDH mutations occur at very early stages of origination of brain gliomas and often precede other genetic lesions (Watanabe *et al.*, 2009). Second, all IDH mutations are missense substitutions and are heterozygous mutations, so a loss of heterozygosity is not characteristic of IDH1 mutations (Cairns and Mak, 2013; Reitman and Yan, 2010). Finally, most IDH1 and IDH2 mutations occur only in specific arginine residues and the most common mutation is IDH1 R132H, comprising more than 80% of all IDH1 mutations (Balss *et al.*, 2008; Parsons *et al.*, 2008; Yan *et al.*, 2009b). Other rarer IDH1 mutations can also occur in gliomas, including R132S, R132C, R132L and R132G (Hartmann *et al.*, 2010; Yan *et al.*, 2009b).

IDH1 mutations were found to produce significant metabolic changes and reduced concentrations of a-ketoglutarate. However, it is surprising that mutant IDH1 not only loses oxidative activity but also gains a new unique function. Therefore, the direct effect of IDH1 mutation is the production of D-2-hydroxyglutarate (D2HG), which is an oncometabolite that contributes to the causation and progression of glioma (Dang *et al.*, 2010; Sonoda and Tominaga, 2010). D2HG is structurally similar to a-ketoglutarate and can act as a competitive inhibitor of some a-ketoglutarate-dependent dioxygenases, such as histone demethylases (Chowdhury *et al.*, 2011; Xu *et al.*, 2011). Inhibition of demethylases induces significant epigenomic changes such as histone and DNA hypermethylation (the so-called "hypermethylator phenotype") that inhibit normal cellular differentiation and are triggers of subsequent oncogenic effects (Lu *et al.*, 2012; Turcan *et al.*, 2012). Sasaki *et al.* reported that D2HG also blocks hydroxylation of collagen, causing impairment of collagen maturation (Sasaki *et al.*, 2012).

IHC is currently the alternative sensitive method of IDH R132H mutation detection. Monoclonal antibody against the mutant protein IDH1 R132H is a highly sensitive and specific method of evaluation that can be used for both diagnostic and prognostic purposes (Capper *et al.*, 2010). The sensitivity and specificity of the IHC approach in determining the genetic status of IDH1 R132H mutation is 94% and 100%, respectively (Capper *et al.*, 2010). Other authors have even found 100% sensitivity and 100% specificity of the IDH1 R132H antibody against this mutation verified by DNA sequencing (Loussouarn *et al.*, 2012).

It was observed that IDH1 mutations occurred in almost all patients with secondary GBMs, mainly young patients, and a high association was shown with increased survival. The median overall survival rates of mutated and non-mutated IDH 1 patients were 3.8 and 1.1 years, respectively (Parsons *et al.*, 2008). The correlation between the presence of IDH1 mutation and survival is so impressive that some researchers suppose that primary GBMs with IDH1 mutation are underdiagnosed secondary GBMs and vice versa (Kim and Liau, 2012). Thus routine IHC is a perfect method for detecting IDH1 R132H mutation and can be used to differentiate primary GBMs from secondary GBMs (Gondim *et al.*, 2019). Due to the high frequency of IDH1 R132H mutation in low-grade gliomas and its absence in reactive astrogliosis, IHC can also be used in differentiating between these processes (Camelo-Piragua *et al.*, 2010).

CD34 is a monomeric cell surface antigen that is widely expressed on immature haematopoietic stem cells as well as in endothelium and some soft tissue tumours (Baumhueter *et al.*, 1994; Fina *et al.*, 1990; Young *et al.*, 1995). CD34 has been widely used in haematology to assist with identification of immature haematopoietic stem cells for bone marrow transplantation (Nielsen and McNagny, 2008). CD34 has also been identified in some other tissue-specific precursor cells, such as muscle satellite cells and epidermal stem cells (Nielsen and McNagny, 2008). Besides normal endothelium and some sorts of stem cells, CD34 expression is preserved in neoplastic endothelium: for example, haemangiomas, angiosarcomas, Kaposi sarcomas and other vascular origin tumours express this molecule. CD34 expression has also been described in different soft tissue tumours, such as solitary fibrous tumours, gastrointestinal stromal tumours, dermatofibrosarcoma protuberans, epitheloid sarcomas and others (Ardeleanu *et al.*, 2005; Bandarchi *et al.*, 2010; Cummings *et al.*, 2001; Miettinen *et al.*, 2000; Tardio, 2008). Some brain tumours, such as pleomorphic xanthoastrocytomas, gangliogliomas and meningeal solitary fibrous tumours, frequently express CD34 antigen (Blumcke and Wiestler, 2002; Cummings *et al.*, 2001; Reifenberger *et al.*, 2003).

CD34 as an endothelial marker is useful in studies of angiogenesis to determine microvascular density (MVD) and vascular patterns of neoplasms (Foote *et al.*, 2005; Weidner, 2008). Many studies have found a correlation between intratumoural MVD and aggressive behaviour.

Weidner *et al*. were the first researchers to describe the new method of evaluation of MVD in 1991 (Weidner *et al.*, 1991). According to their approach, the first step is to identify any "hot spot" of increased microvascular density by light microscopy at low power magnification. Then individual microvessels are counted at a higher magnification power (200x) in an adequate area

(0.74 mm^2 per field at 200x or 400x). Each count is expressed as the highest number of microvessels found within a 200x or 400x magnification (Weidner *et al.*, 1991).

Assessment of MVD was first widely used with patients with breast carcinomas by different authors (Bevilacqua *et al.*, 1995; Bosari *et al.*, 1992; Obermair *et al.*, 1995; Weidner *et al.*, 1991).

Some studies have evaluated MVD in gliomas. MVD in GBMs was very variable and was distributed within a median MVD count range of 34 to 85 and interobserver variability was high on counting MVD (Preusser *et al.*, 2006). The mean MVD density correlated with the glioma grade and was significantly higher in high-grade gliomas than low-grade gliomas: 45 ± 6.2 vessels/field versus 28 ± 7.2 vessels/field (Zhang *et al.*, 2014). Heike *et al*. evaluated the mean MVD in different grades of astrocytomas and found the following results: grade II – 25, grade III – 33 and grade IV – 35 (Heinke *et al.*, 2013). Another study described mean MVD values in astrocytomas as follows: grade II – 14.5, grade III – 42.3 and GBM – 50.2 (Assimakopoulou *et al.*, 1997). The MVD in gliomas appeared to be independent of the age and gender of patients (Assimakopoulou *et al.*, 1997).

There were few studies regarding the prognostic role of MVD in brain tumours. No correlation was found between MVD value and prognosis in medulloblastomas (Tural *et al.*, 2009). In one study, MVD was found to be strongly associated with worse survival in astrocytomas (Leon *et al.*, 1996). Abdulrauf *et al.* evaluated MVD in low-grade astrocytomas and found that patients with more than seven microvessels in tumour tissue had a shorter survival time (mean 3.8 years) than those with seven or fewer microvessels (mean survival 11.2 years) (Abdulrauf *et al.*, 1998).

Fan *et al.* also showed that higher MVD was associated with worse OS in patients with glioma (Fan *et al.*, 2019).

1.6. Survival characteristics and prognostic factors

The most relevant prognosticator of gliomas is grade. Patients with GBM, which is a grade IV glioma, have one of the worst prognoses among other types of cancers. Those patients who survive for three or more years after initial diagnosis of GBM in the literature are referred to as "long-term survivors" (Krex *et al.*, 2007). Only about 2–5 % of patients are long-term survivors (Krex *et al.*, 2007; Naydenov *et al.*, 2011; Poon *et al.*, 2020; Senger *et al.*, 2003).

In the United States, the five-year survival rate for GBM patients has been reported to be about 3% (Ostrom *et al.*, 2013). Median survival rates at six months and one year have been reported to be 42.4% and 17.7%, respectively (Ohgaki and Kleihues, 2005b). Overall median survival rates in all GBM patients have been reported to range from 9.7 months to 13.6 months

(Ahmadloo *et al.*, 2013; Back *et al.*, 2007; Delgado-Lopez and Corrales-Garcia, 2016; Johnson and O'Neill, 2012; Kumar *et al.*, 2013; Ulutin *et al.*, 2006). One-year survival rates in GBM patients from different studies ranged from 28% to 62%, while two-year survival rates ranged from 5% to 25% (Ahmadloo *et al.*, 2013; Filippini *et al.*, 2008; Li *et al.*, 2010; Ma *et al.*, 2009; Paszat *et al.*, 2001; Piroth *et al.*, 2007; Scoccianti *et al.*, 2010).

In contrast, DAs have more indolent behaviour with a median overall survival rate of five to eight years (Claus *et al.*, 2015; Tove *et al.*, 2012). Median OS rates of 10.5 years, with five-year and 10-year survival rates of 72% and 50%, respectively, have also been reported (Leighton *et al.*, 1997). In one other study, a team of researchers from the USA (Mayo clinic) evaluated long-term survival in patients with diffuse gliomas, with a median follow-up time of 13.6 years. They described a median OS of 6.9 years and 10-year and 15-year survival rates of 36% and 23%, respectively (Schomas *et al.*, 2009).

Due to the widely infiltrative growth of DAs, they typically relapse and potentially progress into high-grade gliomas over time (Ohgaki and Kleihues, 2007).

Today, the best that we can offer to GBM patients is multimodal therapy according to Stupp's regimen, which includes surgical resection followed by radiotherapy and chemotherapy with temozolomide (Lakomy *et al.*, 2020; Stupp *et al.*, 2005). Treatment with temozolomide has a mild beneficial effect on patients' survival compared with radiotherapy alone: 14.6 months versus 12.1 months (Stupp *et al.*, 2005). The two-year survival rates of treated GBM patients were 26.5% (radiotherapy and temozolomide) and 10.4% (radiotherapy only) (Stupp *et al.*, 2005). Some other authors compared survival differences in GBM patients in pre-temozolomide (before temozolomide was introduced) and post-temozolomide eras. For example, in one population-based research from the Cancer Registry of Norway, the median overall survival in pre-temozolomide and post-temozolomide eras was 8.3 and 10.1 months, respectively (Rønning *et al.*, 2012). According to the type of treatment, the median OS in patients with surgery only, surgery plus radiotherapy and surgery plus radiotherapy and temozolomide was 2.5 months, 9.0 months and 16.2 months, respectively (Rønning *et al.*, 2012). Other authors from the USA described a median overall survival of 8.1 and 9.7 months in pre-temozolomide and post-temozolomide eras (Johnson and O'Neill, 2012).

Among the clinical parameters that affect prognosis, old age and poor performance status of GBM patients have been reported to be associated with worse prognosis (Buckner, 2003; Scott *et al.*, 2012). The median survival rate in elderly people is shorter, reaching only 8.5 months, thus old age (> 70 years) is also an important prognostic factor of decreased survival (Scott *et al.*, 2012). In some other studies, the median survival rate of older GBM patients (> 65 years) is about six months (Barnholtz-Sloan *et al.*, 2008; Kita *et al.*, 2009).

Some studies have shown that gender may also influence the prognosis of GBM, however these data are contradictory. Some authors found worse prognosis in females (Reavey-Cantwell *et al.*, 2001; Verger *et al.*, 2011), however others reported worse prognosis in males (Caloglu *et al.*, 2009). Female gender and young age were more frequently associated with long-term survival in GBM patients (Krex *et al.*, 2007; Tian *et al.*, 2018; Yang *et al.*, 2019).

Some studies proved the impact of glioma anatomical localization on prognosis. GBM limited to frontal lobes showed only some trend toward better survival ($p = 0.060$). However, when patient groups were limited to young patients (less than 40 years) the result was statistically significant ($p = 0.005$) and frontal lobe-only tumours showed better prognosis (Lamborn *et al.*, 2004). Compared with gliomas in other locations, frontal lobe GBMs were associated with better prognosis in some other studies (Jeremic *et al.*, 1994; Paldor *et al.*, 2016; Simpson *et al.*, 1993). Patients with multifocal GBMs have much worse prognosis than patients with GBM in a single location (Patil *et al.*, 2012). Paldor *et al.* reported that frontal GBMs could even be biologically distinct from other non-frontal and multilobar tumours, supported by their findings that IDH1 mutated tumours were more frequent in frontal lobes and tumours with a higher Ki-67 proliferation index were more likely to be localized in frontal lobes (Paldor *et al.*, 2016).

One of the least described and most controversial prognostic factors is tumour size. Few studies proved the impact of tumour size on prognosis in glioma patients. Sarica *et al.* did not find any prognostic association between anaplastic astrocytomas whose largest diameter was more than, or equal to, 4 cm and those for which it was less than 4 cm ($p = 0.273$) (Sarica *et al.*, 2012). However, Raysi Dehcordi *et al.* found that tumours whose largest size exceeded 5 cm were associated with worse prognosis in GBM patients ($p = 0.01$) (Raysi Dehcordi *et al.*, 2012). A tumour size of less than 5 cm was also associated with better prognosis in low-grade gliomas ($p = 0.001$) (Kashi *et al.*, 2015; Wang *et al.*, 2019).

Although GBMs can present in morphologically different forms, morphology alone is not a prognostic factor and the prognostic role of distinct morphological variants is disputable and thought to be of minor clinical importance (Miller and Perry, 2007). Because of possible morphological overlaps with other tumours with more favourable prognosis, it is important to distinguish these GBM variants (Louis *et al.*, 2007; Miller and Perry, 2007). For example, giant-cell GBMs can be confused with low-grade pleomorphic xanthoastrocytomas. However, some authors found that giant-cell GBMs have a slightly better prognosis than conventional GBMs, and this is probably due to their better circumscription (Kozak and Moody, 2009; Ogawa *et al.*, 2020; Shinojima *et al.*, 2004).

Although clinical and morphological features and their prognostic impact on glioma patients have been widely described by various authors, nowadays all attention is focused on

understanding and evaluating molecular prognostic factors. The prognostic role of immunohistochemical parameters evaluated in this study was described together with general features and characteristics of selected markers in Section 1.5.

2. Materials and methods

2.1. The essence of study and ethical principles

This thesis was performed as a retrospective study that is based on analysis of formalin-fixed paraffin-embedded surgically treated human glioma tissues. 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 (Pauls Stradins Clinical University Hospital, Riga). Comprehensive morphological and immunohistochemical evaluations of 172 gliomas were performed, including 146 GBMs (WHO grade IV) and 26 DAs (WHO grade II). Anaplastic astrocytomas (WHO grade III) were excluded from the study because we evaluated the two most contrasting grades of diffuse gliomas. The research was carried out in accordance with the Declaration of Helsinki and received approval from the Ethics Committee of Rīga Stradiņš University, No E-9 (2), 12.09.2013.

2.2. Patient cohort and sample collection

The study included 172 gliomas: 146 consecutive cases of GBMs and 26 cases of DAs were identified by an archive search from 2009 to 2014. Each tumour was classified according to the 2016 WHO classification of tumours of the CNS. Only patients with a DA or GBM diagnosis that met the following inclusion criteria were included in the study.

The inclusion criteria for the study were as follows:

1) Patients with histologically proved GBMs and DAs that were diagnosed according to the criteria defined by the 2016 WHO classification of tumours of the CNS.
2) Sufficient amount of tissue material (only surgical resection material).
3) Adequate tumour tissue material (comprised of at least 10% of tissue material composed of morphologically intact, non-necrotic tumour cells).
4) Newly diagnosed glioma cases without any preceding adjuvant therapy.

The exclusion criteria for the study were as follows:

1) Other histological types of glial tumours and tumours with doubtful histological appearance.
2) Small amount of tissue material or material acquired from brain stereotactic biopsies.
3) Seriously damaged and non-adequate tissue material: necrosis and/or tissue damage artefacts constitute more than 90% of tissue material.

Patients' demographic information (gender, age), data about tumour size and localization, as well as data about the adjuvant treatment (chemotherapy with temozolomide

and/or radiotherapy) were acquired from medical case histories. Data about tumour size and localization were also verified from MRI descriptive reports.

2.3. Tissue processing and microscopy

Gross description is not essential when grossing brain tumours because the WHO classification system and grading of CNS tumours relies mainly on microscopical, immunohistochemical and molecular features but not gross examination. Thus, during grossing, only the amounts of received tissue material were recorded and all available fragments of tissue material obtained from patients who were subjected to neurosurgical treatment were submitted for subsequent processing and microscopical analysis.

The tumour tissue samples were fixed in neutral buffered 10% formalin (Sigma-Aldrich, United States of America), processed in vacuum infiltration processor Tissue-Tek® VIPTM 6 (Sakura Seiki Co., Ltd., Nagano, Japan) and embedded in paraplast (Diapath S.r.l., Belgamo, Italy) using the tissue embedding system TES 99 (Medite GmbH, Burgdorf, Germany). After embedding, tissue samples from paraffin blocks were cut in 4-micron-thick sections by microtome (Accu-cut SRM 200CW, Sakura Finetek Europa B.V., the Netherlands), placed on glass slides (Menzel-Glaser, Braunschweig, Germany) and stained with haematoxylin and eosin (H&E) using an automated tissue stainer (TST 44, Medite Medizintechnik, Germany). Stained slides were covered by cover glass (Prestige, Vemi S.R.L., Milano, Italy) employing an automated coverslipper (Dako Coverslipper, Dako Denmark A/S, Glostrup, Denmark). Standard slides, stained by haematoxylin and eosin, were examined under a light microscope to obtain morphological data about the tumour histological type according to criteria defined by the 2016 WHO classification of tumours of the CNS, as well as to evaluate the quality and quantity of tumour tissue material.

2.4. Immunohistochemistry

Immunohistochemical visualization was performed on formalin-fixed paraffin-embedded tissues of CNS glial tumours. First, each case stained by haematoxylin and eosin was evaluated under a light microscope to select the most qualitative and representative tissue block-based assessment of viable, non-necrotic tumour tissue assessible for immunostaining and subsequent microscopical analysis. Complete necrosis or disappearance of tumour tissue in the deeper sections prompted exclusion from the immunohistochemical evaluation.

For IHC analysis, 3-micrometer-thick sections were cut by an electronic rotary microtome Microm HM 360 on electrostatically charged glass slides (Histobond, Marienfeld, Germany) followed by deparaffinization in graded alcohols (Sigma-Aldrich). Heat-induced antigen epitope retrieval was performed according to the manufacturer's instructions in TEG

buffer at pH 9.0 using a microwave oven for 3x5 min. After blocking endogenous peroxidase (Sigma-Aldrich), the sections were incubated with primary antibodies at room temperature. Each antibody used in immunohistochemistry was initially standardized using control tissue as recommended on their specification sheets. Positive controls were performed in accordance with data provided by the company; negative controls were performed when the primary antibody was not applied. When an optimal concentration of antibody was found, it was tested on glioma tissues. The clonality, species origin and specificity, as well as the working dilution and manufacturer, are shown in Table 2.1.

Table 2.1.

Characteristics of primary antibodies

Antigen	Antibody characteristics	Clone	Dilution	Manufacturer
Ki-67	Monoclonal mouse Ab against human Ag	MIB-1	1:100	Dako
p53	Monoclonal mouse Ab against human Ag	DO-7	1:400	Dako
$p21^{WAF1/Cip1}$ protein	Monoclonal mouse Ab against human p21	SX118	1:25	Dako
$p27^{Kip1}$ protein	Monoclonal mouse Ab against human p27	SX53G8	1:50	Dako
Mutant IDH R132H	Monoclonal mouse antibody against human mutant protein IDH R132H	H09	1:50	Dianova
PDGFRA	Rabbit polyclonal anti-PDGFR alpha antibody. Reacts with human Ag	Polyclonal	1:200	Abcam
CD44	Monoclonal mouse Ab against human Ag	DF1485	1:50	Dako
CD34	Monoclonal mouse Ab against human Ag	QBEnd10	1:50	Dako

* Abbreviations in the table: Ab, antibody; Ag, antigen; IDH01, isocitrate dehydrogenase 1; PDGFRA, platelet-derived growth factor receptor alpha.

After immunostaining, the expression of markers was evaluated by light microscopy under high-power magnification (400x).

The expression of marker was considered positive only if the expression intensity was moderate or high (Ryu *et al.*, 2018; van Diest *et al.*, 1997). The intensity levels are shown in Figure 2.1 in the example of CD44 expression.

For most markers (Ki-67, p53, p21, p27, CD44, CD34) the presence of nuclear, cytoplasmic or membranous staining was assessed quantitatively as the relative number of positive neoplastic cells (%).

For PDGFRA we used both quantitative and semi-quantitative methods (0–9 % of immunoreactive cells were considered as a negative sample, 10–50 % as focally positive and > 50 % positive). The expression of IDH1 R132H mutant protein was assessed only as positive (+) and negative (-) (Figure 2.2).

Immunolocalization of evaluated antigens is shown in Table 2.2.

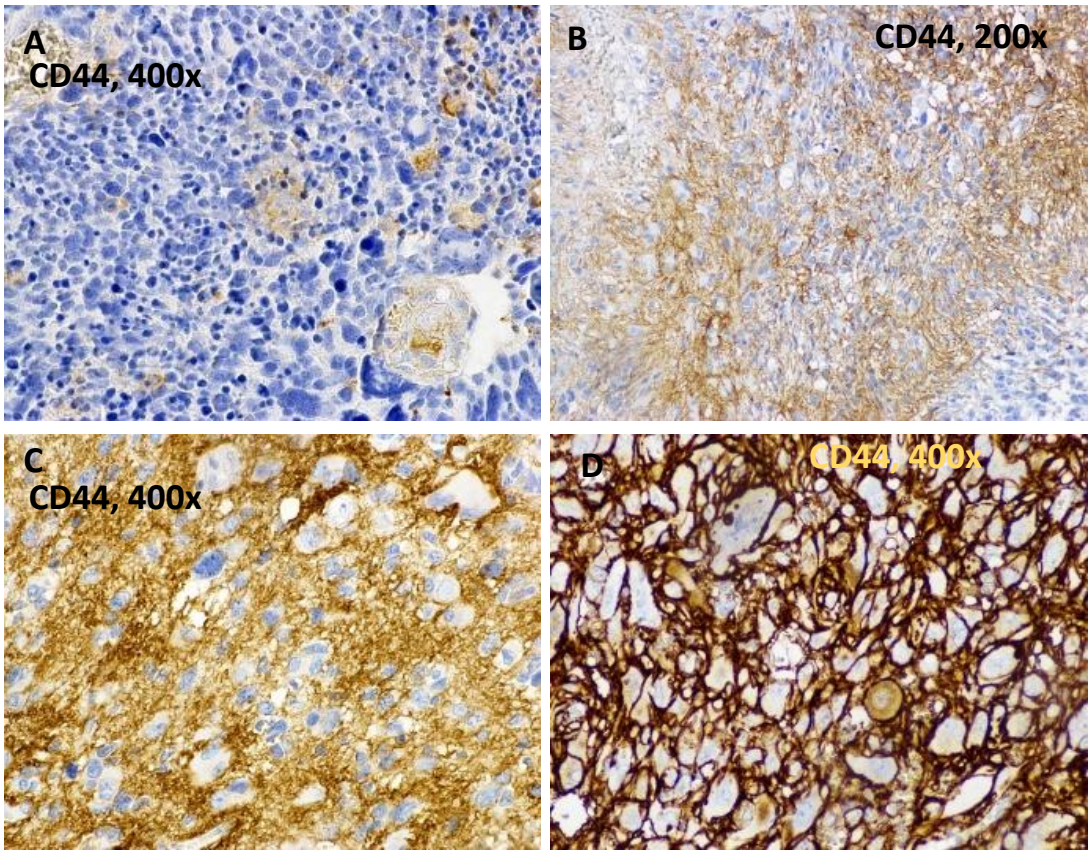


Figure 2.1. **The intensity levels of CD44 expression in GBM. A, no expression; B, low expression; C, moderate intensity; D, high intensity. Immunoperoxidase, CD44, original magnification 200× (B) and 400x (A, C, D).**

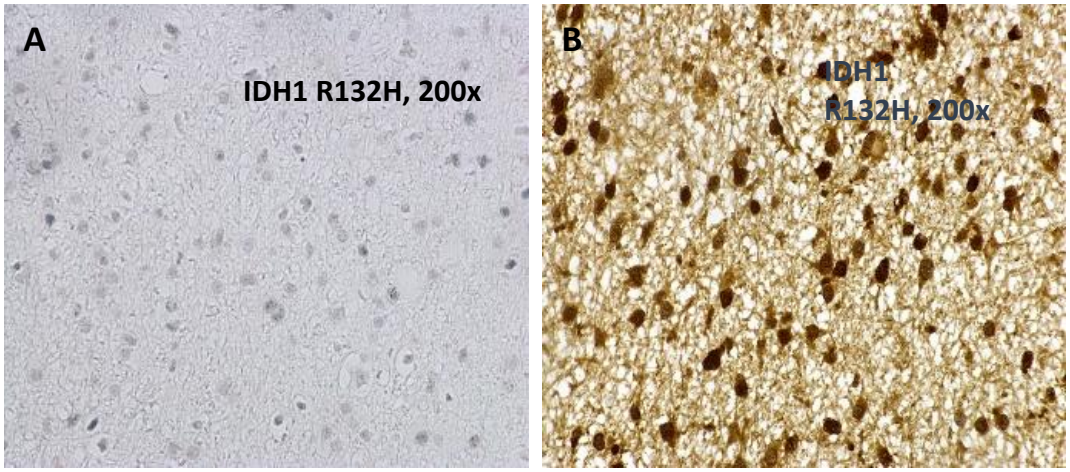
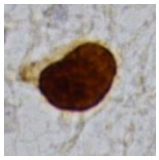
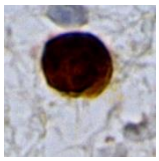
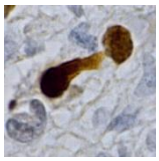
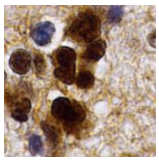
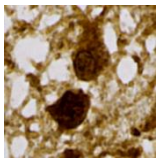
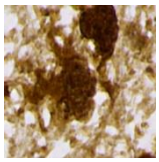
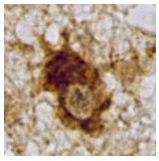
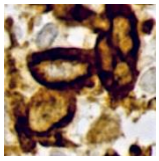
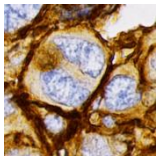
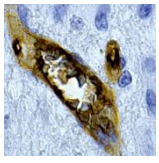


Figure 2.2. **Immunohistochemical visualization of IDH1 R132H mutant protein in DAs. A, negative; B, positive; Immunoperoxidase, IDH1 R132H, original magnification 200×.**

Table 2.2.

Immunolocalization of evaluated antigens

Antigen	Cellular localization		
	Nuclear	**Cytoplasmic**	**Membranous**
Ki-67	+ 	-	-
p53	+ 	-	-
p21$^{WAF1/Cip1}$ protein	+ 	-	-
p27 ^{Kip1} protein	+ 	-	-
Mutant IDH R132H	+ 	+ 	-
PDGFRA	-	+ 	+ 
CD44	-	-	+ 
CD34	-		+ 

To detect the MVD, endothelial differentiation was highlighted by CD34 expression. MVD was assessed according to Weidner's approach (Weidner *et al.*, 1991). The first step was identifying any "hot spot" of increased microvascular density by light microscopy at low power magnification (Figure 2.3). Then individual microvessels were counted at high power in an adequate area (0.74 mm^2 per field at 400x) (Figure 2.4). Each count was expressed as the highest number of microvessels found within a 400x magnification. Vessels with muscular walls were not counted.

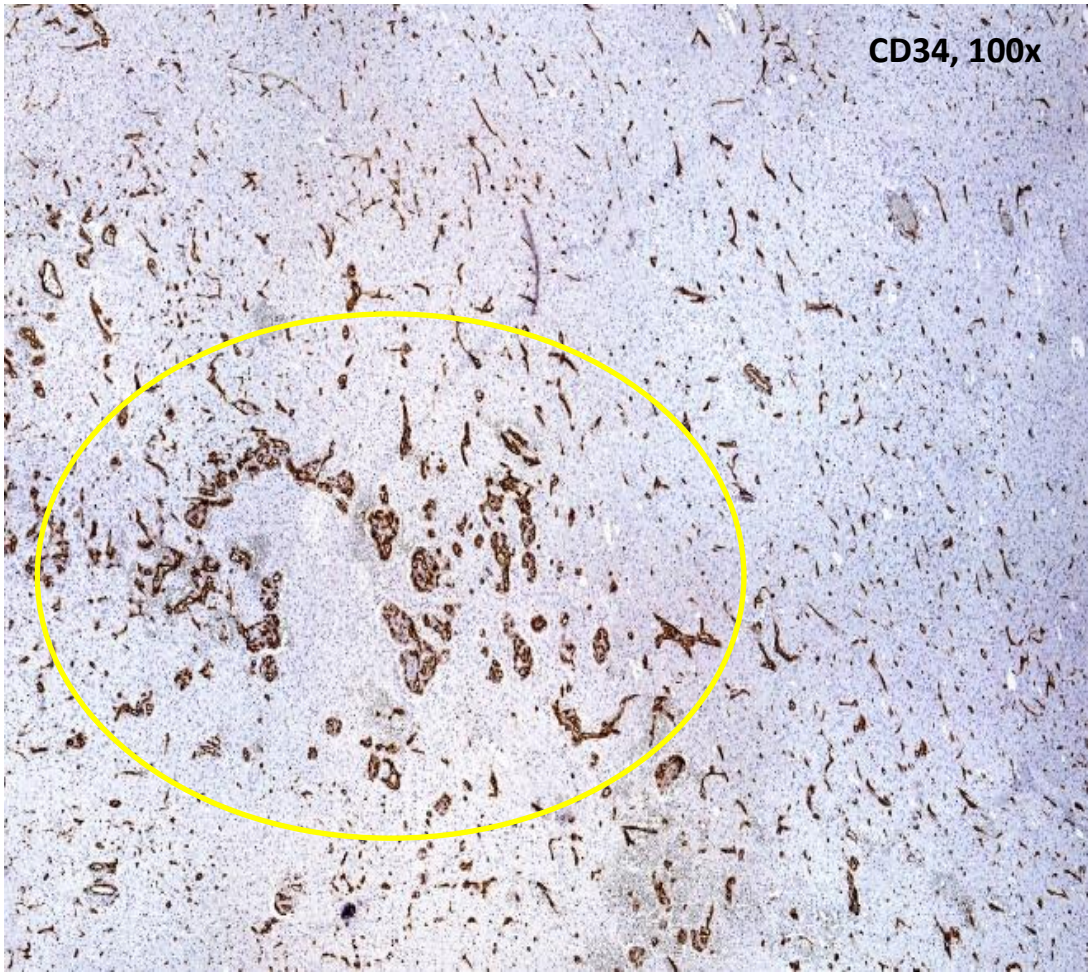


Figure 2.3. **Evaluation of MVD in GBM. Hot spot area in yellow circle. Immunoperoxidase, CD34, original magnification 100×.**

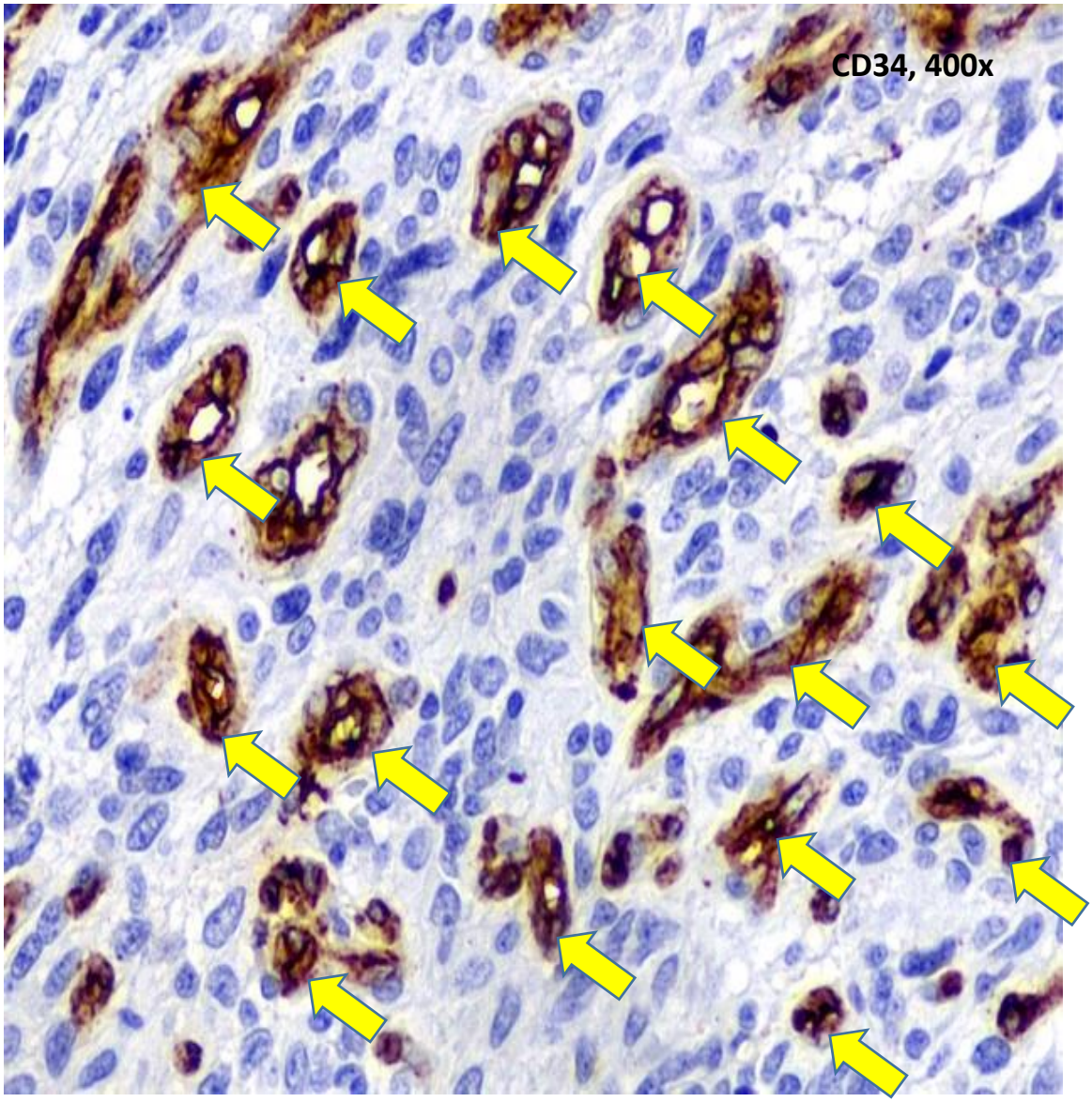


Figure 2.4. **Microvessels (arrows) in GBM. Immunoperoxidase, CD34, original magnification 400×.**

For each case to be considered positive, the relative number of positive cells had to reach a certain cut-off value. In the literature there were many disagreements regarding the best cut-off values in gliomas, and many authors used very different cut-offs. Thus, we used two cut-offs for each marker: the first cut-off value was based on other studies published in international journals, and in addition we used our own cut-off value based on the median expression value of the immunohistochemical marker. For MVD the cut-off was only based on the median expression value because of the few studies in literature regarding the cut-off for CD34. The chosen cut-off values for immunohistochemical markers for both GBMs and DAs are shown in Table 2.3.

Table 2.3.

The cut-off values of immunohistochemical markers for GBMs and DAs

Marker	GBM	DA	References
Ki-67	25% and 41% (median)	3% and 5.5% (median)	(Jin *et al.*, 2011; Neder *et al.*, 2004)
p53	10% and 15% (median)	10% and 52% (median)	(Hilton *et al.*, 2002; Popova *et al.*, 2014; Takano *et al.*, 2012; Wang *et al.*, 2014)
CD44	50% and 86.5% (median)	50% and 8.5% (median)	(Popova *et al.*, 2014)
PDGFRA	50% and 1% (median)	50% and 42% (median)	(Popova *et al.*, 2014)
p21	20% and 19% (median)	20% and 2.5% (median)	(Trabelsi *et al.*, 2016)
p27	70% and 74% (median)	70% and 92% (median)	(Faria *et al.*, 2007; Yang *et al.*, 2011)
MVD	35% (median)	13.0% (median)	NA

* Abbreviations in the table: NA, not applicable; MVD, microvascular density; PDGFRA, platelet-derived growth factor receptor alpha; GBM, glioblastoma; DA, diffuse astrocytoma

The immunohistochemical data of p53, IDH1 R132H, PDGFRA and CD44 expression was used to determine the subtype of GBMs (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, referred to as "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, IDH1 R132H). All remaining cases that do not fit the proneural or mesenchymal subtype category were referred to as "Other" or not otherwise classified (see Figure 2.5).

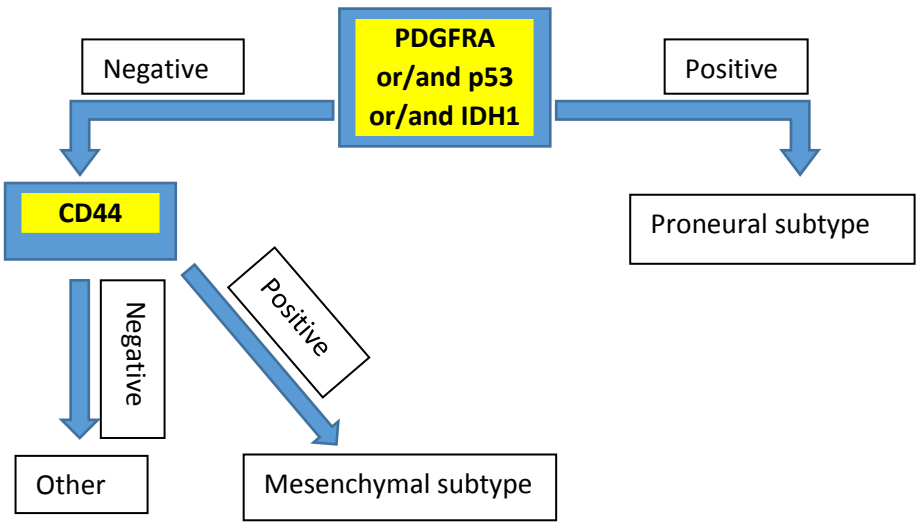


Figure 2.5. **Immunohistochemical subtyping of gliomas**

Analysis of immunostained specimens was performed using a Carl Zeiss Axiolab (Germany) microscope. Nuclear immunostaining of Ki-67, p53, p21 and p27 was evaluated by computed morphometry using the Kappa image base program (KAPPA opto-electronics Inc., United States of America).

2.5. Statistical analysis

All statistical analysis was performed using the IBM SPSS Statistics version 20.0 statistical software package (International Business Machines Corp., Armonk, New York, USA). An assumption of normality check using the Shapiro-Wilk test was performed before statistical calculations. Descriptive statistics were calculated as mean ± standard deviation (SD), median with interquartile range (IQR) and/or frequency (%) with 95% confidence interval (CI). Descriptive statistical methods, including descriptive and cross-tabulation with Pearson's chi-square, Fisher's exact test, bivariate correlation with Spearman's rank correlation coefficient, non-parametric method, including the Mann-Whitney U test and Kruskal-Wallis one-way analysis of variance by ranks, were used.

Survival was evaluated by Kaplan-Meier analysis. A value of $p < 0.05$ was considered statistically significant.

3. Results

3.1. Basic characteristics of research group

3.1.1. Characteristics of GBM cases

The study included 146 patients diagnosed with GBM during the evaluated period of time (2009–2014). GBM was diagnosed in 75/146 (51.4%; 95% CI = 43.3–59.5) females and in 71/146 (48.6%; 95% CI = 40.5–56.7) males. The age of patients ranged from 34 to 89. The mean age ± standard deviation (SD) was 62.0 ± 11.2 (95% CI = 60.2–63.8). The median age was 62.0 (IQR = 18). The mean age of males ± SD was 60.8 ± 11.5 (95% CI = 58.1–63.5); the median age was 60.7 (IQR = 19). The mean age of females ± SD was 63.1 ± 10.9 (95% CI = 60.7–65.7); the median age was 65.0 (IQR = 17).

The age distribution of GBM patients is shown in Figure 3.1.

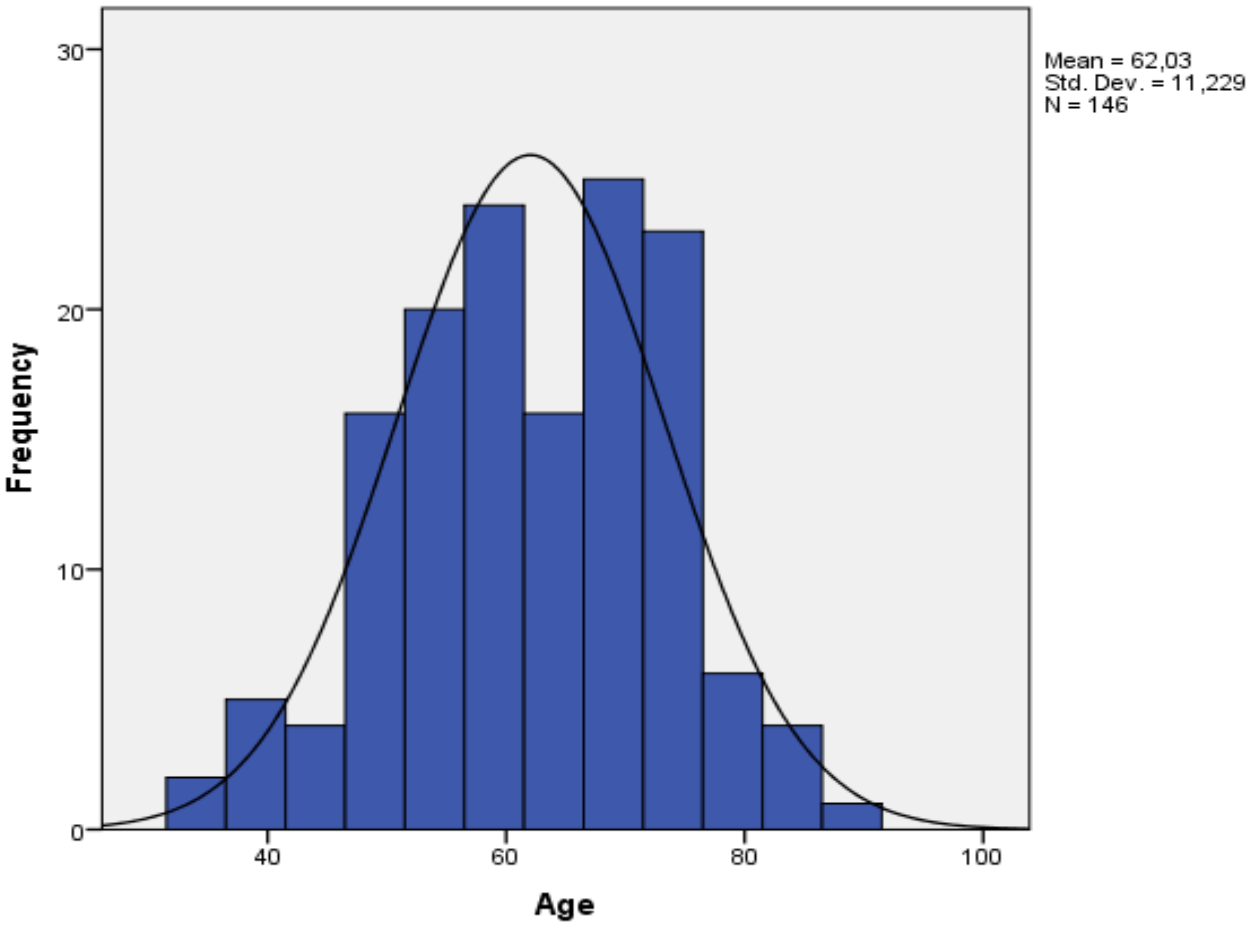


Figure 3.1. **The age distribution of GBM patients**

The most frequent localization of GBM was the frontal lobe – 56/146 (38.4%; 95% CI = 30.5–46.3), followed by the temporal lobe – 41/146 (28.1%; 95% CI = 20.8–35.4), the parietal lobe – 21/146 (14.1%; 95% CI = 8.5–19.7) and the occipital lobe 2/146 (1.4%; 95% CI = 0–

3.3);26/146 (17.8%; 95% CI = 11.6–24.0) of GBM cases diffusively infiltrate several lobes in the same hemisphere. The relevant data are shown in Figure 3.2.

In terms of GBMs, 78/146 (53.4%; 95% CI = 45.3–61.5) were localized in the right cerebral hemisphere, while 62/146 (42.5%; 95% CI = 34.5–50.5) GBMs were found in the left hemisphere; 6/146 (4.1%; 95% CI = 0.9–7.3) GBMs were bilateral, involving both frontal lobes. Multifocal involvement was recognized in 16/146 (11.0%; 95% CI = 5.9–16.1) GBMs.

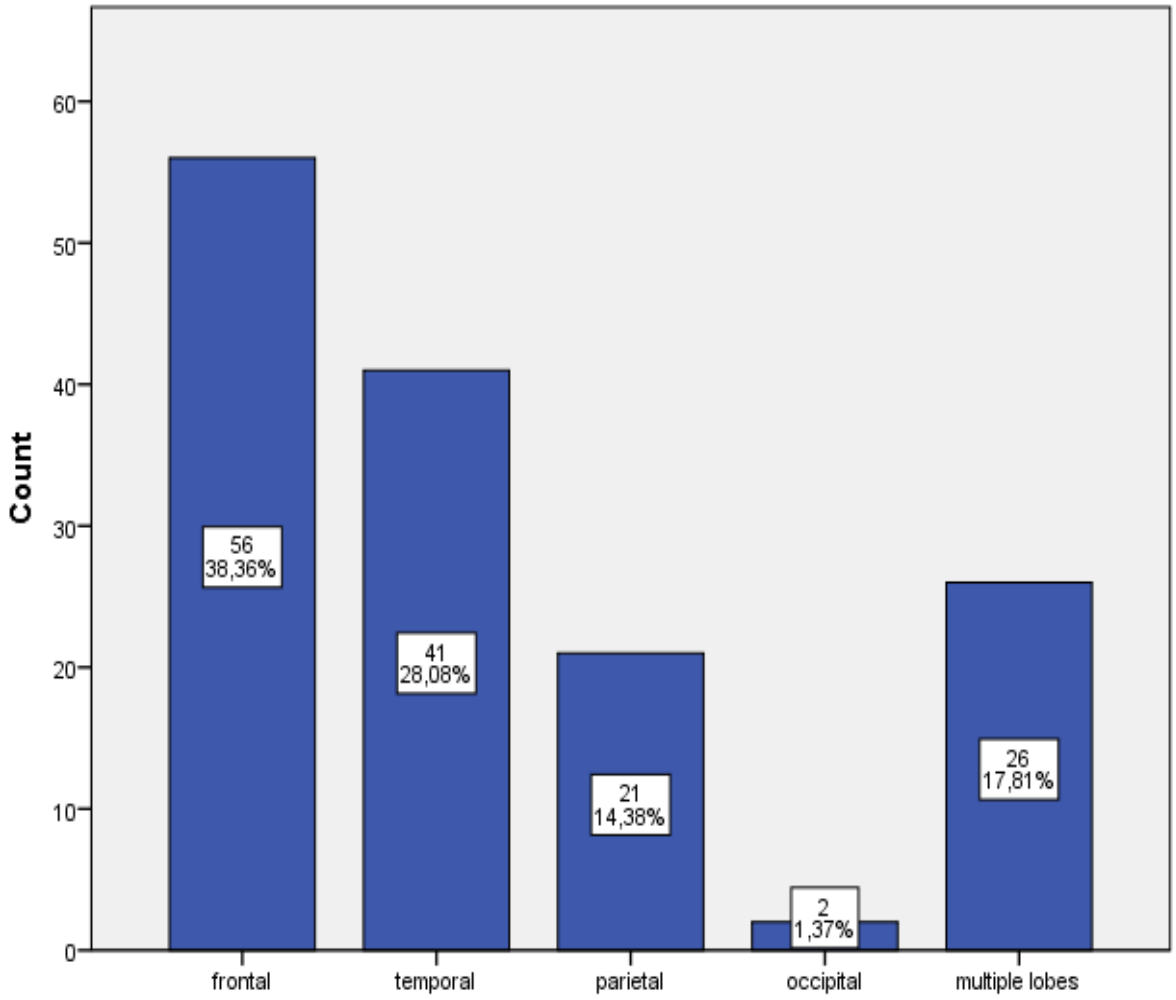


Figure 3.2. **The distribution of GBMs by localization**

The maximum tumour diameter measured from MRI series ranged from 1 to 9 cm; mean ± SD was 5.1 ± 1.4 (95% CI 4.85–5.36); the median was 5.1 (IQR = 2). In 101/123 (29.5%; 95% CI = 21.4–37.6) GBM cases the maximum diameter exceeded 4 cm. In 22/123 (15.1%; 95% CI = 8.8–21.4) GBMs the maximum diameter was less than 4 cm. The MRI data were missing for 23/146 (15.7%; 95% CI = 9.8–21.6) cases from the sample. The distribution of GBMs by size shown as maximum diameter is shown in Figure 3.3.

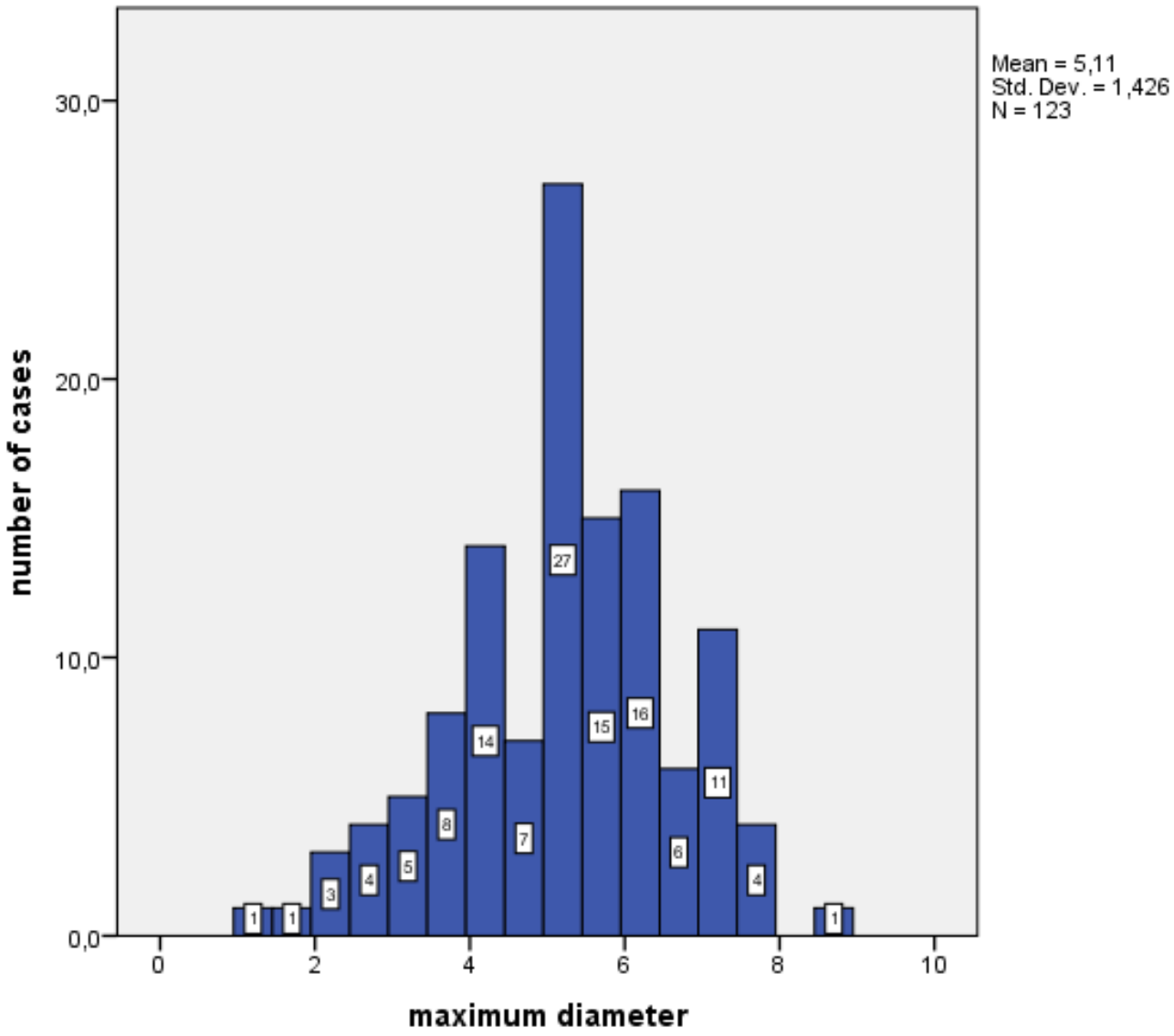


Figure 3.3. **The distribution of GBM by size (maximum diameter)**

Surgical resection was performed in all GBMs (146/146), and in addition, adjuvant therapy such as radiotherapy and chemotherapy with temozolomide was used. The data concerning type of therapy was not available in 11/146 (7.5%; 95% CI = 4.3–12.9) cases.

In the remaining 135 patients with GBM, the most frequent therapy was the standard type of treatment with surgery followed by adjuvant radiotherapy and chemotherapy with temozolomide in 56/135 (41.5%; 95% CI = 33.5–49.9) patients, while surgery plus radiotherapy was used in 50/135 (37.0%; 95% CI = 29.3–45.4) patients and 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 than those who received adjuvant radiotherapy or were treated with surgical resection alone without adjuvant treatment (one-way ANOVA, $p < 0.001$). The mean ages of patients receiving different types of treatment were 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. There was no association between the size of tumour and type of treatment (Kruskal-Wallis H; p = 0.708).

The types of treatment in GBMs are shown in Figure 3.4.

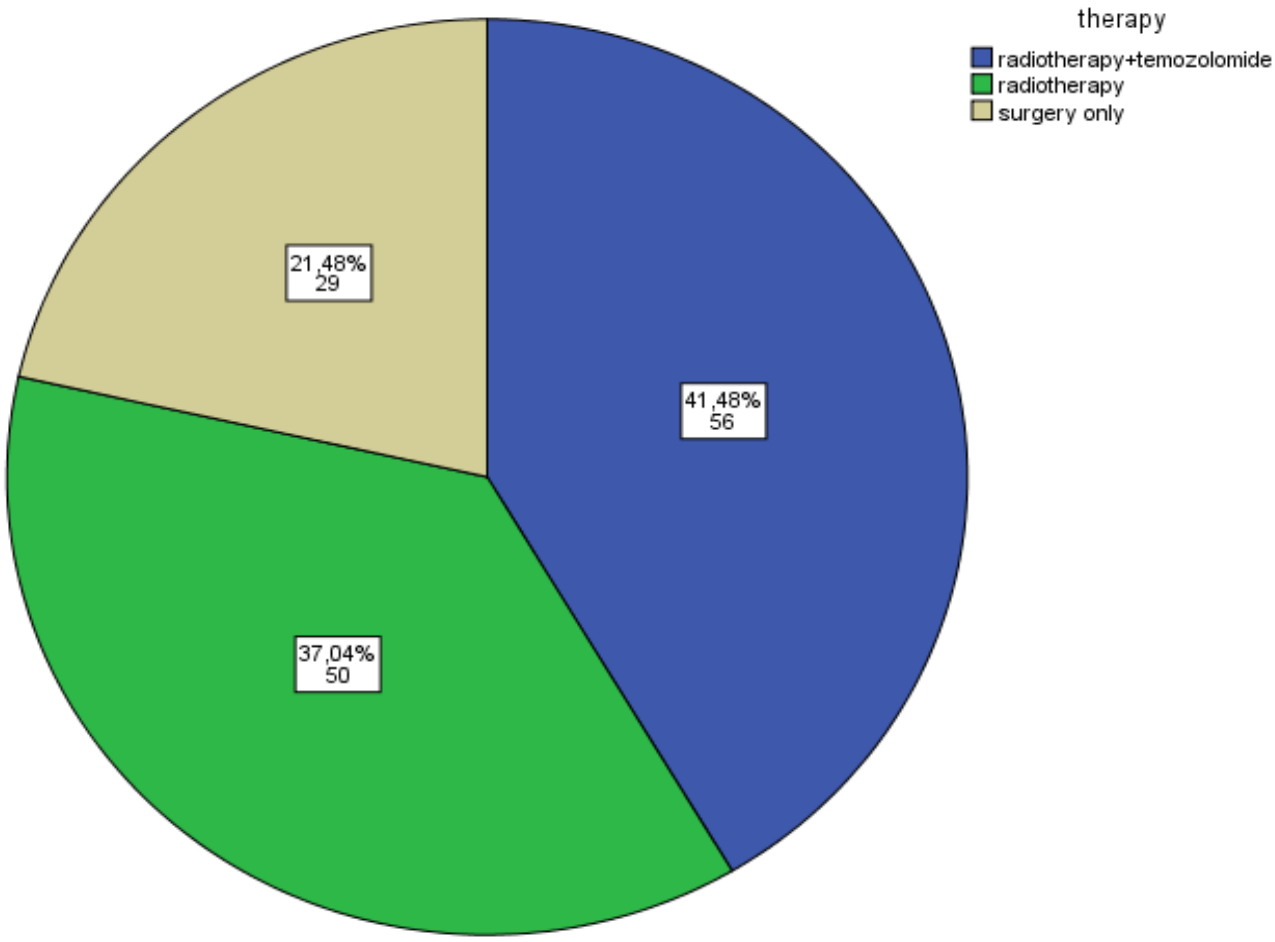


Figure 3.4. **The types of treatment in GBMs**

3.1.2. Characteristics of DA cases

This study included 26 patients diagnosed with DA during the evaluated period of time (2009–2014). DAs were 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. The mean age ± SD was 37.5 ± 11.2 (95% CI = 33.0–42.0). The median age was 35.5 (IQR = 19). The mean age of males ± SD was 37.6 ± 12.3 (95% CI = 29.8–45.5); the median age was 34.0 (IQR = 17). The mean age of females ± SD was 37.4 ± 10.5 (95% CI = 31.3–43.4); the median age was 36.0 (IQR = 20). The age distribution of DA patients is shown in Figure 3.5.

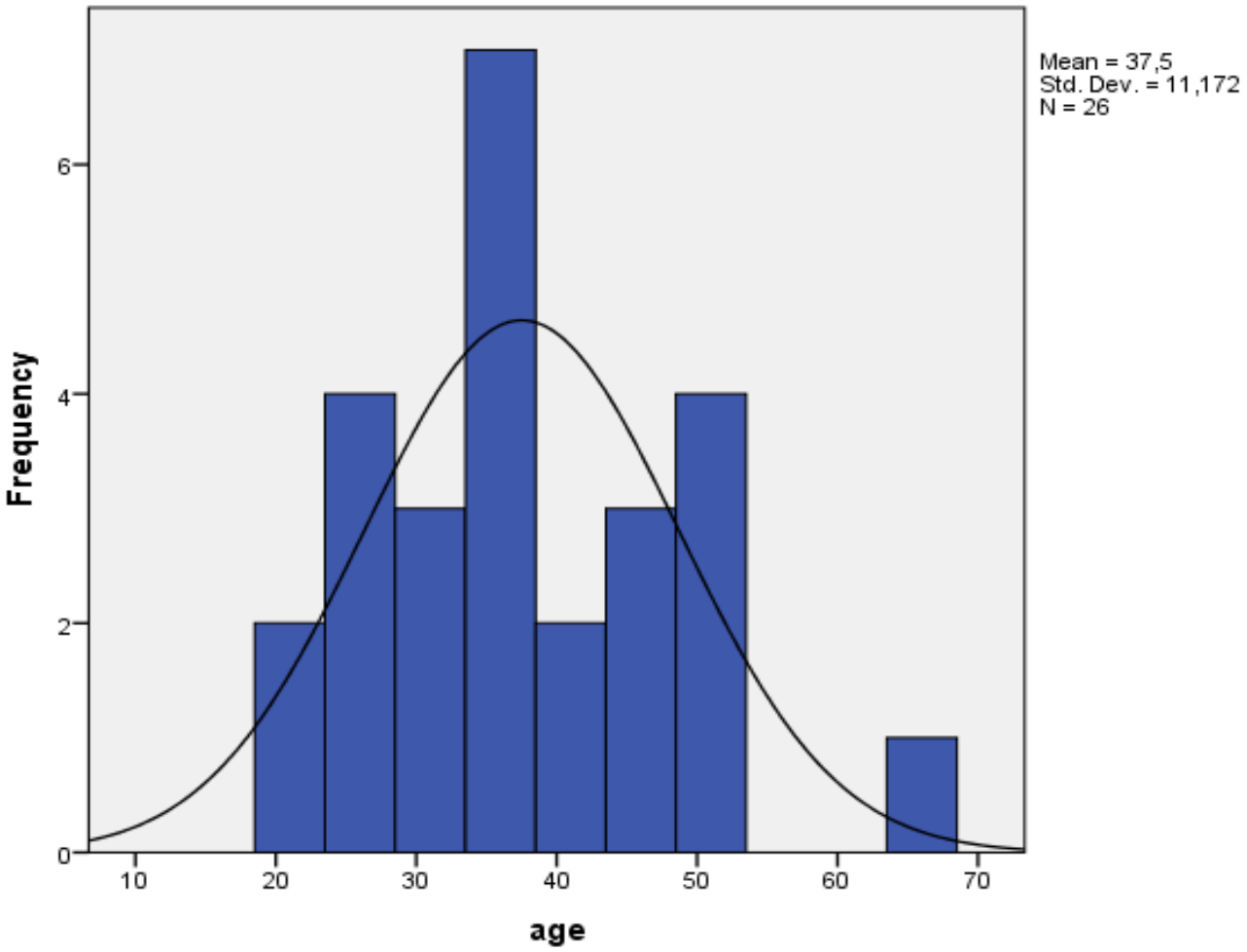


Figure 3.5. **The age distribution of DA patients**

The most frequent localization of DA was the frontal lobe – 13/26 (50%; 95% CI = 30.8–69.2), followed by the temporal lobe – 6/26 (23.1%; 95% CI = 6.9–39.3); 7/26 (26.9%; 95% CI = 9.9–43.9) DAs involve several cerebral lobes. The data illustrating the localization of DAs is shown in Figure 3.6.

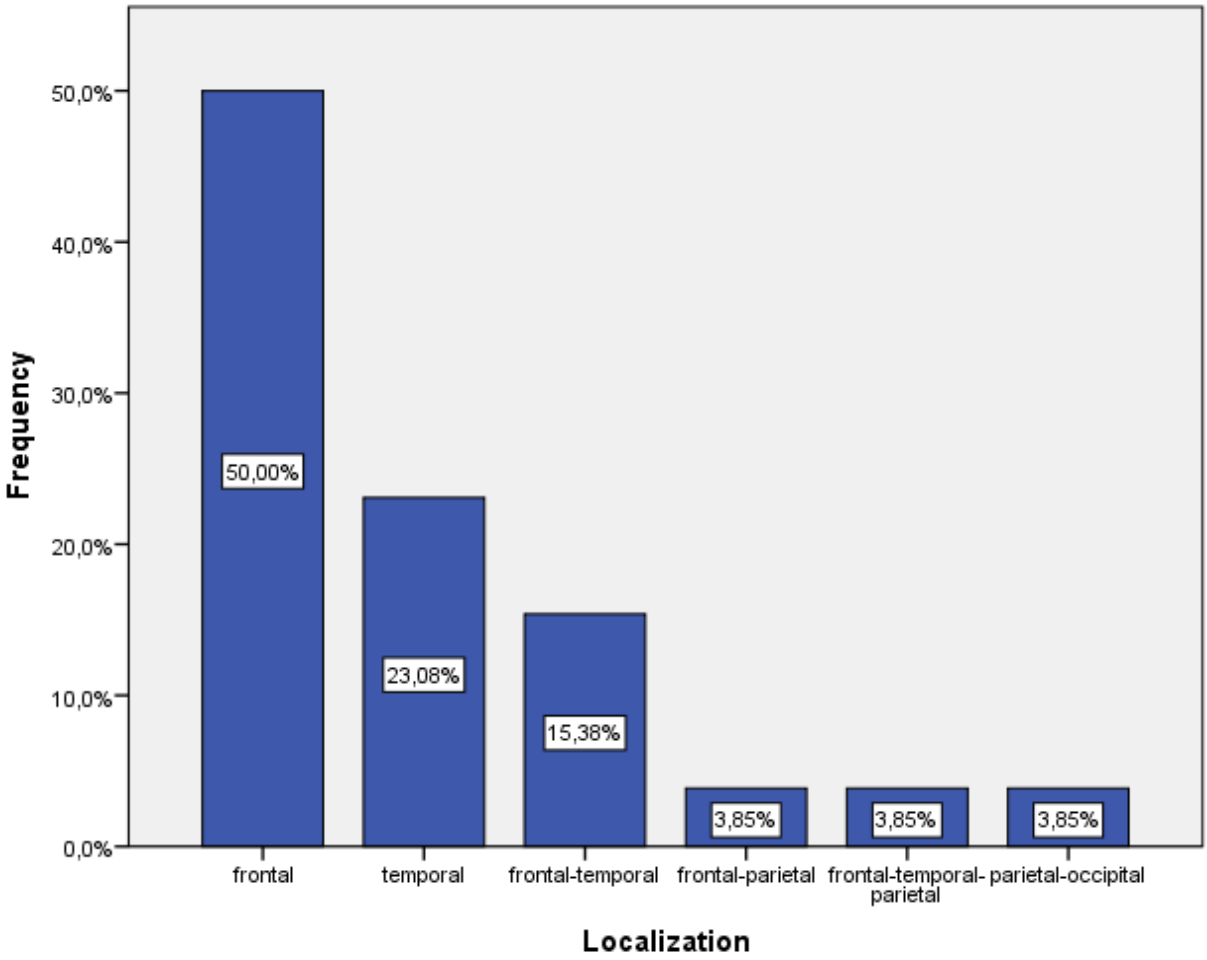


Figure 3.6. **The distribution of DAs by localization**

In terms of DAs, 14/26 (53.8%; 95% CI = 34.6–72.0) were localized in the right cerebral hemisphere, while 12/26 (46.2%; 95% CI = 27.0–65.4) were found in the left hemisphere.

The maximum tumour diameter measured from MRI series ranged from 4.7 to 9 cm; the mean ± SD was 6.1 ± 0.9 (95% CI = 5.6–6.7); the median was 6.0 (IQR = 1). The MRI data and information about the tumour size were missing for 13/26 (50.0%; 95% CI = 30.8–69.2) of the sample. The distribution of DAs by size shown as maximum diameter is shown in Figure 3.7.

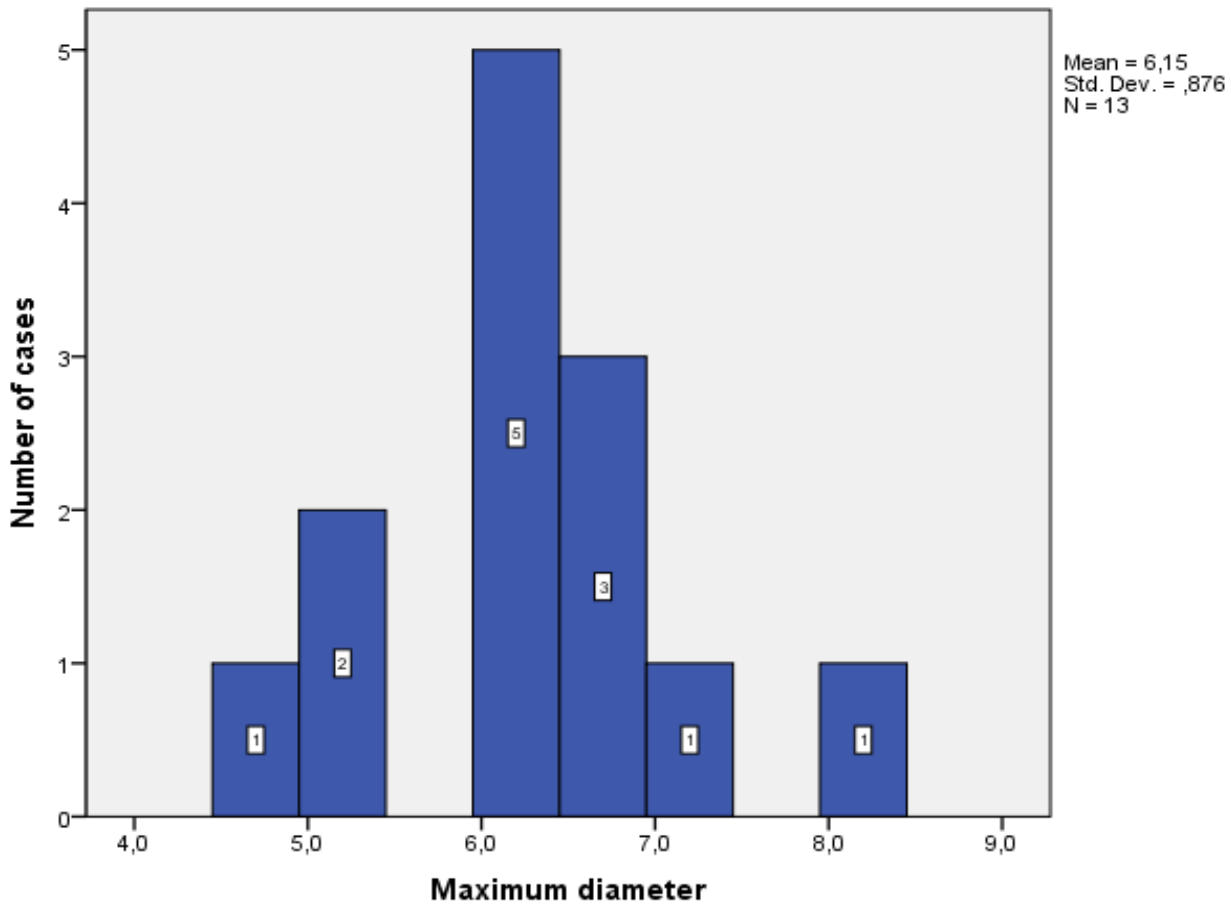


Figure 3.7. **The distribution of DAs by size**

Surgical resection was performed in all DAs (26/26), and in addition, all patients received adjuvant radiotherapy.

3.2. Morphology

3.2.1. Characteristics of GBM cases

All GBM cases were diagnosed according to the 2016 WHO classification of CNS tumours and thus all cases showed necrosis (ischaemic and/or pseudopalisading) and microvascular proliferation (Figure 3.8), and IDH1 status was assesed by IHC (Table 3.1.).

In 16/146 (10.9%; 95% CI = 5.8–16.0) GBM cases, mild initial microvascular proliferation was found; however, the presence of necrosis and cellular atypia supported the diagnosis of GBMs. In 130/146 (89.0%; 95% CI = 83.9–94.1) GBM cases, microvascular proliferation was prominent, frequently with the formation of glomeruloid-like vascular structures.

Most GBM cases – 141/146 (96.6%; 95% CI = 93.7–99.5) – belong to conventional GBMs. There were only 2/146 (1.4%; 95% CI = 0–3.3) gliosarcomas and 3/146 (2.1%; 95% CI = 0–4.4) giant-cell GBMs. Morphological features of GBMs are shown in Figure 3.8 (A–D).

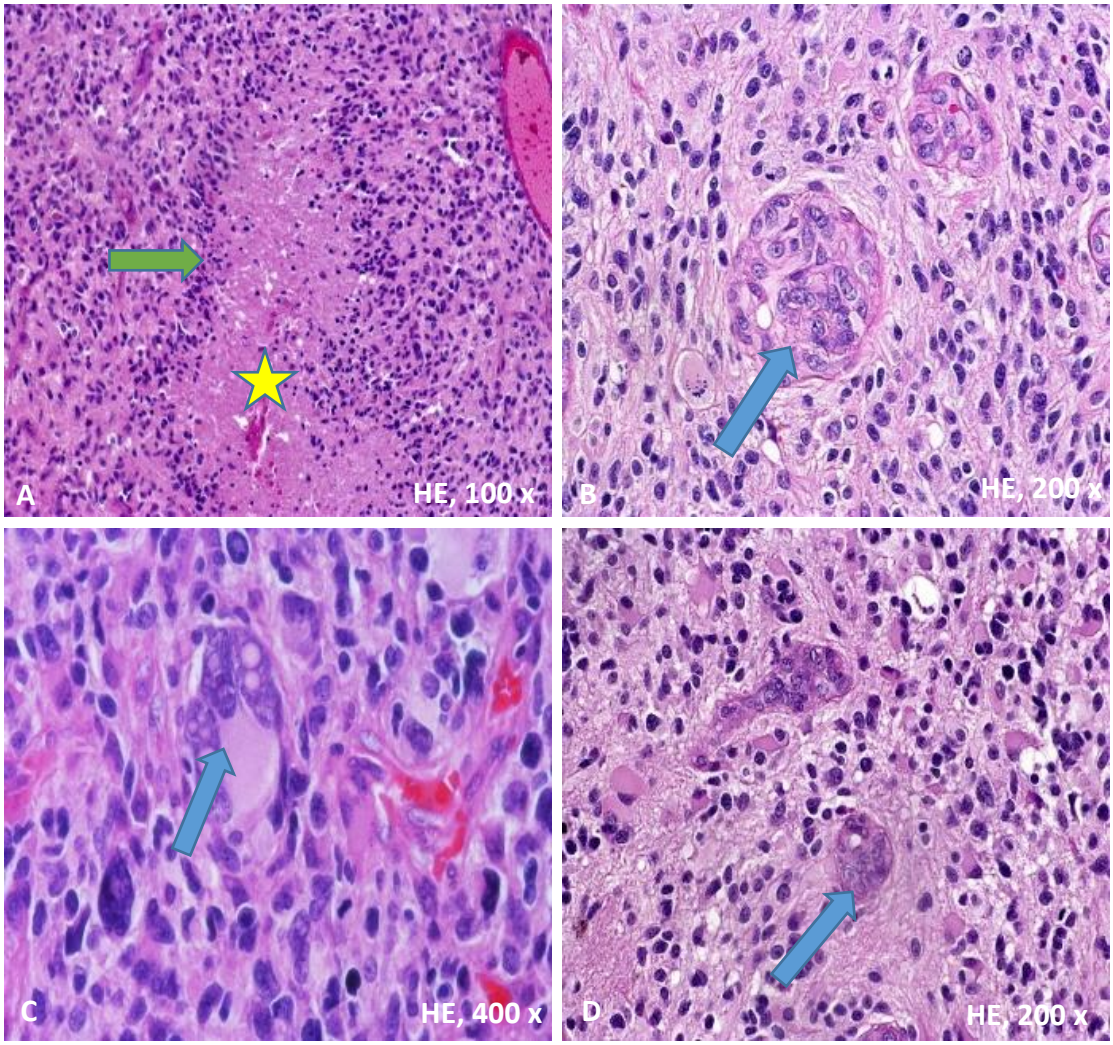


Figure 3.8. **Morphology of GBM. A, pseudopalisading necrosis. Pseudopalisade is shown by the green arrow; necrosis in the centre is shown by the yellow star. B, glomeruloid microvascular proliferation (arrow). C, prominent cellular atypia and giant atypical cell (arrow). D, initial microvascular proliferation forming multilayer tufts of endothelial cells. Haematoxylin and eosin (HE), original magnification 100x (A), 200× (B, D) and 400× (C).**

3.2.2. Characteristics of DA cases

All 26/26 (100%) DAs were diagnosed as diffuse fibrillary astrocytomas on morphological grounds according to the 2016 WHO classification of CNS tumours (Figure 3.9). All DAs showed mild to moderate cellularity; however, 3/26 (11.5%; 95% CI = 0–23.7) of the DAs showed focal areas of increased cellular density compared with the rest of the tumour, and rare mitotic figures up to 2 mitoses per 10 high-power field were found. All DAs showed mild nuclear atypia, no necrosis and no microvascular proliferation. In 2/26 (7.7%; 95% CI =

0–17.8) of the DAs, prominent gemistocytic astrocytes were found, and 12/26 (46.1%; 95% CI = 26.0–65.3) DAs showed microcystic changes. Morphological features of DAs are shown in Figure 3.9 (A–D).

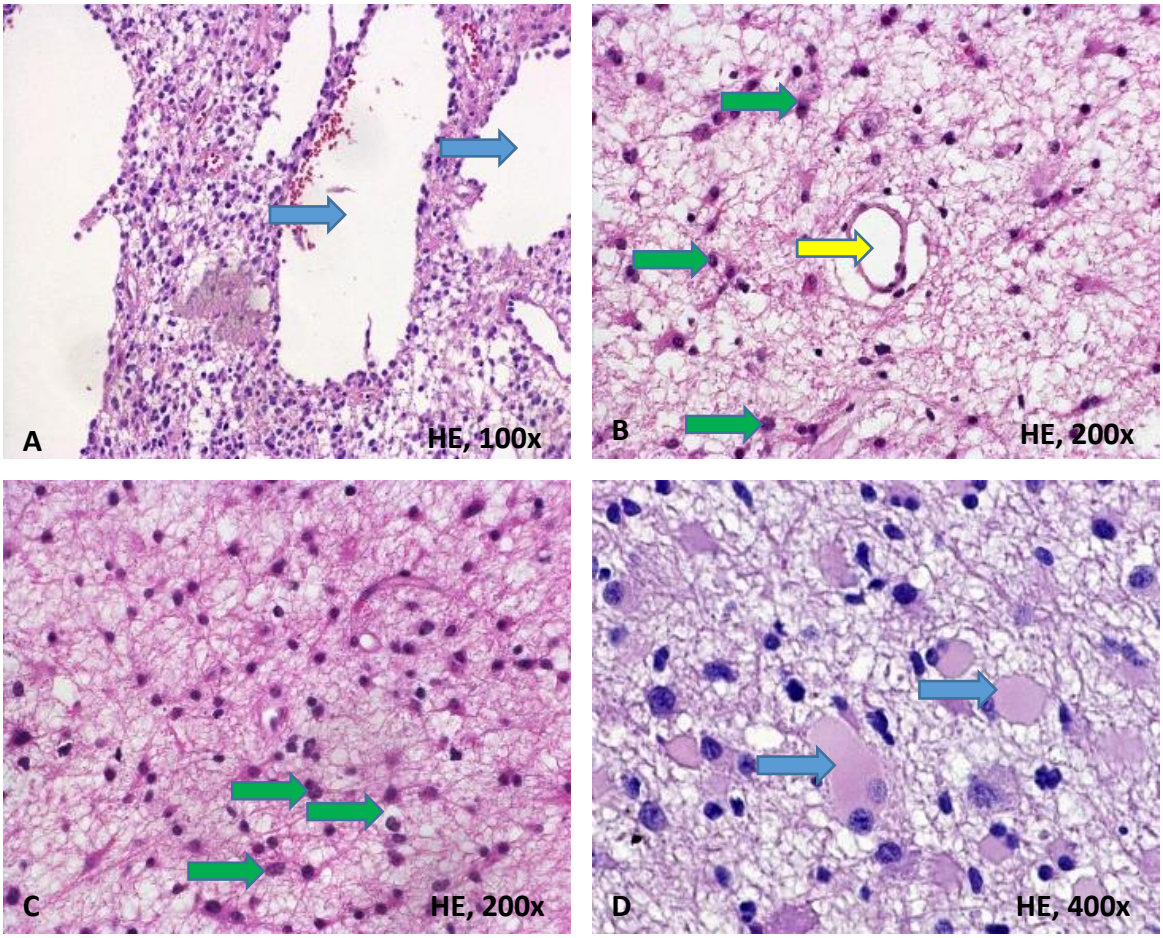


Figure 3.9. **Morphology of DA. A, microcystic changes (blue arrows). B, C, mild cellularity; note small, uniform nuclei of neoplastic cells (green arrows) and microvessel (yellow arrow). D, gemistocytic astrocytes; note enlarged, plump cells with abundant eosinophilic cytoplasm (blue arrows). Haematoxylin and eosin (HE), original magnification 100× (A), 200x (B, C) and 400× (D).**

3.3. Immunohistochemical findings in GBMs and DAs

The expression of the following immunohistochemical markers was assessed in GBMs and DAs: Ki-67, p53, p21, p27, CD44, PDGFRA, IDH1 R132H and CD34.

The expression of p53 and Ki-67 was confined to the nuclei of neoplastic cells. GBM demonstrated a marked increase of Ki-67 proliferation activity compared with DAs: 44.4% [95% CI = 41.1–47.6] versus 6.4% [95% CI = 4.7–8.0]. Ki-67 proliferation indices ranged from 13 to 95 % in GBMs and from 2 to 15 % in DAs.

A box plot illustrating and comparing Ki-67 proliferation indices in GBMs and DAs is shown in Figure 3.9.

The expression of the aberrant p53 protein varied significantly in both groups from absence of any immunoreactivity (0%) to strong labelling of almost all cells (99%). Some level of p53 immunoreactivity (> 1%) was found in 87.3% (95% CI = 81.5–93.1) of GBMs and 87.5% (95% CI = 74.3–100) of DAs. However, strong p53 immunoreactivity (> 50%) was found in 31.7% (95% CI = 23.6–39.8) of GBMs and 50% (95% CI = 30.8–69.2) of DAs. There was no statistically significant difference in p53 protein expression in analysis of the mean amount of positive cells between DAs and GBMs (p = 0.416). Using the selected cut-off of 10%, p53 expression was found in 64.3% (95% CI = 55.6–72.1) of GBMs and 75.0% (95% CI = 55.1–88) of DAs.

All secondary GBMs, i.e. 100% (95% CI = 56.5–100), had a high level of p53 expression (with a cut-off of 10%) compared with 51.5% (95% CI = 44.8–62.3) of primary GBMs. There was also a statistically significant difference in p53 protein expression by analysis of the mean amount of positive cells between primary and secondary GBMs – 32.7 (95% CI = 26.2–39.2) versus 98.6 (95% CI = 96.8–99.1) (p = < 0.001).

A box plot illustrating and comparing p53 protein expression in GBMs and DAs is shown in Figure 3.10.

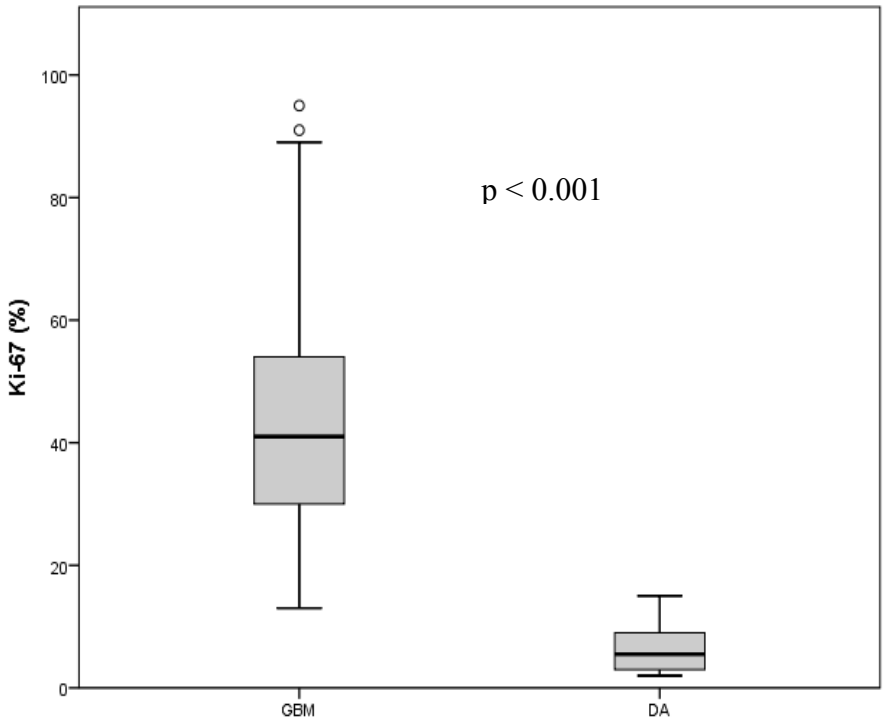


Figure 3.10. **Box plot of Ki-67 proliferation indices in GBMs and DAs**

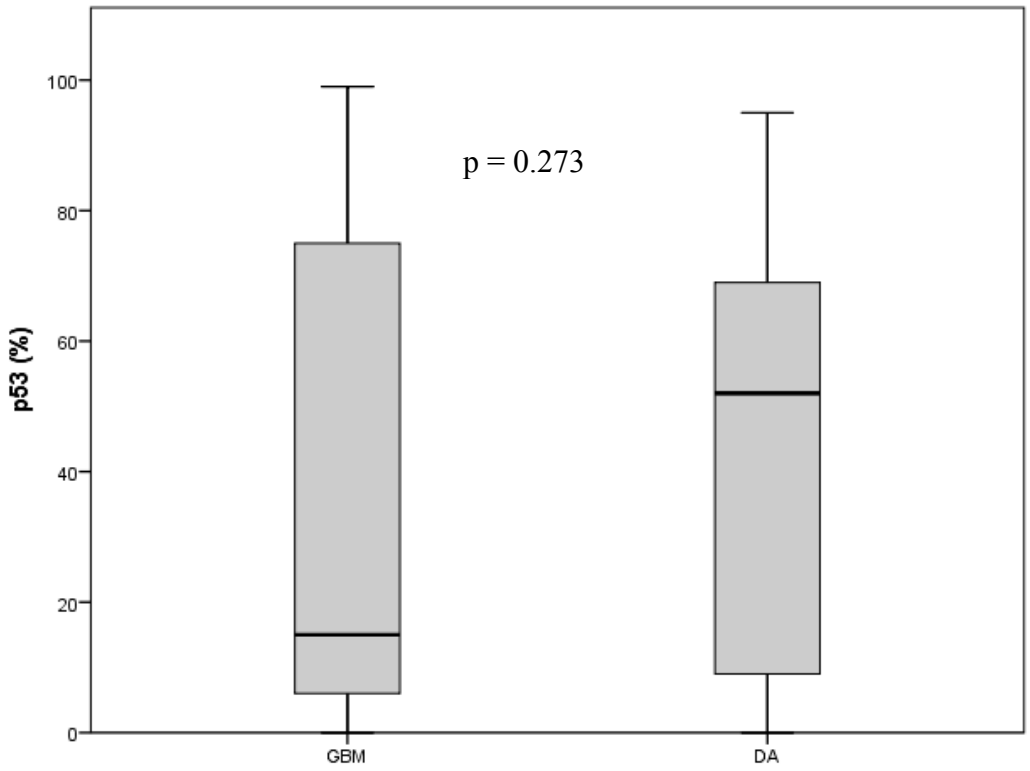


Figure 3.11. **Box plot of p53 expression in GBMs and DAs**

Expression of p21 was significantly more frequent in GBMs than in DAs: 21.2% (95% CI = 18.7–23.6) versus 6.9% (95% CI = 2.4–11.4). Also, using a cut-off of 20%, 49.3% (95% CI = 41.3–57.4) of GBMs and only 15% (95% CI = 5.2–36.0) of DAs had a high 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) versus 86.6% (95% CI = 81.6–91.7). Using a cut-off of 70%, 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. Box plots illustrating and comparing the expression of proteins p21 and p27 in GBMs and DAs are shown in Figure 3.12 and Figure 3.13.

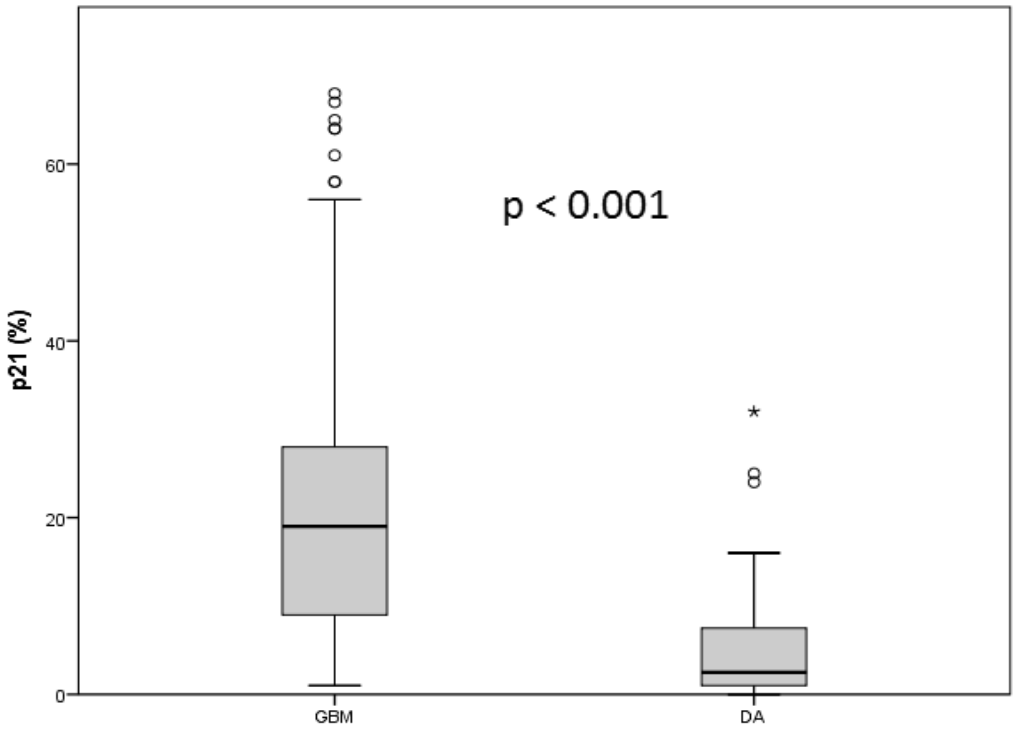


Figure 3.12. **Box plot of p21 expression in GBMs and DAs**

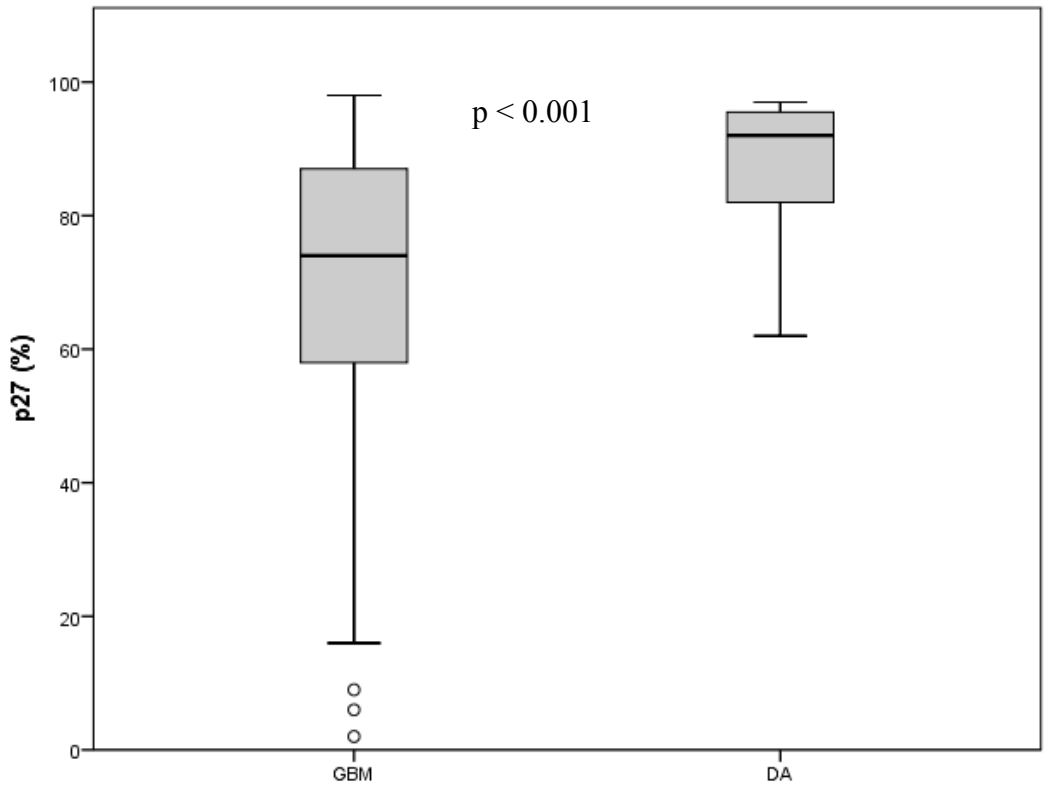


Figure 3.13. **Box plot of p27 expression in GBMs and DAs**

CD44 protein was expressed in a significantly greater percentage of cells in GBMs than in DAs: 74.1% (95% CI = 69.6–78.7) versus 13.5% (95% CI = 7.7–19.2). All cases of GBMs and DAs showed some level of CD44 expression. Strong expression of CD44 in more than 50% of neoplastic cells was found in 81.5% [95% CI = 74.6–87.4] of GBMs compared with only one DA that reached this level of expression. Intense, diffuse membranous expression was the predominant pattern of CD44 immunoreactivity in GBMs. However, the expression of CD44 in DAs was weak and mostly limited to numerous cytoplasmic processes of astrocytes creating a richly branched, delicate network of low CD44 immunoreactivity. In addition to this weak CD44 expression, all DAs had patchy areas with at least a moderate expression level of CD44. A box plot illustrating and comparing the expression of CD44 in GBMs and DAs is shown in Figure 3.14.

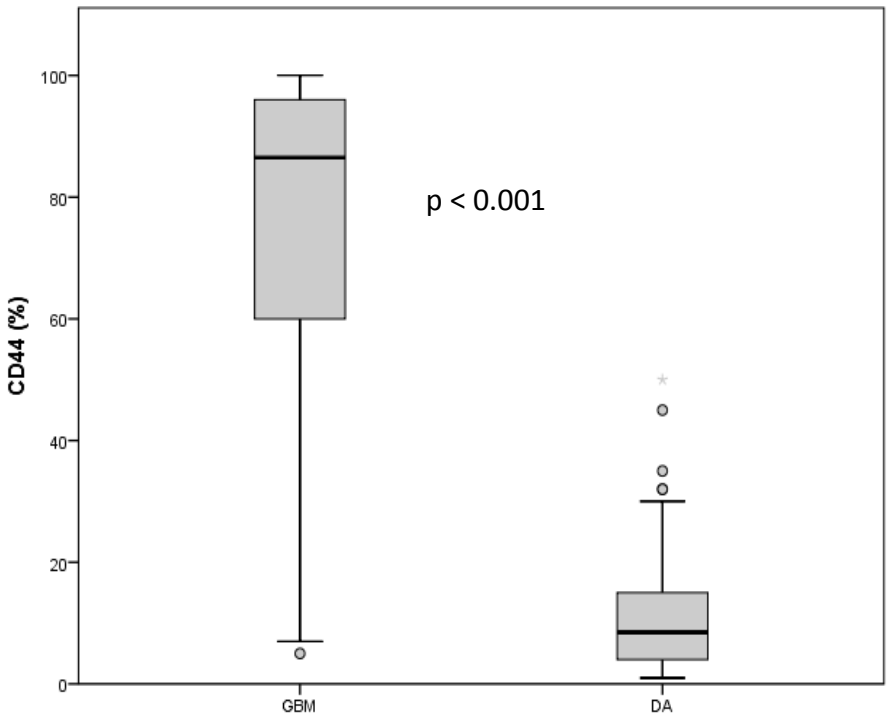


Figure 3.14. Box plot of CD44 expression in GBMs and DAs

Significantly, increased expression of PDGFRA was observed in DAs compared with GBMs ($p < 0.001$). A high PDGFRA protein expression was observed (with a cut-off of 50%) in 6.2% (95% CI = 5.0–10.6) of GBMs and 52.6% (95% of CI = 30.1–75.0) of DAs. With the selected cut-off level of 10%, 80.8% (95% CI = 74.4–87.2) of GBMs were negative for PDGFRA, in contrast to 26.3% (95% CI = 6.5–46.1) of DAs. A focal expression of PDGFRA (10–50 % of cells) was observed in 13.0% (95% CI = 7.5–18.5) of GBMs and 21.1% (95% CI = 2.7–39.4) of DAs. PDGFRA in most cases was expressed in cytoplasm and membrane of

neoplastic cells; however, some rare cases showed nuclear labelling. A box plot illustrating and comparing the expression of PDGFRA in GBMs and DAs is shown in Figure 3.15.

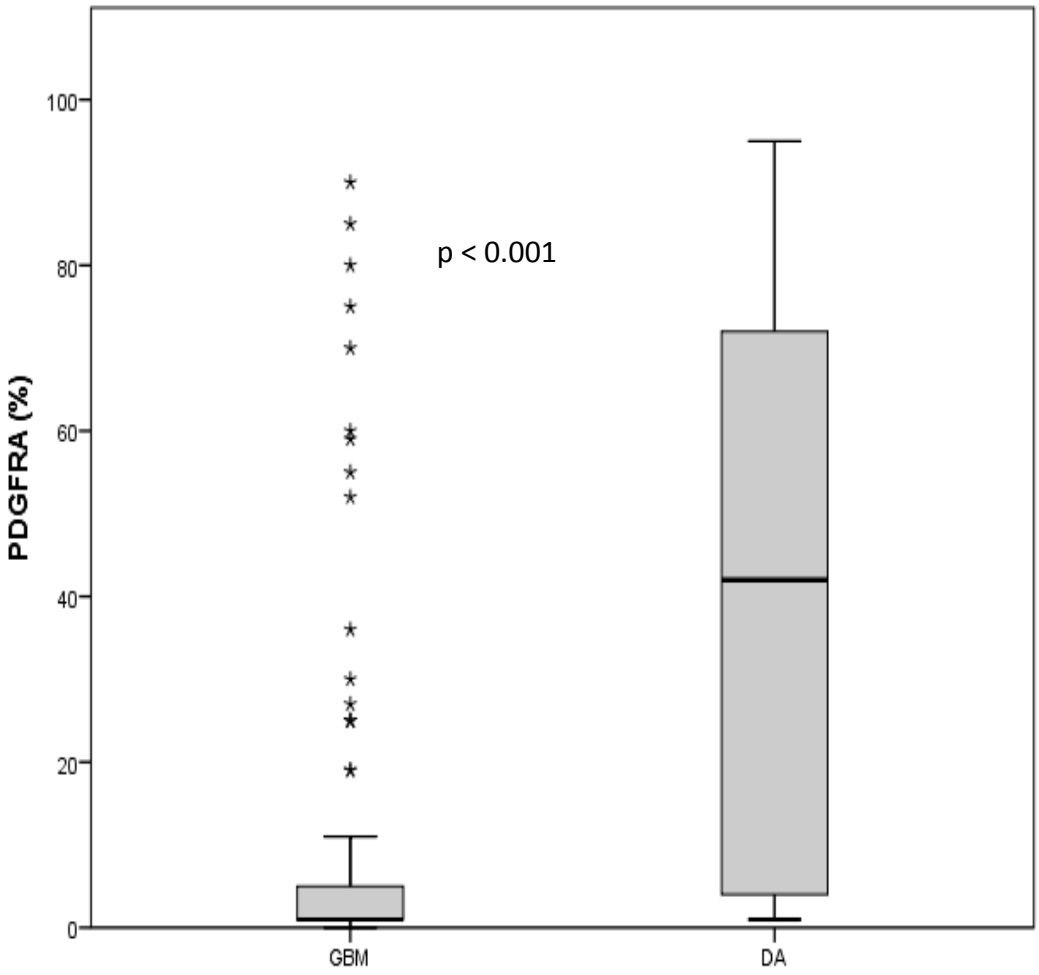


Figure 3.15. Box plot of PDGFRA expression in GBMs and DAs

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 component of lower-grade glioma, thus confirming secondary GBMs on morphological grounds. All IDH1 R132H positive GBMs (n = 5) lacked 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 with primary GBMs (IDH1 R132H negative) – 62.4 (95% CI = 60.7–64.0) years.

The MVD was assessed by CD34 immunostaining to highlight the endothelial cells. In GBMs the MVD ranged from 6 to 130 microvessels per one high-power field. The mean MVD ± SD was 40.7 ± 25.4 (95% CI = 35.8–45.6) and the median value was 35.0 (IQR = 29). In DAs, the MVD ranged from 4 to 49 microvessels per one high-power field. The mean MVD ± SD was 18.1 ± 12.1 (95% CI = 12.9–23.3) and the median value was 13.0 (IQR = 16). Thus,

the MVD in GBMs was significantly higher than in DAs ($p < 0.001$). A box plot illustrating and comparing MVD values in GBMs and DAs is shown in Figure 3.16.

Immunohistochemical results of all evaluated markers are summarized in Table 3.1.

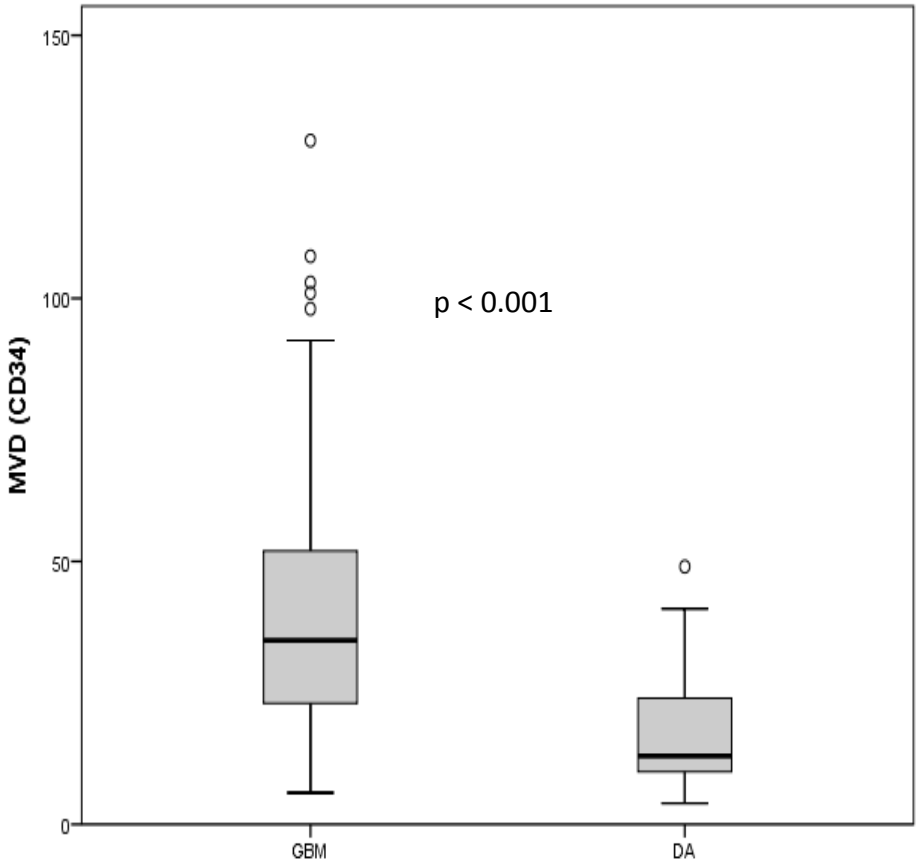


Figure 3.16. **Box plot of MVD values in GBMs and DAs**

Table 3.1.

IHC profile of GBMs and DAs

Variable	GBM	DA
Ki-67		
Number of evaluated cases	126	24
Range	13–95	2–15
Mean ± SD; 95% CI	44.4% ± 18.5; 41.1–47.6	6.4% ± 3.9; 4.7–8.0
Median; IQR	41.0; 24	5.5; 6
High Ki-67 expression; No; %; 95% CI (cut-off 25%)	111; 88.1 81.2–92.6	0; 0 0–10.3
Low Ki-67 expression; No; %; 95% CI (cut-off 25%)	15; 11.9 7.3–18.7	24; 100 86.2–100

Table 3.1 (continued)

Variable	GBM	DA
High Ki-67 expression; No; %; 95% CI (cut-off 41%)	61; 48.4 39.8–57.0	0; 0 0.0–13.8
Low Ki-67 expression; No; %; 95% CI (cut-off 41%)	65; 51.6 42.9–60.1	24; 100 86.2–100
High Ki-67 expression; Number of cases; %; 95% CI (cut-off 3%)	126; 100% 97.0–100	16; 66.7 46.7–82.0
Low Ki-67 expression; No; %; 95% CI (cut-off 3%)	0; 0; 0 0.0–2.0	8; 33.3 17.8–53.2
High Ki-67 expression; No; %; 95% CI (cut-off 5.5%)	126; 100% 97.0–100	12; 50.0 31.4–68.5
Low Ki-67 expression; No; %; 95% CI (cut-off 5.5%)	0; 0; 0 0.0–2.0	12; 50.0 31.4–68.5
p53		
Number of evaluated cases	126	24
Range	0–99	0–95
Mean ± SD; 95% CI	35.3 ± 37.6 28.7–42.0	43.4 ± 31.7 30.0–56.8
Median; IQR	15.0; 71	52.0; 63
0–5 % of p53 positive cells; No; %; 95% CI	30; 23.8 17.2–31.9	6; 25.0 12.0–44.9
6–10 % of p53 positive cells; No; %; 95% CI	25;19.8 13.8–27.6	0; 0 0–13.8
11–50 % of p53 positive cells; No; %; 95% CI	31; 24.6 17.9–33.2	6; 25.0 12.0–44.9
> 50% of p53 positive cells; No; %; 95% CI	40; 31.7 24.2–40.3	12; 50.0 31.4–68.6
High p53 expression; No; %; 95% CI (cut-off 10%)	81; 64.3 55.6–72.1	18; 75 55.1–88
Low p53 expression; No; %; 95% CI (cut-off 10%)	45; 35.7 27.9–44.4	6; 25% 12–44.9
High p53 expression; No; %; 95% CI (cut-off 15%)	67; 53.2 44.5–53.2	17; 70.8 50.8–85.1
Low p53 expression; No; %; 95% CI (cut-off 15%)	59; 46.8 38.3–55.5	7; 29.2 14.9–49.2
High p53 expression; No; %; 95% CI (cut-off 52%)	39; 30.9 23.5–39.5	12; 50% 31.4–68.6
Low p53 expression; No; %; 95% CI (cut-off 52%)	87; 69.1 60.5–76.5	12; 50% 31.4–68.6
CD44		
Number of evaluated cases	146	26
Range	5–100	1–50
Mean ± SD; 95% CI	74.1 ± 27.8 69.6–78.7	13.5 ± 14.3 7.7–19.2
Median; IQR	86.5; 36	8.5; 15
High CD44 expression; No; %; 95% CI (cut-off 50%)	119; 81.5 74.4–86.9	0; 0 0–12

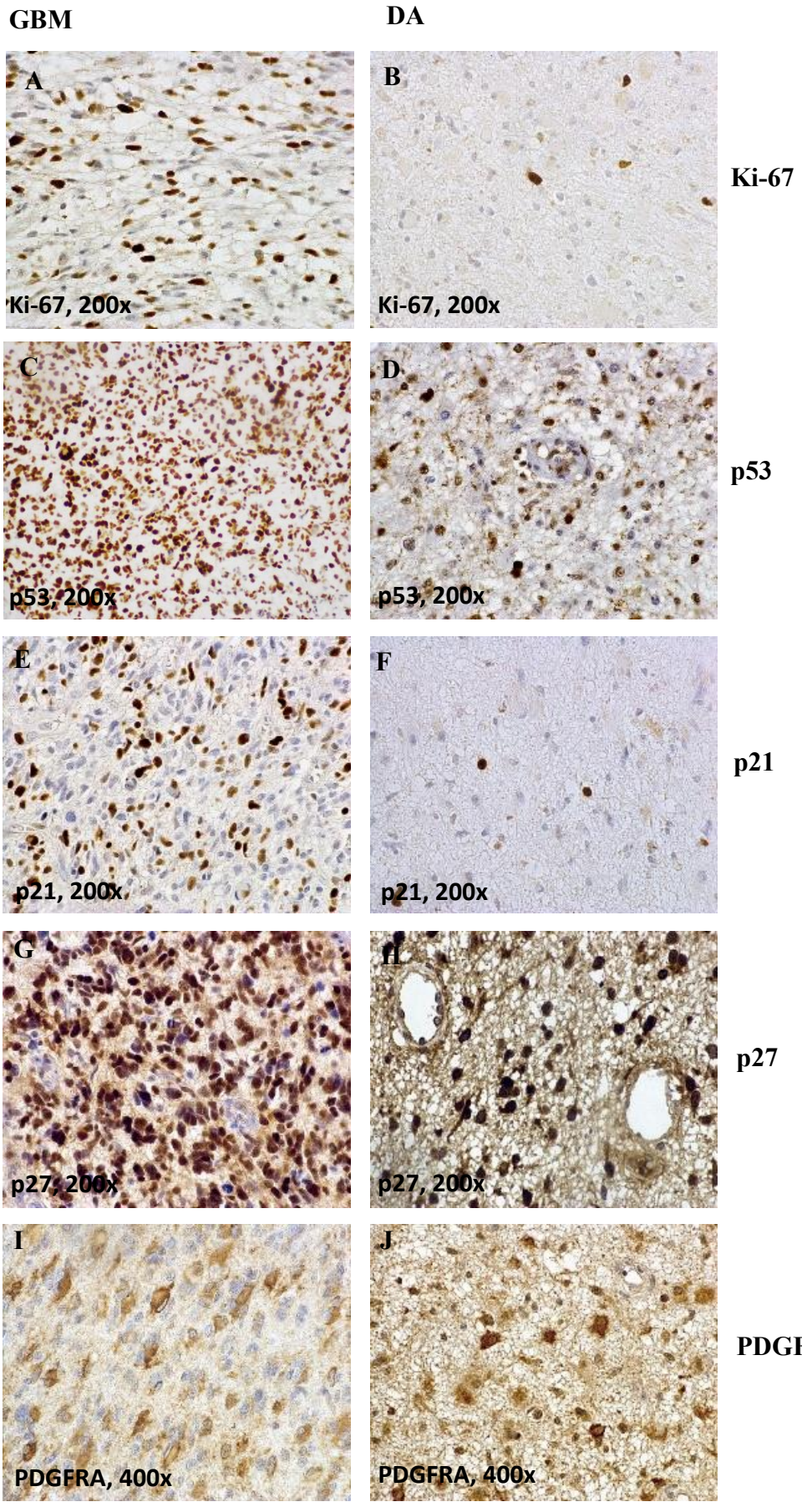
Table 3.1 (continued)

Variable	GBM	DA
Low CD44 expression; No; %; 95% CI (cut-off 50%)	27; 18.5 13.0–25.7	26; 100 87.1–100
High CD44 expression; No; %; 95% CI (cut-off 86%)	75; 51.4 43.3–59.3	0; 0 0–12
Low CD44 expression; No; %; 95% CI (cut-off 86%)	71; 48.6 40.6–56.6	26; 100 87.1–100
High CD44 expression; No; %; 95% CI (cut-off 8%)	144; 98.6 95.1–99.6	14; 53.8 35.4–71.2
Low CD44 expression; No; %; 95% CI (cut-off 8%)	2; 1.4 0–4.8	12; 46.1 28.7–64.5
p21		
Number of evaluated cases	146	20
Range	1–68	0–32
Mean ± SD; 95% CI	21.2 ± 15.0 18.7–23.6	6.9 ± 9.5 2.4–11.4
Median; IQR	19.0; 19	2.5; 7
High p21 expression; No; %; 95% CI (cut-off 20%)	72; 49.3 41.3–57.4	3; 15 5.2–36.0
Low p21 expression; No; %; 95% CI (cut-off 20%)	74; 50.7 52.6–58.6	17; 85 63.9–94.7
High p21 expression; No; %; 95% CI (cut-off 2.5%)	145; 99.3 96.2–99.9	12; 60 38.6–78.1
Low p21 expression; No; %; 95% CI (cut-off 2.5%)	1; 0.7 0–3.7	8; 40 21.8–61.3
p27		
Number of evaluated cases	113	23
Range	2–98	62–97
Mean ± SD; 95% CI	69.7 ± 21.2 65.8–73.7	86.6 ± 11.6 81.6–91.7
Median; IQR	74; 31	92; 17
High p27 expression; No; %; 95% CI (cut-off 70%)	68; 60.1 50.9–68.7	20; 86.9 67.8–95.4
Low p27 expression; No; %; 95% CI (cut-off 70%)	45; 39.9 31.2–49.6	3; 13.1 4.5–32.1
High p27 expression; No; %; 95% CI (cut-off 92%)	11; 9.7 5.2–17.1	12; 52.2 32.9–70.7
Low p27 expression; No; %; 95% CI (cut-off 92%)	102; 90.3 83.4–94.5	11; 47.8 29.2–67.0
PDGFRA		
Number of evaluated cases	146	20
Range	0–90	1–95
Mean ± SD; 95% CI	7.9 ± 17.3 5.0–10.7	42.3 ± 35.5 25.7–59.0
Median; IQR	1.0; 4	42.0; 68

Table 3.1 (end)

Variable	GBM	DA
0–10 % of PDGFRA positive cells; No; %; 95% CI	118; 80.8 74.4–87.2	5; 26.3 6.5–46.1
10–50 % of PDGFRA positive cells; No; % 95% CI	19; 13.0 7.5–18.5	4; 21.1 2.7–39.4
> 50% of PDGFRA positive cells; No; % 95% CI	9; 6.2 2.3–10.1	10; 52.6 30.1–75.0
High PDGFRA expression; No; %; 95% CI (cut-off 50%)	9; 6.1 3.2–11.3	10; 50 29.9–70.1
Low PDGFRA expression; No; %; 95% CI (cut-off 50%)	137; 93.8 88.7–96.7	10; 50 29.9–70.1
High PDGFRA expression; No; %; 95% CI (cut-off 1%)	130; 89.0 82.9–93.1	20;100 83.9–100
Low PDGFRA expression; No; %; 95% CI (cut-off 1%)	16; 11.0 6.8–17.0	0; 0 0–18.1
High PDGFRA expression; No; %; 95% CI (cut-off 42%)	9; 6.2 3.2–11.2	10; 50 29.9–70.1
Low PDGFRA expression; No; %; 95% CI (cut-off 42%)	137; 93.8 88.7–96.7	10; 50 29.9–70.1
IDH1 R132H status		
Number of evaluated cases	146	26
Negative (Nr of cases; %; 95% CI)	141; 96.6 93.7–99.5	6; 23.1 6.9 – 39.3
Positive (Nr of cases; %; 95% CI)	5; 3.4 0.5–6.3	20; 76.9 60.7–93.1
MVD (CD34)		
Number of evaluated cases	107	23
Range	6–130	4–49
Mean ± SD; 95% CI	40.7 ± 25.4 35.8–45.6	18.1 ± 12. 12.9–23.3
Median; IQR	35.0; 29	13.0; 16
High MVD; Number of cases; %; 95% CI (cut-off 35%)	55; 51.4 42.0–60.7	3;1 3.0 4.5–32.1
Low MVD; Number of cases; %; 95% CI (cut-off 35%)	52; 48.6 39.3–57.9	20; 87.0 67.9–95.5
High MVD; Number of cases; %; 95% CI (cut-off 13%)	98; 91.6 84.8–95.5	13; 56.5 36.8–74.4
Low MVD; Number of cases; %; 95% CI (cut-off 13%)	9; 8.4 4.5–15.2	10; 43.5 25.6–63.2

* Abbreviations in the table: NA, not applicable; CI, confidence interval; MVD, microvascular density; PDGFRA, platelet-derived growth factor receptor alpha; IDH1, isocitrate dehydrogenase 1


GBM
DA
A
Ki-67, 200x
B
Ki-67, 200x
Ki-67
C
p53, 200x
D
p53, 200x
p53
E
p21, 200x
F
p21, 200x
p21
G
p27, 200x
H
p27, 200x
p27
I
PDGFRA, 400x
J
PDGFRA, 400x
PDGFRA

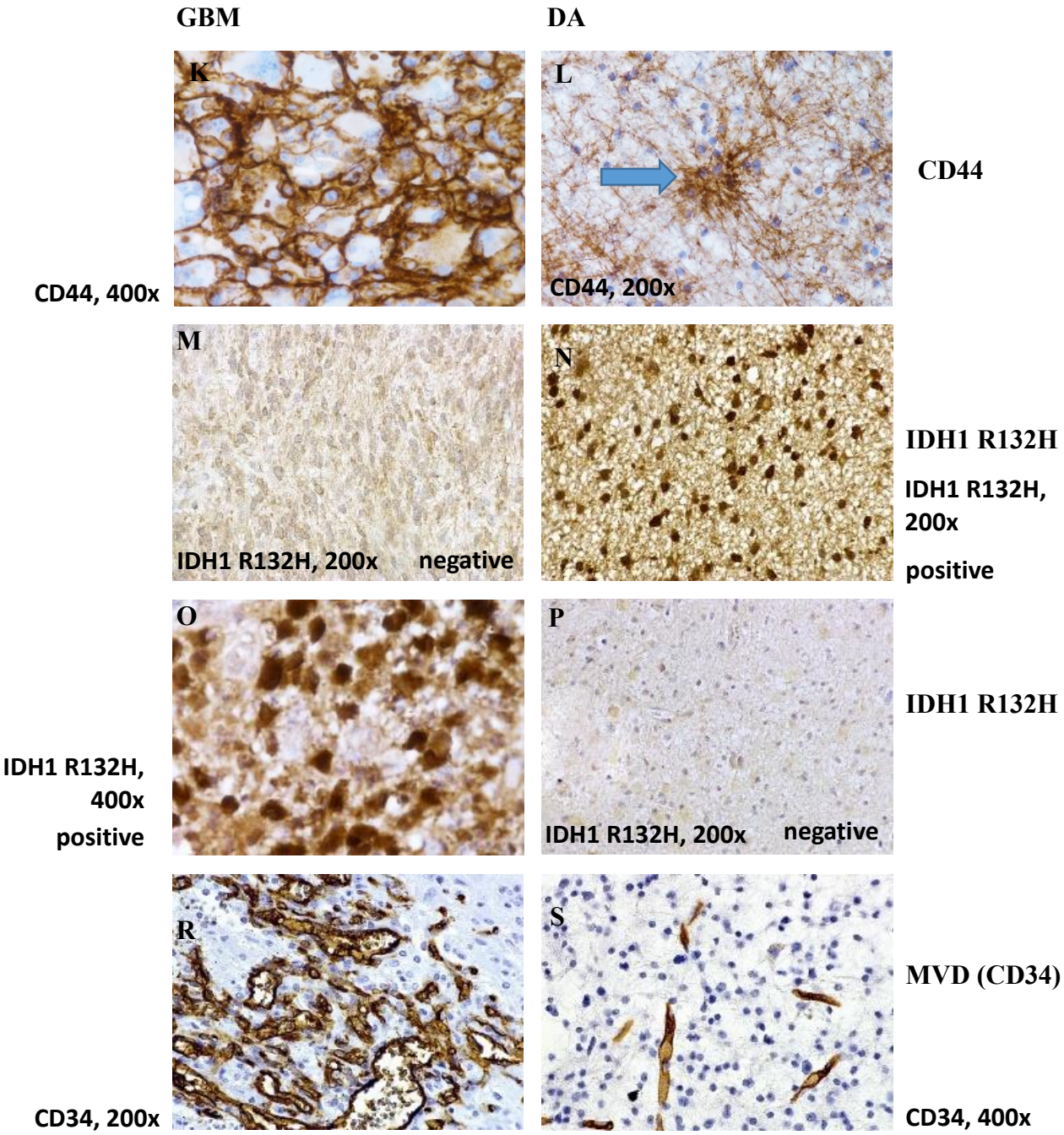


Figure 3.17. **Immunohistochemical visualization of evaluated markers in GBMs and Das**
A and B, Ki-67 proliferation fraction: markedly increased proliferation in GBMs (A) and low proliferation in DAs (B). C and D, nuclear expression of p53 protein: C, in GBM; D, in DA. E and F, nuclear expression of p21 protein: E, in GBM; F, in DA. G and H, nuclear expression of p27 protein: G, in GBM; H, in DA. I and J, cytoplasmic expression of PDGFRA: I, in GBM; J, in DA. K and L, expression of CD44: K, in GBM, note intense, membranous expression; L, in DA, very weak, faint background expression in fibrillary processes of neoplastic cells, and more intense focus of expression is seen (arrow). M–P, status IDH1 R132H mutation by IDH1 R132H immunohistochemistry: M, IDH1 R132H negative or primary GBM; O, IDH1 R132H positive or secondary GBM; N, IDH1 R132H positive DA; P, IDH1 R132H negative DA. K and S, evaluation of MVD by CD34: K, in GBM; S, in DA.

3.4. Associations and correlations between clinical and immunohistochemical variables

3.4.1. Characteristics of GBM cases

3.4.1.1. Associations between clinical variables

A statistically significant association was found between glioma size and tumour localization within certain lobes (Fisher's exact test; p = 0.004). Thus, all GBMs involving multiple lobes were large tumours with a maximum size exceeding 4 cm. Furthermore, the proportion of large tumours was highest in the frontal lobes (ratio 1:5.7) (Figure 3.18).

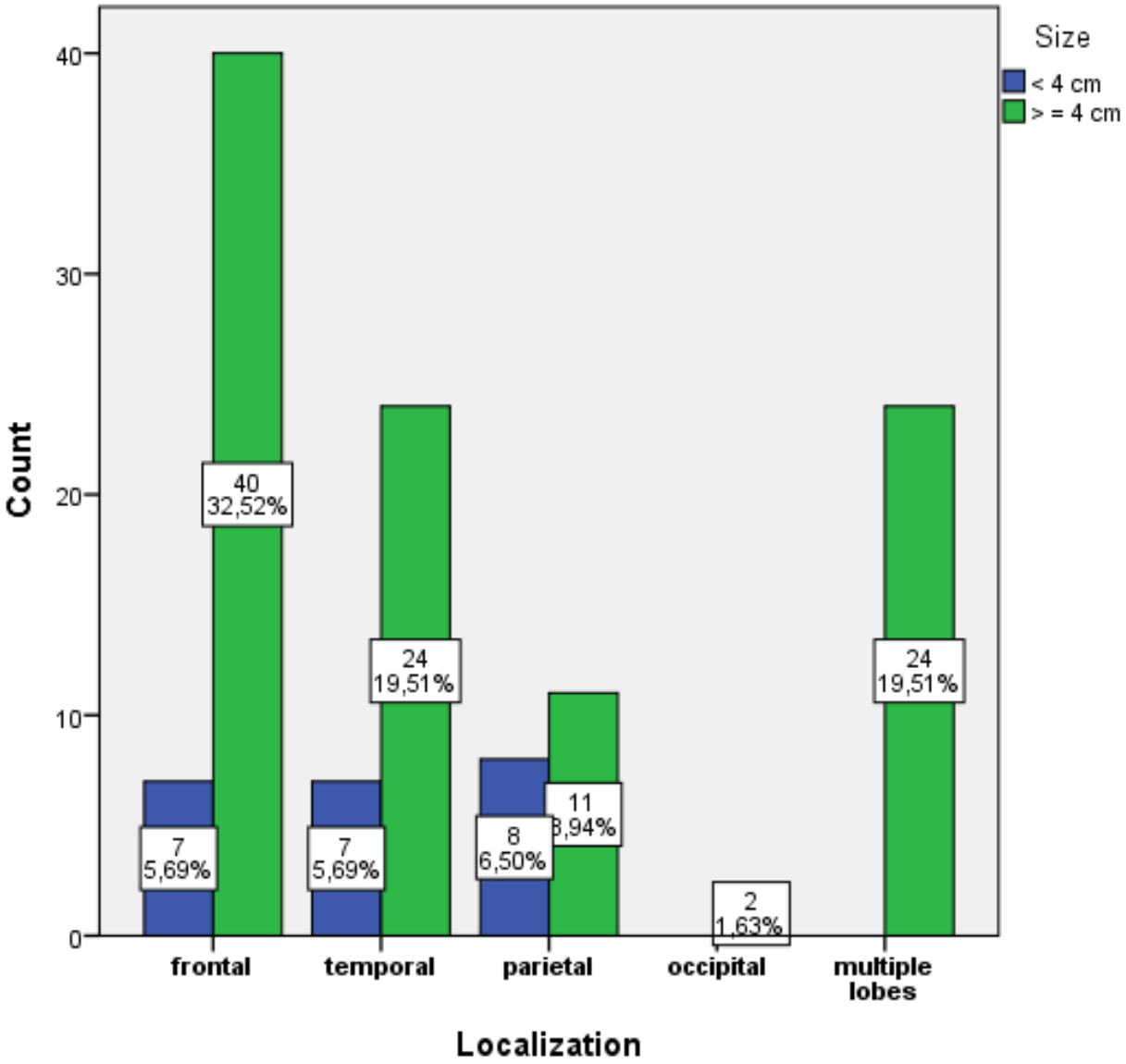


Figure. 3.18. **The relevance between the localization and size of GBMs**

There was a relationship between multifocality and tumour size (Fisher's exact test; p = 0.034). Thus, all multifocal gliomas were large with a maximum size exceeding 4 cm (Figure 3.19). All results of associations and correlations between clinical variables are shown in Table 3.2.

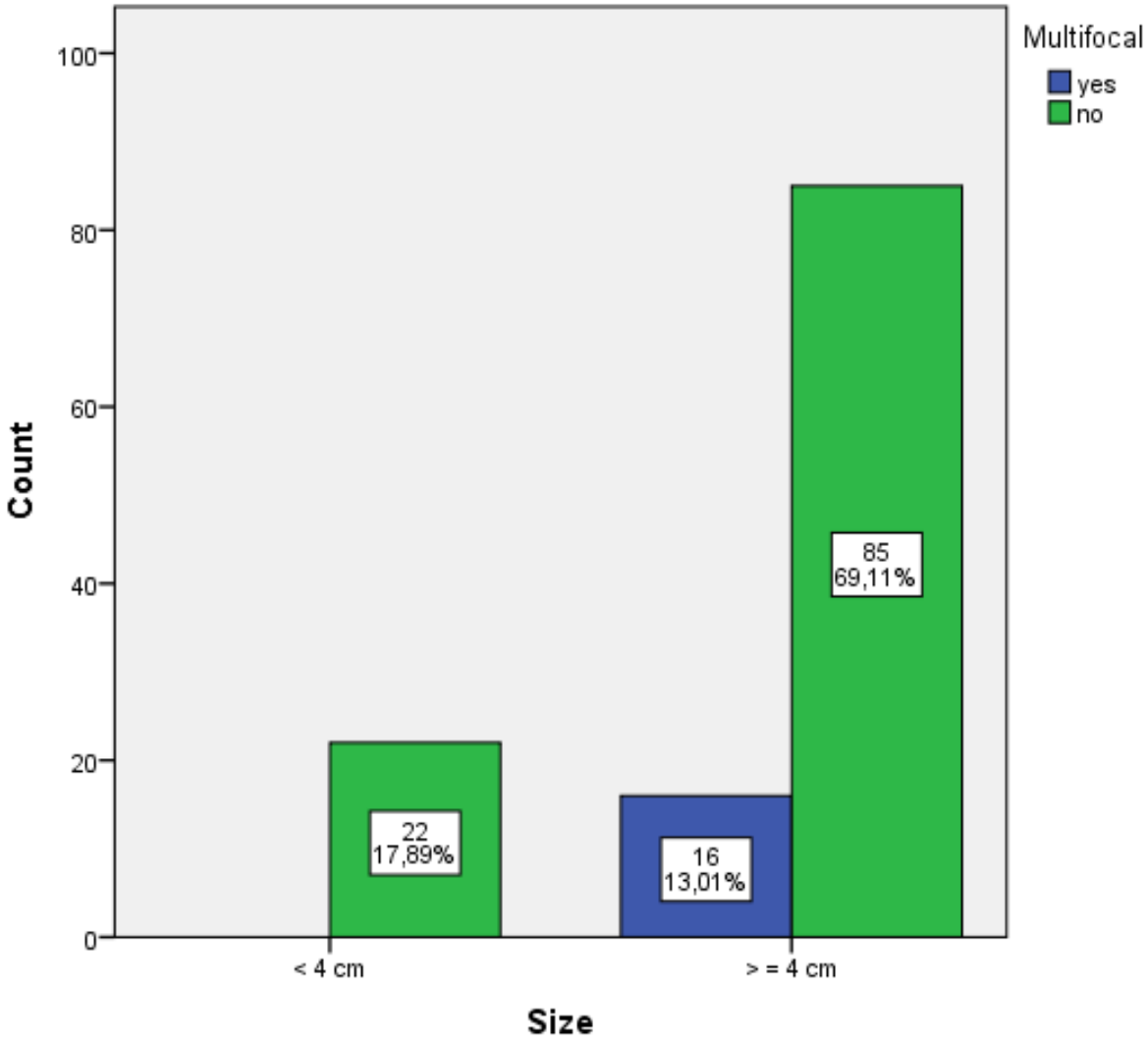


Figure. 3.19. **The relevance between the multifocality and size of GBMs**

Table 3.2.

The associations and correlations between clinical findings in GBMs by Mann-Whitney U, Kruskal-Wallis H, Fisher's exact test and Spearman's rank order correlation

Variables	Age	Gender	Localization (cerebral lobes)	Hemisphere (right, left)	Multifocal (yes, no)	Size (≥ 4 cm or < 4 cm)	Maximum diameter of tumour (cm)
Age	NA	−1.467 0.142	13.560 0.257	0.274 0.872	0.288 0.773	0.191 0.848	−0.004 0.963
Gender	−1.467 0.142	NA	0.352	0.455	0.601	0.356	1.579 0.114

Table 3.2. (continued)

Variables	Age	Gender	Localization (cerebral lobes)	Hemisphere (right, left)	Multifocal (yes, no)	Size (≥ 4 cm or < 4 cm)	Maximum diameter of tumour (cm)
Localization (lobes)	13.560 0.257	0.352	NA	0.221	NA	**0.004**	**8.614** **0.001**
Hemisphere (right, left)	0.274 0.872	0.455	0.221	NA	NA	0.533	0.092 0.953
Multifocal (yes, no)	0.288 0.773	0.418 0.518	NA	NA	NA	**0.034**	−1.356 0.175
Size (≥ 4 cm or < 4 cm)	0.191 0.848	0.356	**0.004**	0.533	**0.034**	NA	NA
Maximum diameter of tumour (cm)	0.004 0.963	1.579 0.114	**8.614** **0.001**	0.092 0.953	−1.356 0.175	NA	NA

* z, r_s and p values are shown in the table. Statistically significant associations and correlations ($p < 0.05$) are marked in bold.

* Abbreviations in the table: NA, not applicable.

3.4.1.2. Associations and correlations between clinical and immunohistochemical variables

No significant associations were found between patient age and any of the evaluated markers, although there was a trend towards a younger age in secondary (IDH1 R132H positive) GBMs (Mann-Whitney U test, z = -1.632; p = 0.060) as depicted in Figure 3.20.

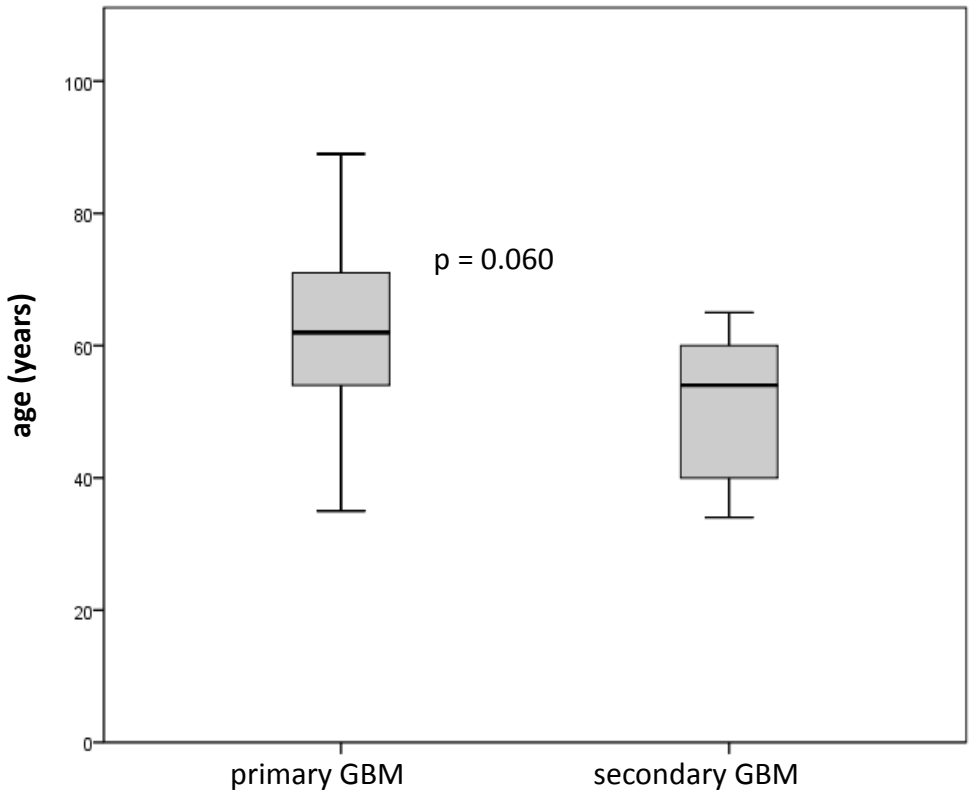


Figure 3.20. **The difference in age in patients with primary and secondary GBMs**

A significant difference was found between the gender and expression of CD44 protein in GBMs. Thus, a significantly higher expression of CD44 was found in females according to the Mann-Whitney test (z = -2.224; p = 0.026) (Figure 3.21). CD44 also showed a weak, significant, negative correlation with GBM size (r_s = - 0.314; p < 0.0001). In addition, higher CD44 expression values were more frequently found in GBMs of a smaller size (< 4 cm) (Mann-Whitney U test, z = -2.364; p = 0.018) as depicted in Figure 3.22. Immunohistochemical visualization of CD44 expression in GBM is shown in Figure 3.23 (A, B).

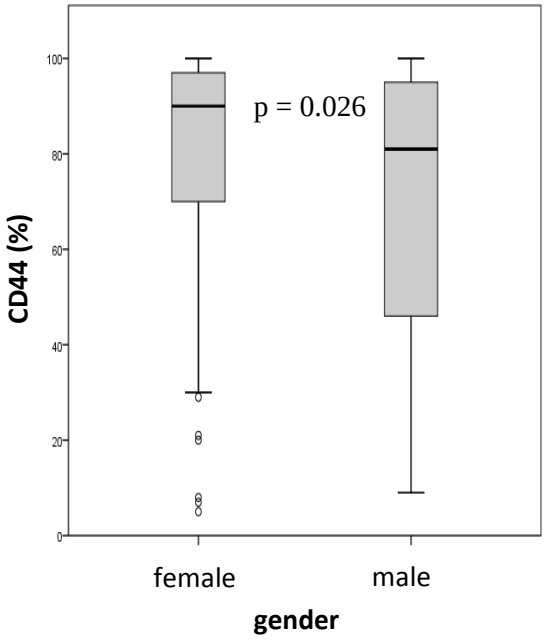


Figure 3.21. **The difference in CD44 expression between patients with GBM by gender**

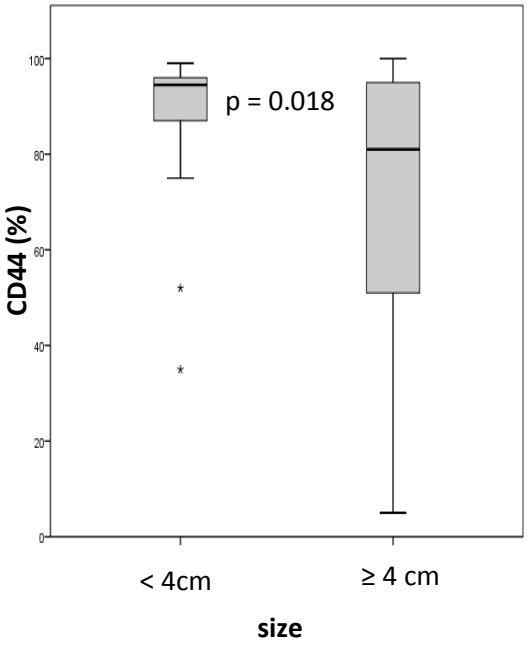


Figure 3.22. **The difference in CD44 expression between patients with GBM by size (< 4 cm versus ≥ 4 cm)**

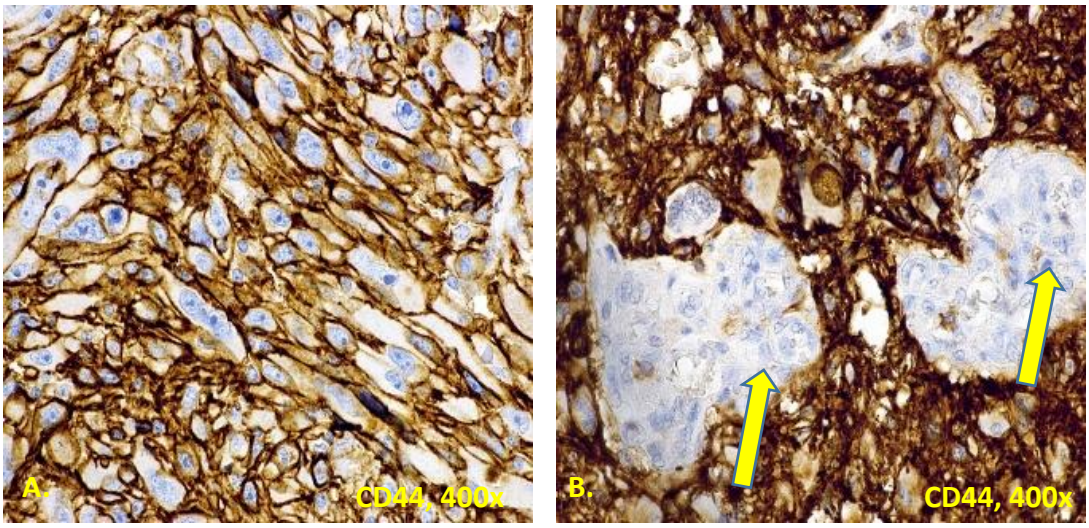


Figure 3.23. **Intense membranous expression of CD44 in GBM. Note prominent, proliferative blood vessels negative for CD44 (internal negative control) (arrows). Immunoperoxidase, anti-CD44, original magnification 400×.**

There was a trend towards lower Ki-67 labelling indices in GBMs in males (Mann-Whitney U test, z = −1.913; p = 0.056) (Figure 3.24). Immunohistochemical visualization of Ki-67 proliferation fraction is shown in Figure 3.28. There was also a weak but statistically significant correlation between Ki-67 and maximum diameter in GBMs (r_s = 0.243; p = 0.013).

p21 protein expression showed a very weak but significant negative correlation with maximum diameter in GBMs (r_s = −0.181; p = 0.045). A higher p21 protein expression was also observed in GBMs of a smaller size (< 4 cm) by the Mann-Whitney U test (z = −2.460; p = 0.014) (Figure 3.25).

p27 protein expression showed a significant difference between gender and multifocality in GBMs. Thus, a higher expression of p27 was observed in males (Mann-Whitney U test; z = −2.174; p = 0.030) and multifocal GBMs (Mann-Whitney U test; z = −2.1; p = 0.037) as depicted in Figures 3.25 and 3.26. Immunohistochemical visualization of p27 expression is shown in Figure 3.29.

Mean ranks of PDGFRA expression had a trend toward a higher expression in multifocal GBMs (mean ranks: 92.34 versus 71.18) (Mann-Whitney U test; z = −1.9; p = 0.049). Immunohistochemical visualization of PDGFRA expression in GBM is shown in Figure 3.30.

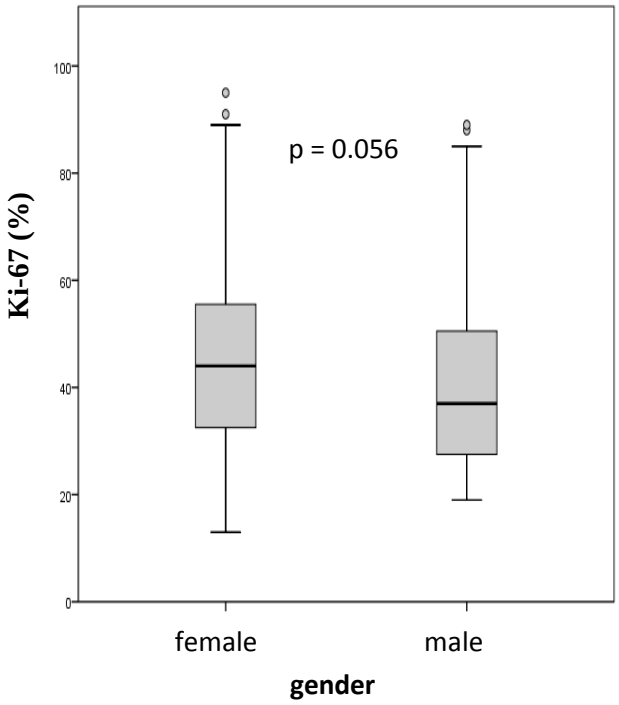


Figure 3.24. **The difference in Ki-67 expression between patients with GBM by gender**

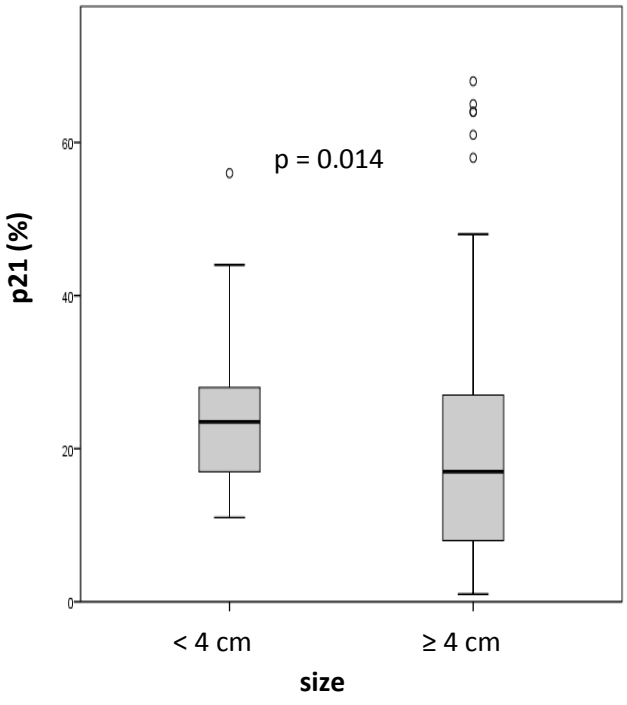


Figure 3.25. **The difference in p21 expression between patients with GBM by size of tumour (< 4 cm versus ≥ 4 cm)**

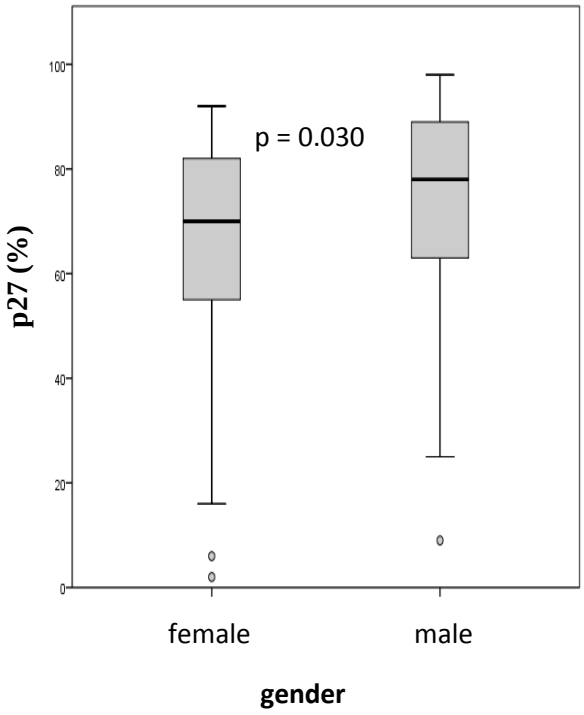


Figure 3.26. **The difference in p27 expression in patients with GBM by gender**

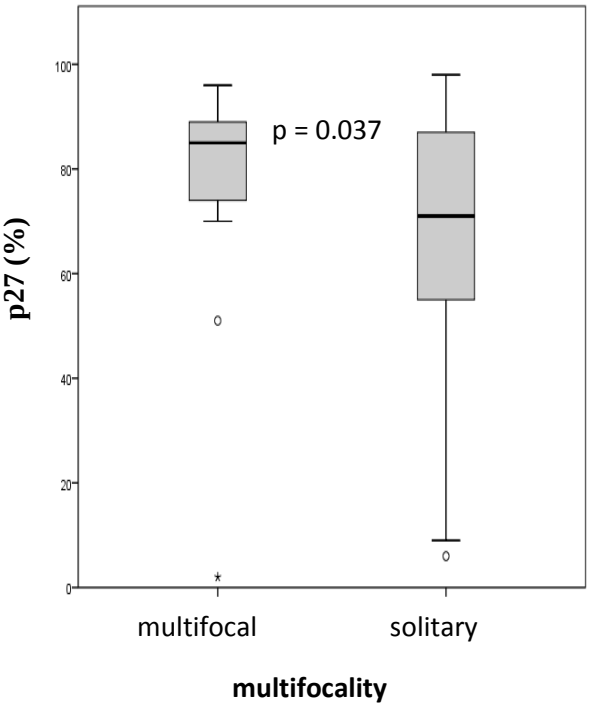


Figure 3.27. **The difference in p27 expression between patients with GBM by multifocality (multifocal vs solitary)**

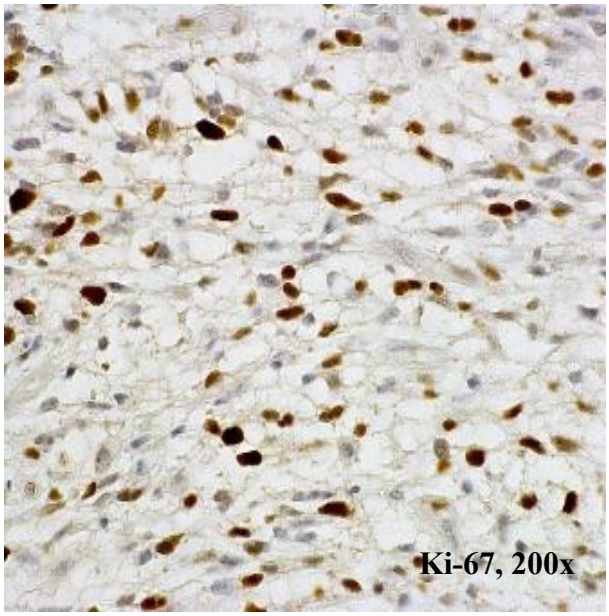


Figure 3.28. **Ki-67 proliferation fraction in GBM. Immunoperoxidase, MIB-1, original magnification 200×.**

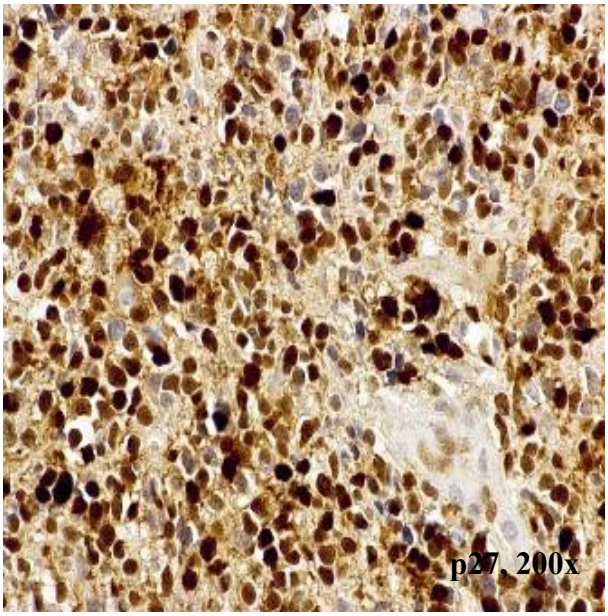


Figure 3.29. **Nuclear expression of p27 in GBM. Immunoperoxidase, anti-p27, original magnification 200×.**

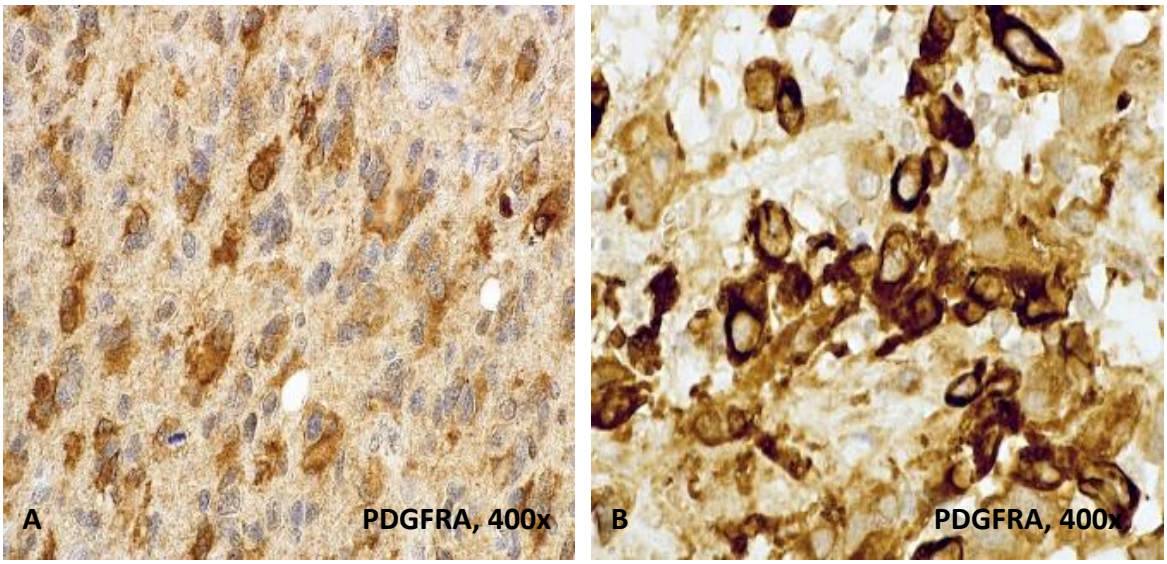


Figure 3.30. **Membranous and cytoplasmic expression of PDGFRA in GBM. Immunoperoxidase, anti-PDGFRA, original magnification 400×.**

Table 3.3.

The associations and correlations between clinical findings and IHC results in GBMs by Mann-Whitney U, Kruskal-Wallis H, Fisher's exact test and Spearman's rank order correlation

	Age	Gender (male, female)	Localization (lobes)	Hemisphere	Multifocal (yes, no)	Size ≥ 4 or < 4 cm	Maximum diameter (cm)
Ki-67	0.100 0.267	**−1.913** **0.056**	4.694 0.320	0.203 0.903	0.281 0.779	−0.722 0.470	**0.243** **0.013**
p53	0.157 0.079	−0.938 0.348	9.954 0.535	0.428 0.669	−1.157 0.247	−0.259 0.796	−0.045 0.646
p21	0.028 0.740	−0.335 0.738	13.737 0.248	0.206 0.837	−0.238 0.812	**−2.460** **0.014**	**−0.181** **0.045**
p27	0.158 0.095	**−2.174** **0.030**	9.775 0.460	0.021 0.983	**−2.087** **0.037**	−0.546 0.585	−0.046 0.654
CD44	0.035 0.671	**−2.224** **0.026**	6.574 0.832	−0.411 0.681	−0.304 0.761	**−2.364** **0.018**	**−0.314** **0.0001**
PDGFRA	0.040 0.632	−0.881 0.378	15.452 0.163	−0.400 0.689	**−1.968** **0.049**	0.034 0.973	−0.079 0.388
IDH1 R132H	**−1.632** **0.060**	0.606	0.047	0.217	0.555	0.367	−0.629 0.529
MVD	0.025 0.797	0.761 0.447	6.571 0.765	−0.363 0.717	−1.037 0.300	−1.329 0.184	−0.085 0.423

* z, r_s and p values are shown in the table. Statistically significant associations and correlations ($p < 0.05$) are marked in bold.

* Abbreviations in the table: NA, not applicable; MVD, microvascular density; PDGFRA, platelet-derived growth factor receptor alpha; IDH1, isocitrate dehydrogenase 1

3.4.1.3. Associations and correlations between the studied immunohistochemical variables

In GBMs, very weak positive correlations between p53 and proliferation fraction by Ki-67 (r_s = 0.196; p = 0.027) and PDGFRA (r_s = 0.181; p = 0.043) were found. A weak positive correlation between p53 and MVD was found (r_s = 0.228; p = 0.031). Ki-67 tended toward a very weak, negative correlation with p27 (r_s = 0.199; p = 0.055). The mean rank of p53 expression was significantly higher in secondary GBMs (IDH1 R132H positive) (Mann-Whitney U test, z = −3.555; p = 0.0001)

The full results of correlations and associations between IHC markers in GBMs are summarized in Tables 3.4 and 3.5.

Table 3.4.

The correlations between IHC markers in GBMs by Spearman's rank order correlation

Variables	Ki-67	p53	CD44	PDGFRA	p21	p27	MVD
Ki-67	NA	**0.196** **0.027**	−0.162 0.070	0.098 0.274	−0.060 0.503	**−0.199** **0.055**	−0.038 0.723
p53	**0.196** **0.027**	NA	−0.073 0.414	**0.181** **0.043**	0.019 0.829	0.037 0.725	**0.228** **0.031**
CD44	−0.162 0.070	−0.073 0.414	NA	−0.141 0.090	0.037 0.659	−0.144 0.128	0.038 0.694
PDGFRA	0.098 0.274	**0.181** **0.043**	−0.141 0.090	NA	−0.152 0.067	−0.056 0.555	0.132 0.176
p21	−0.060 0.503	0.019 0.829	0.037 0.659	−0.152 0.067	NA	0.119 0.211	−0.054 0.580
p27	**−0.199** **0.055**	0.037 0.725	−0.144 0.128	−0.056 0.555	0.119 0.211	NA	−0.121 0.214
MVD	−0.038 0.723	**−0.228** **0.031**	0.038 0.694	0.132 0.176	−0.054 0.580	−0.121 0.214	NA

* r_s and p values are shown in the table. Statistically significant correlations (p < 0.05) are marked in bold.

* Abbreviations in the table: NA, not applicable; MVD, microvascular density; PDGFRA, platelet-derived growth factor receptor alpha; IDH1, isocitrate dehydrogenase 1

Table 3.5.

The associations between IDH1 R132H and other IHC markers in GBMs by Mann-Whitney U test

Variables	Ki-67	p53	CD44	PDGFRA	p21	p27	MVD
IDH1 R132H	−0.656 0.512	**−3.555** **0.0001**	−1.201 0.230	−0.449 0.654	−1.545 0.122	−1.632 0.103	−1.281 0.200

* z and p values are shown in the table. Statistically significant associations (p < 0.05) are marked in bold.

* Abbreviations in the table: MVD, microvascular density; PDGFRA, platelet-derived growth factor receptor alpha; IDH1, isocitrate dehydrogenase 1

3.4.2. Characteristics of DA cases

3.4.2.1. Associations and correlations between clinical variables

No associations and correlations were found between clinical variables in DAs (Table 3.6.). The clinical data about multifocality have not been included in the analysis because such data were not reported for DAs. The size categories (≥ 4 cm or < 4 cm) were also excluded from the analysis because all DAs were large tumours exceeding 4 cm in diameter. Instead of that, the maximum tumour diameters were more appropriate for statistical analysis.

Table 3.6.

The associations and correlations between clinical findings in DAs by Mann-Whitney U, Kruskal-Wallis H, Fisher's exact test and Spearman's rank order correlation

Variables	Age	Gender (male, female)	Localization (cerebral lobes)	Hemisphere	Maximum diameter (cm)
Age	NA	−0.258 0.820	5.047 0.410	0.309 0.781	−0.219 0.473
Gender (male, female)	−0.258 0.820	NA	0.405	0.713	6.805 0.659
Localization (cerebral lobes)	5.047 0.410	0.405	NA	0.486	3.715 0.446
Hemisphere	0.309 0.781	0.713	0.486	NA	21.000 1.000
Maximum diameter (cm)	−0.219 0.473	6.805 0.659	3.715 0.446	21.000 1.000	NA

* z, r_s and p values are shown in the table. Statistically significant associations and correlations ($p < 0.05$) are marked in bold. * Abbreviations in the table: NA, not applicable.

3.4.2.2. Associations between clinical and immunohistochemical variables

There was a statistically significant gender difference in the mean ranks of Ki-67 expression in DAs. Thus, the mean rank of Ki-67 expression was statistically significantly higher in males (mean rank = 16.8) than in females (mean rank = 9.3) in DAs (Mann-Whitney U test, $z = -2.201$; $p = 0.010$) (Figure 3.30).

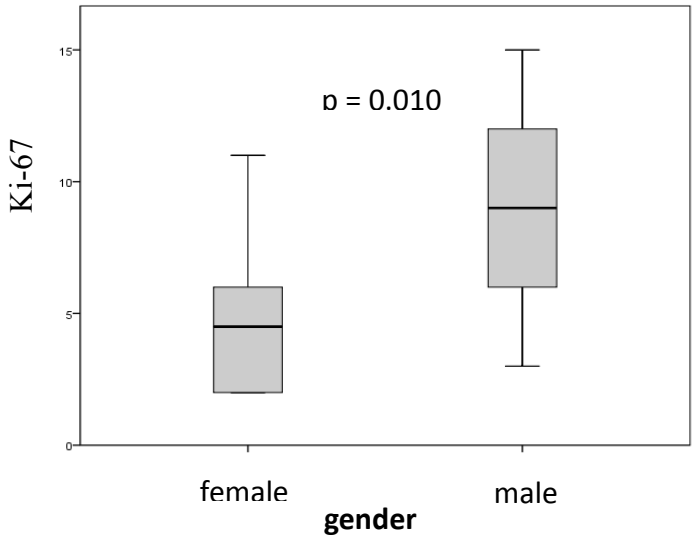


Figure 3.31. **The difference in Ki-67 proliferation fraction between patients with DA by gender**

There was a moderate, positive correlation of p27 with age (r_s = 0.519; p = 0.011). Immunohistochemical visualization of Ki-67 proliferation fraction and p27 expression is shown in Figures 3.31 and 3.32.

The full results of associations between IHC markers and clinical parameters in DAs are summarized in Table 3.7.

Table 3.7.

The associations and correlations between clinical findings in DAs by Mann-Whitney U, Kruskal-Wallis H, Fisher's exact test and Spearman's rank order correlation

Variables	Age	Gender (male, female)	Localization (cerebral lobes)	Hemisphere (right, left)	Maximum diameter of tumour (cm)
Ki-67	0.136 0.526	**2.563** **0.010**	4.486 0.482	−0.583 0.560	−0.380 0.201
p53	0.049 0.819	0.235 0.815	6.838 0.233	1.015 0.310	−0.192 0.529
p21	−0.041 0.865	−0.626 0.531	8.971 0.072	−0.578 0.568	0.550 0.158
p27	**0.519** **0.011**	0.464 0.643	2.140 0.829	−0.358 0.728	−0.244 0.470
CD44	0.148 0.472	0.387 0.699	5.664 0.340	0.697 0.494	0.148 0.678
PDGFRA	−0.157 0.507	−0.476 0.634	4.286 0.369	−1.521 0.131	0.057 0.877
IDH1 R132H	−2.076 0.089	0.653	0.471	0.829	1.043 0.371
MVD	0.049 0.824	0.678 0.498	5.352 0.374	−0.789 0.439	−0.497 0.120

* z, r_s and p values are shown in the table. Statistically significant associations and correlations ($p < 0.05$) are marked in bold.

* Abbreviations in the table: NA, not applicable; MVD, microvascular density; PDGFRA, platelet-derived growth factor receptor alpha; IDH1, isocitrate dehydrogenase 1

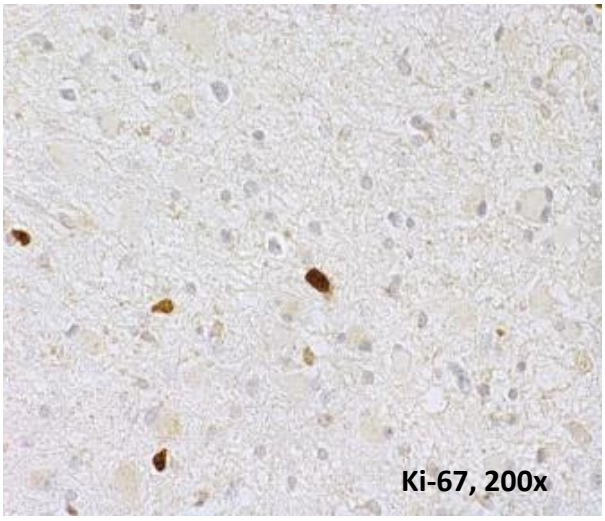


Figure 3.32. **Ki-67 proliferation fraction in DA. Immunoperoxidase, MIB-1, original magnification 200×.**

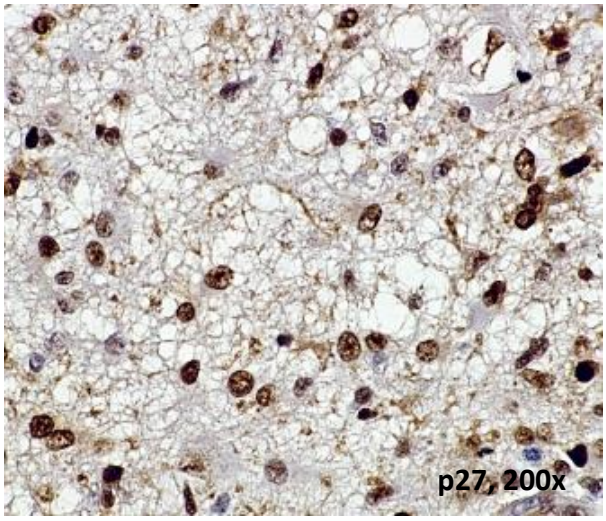


Figure 3.33. **Nuclear expression of p27 in DA. Immunoperoxidase, anti-p27, original magnification 200×.**

3.4.2.3. Associations and correlations between immunohistochemical variables

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, while also reaching moderate strength (r_s = -0.592; p = 0.006). There was also a trend towards an association between PDGFRA expression groups and CD44, thus CD44 mean rank values tend to be higher in negative (Kruskal-Wallis H; p = 0.068) and focally positive tumours (Kruskal-Wallis H; p = 0.053).

There was a strong, negative correlation between PDGFRA and p21 in DAs (r_s = -0.603; p = 0.008). A moderate, negative correlation was also found between PDGFRA and MVD (r_s = -0.501; p = 0.034). p21 had a positive, moderate correlation with MVD in DAs (r_s = 0.458; p = 0.049). The full 3.9. Immunohistochemical visualization of CD44, PDGFRA expression and MVD is shown in Figures 3.33, 3.34 and 3.35.

Table 3.8.

The correlations between IHC markers in DAs by Spearman's rank order correlation

Variables	Ki-67	p53	CD44	PDGFRA	p21	p27	MVD
Ki-67	NA	0.339 0.106	−0.130 0.544	−0.002 0.992	0.146 0.551	0.276 0.226	0.215 0.350
p53	0.339 0.106	NA	−0.382 0.066	**0.544** **0.013**	−0.260 0.282	0.149 0.518	0.274 0.230
CD44	−0.130 0.544	−0.382 0.066	NA	**−0.592** **0.006**	0.170 0.474	0.302 0.162	**0.490** **0.018**
PDGFRA	−0.002 0.992	**0.544** **0.013**	**−0.592** **0.006**	NA	**−0.603** **0.008**	0.149 0.555	**−0.501** **0.034**

Table 3.8. (continued)

Variables	Ki-67	p53	CD44	PDGFRA	p21	p27	MVD
p21	0.146 0.551	−0.260 0.282	0.170 0.474	**−0.603** **0.008**	NA	−0.290 0.229	**0.458** **0.049**
p27	0.276 0.226	0.149 0.518	0.302 0.162	0.149 0.555	−0.290 0.229	NA	0.204 0.350
MVD	0.215 0.350	−0.274 0.230	**0.490** **0.018**	**−0.501** **0.034**	**0.458** **0.049**	0.204 0.350	NA

* r_s and p values are shown in the table. Statistically significant correlations ($p < 0.05$) are marked in bold.

* Abbreviations in the table: NA, not applicable; MVD, microvascular density; PDGFRA, platelet-derived growth factor receptor alpha; IDH1, isocitrate dehydrogenase 1

Table 3.9.

The associations between IDH1 R132H and other IHC markers in DAs by Mann-Whitney U test

Variables	Ki-67	p53	CD44	PDGFRA	p21	p27	MVD
IDH1 R132H	0.973 0.343	−0.234 0.815	−0.794 0.457	−0.568 0.612	1.284 0.230	−1.589 0.112	−0.122 0.907

* z and p values are shown in the table.

* Abbreviations in the table: MVD, microvascular density; PDGFRA, platelet-derived growth factor receptor alpha; IDH1, isocitrate dehydrogenase 1

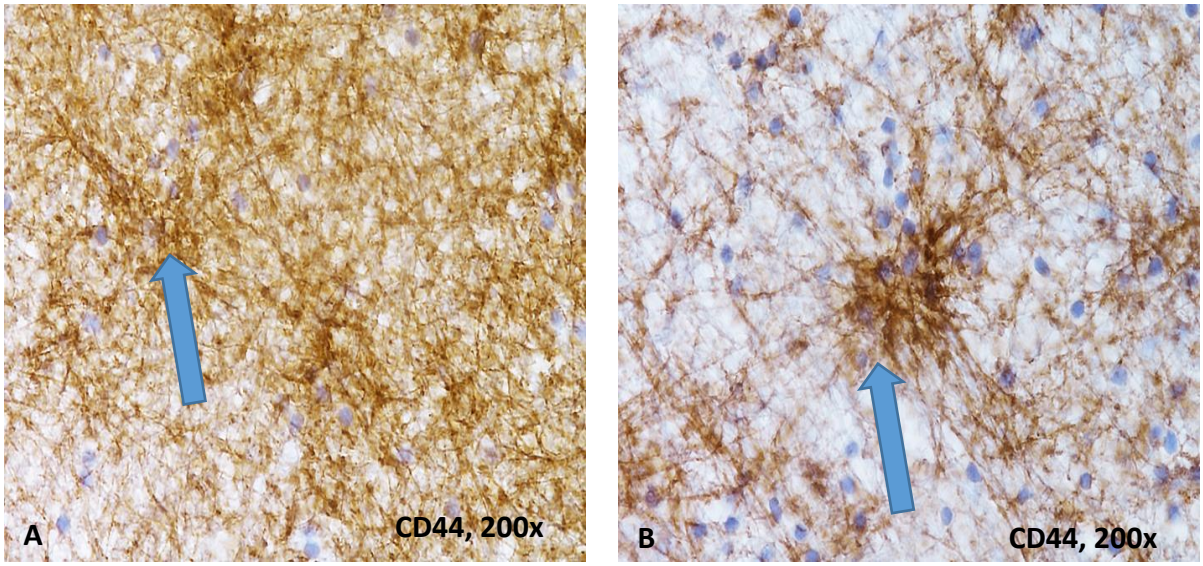


Figure 3.34. **Expression of CD44 in DAs. Weak background expression in fibrillary cytoplasmic processes of astrocytes is seen together with foci of higher expression intensity (arrows). Immunoperoxidase, anti-CD44, original magnification 200×.**

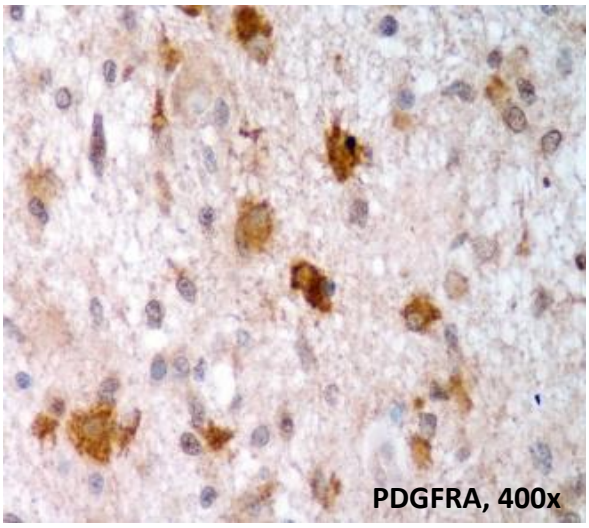


Figure 3.35. **Cytoplasmic expression of PDGFRA in DA. Immunoperoxidase, anti-PDGFRA, original magnification 400×.**

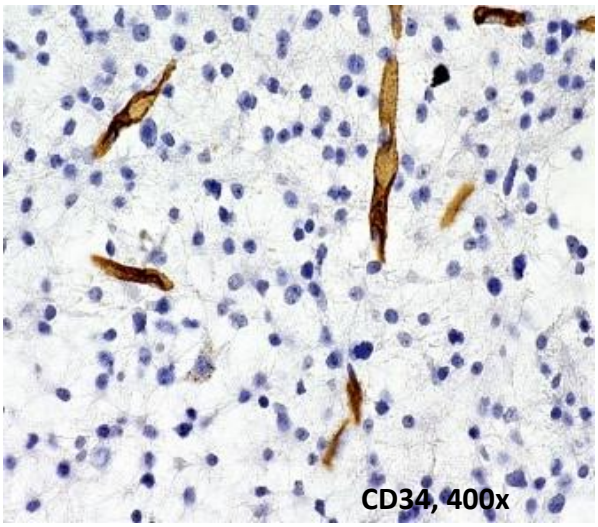


Figure 3.36. **Microvessels in DA. Immunoperoxidase, anti-CD34, original magnification 400×.**

3.5. Survival

3.5.1. Characteristics of GBM cases

The survival data was available for 135 patients that have been included in 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). The survival plot by Kaplan-Meier is shown in Figure 3.36.

Within the first month after their surgical operation, 6/135 (4.5%; 95% CI = 2.0–9.3) patients had died, but 129/135 (95.5%; 95% CI = 90.6–97.9) were alive. Three months after their operation, 30/135 (22.2%; 95% CI = 16.0–29.9) patients had died, but 105/135 (77.8%; 95% CI = 70.0–83.0) were alive. Six months after their operation, 53/135 (39.3%; 95% CI = 31.4–47.7) patients had died, but 82/135 (60.7%; 95% CI = 52.3–68.6) were alive. One year after their operation, 86/135 (63.7%; 95% CI = 55.3–71.3) patients had died, but 49/135 (36.3%; 95% CI = 28.6–44.7) were alive. Two years after their operation, 122/135 (90.4%; 95% CI = 84.2–94.3) patients had died, but 13/135 (9.6%; 95% CI = 5.7–15.8) were alive. Three years after their operation, 133/135 (98.5%; 95% CI = 94.8–99.6) patients had died, but 2/135 (1.5%; 95% CI = 0–5.8) were alive.

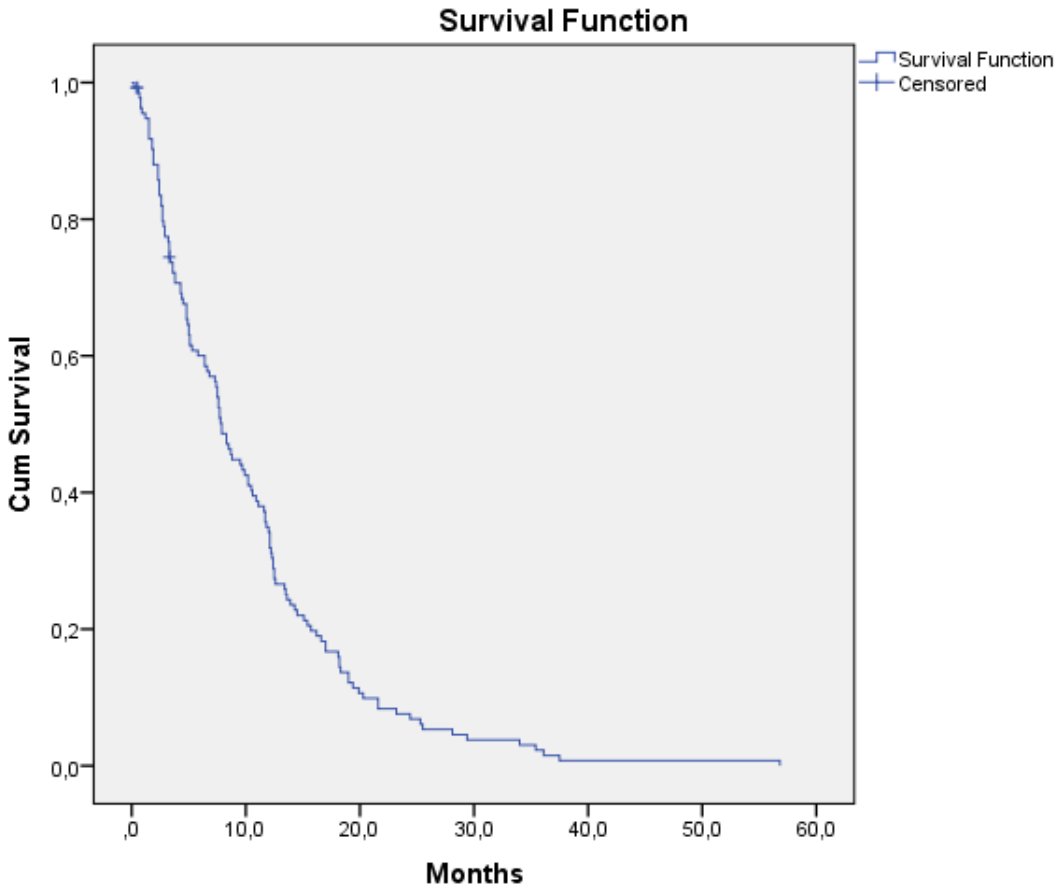


Figure 3.37. **Kaplan-Meier survival plot of patients with GBMs**

3.5.2. Characteristics of DA cases

The survival data was available for 25 patients that have been included in 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) had died during the observation period. Because of the small study group and small number of death cases, statistical calculations are embarrassing and the overall median survival time could not be calculated. The survival plot by Kaplan-Meier is shown in Figure 3.37. As can be seen from the plot, the survival curve does not drop below 0.5.

Within the first year after their surgical operation all patients were alive (25/25). Two years after their 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 their 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.

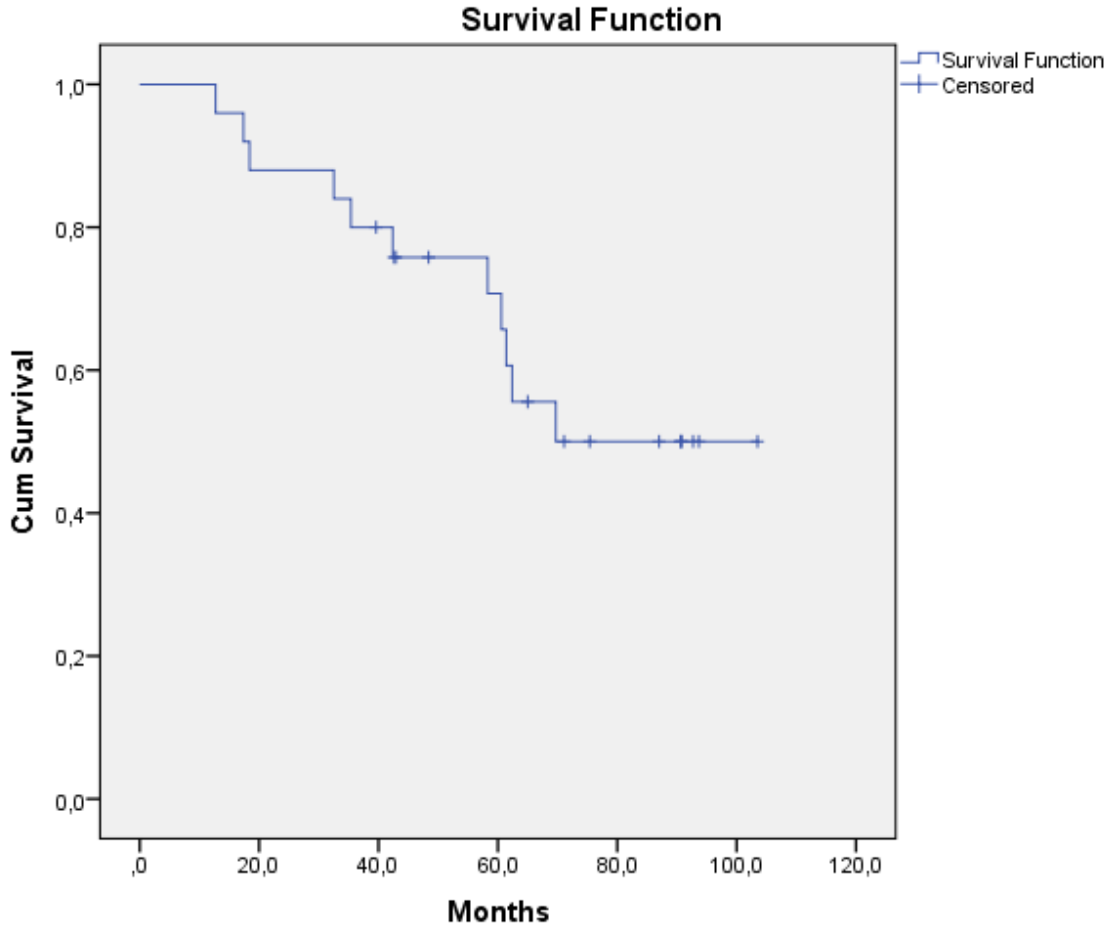


Figure 3.38. **Kaplan-Meier survival plot of patients with DAs**

3.6. Associations between survival and clinical variables

3.6.1. Prognostic characteristics of GBMs

There was a statistically significant difference in overall survival (OS) regarding patient age (log-rank, $p < 0.001$). The median OS of patients ≤ 65 years old was 11.7 (95% CI = 8.1–15.3) months, however the median OS of patients older than 65 years was 5 (95% CI = 3.2–6.8) months. Thus, younger age at diagnosis is associated with a significantly longer survival rate in GBM patients (Figure 3.38).

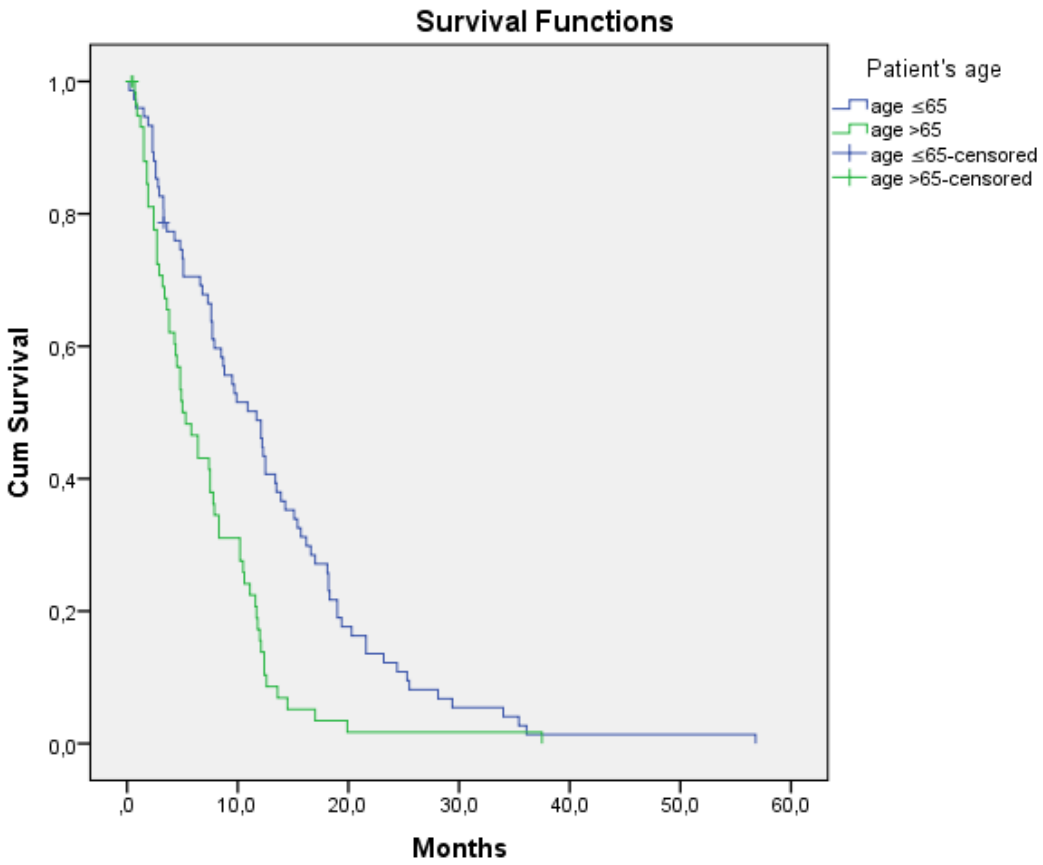


Figure 3.39. **Kaplan-Meier survival curves by patient's age in patients with GBMs**

There was a statistically significant difference in median OS regarding tumour localization (log-rank, p = 0.018). GBMs localized in occipital lobes were excluded from survival analysis because of the rarity of cases (n = 2). There was a tendency toward higher survival rates in GBMs localized in parietal lobes over frontal localization (p = 0.06). GBMs located in multiple lobes also had the worst prognosis. The median OS rates in patients with tumours localized in frontal, temporal or parietal lobes were, respectively, 8.3 (95% CI = 6.7–9.8) months, 7.7 (95% CI = 4.0–11.4) months and 12.6 (95% CI = 10.4–14.8) months. In comparison, the median survival of patients with tumours involving more than one lobe was 6.4 (95% CI = 3.7–9.0) months. The corresponding Kaplan-Meier curves are shown in Figure 3.39.

However, survival differences between cerebral lobes existed only in younger patients (< 65 years), while in older patients (≥ 65 years) there were no survival differences at all. Thus, in patients of a younger age (< 65 years) the survival difference between parietal lobe and frontal lobe was statistically significant (p = 0.018). The corresponding Kaplan-Meier curves comparing the patient age groups are shown in Figure 3.40.

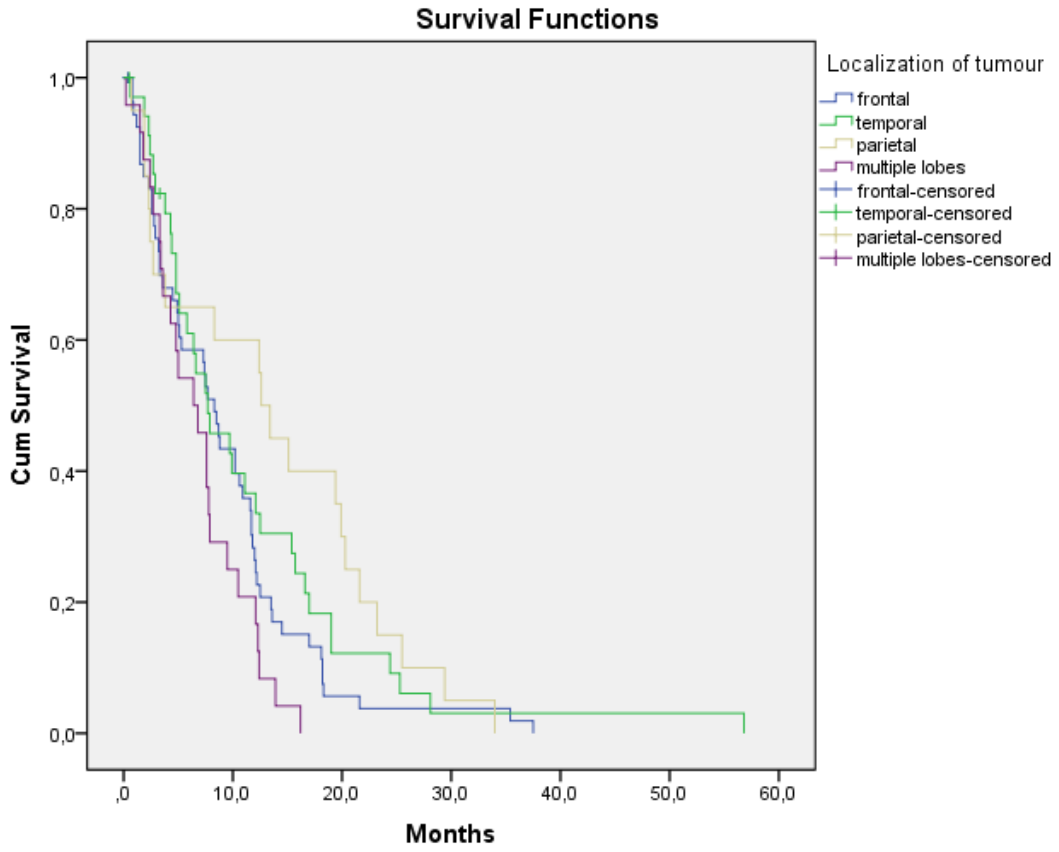


Figure 3.40. **Kaplan-Meier survival curves by localization of tumour in patients with GBMs**

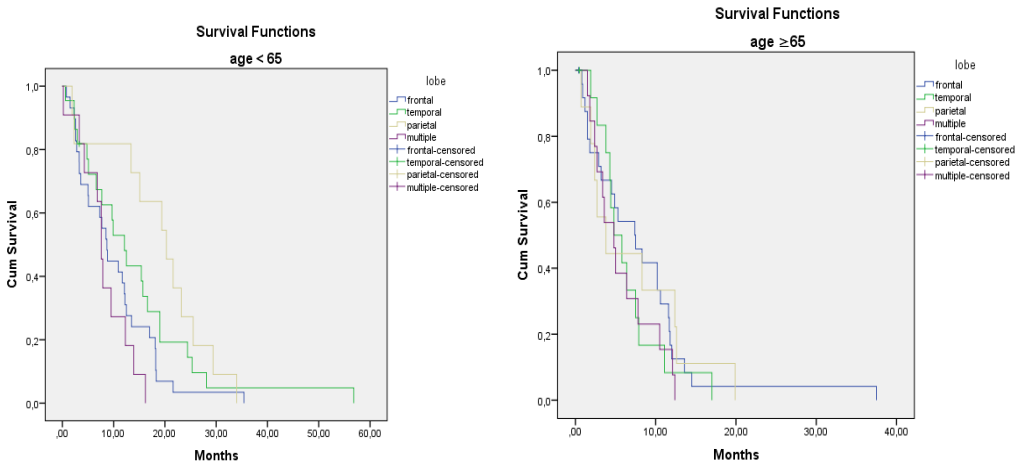


Figure 3.41. **Kaplan-Meier survival curves by localization of tumour in patients with GBMs in different age groups**

There was a statistically significant difference in median OS regarding tumour size (log-rank, p = 0.018). The median OS rates in patients with tumours ≤ 4 cm and > 4 cm were, respectively, 11.8 (95% CI = 8.1–15.5) months and 6.8 (95% CI = 4.7–8.8) months. The corresponding Kaplan-Meier curves are shown in Figure 3.41.

Patients diagnosed with multifocal GBMs had significantly worse survival than those with solitary tumours (log-rank, p = 0.002). The median OS survival in patients with multifocal GBMs was 3.4 (95% CI = 0–6.9) months compared with a median OS of 8.7 (95% CI = 6.8–10.6) months in patients with solitary tumours. The corresponding Kaplan-Meier curves are shown in Figure 3.42.

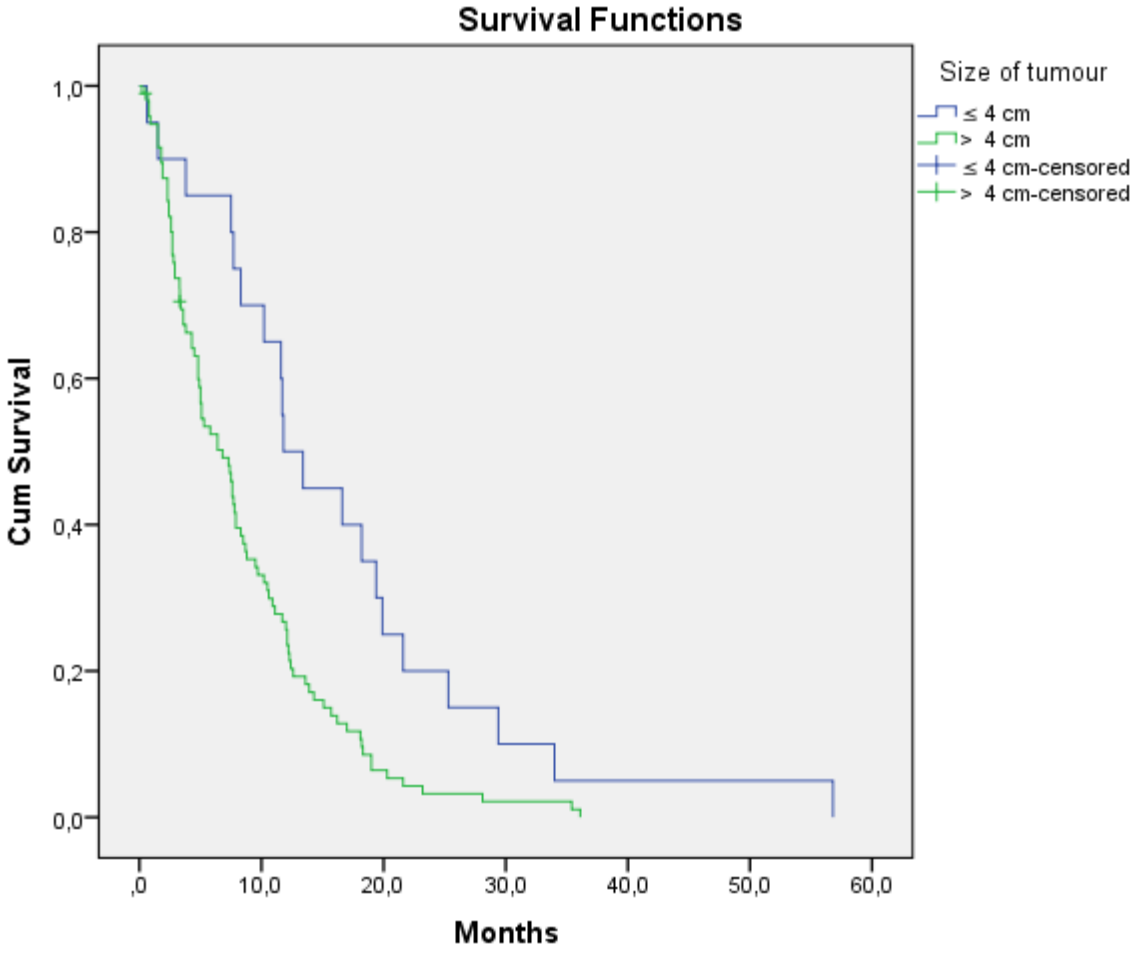


Figure 3.42. **Kaplan-Meier survival curves by size of tumour in patients with GBMs**

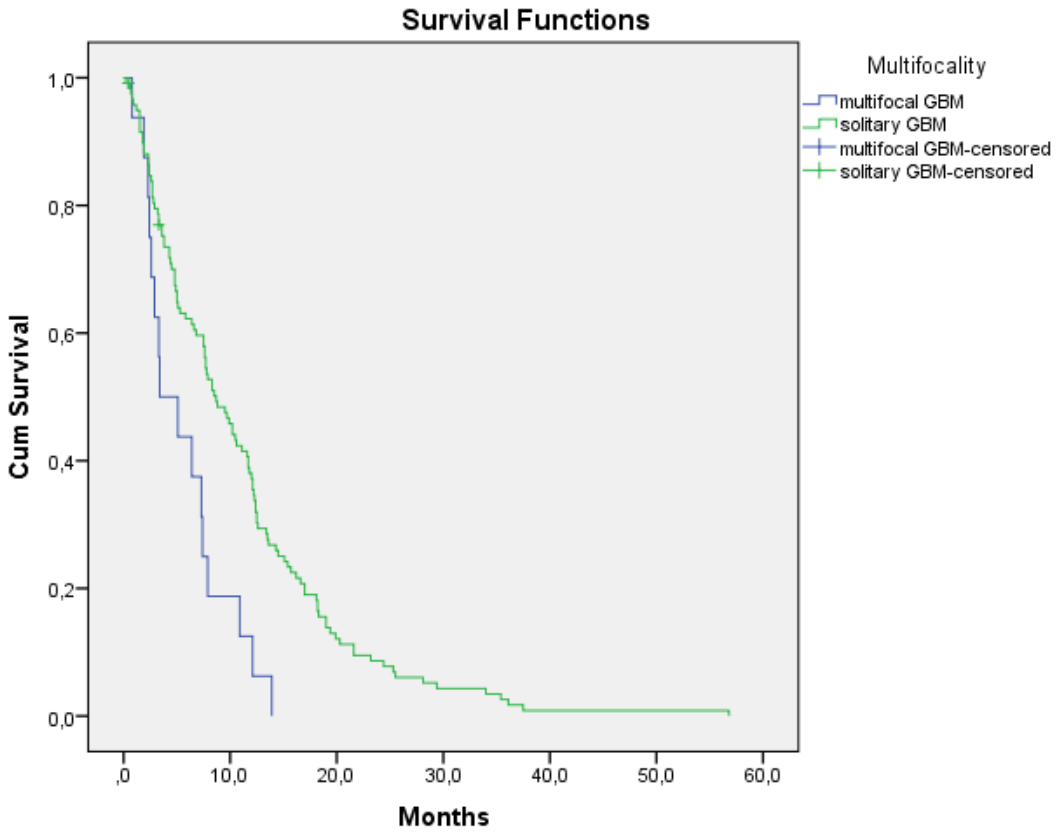


Figure 3.43. **Kaplan-Meier survival curves by multifocal versus solitary tumours in patients with GBMs**

A significant difference in median OS was observed in GBMs by type of treatment (log-rank, $p < 0.001$). Thus, tumours treated with the current standard of care with surgery followed by radiotherapy and chemotherapy with temozolomide had a median OS of 12.1 (95% CI = 11.2–13.0) months, versus surgery plus radiotherapy – 7.5 (95% CI = 5.4–9.6) months, versus surgery only – 2.9 months (95% CI = 1.4–4.4). The corresponding Kaplan-Meier curves are shown in Figure 3.43.

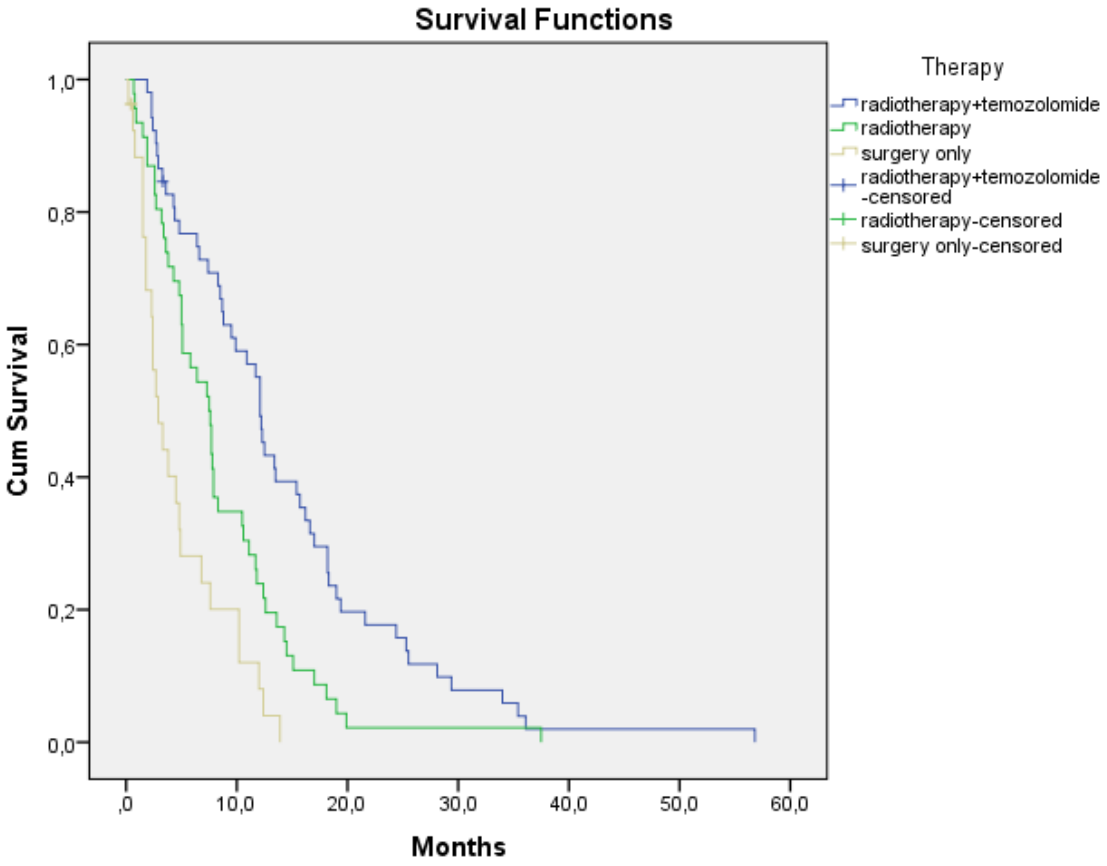


Figure 3.44. **Kaplan-Meier survival curves by therapy in patients with GBMs**

There were no statistically significant survival differences in median OS by gender (p = 0.560) and localization within the left or right cerebral hemisphere (p = 0.876).

3.6.2. Prognostic characteristics of DAs

In DAs, there was a statistically significant difference in survival regarding gender (log-rank, p = 0.002). Females showed better survival than males, as can be seen in Figure 3.44. At the end of the study, 11/13 (84.6%; 95% CI = 57.8% – 95.7%) of the females and only 3/12 (25%; 95% CI = 8.9% – 57.2%) of the males were alive. The median OS of males with DAs was 58.3 (95% CI = 31.1–85) months. The median OS in females cannot be calculated because of the few cases of death (n = 2) and small study group.

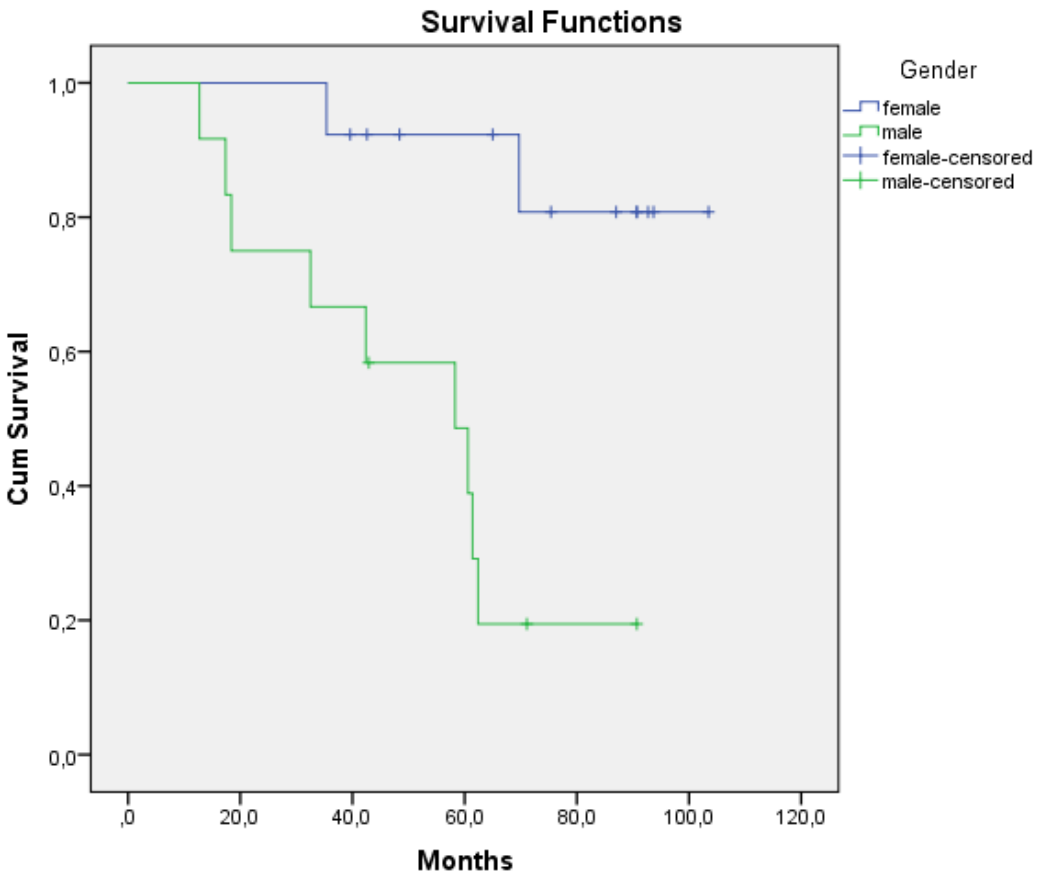


Figure 3.45. **Kaplan-Meier survival curves by gender in patients with DAs**

There were no statistically significant survival differences by localization (p = 0.812) or by hemisphere (right versus left) (p = 0.728) in patients with DAs.

All DAs were large tumours with a size exceeding 4 cm, thus tumour size was not included in survival analysis.

3.7. Associations between survival and immunohistochemical variables

3.7.1. Immunohistochemical prognostic markers in GBMs

In GBMs, a statistically significant survival difference was found in patients regarding IDH1 R132H mutant protein expression (log-rank, p = 0.040). Thus, patients with secondary GBMs (IDH1 R132 positive) had a median OS of 18.3 (95% CI = 18.0–18.5) months versus 7.7 (95% CI = 6.3–9.0) months in patients with primary GBMs (IDH1 R132H negative). The corresponding Kaplan-Meier curves are shown in Figure 3.45.

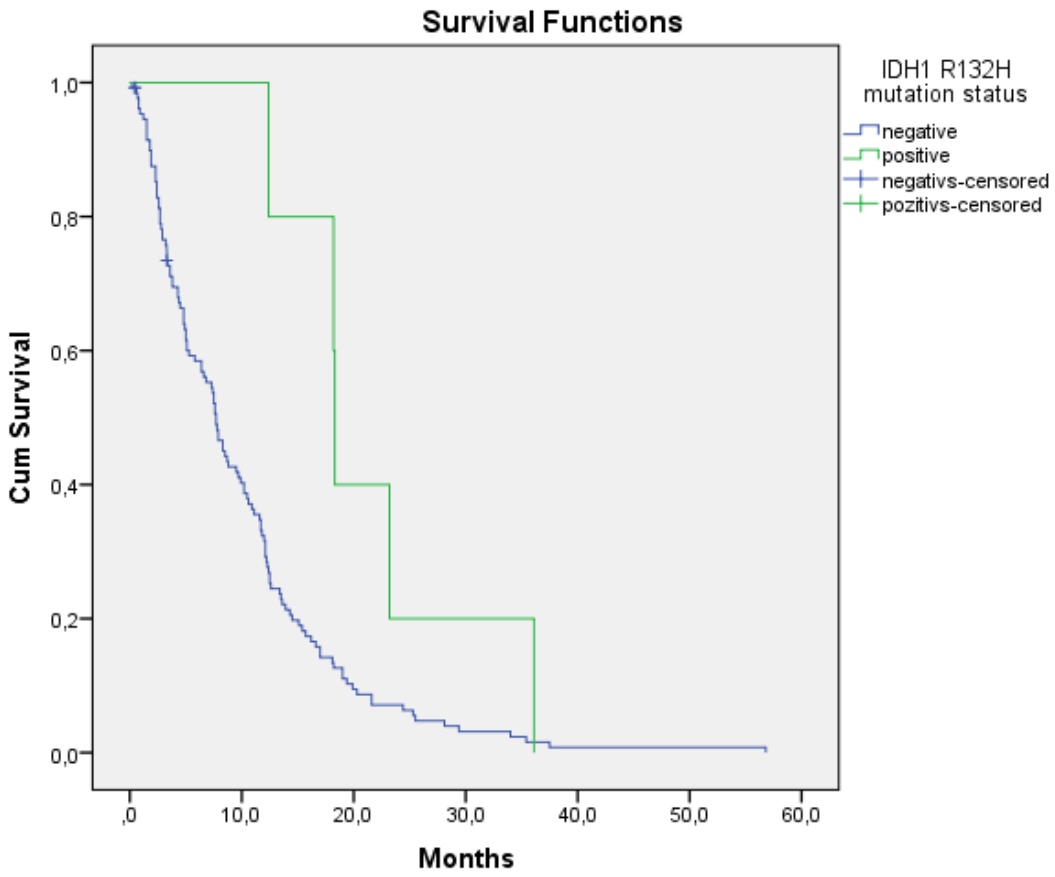


Figure 3.46. **Kaplan-Meier survival curves by IDH1 R132H mutation status in patients with GBMs**

A trend towards a difference in OS was found in patients with GBMs by PDGFRA expression at a cut-off point of 50% (log-rank, p = 0.066). The median OS of patients with high PDGFRA expression was 6.4 (95% CI = 2.8–9.9) months versus 8.3 (6.4–10.1) months in patients with low PDGFRA expression. The corresponding Kaplan-Meier curves are shown in Figure 3.46.

However, if PDGFRA expression is divided into three expression groups (positive, focally positive, negative), the trend towards a difference in OS is lost and the result is not statistically significant (log-rank, p = 0.165). Nonetheless, on examination of the Kaplan-Meier survival plot, a visual tendency toward a difference between negative and positive PDGFRA groups exists. Nevertheless, there was no visual difference between focally positive and negative PDGFRA groups (Figure 3.47).

There were no differences in survival by PDGFRA expression using a cut-off of 1% (cut-off based on median expression value).

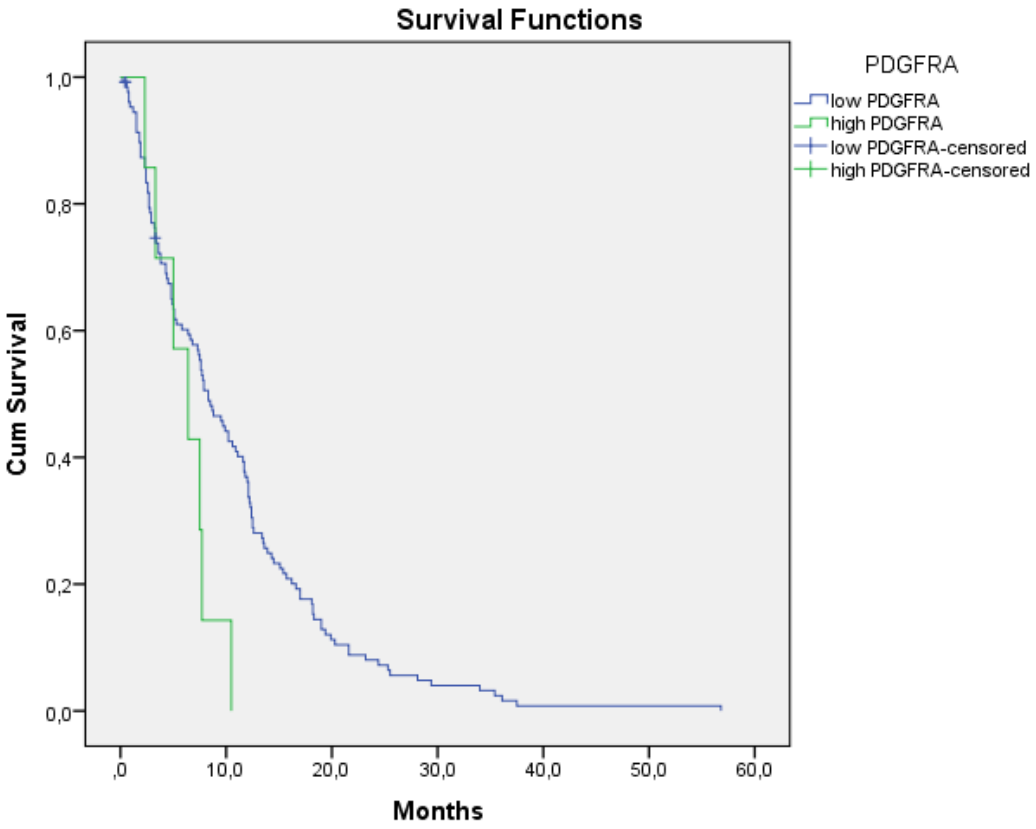


Figure 3.47. **Kaplan-Meier survival curves by PDGFRA expression in patients with GBMs (cut-off value – 50%).**

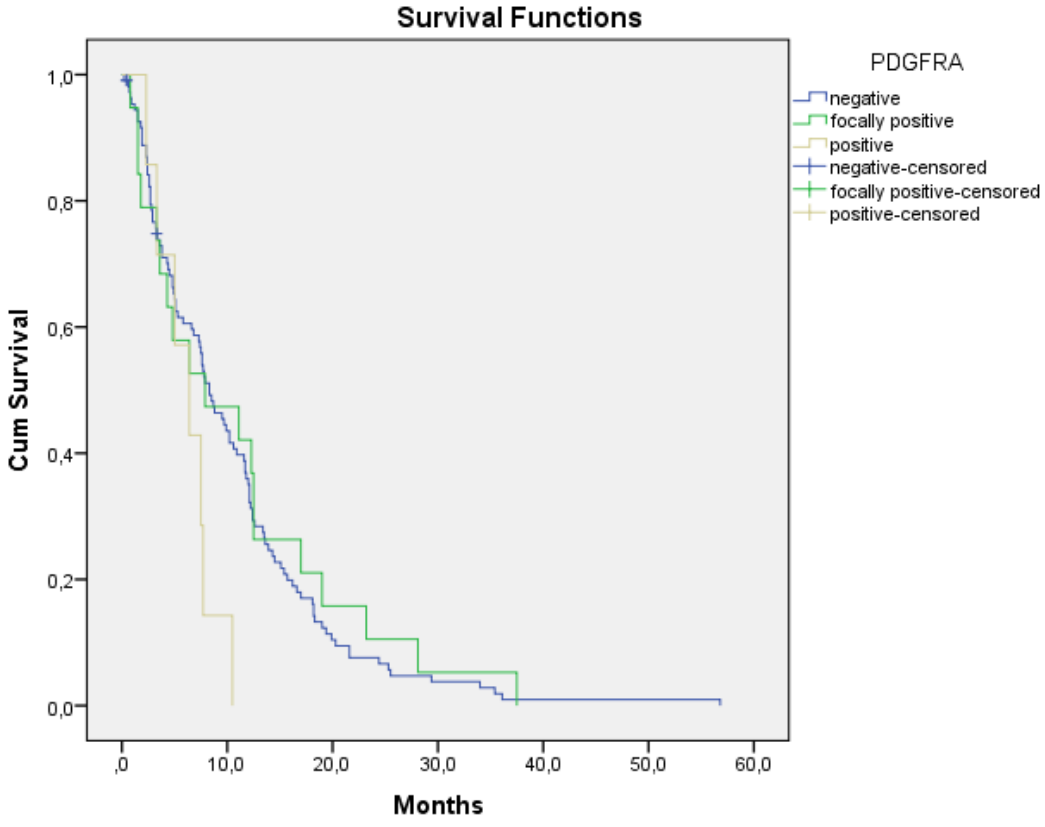


Figure 3.48. **Kaplan-Meier survival curves by PDGFRA expression groups in patients with GBMs**

The proliferation index by Ki-67 showed a statistically non-significant difference with survival (log-rank, p = 0.252). However, using a cut-off of 25% by visual inspection survival curves are different, so the curves overlap in the first five months but diverge thereafter and then cross again after 20 months of follow-up. The median survival of patients with high Ki-67 proliferation indices was 7.4 (95% CI = 5.7–9.1) months versus 13.5 (95% CI = 9.1–17.8) months in patients with low Ki-67 proliferation indices. The corresponding Kaplan-Meier curves are shown in Figure 3.48.

With regard to other immunohistochemical markers, no significant survival differences were found using either cut-off values. All immunohistochemical markers with statistically significant and non-significant survival differences in GBMs are summarized in Table 3.10.

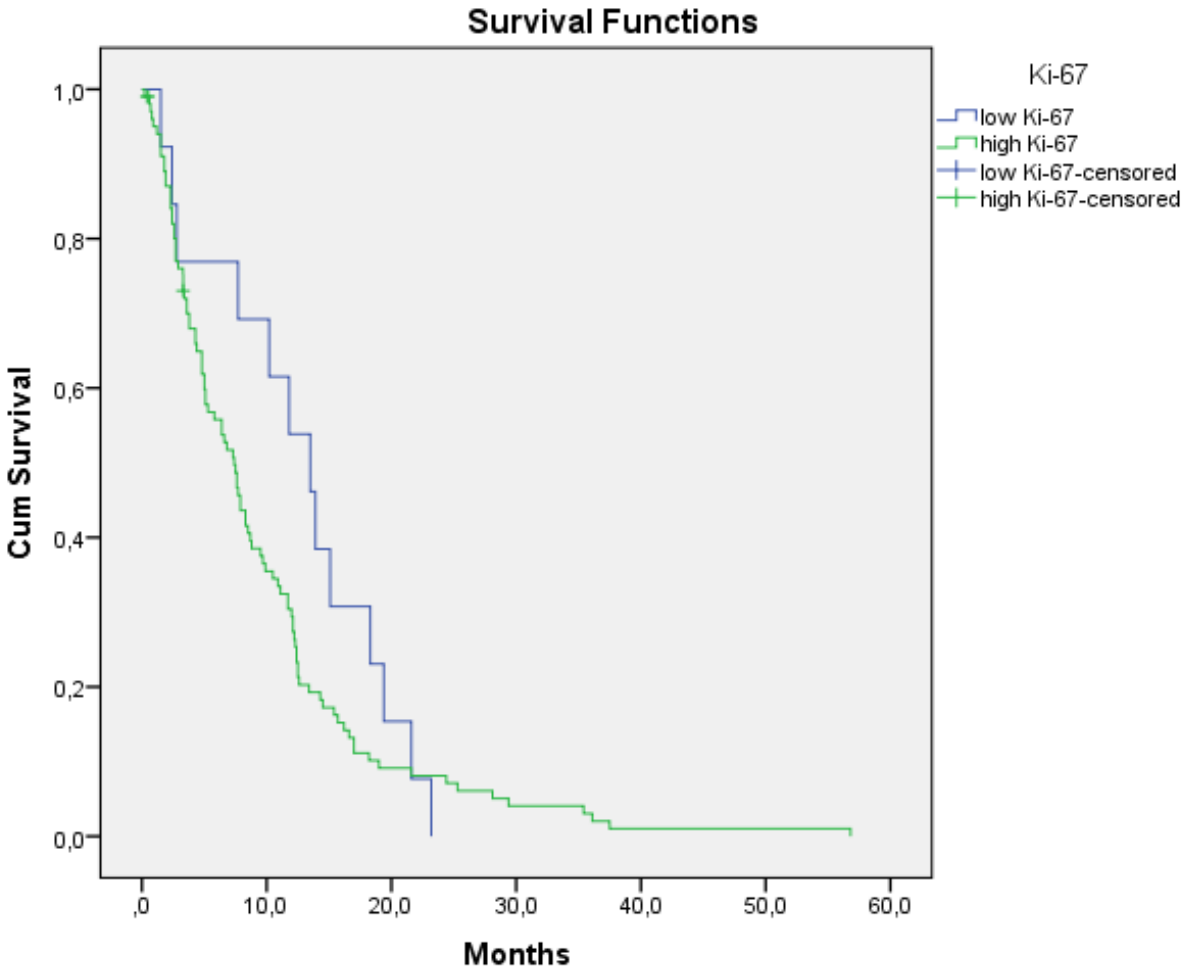


Figure 3.49. **Kaplan-Meier survival curves by Ki-67 expression in patients with GBMs (cut-off value – 25%)**

Table 3.10.

Statistically significant and non-significant survival differences among immunohistochemical markers in GBMs

IHC marker	Cut-off (by literature)	Cut-off (by median)
	p value	p value
IDH1 R132H	**0.040**	NA
Ki-67	0.252	0.454
p53	0.831	0.873
p21	0.464	0.464
p27	0.715	0.715
CD44	0.952	0.772
PDGFRA	0.066	0.981
CD34	NA	0.940

* Abbreviations in the table: NA, not applicable; IHC, immunohistochemistry

3.7.2. Immunohistochemical prognostic markers in DAs

In DAs, a statistically significant survival difference was found in patients regarding Ki-67 expression using a cut-off of 5.5% (log-rank, p = 0.037). A high Ki-67 proliferation index (≥ 5.5%) was present in 12/23 (52.2%; 95% CI = 33.0–70.8) cases of DAs. A low Ki-67 proliferation index (< 5.5%) was present in 11/23 (47.8%; 95% CI = 29.2–67.0) DA cases. At the end of the study, 7/12 (58.3%; 95% CI = 31.9–80.7) of the patients had died within the tumour group with high Ki-67 proliferation indices compared with 2/11 (18.2%; 95% CI = 5.1–47.7) of the patients that had died in the tumour group with low Ki-67 proliferation indices. The median OS in patients with high Ki-67 expression was 60.6 months (95% CI = 35.3–85.9). The median OS in patients with low Ki-67 expression could not be calculated because of the few cases of death and small study group. The corresponding Kaplan-Meier curves are shown in Figure 3.49.

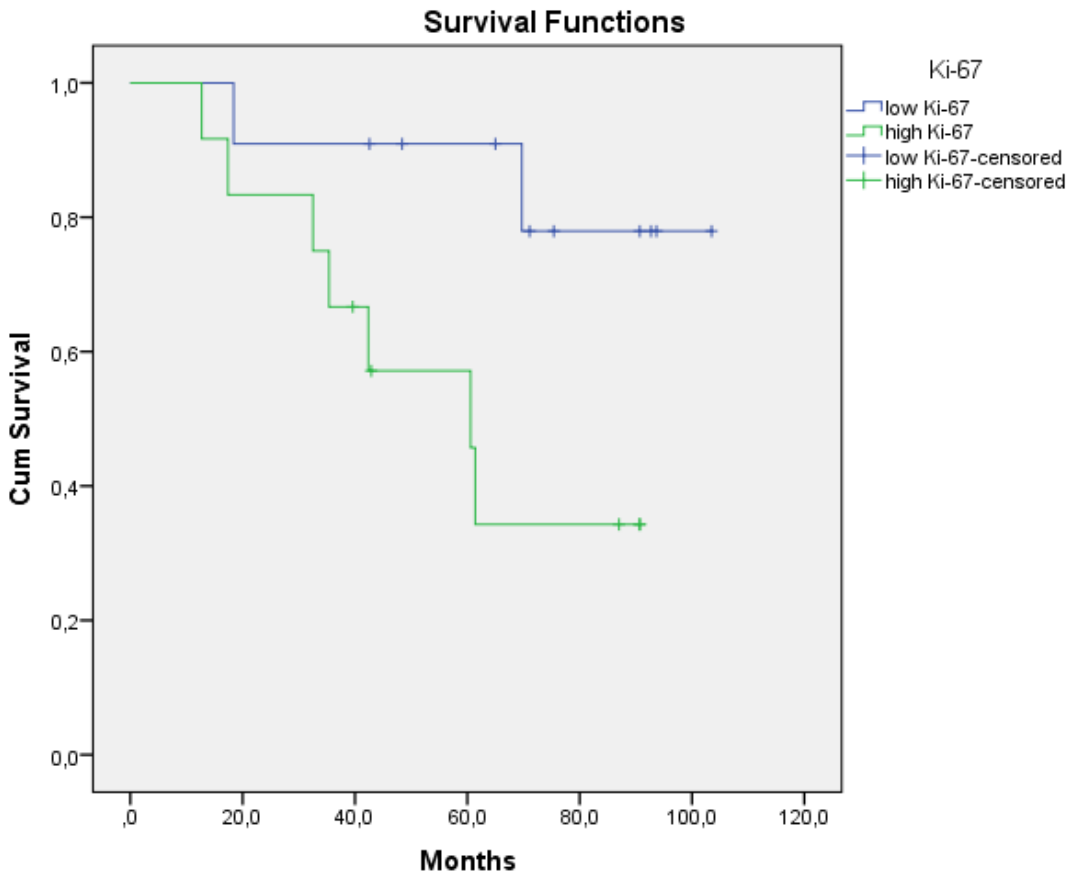


Figure 3.50. **Kaplan-Meier survival curves by Ki-67 expression in patients with DAs (cut-off value – 5.5%)**

A statistically significant survival difference was found in patients regarding PDGFRA expression using a cut-off of 50% (log-rank, p = 0.017). A high PDGFRA expression (≥ 50%) was present in 10/19 (52.6%; 95% CI = 31.7–72.6) of the DA cases. A low PDGFRA expression (< 50%) was present in 9/19 (47.3%; 95% CI = 27.3–68.3) of the DA cases. At the end of the study, 1/10 (10%; 95% CI = 1.8–40.4) patients had died within the tumour group with high PDGFRA expression compared with 5/9 (55.6%; 95% CI = 26.7–81.1) of patients that had died in the tumour group with low PDGFRA expression. The median OS in patients with low PDGFRA expression was 61.4 months (95% CI = 9.9–112.0). The median OS in patients with high PDGFRA expression could not be calculated because of the few cases of death and small study group.

The corresponding Kaplan-Meier curves are shown in Figure 3.50.

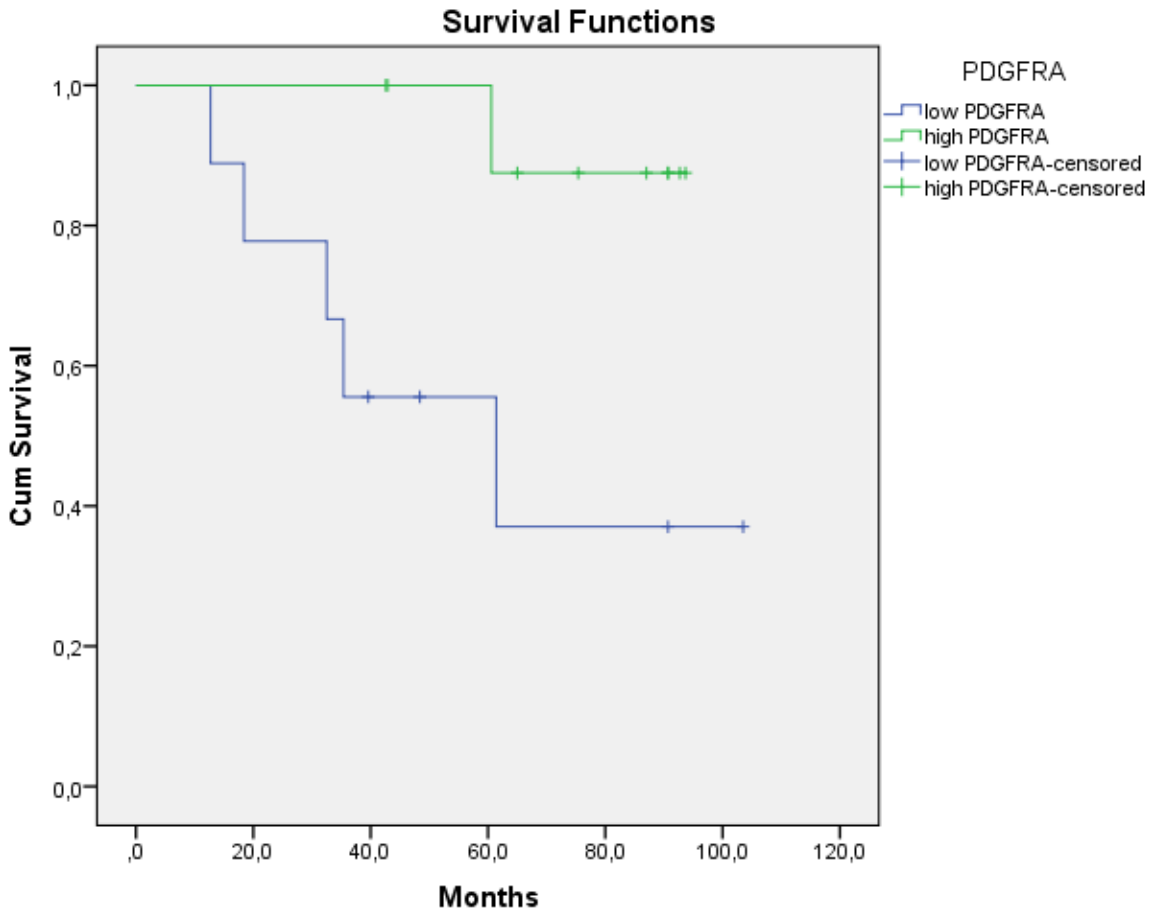


Figure 3.51. **Kaplan-Meier survival curves by PDGFRA expression in patients with DAs (cut-off value – 50%)**

As regards other immunohistochemical markers, no significant survival differences were found using both cut-off values. Cut-off values defined from the literature studies were not applicable to p21 and CD44 because all expression values were below these cut-off points. Thus, for p21 and CD44 only cut-off points based on median expression of markers were used.

All immunohistochemical markers with statistically significant and non-significant survival differences are summarized in Table 3.11.

Table 3.11.

Statistically significant and non-significant survival differences among immunohistochemical markers in DAs

IHC marker	Cut-off (by literature)	Cut-off (by median)
	p value	p value
IDH1 R132H	0.336	NA
Ki-67	0.256	**0.037**
p53	0.330	0.356
p21	NA	0.307
p27	0.558	0.226
CD44	NA	0.233
PDGFRA	**0.017**	**0.017**
CD34	NA	0.949

* Abbreviations in the table: NA, not applicable; IHC, immunohistochemistry

3.8. Immunohistochemical subtypes and survival

The immunohistochemical data concerning p53, IDH1 R132H, PDGFRA and CD44 expression was used to determine the immunohistochemical subtype of GBMs (see Chapter 2. Materials and Methods). Based on these protein expression signatures, three categories of GBMs were distinguished: proneural, mesenchymal and not otherwise classified, referred to as "Other". For subtyping of GBMs we used two different cut-off levels for immunohistochemical markers based on the literature studies and based on the median value of expression.

3.8.1. Immunohistochemical subtypes of GBMs

3.8.1.1. Immunohistochemical subtypes based on the cut-off levels from the literature studies

The majority of GBM cases were of the proneural subtype – 73/146 (50.0%; 95% CI = 42.0–58.0), followed by other (not otherwise classified) – 46/146 (31.5%; 95% CI = 24.5–39.4) and the mesenchymal subtype – 27/146 (18.5%; 95% CI = 13.0–25.6). All rare morphological subtypes of GBM (gliosarcoma and giant-cell GBM) belong to the proneural subtype.

There were no associations between subtypes of GBMs and any clinical or other immunohistochemical parameters (Ki-67, MVD, p21, p27). The summarized results are shown in Table 3.12.

Table 3.12.

Statistical significance (p values) for associations between subtypes and clinical or immunohistochemical parameters

Clinical and immunohistochemical parameters	p value
Age	0.529
Gender (male/female)	0.373
Multifocal/solitary	0.410
Hemisphere (right/left)	0.682
Localization (frontal/temporal/parietal/occipital/multiple)	0.411
Size (≥4 cm / < 4cm)	0.811
Ki-67	0.145
MVD	0.593
p21 expression	0.740
p27 expression	0.350

* Abbreviations in the table: MVD, microvascular density

There was no difference in OS between immunohistochemical subtypes of GBMs (log-rank, p value = 0.424) (Figure 3.51A).

Furthermore, the response to therapy was evaluated on survival in different GBM subtypes.

As shown in Figure 3.51B, in the proneural subtype there was a tendency for the addition of temozolomide to improve OS compared with radiotherapy alone (p = 0.061) (the median OS ratio was 1.6). However, a visual tendency towards a difference between Kaplain-Maier curves was more perspicuous. Radiotherapy also improved OS in the proneural subtype compared with surgery only (p = 0.008).

In the mesenchymal subtype, the addition of temozolomide significantly improved OS compared with radiotherapy alone (p = 0.002). However, the addition of radiotherapy did not improve the OS of the patients with the mesenchymal subtype compared with surgery only (p = 0.857) (Figure 3.51.C). Thus, the addition of radiotherapy did not have any benefit compared with surgery only in patients with the mesenchymal subtype.

In other GBMs (not otherwise classified), there was a statistically significant OS difference only between those GBMs treated with adjuvant chemotherapy and radiotherapy compared with those only surgically treated (p = 0.031). There were no statistically significant differences between other groups: temozolomide + radiotherapy versus radiotherapy alone (p = 0.319) and radiotherapy versus surgery only (p = 0.080) (Figure 3.51D).

The corresponding Kaplan-Meier curves are shown in Figure 3.51. The median OS rates for GBM subtypes, as well as the number of cases and p values for pairwise comparisons, are summarized in Table 3.13.

Table 3.13.

Median OS rates in GBM subtypes together with p values for pairwise comparisons

Subtype (n = 135*)	**Therapy (n = 135*)**	**Median OS (months)**	**95% CI**	**p values**		
Proneural (n = 66)	Radiotherapy + temozolomide (n = 25)	12.3	8.4–16.2	0.061		< 0.001
	Radiotherapy (n = 27)	7.6	7.1–8.1		0.008	
	Surgery only (n = 14)	2.4	1.1–3.6			
Mesenchymal (n = 26)	Radiotherapy + temozolomide (n = 11)	11.7	6.4–16.9	0.002		0.003
	Radiotherapy (n = 9)	2.7	0.3–5.0		0.857	
	Surgery only (n = 6)	2.7	2.0–3.3			
Other (n = 43)	Radiotherapy + temozolomide (n = 20)	12.2	6.7–17.6	0.319		0.031
	Radiotherapy (n = 14)	8.3	3.7–12.9		0.080	
	Surgery only (n = 9)	4.9	3.9–5.9			

* Data about the therapy were missing in 11 cases. Thus, 135 cases of GBMs were available for this analysis.

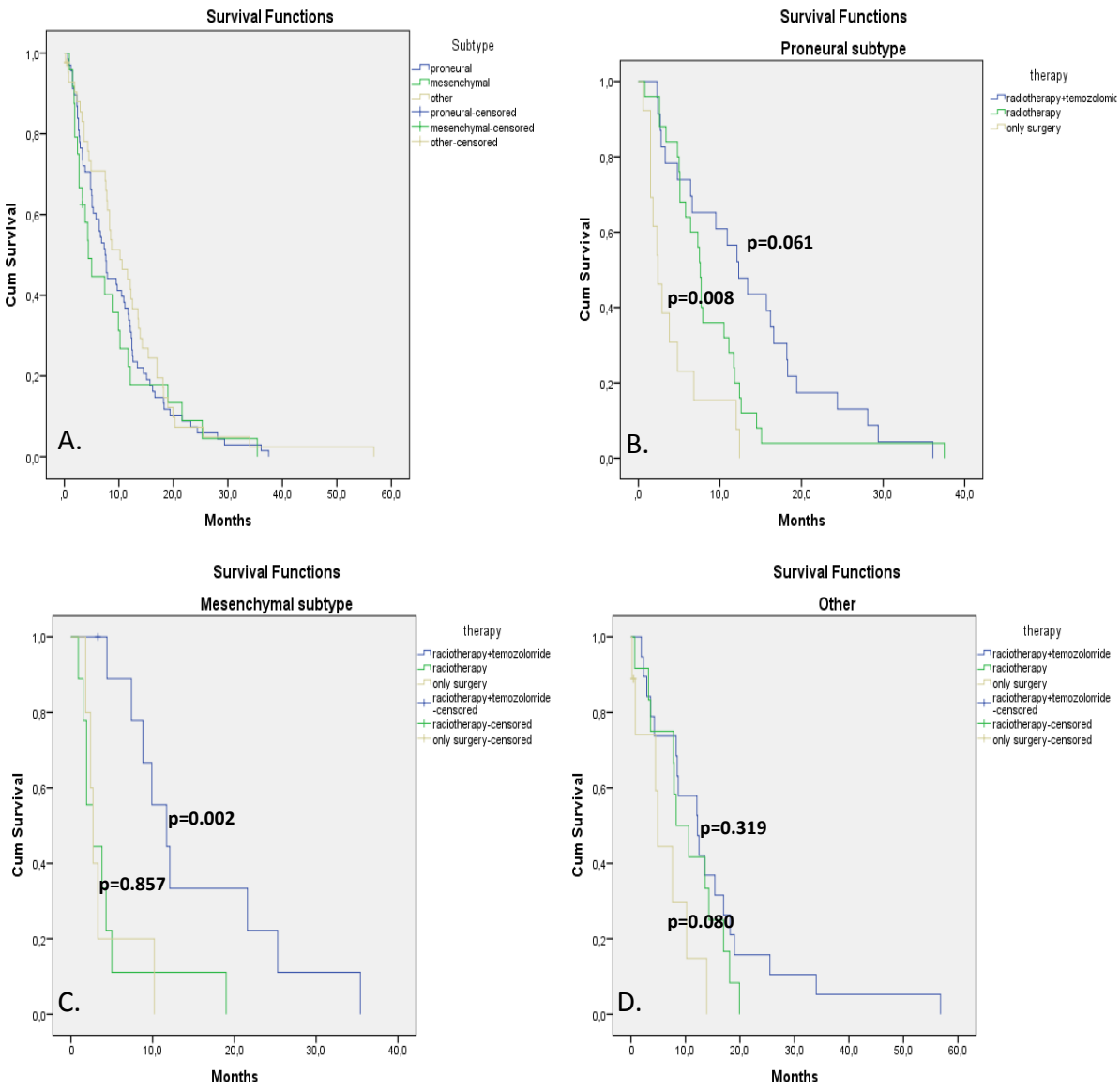


Figure 3.52. **Kaplan-Meier survival curves by GBM subtype and treatment type**

3.8.1.2. Immunohistochemical subtypes based on the cut-off levels from median expression values of evaluated markers

In addition to previously described subtypes, cut-off levels based on median expression values of immunohistochemical markers were also used for immunohistochemical subtyping of GBMs.

According to cut-off levels based on median expression, the proportion of GBMs with the proneural subtype had increased – 95/146 (65.1%; 95% CI = 57.0–72.3), followed by other (not otherwise classified) – 32/146 (21.9%; 95% CI = 15.9–29.3) and the mesenchymal subtype – 19/146 (13.0%; 95% CI = 8.5–19.4).

No associations were found between subtypes of GBMs and any clinical or immunohistochemical parameters (Ki-67, MVD, p21, p27). There was no difference in OS between immunohistochemical subtypes of GBMs (log-rank, p value = 0.511).

When response to treatment is evaluated in GBM subtypes (based on cut-offs from median values), Kaplain-Meier survival lines showed a very similar shape and abruptness of lines between survival plots, analogous to previously described GBM subtypes that were based on cut-offs from the literature studies. The corresponding Kaplan-Meier curves are shown in Figure 3.52.

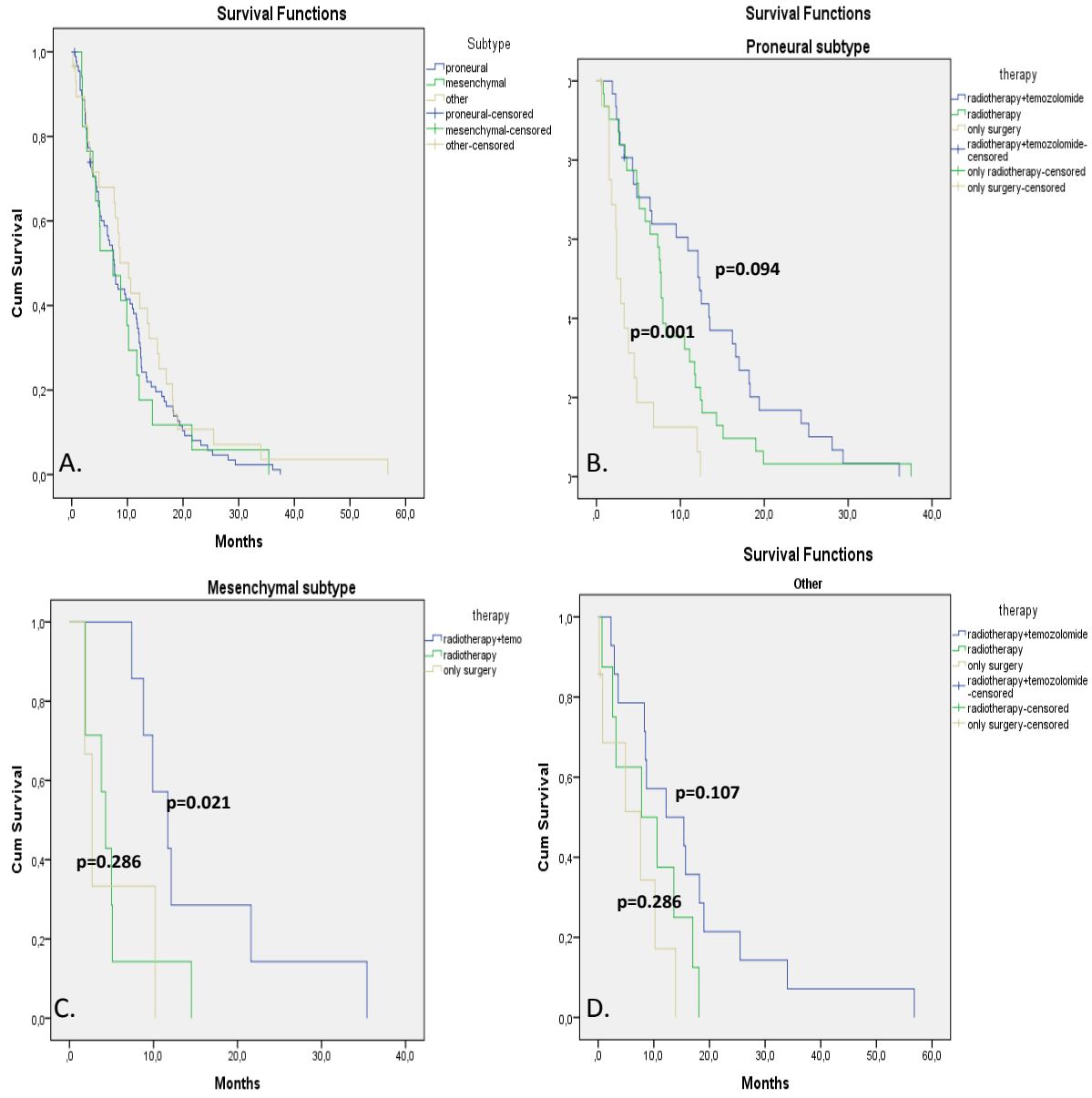


Figure 3.53. **Kaplan-Meier survival curves by GBM subtype (by cut-off based on median expression values) and treatment type**

3.8.2. Immunohistochemical subtypes of DAs

Using both cut-off values most DAs belong to the proneural subtype 24/26 (92.3%; 95% CI = 75.8–97.9), while the remaining 2/26 (7.6%; 95% CI = 2.1–24.1%) DAs were not otherwise classified (“other”) according to cut-off values based on the literature studies. CD44 expression in all DAs was very low, according to the literature-defined cut-off of 50%; CD44 expression reached this level in only one tumour. Because of the few cases and predominance of proneural signature survival analysis by subtype and therapy was not performed.

4. Discussion

Diffuse gliomas do not rank among malignancies with the highest incidence such as lung cancer, breast cancer or colorectal cancer; nevertheless, gliomas rank among the most aggressive human malignancies and have limited treatment options. The most common and the most aggressive type of glioma is GBM, so many international studies have researched this tumour. It is crucial to better understand specific signalling pathways and molecular alterations determining biological features of gliomas such as invasion, proliferation and resistance to current therapy because this is the only thing that might bring a ray of hope for improved target-specific management, as well as the development of personalized therapy. The current standard therapy, which includes chemotherapy with temozolomide and radiotherapy followed by surgery, is the best available treatment nowadays, but prognosis is still very bad (Stupp *et al.*, 2014, 2005). Fortunately, over the last 10 years, huge scientific progress has been made in the field of molecular biology of gliomas, creating a completely new understanding of these lethal tumours. One such breakthrough was the complete sequencing of GBM whole genomes in a large group of patients with GBMs by the TCGA project (Cancer Genome Atlas Research, 2008). Due to comprehensive high-throughput analysis of GBM genome, better characterization of the genomic landscape of glioma was possible and several critical pathogenesis-driven mutations were identified in such genes as IDH1 and IDH2, EGFR, PDGFRA, ATRX and others (Brennan *et al.*, 2013; Parsons *et al.*, 2008; Verhaak *et al.*, 2010; Yan *et al.*, 2009b).

In was also found that GBM is not a single entity as previously thought, but is composed of molecularly and biologically distinct subsets of the tumours, albeit with the same morphological appearance. Several studies have been performed to identify subtypes of gliomas based on molecular and proteomic signatures (Motomura *et al.*, 2012; Parsons *et al.*, 2008; Verhaak *et al.*, 2010). For example, Verhaak *et al.* described four glioblastoma molecular subtypes based on gene expression analysis – classical, proneural, mesenchymal and neural (Verhaak *et al.*, 2010). The tumours that possess similar molecular signatures and expression patterns likely share a common pathogenesis reflecting similar therapy responses and prognosis.

Some of these identified genomic and proteomic signatures can potentially be used to develop new molecularly targeted therapies; in addition, they can be used as prognostic or predictive factors that provide some clues for a more personalized treatment approach.

Molecular profiling of gliomas is a time-consuming, very expensive technique that also requires unfixed tumour tissues. For practical needs in terms of routine, analysis of prognostic

factors needs to be cheaper and easy to replicate, thus immunohistochemistry could be a good surrogate for other, more expensive methods. Recent molecular advantages have enabled wider implementation of immunohistochemistry in glioma profiling and new immunohistochemical markers have been developed for routine use, such as IDH1 132H.

There have been several attempts at immunohistochemistry-based subtyping of gliomas (Le Mercier *et al.*, 2012; Popova *et al.*, 2014; Trabelsi *et al.*, 2016). Within this study, we used IHC to assess the expression of some of the proteins that have been reported to have a prognostic importance or determine basic biological features of malignant tumours such as invasion, regulation of cell cycle and proliferation. Inspired by the molecular classification of gliomas described by Verhaak *et al.*, we also tried to subdivide GBMs into several groups and assess their prognostic significance. In addition, the immunohistochemistry prognostic impact of clinical parameters as well as possible correlations between any of the examined parameters were assessed.

4.1. Overall survival and treatment

In this study, the median OS of all patients with GBM was 7.9 months (95% CI = 6.8–9.0), which is shorter than described in other studies by different authors where the median OS ranged from 9.7 to 13.6 months (Back *et al.*, 2007; Johnson and O'Neill, 2012; Kumar *et al.*, 2013; Ulutin *et al.*, 2006). Only in a few studies that described survival in the pre-temozolomide era or were limited to the management of GBM in elderly patients was the OS rate reported to be from five to eight months (Chaichana *et al.*, 2011; Ewelt *et al.*, 2011; Johnson and O'Neill, 2012; Rønning *et al.*, 2012; Stark *et al.*, 2005). In the present study, the one-year and two-year survival rates for patients with GBM were 36.3% and 9.6%, respectively. These survival rates are comparable to those in other studies, but on average they are shorter than those published by the majority of researchers (Ahmadloo *et al.*, 2013; Filippini *et al.*, 2008; Li *et al.*, 2010; Ma *et al.*, 2009; Paszat *et al.*, 2001; Piroth *et al.*, 2007; Scoccianti *et al.*, 2010; Shah *et al.*, 2020). In this study there were only two (1.5%; 95% CI = 0–5.8) patients who survived more than three years. The longest survival in this study was 56 months or 4.6 years.

By the type of treatment in the current study the median OS rate was 2.9 months for the patients who did not receive any adjuvant oncological treatment, 7.9 months for the patients who received adjuvant radiotherapy in addition to surgery and 12.1 months for the patients with maximum standard therapy, including surgery and adjuvant radiotherapy together with temozolomide. In comparison to the Norwegian study, these survival rates were 2.5 months, 9.0 months and 16.2 months, respectively (Rønning *et al.*, 2012). However, Stupp *et al.*, after the publication of a randomized phase III trial to compare the effectiveness of radiotherapy with

and without temozolomide, reported median OSs of 12.1 months and 14.6 months, respectively. Overall, the survival rates by treatment type described in the literature are higher than in this study in Latvian patients affected by GBM.

Patients with DAs had a significantly better prognosis than those with GBMs. In the current study, 14/25 (56.0%; 95% CI = 37.0–73.3) of patients were alive, but 11/25 (44.0%; 95% CI = 26.6–62.9) of patients had died during the observation period. The median OS could not be calculated because of the small study group and short follow-up time. In this study, the two-year and three-year survival of patients with DAs was 88% and 80%, respectively.

The median OS of patients with DAs in different studies has been reported as ranging from 5 to 10.5 years (Claus *et al.*, 2015; Leighton *et al.*, 1997; Tesileanu *et al.*, 2020; Tove *et al.*, 2012). Leighton *et al.* observed five-year and 10-year survival rates of 72% and 50% (Leighton *et al.*, 1997). Researchers from the USA (Mayo clinic) conducted a large study on the survival of patients with diffuse gliomas with a median follow-up time of 13.6 years; they reported a median OS of 6.9 years and 10-year and 15-year survival rates of 36% and 23%, respectively (Schomas *et al.*, 2009).

In this study, the median follow-up time of patients is 60.2 months or 5.0 years. This is an adequate follow-up time for patients with GBMs who have a very poor prognosis. However, due to the relatively long survival time in patients with DAs, a longer duration of follow-up might be necessary for more precise survival time evaluation.

4.2. Clinical and morphological findings

Gender. In this study, both females and males were affected by GBMs and DAs in approximately equal proportions, so there were no gender differences in regard to gliomas.

In contrast to our study, a predominance of gliomas in males was reported in the literature; however, in most studies gender differences are inconspicuous and the male:female ratio usually does not exceed 1.3 (Dobes *et al.*, 2011; Kushnir and Tzuk-Shina, 2011; Sun *et al.*, 2015). A higher male:female ratio of about 1.6 has been reported by some authors (Brodbelt *et al.*, 2015; Dubrow and Darefsky, 2011).

Differences in survival by gender were not found in this study. Although various articles have noted that male gender might be associated with a better prognosis, multivariate analysis showed that women in the whole study group were older and that may explain these survival differences (Tian *et al.*, 2018; Tugcu *et al.*, 2010; Verger *et al.*, 2011). In our study there were no age differences by gender.

Age. In this study, the mean (62.0 and 37.5 years) and median (62.0 and 35.0 years) ages of GBMs and DAs were similar to those found in other studies (Ladomersky *et al.*, 2019;

Oszvald *et al.*, 2012; Schomas *et al.*, 2009; Schwartzbaum *et al.*, 2006). In our study, we have only five patients with secondary GBM, with a mean age of 50.6 years, which is slightly above the mean ages reported in the literature, but also the number of patients with secondary GBMs recruited in studies was higher (Juratli *et al.*, 2013; Ohgaki and Kleihues, 2007).

In this study, older age was found to be associated with a worse prognosis, which is supported by data from other publications (Buckner, 2003; Scott *et al.*, 2012; Smrdel *et al.*, 2018).

However, as found in the current study and supported by others, older patients tend to have a different pattern of care, and they receive less aggressive management (Iwamoto *et al.*, 2009; Ladomersky and Zhai, 2020; Lutterbach *et al.*, 2005). Older patients may also have a decreased ability to cope with neurological damage caused by glioma, surgery or adjuvant therapy, and numerous co-morbidities may affect their speed of recovery (Chaichana *et al.*, 2013).

Tumour localization. In the present study, the most frequent location of GBM was the frontal lobe – 56/146 (38.4%; 95% CI = 30.5–46.3), followed by the temporal lobe – 41/146 (28.1%; 95% CI = 20.8–35.4). The data is comparable to other studies where the frontal lobe was affected in 40% and 43% of cases and the temporal lobe in 28% and 29% of cases (Larjavaara *et al.*, 2007; Li *et al.*, 2018; Simpson *et al.*, 1993). Other localizations of these tumours were less frequent (Larjavaara *et al.*, 2007).

Some studies showed that GBMs in the frontal lobe were characterized by better prognosis than those in other localizations (Lamborn *et al.*, 2004; Simpson *et al.*, 1993). In this study, better survival was found in GBMs localized in parietal lobes and only in younger patients.

With regard to DAs, in this study the most frequent localization is within frontal and temporal lobes – 13/26 (50%; 95% CI = 30.8–69.2) and 6/26 (23.1%; 95% CI = 6.9–39.3), respectively. This data also corresponds with other studies (Capelle *et al.*, 2013; Duffau and Capelle, 2004; Larjavaara *et al.*, 2007).

In this study, multifocal involvement in GBM was identified in 11% of cases, which is very similar to other published articles where the frequency of multifocal gliomas was about 8–10 % (Barnard and Geddes, 1987; Djalilian *et al.*, 1999; Giannopoulos and Kyritsis, 2010; Li *et al.*, 2020).

Tumour size. As gliomas generally grow and invade extensively brain parenchyma before patients experience any symptoms, almost all gliomas are large tumours at the time of diagnosis. In this study, most of the GBMs and all DAs were large tumours whose size exceeded 4 cm. Very few studies have described the relationship between glioma size and prognosis, and

in some of these reports a large tumour size is associated with a worse prognosis (Kashi *et al.*, 2015; Raysi Dehcordi *et al.*, 2012; Wang *et al.*, 2019). In this study, the largest tumours exceeding 4 cm had less favourable outcomes than smaller tumours of less than 4 cm.

Morphology. All GBMs in this study were diagnosed according to the 2016 WHO classification of CNS tumours and thus showed necrosis (ischaemic and/or pseudopalisading) and microvascular proliferation. Most of the GBMs in this study belong to typical or conventional morphology – 141/146 (96.6%; 95% CI = 93.7–99.5), and a minority of GBMs had giant-cell – 2/146 (1.4%; 95% CI = 0–3.3) or gliosarcoma morphology 3/146 (2.1%; 95% CI = 0–4.4). The frequency of these rare GBM variants in our study was similar to that in other published reports where gliosarcomas and large-cell glioblastomas comprised 2–8 % and 1–5 % of cases, respectively (Castelli *et al.*, 2016; Kozak and Moody, 2009; Meis *et al.*, 1991; Valle-Folgueral *et al.*, 2008). In terms of the prognostic role of these morphological subtypes, concerning only giant-cell GBMs, there are some data in the literature indicating that it can be associated with a slightly better prognosis and a higher probability of long-term survival (Kozak and Moody, 2009; Mallya *et al.*, 2015; Naydenov *et al.*, 2009). In our study, there were no long-term survivors among giant-cell GBMs: all three patients with this subtype survived 7.7, 13.4 and 1.5 months. Due to the very small number of patients with gliosarcomas and giant-cell GBMs, with the amount of data available, statistical calculations are not sufficiently powered and reasonable to precisely characterize these rare GBM variants. High statistical power and clinically important differences between these rare morphologies may appear with examination of larger groups of patients.

With regard to five patients with immunohistochemically proven secondary GBMs (IDH1 R132H positive), only one GBM showed a component of lower-grade glioma, thus confirming secondary GBM on morphological grounds. In addition, one other secondary GBM showed a focally increased number of gemistocytic astrocytes. The remaining three cases of secondary GBM did not differ from other conventional GBMs. In the literature, some articles reported that secondary GBMs are characterized by more frequent presence of gemistocytes, but this is not a reliable indicator of secondary origin (Reis *et al.*, 2001; Watanabe *et al.*, 1997).

All 26 cases of DAs were diagnosed as diffuse fibrillary astrocytomas on morphological grounds according to the 2016 WHO classification of CNS tumours. All tumours in this study showed mild to moderate cellularity, individual tumour cells showed mild nuclear and cellular atypia, rare mitotic figures in some cases up to 2 mitoses per 10 high-power field was found.

4.3. Immunohistochemical profile of GBMs and DAs

The **Ki-67** proliferation index directly reflects the biological potential of the tumours and increasing values of Ki-67 strongly correlate with a higher grade of glioma (Arshad *et al.*, 2010; Johannessen and Torp, 2006; Skjulsvik *et al.*, 2014). Although the grading of gliomas is based on morphology and not on proliferation fraction, Ki-67 can be used as a useful supplement to the histopathological diagnosis and grading of gliomas (Thotakura *et al.*, 2014). In our study there was a considerable difference in the proliferation rate between GBMs and DAs – 44.4% (95% CI = 41.1–47.6) versus 6.4% (95% CI = 4.7–8.0). Several researchers have reported that overlapping of Ki-67 indices may occur between different grades of gliomas, most frequently between grade III and grade IV astrocytomas, but usually not between grade II and grade IV. However, a small fraction of GBMs may have a very low proliferation rate comparable with grade II tumours: for example, Shivaprasad *et al.* reported two cases of gliosarcomas with Ki-67 fractions of 5% and 12% (Shivaprasad *et al.*, 2016). In this study we had one case of GBMs with conventional morphology with a proliferation rate of 13%, and one case of DAs with quite low cellularity and typical morphology that had a proliferation rate of 15%. These findings indicate that Ki-67 should be interpreted carefully and can only be used together with appropriate morphology and in a clinical context but not alone as a separate diagnostic marker. Other authors supported that and noted that some rare cases of DAs with a typical low-grade histological appearance may have higher proliferation indices than usual and such cases should be reported as astrocytomas with elevated proliferative indices because these patients might be needed for closer follow-up (Tove *et al.*, 2012).

In this study, the mean Ki-67 proliferation index value in GBMs was relatively high at 44.4% (95% CI = 41.1–47.6), compared to other studies that reported lower mean proliferation rates that ranged from 12% to 32% (Di *et al.*, 1997; Hsu *et al.*, 1997; Kleinschmidt-DeMasters *et al.*, 2005; Shivaprasad *et al.*, 2016; Wakimoto *et al.*, 1996). However, in one study the proliferation fraction of 44% was also reported in GBMs (Kirkegaard *et al.*, 1998). In DAs, Ki-67 was observed with a mean rate of 6.4% (95% CI = 4.7–8.0) and a median of 5.5 (IQR = 6). In comparison, Norwegian authors reported mean and median Ki-67 proliferation rates in DAs of 5.2% and 4.5% with a range of 1% – 16% (Tove *et al.*, 2012). Wakimoto *et al.* and Ralte *et al.*, however, reported lower proliferation indices in DAs with a mean proliferation rate of 3.8% and 3.7% (Ralte *et al.*, 2001; Wakimoto *et al.*, 1996). There were also studies where a mean Ki-67 proliferation fraction of about 1% was reported in DAs (Di *et al.*, 1997; Hilton *et al.*, 1998; Shivaprasad *et al.*, 2016).

The variations in the proliferation fraction in various studies can be associated with many factors, such as immunohistochemical procedures, the fixatives used and the

interpretation of immunostaining, and could also be the result of the biological heterogeneity of the gliomas.

The prognostic role of the Ki-67 proliferation index in GBMs remains inconclusive, with some studies showing a prognostic role (Ho *et al.*, 2003; Jin *et al.*, 2011) and others not finding any significant association with prognosis (Moskowitz *et al.*, 2006; Yang *et al.*, 2013). However, in many survival studies researchers used different cut-off levels: 12.5%, 25% and 35% (Ho *et al.*, 2003; Jin *et al.*, 2011; Moskowitz *et al.*, 2006). In one large meta-analysis where different possible prognostic factors in GBMs were analysed, Ki-67 was put into the category of weakly prognostic factors (Thuy *et al.*, 2015). In the current study, the Ki-67 proliferation index, using both cut-offs (25% and 41%), did not have a significant prognostic value. However, using a cut-off of 25% by visual inspection survival curves are different, but not statistically significantly so ($p = 0.252$).

In this study, a correlation was found between higher Ki-67 expression and the size of the tumour measured as the largest diameter ($r_s = 0.243$; $p = 0.013$); however, this correlation was weak and furthermore in the analysis of the association between proliferation rate and tumour size groups (≥ 4 cm and < 4 cm) no association was identified. Interestingly, in GBMs there was also a tendency towards an association between higher Ki-67 values and gender ($z = -1.913$; $p = 0.056$), and there was a slightly higher mean rank of proliferation fraction in females (mean rank = 69) than in males (mean rank = 57). Chaloob *et al.* did not find any association between Ki-67 and gender ($p = 0.909$), but a significant correlation between the Ki-67 labelling index and patient age was described in GBMs ($r_s = 0.407$; $p = 0.003$) (Chaloob *et al.*, 2012). The correlation of the Ki-67 labelling index with age in gliomas was also supported in one other research ($r = 0.374$, $p = 0.007$) (Zolota *et al.*, 2008). In the current study, Ki-67 did not correlate with patient age, however the Ki-67 proliferation fraction correlated with p53 protein expression ($r_s = 0.196$; $p = 0.027$), which indicates oncogenic properties of p53 upregulating the proliferation in neoplastic cells. Furthermore, another cell cycle inhibitor, p27, had a tendency towards an inverse correlation with Ki-67. Thus, loss of p27 and upregulation of p53 may be indicators of more proliferative features in GBMs.

Interestingly, in DAs, the Ki-67 proliferation index also showed an association with patient gender, thus the mean rank of Ki-67 expression was statistically significantly higher in males (mean rank = 16.8) than in females (mean rank = 9.4) in Das ($z = 2.563$; $p = 0.010$). Although such an association between gender and proliferation fraction is poorly evaluated in glioma studies and the explanation is not clear, a growing number of studies have supported the role of sex hormones such as oestrogens, progesterone and androgens in a variety of brain tumours including gliomas (Hartman *et al.*, 2009; Paruthiyil *et al.*, 2004; Sareddy *et al.*, 2016;

Yu *et al.*, 2013). Recent studies emphasize in particular the role of oestrogen receptor beta (ERβ), which is considered a tumour suppressor and thus is associated with inhibition of tumour proliferation. Downregulation of ERβ is associated with progression and a higher malignancy grade in many human cancers, including gliomas (Bardin *et al.*, 2004; Burns and Korach, 2012). Some recent studies supported this fact by showing that high-grade gliomas usually express low levels of ERβ; however, low-grade gliomas are characterized by high levels of ERβ expression (Batistatou *et al.*, 2006, 2004; Li *et al.*, 2013; Liu *et al.*, 2014; Sareddy *et al.*, 2012). Some recent studies also showed that higher expression of ERβ is associated with a more favourable prognosis in both low-grade and high-grade gliomas. Many of these reports suggest that oestrogens, by binding with Erβ, exert an antiproliferative effect inhibiting tumour growth, which is also supported in clinical studies where beneficial effects of oestrogen agonist drugs were found in patients with gliomas (Paterni *et al.*, 2015; Sareddy *et al.*, 2012). In this study, DAs of females had lower proliferation rates. This can be explained by the inhibitory effect of ERβ and oestrogens on the growth rate of tumour cells: firstly, DAs usually have higher expression of ERβ, and secondly, because these tumours are more frequent in younger patients (median age = 35.5), and high circulating levels of oestrogens in females may promote the ERβ signalling-dependent suppressive effect and decrease the proliferation rate. However, the association between higher Ki-67 expression rates in females with GBMs may indicate a loss of tumour suppressive activity exerted by oestrogens and ERβ, firstly because GBMs have lower levels of ERβ, and secondly, GBMs are more common in elderly patients when circulating oestrogen levels are diminished. To prove this hypothesis, more studies on the role of sex hormones in gliomas are necessary with a higher number of patients, and all significant correlations should be reported.

In the current study, in DAs, Ki-67 was found to be a useful indicator of worse prognosis by using a cut-off of 5.5%. Other studies also support a worse outcome in patients with DA harbouring a high Ki-67 proliferation fraction (Johannessen and Torp, 2006; Tove *et al.*, 2012). Although the Ki-67 proliferation fraction is not included in the latest WHO classification system, assessment of Ki-67 may be useful in identifying increased proliferative activity in DAs with an otherwise typical low-grade glioma appearance, because such cases of DAs might need more careful follow-up and have a worse prognosis (Trembath *et al.*, 2008).

p53. Because p53 has a very short half-life time, it is present in cells at low levels under normal conditions, and accumulation of p53 is a direct result of DNA damage, hypoxia or oncogene activation (Ashcroft and Vousden, 1999). In contrast to the wild-type p53 protein, which is quickly degraded within cells, mutations in the TP53 gene lead to overexpression of more stable mutant p53 protein, which accumulates in the nucleus and can be detected

immunohistochemically. Many studies show a good correlation between the presence of TP53 mutation and overexpression of p53 protein detected by IHC, therefore IHC can be used as a surrogate for mutational analysis (Hall and Lane, 1994; Newcomb *et al.*, 1998; Simmons *et al.*, 2001; Wang *et al.*, 2014; Yemelyanova *et al.*, 2011; Zhang *et al.*, 2018). Upregulation of p53 protein expression is a characteristic feature of gliomas and p53 protein is usually absent in the normal brain tissues (Hussein *et al.*, 2006). In regard to p53 expression, many different cut-off values have been used in the literature by different authors, ranging from 5% to 50% (Houillier *et al.*, 2006; Popova *et al.*, 2014; Simmons *et al.*, 2001; Takano *et al.*, 2012; Wang *et al.*, 2014). In this study, 64.3% (95% CI = 55.6–72.1) of GBMs were positive for p53 protein using a cut-off value of 10%. Popova *et al*. and Takano *et al.*, by using a cut-off of 10%, reported lower p53 positivity rates: 40% and 41% (Popova *et al.*, 2014; Takano *et al.*, 2012). However, Kawasoe *et al.*, using a cut-off of 10%, identified p53 positivity in 75% of GBMs (Kawasoe *et al.*, 2015). In our study, 31.7% (95% CI = 24.2–40.3) of GBMs showed p53 protein expression in more than 50% of tumour cells, in compliance with Houillie *et al.*, and a high p53 protein expression was found in 37% of GBMs using a cut-off value of 50% (Houillier *et al.*, 2006). In this study, high p53 protein expression was found in all cases of secondary GBMs compared with only half of primary GBMs; in addition, the mean p53 labelling index was considerably higher in secondary GBMs (98.6 versus 32.7). This observation is also supported by other researches indicating a high rate of TP53 gene mutations and p53 protein overexpression in secondary GBMs (Ohgaki and Kleihues, 2007; Takano *et al.*, 2012; Watanabe *et al.*, 1996). In addition, TP53 gene mutations were found more frequently in the so-called "proneural molecular subtype" of GBMs in 54% of cases (Verhaak *et al.*, 2010). The limitation of our study is the small number of secondary GBMs (n = 5), but even with such a small group the difference in p53 positivity rates between primary and secondary GBMs was evident.

Using a cut-off of 10%, in this study 75.0% (95% CI = 55.1–88.0) of DAs showed high p53 expression in close agreement with Gillet *et al.*, where, with a cut-off of 10%, 75% of DA cases also showed p53 overexpression (Gillet *et al.*, 2014). However, Takano *et al.* observed p53 expression in only 30% of DAs (with a cut-off of 10%) (Takano *et al.*, 2012).

In this study, there was no statistically significant difference in p53 protein expression between GBMs and DAs (p = 0.416) in relation to other studies with similar results (Ali and Jalal, 2013; Nayak *et al.*, 2004). However, some researchers have reported even higher expression of p53 protein in high-grade gliomas compared with low-grade gliomas (Lin *et al.*, 2015; Ogura *et al.*, 2015; Sengupta *et al.*, 2012). Since most studies did not find any p53 expression differences between glioma grades and some even found higher p53 positivity rates in high-grade gliomas, the logical question emerges: why do secondary GBMs have such a high

rate of TP53 gene mutations and p53 protein expression but low-grade gliomas, which are the precursors of secondary GBMs, don't? The answer may be related to the timing of TP53 and IDH1 gene mutations in gliomas. Thus, it was found that TP53 gene mutations occur in later stages of glioma progression and are more frequent in secondary GBMs; however, IDH1 mutations precede the acquisition of TP53 mutations in diffuse gliomas (Cohen *et al.*, 2013a; Guo *et al.*, 2020; Watanabe *et al.*, 2009; Yan *et al.*, 2009a).

In the current study, p53 protein expression had no prognostic significance either in GBMs ($p = 0.831$) or in DAs ($p = 0.330$); in addition, many studies have failed to identify the prognostic role of p53 overexpression or TP53 gene status (Gross *et al.*, 2005; Houillier *et al.*, 2006; Newcomb *et al.*, 1998; Shiraishi *et al.*, 2002; Simmons *et al.*, 2001; Takano *et al.*, 2012). However, a few authors have found that p53 overexpression predicts better prognosis in GBMs (Ohgaki *et al.*, 2004; Schmidt *et al.*, 2002). In addition, a comprehensive meta-analysis including 32 studies and 2979 patients classified p53 protein as a non-prognostic factor in GBMs (Thuy *et al.*, 2015).

In the current study, p53 expression had correlations with Ki-67 ($r_s = 0.196$; $p = 0.027$), PDGFRA ($r_s = 0.181$; $p = 0.043$) and MVD ($r_s = 0.228$; $p = 0.031$) in GBMs. Correlations between p53 protein and Ki-67 were described before. As regards p53 correlation with PDGFRA, both TP53 and PDGFRA gene mutations are more frequently associated with the proneural subtype of GBMs as described by Verhaak *et al.* (Verhaak *et al.*, 2010).

Besides the central role of p53 in the tumour suppressor function, many studies have shown that p53 may play other roles in tumorogenesis, for example the induction of angiogenic switch (Baeriswyl and Christofori, 2009; Ravi *et al.*, 2000; Teodoro *et al.*, 2007). It was shown that wild-type p53 protein has anti-angiogenic properties via transcriptional upregulation of anti-angiogenic and downregulation of angiogenic molecules, thus a loss of p53 protein may lead to consequent enhancement of vasculogenesis in TP53-mutated tumours (Baeriswyl and Christofori, 2009; Nishimori *et al.*, 1997). As for p53 correlation with MVD in this study, it could support the role of p53 in tumour angiogenesis. In addition, Gaiser *et al.* found an increased number of microvessels in TP53-mutated low-grade astrocytomas (Gaiser *et al.*, 2009). However, in this study, p53 did not correlate with MVD in DAs.

p21 is an important cell cycle inhibitor that can bind to cyclin-dependent kinase (CDK) complexes and induce cell cycle arrest (Coqueret, 2003). Furthermore, p21 is also involved in other important processes, such as apoptosis, DNA replication and cellular motility (Abbas and Dutta, 2009; Gartel and Tyner, 2002; Stivala *et al.*, 2012). A large number of studies have shown that p21 may have a dual role and together with the tumour suppressor effect it may also

act as an oncoprotein because of its antiapoptotic properties (De la Cueva *et al.*, 2006; Gartel, 2006; Xiao *et al.*, 2020).

p21 expression is very low in normal or reactive brain tissue, however p21 is significantly upregulated in gliomas of different malignancy grades (Jung *et al.*, 1995; Korshunov and Golanov, 2001).

In the current study, high p21 expression (cut-off of 20%) was found in 49.3% (95% CI = 41.3–57.4) of GBMs, compared to another study that demonstrated p21 expression in 58% of high-grade gliomas using the same cut-off value of 20% (Trabelsi *et al.*, 2016). However, the main limitation of the study by Trabelsi *et al.* is the very small sample size: out of 60 GBMs p21 status was assessed in only 12 tumours, resulting in seven positive (> 20% of cells) and five negative (< 20% of cells) cases of GBM. In addition, Khalid *et al.* reported p21 positivity in 57% of GBMs (Khalid *et al.*, 1998).

To date, there have been only a few studies that have assessed p21 expression in gliomas and the cut-off values for p21 positivity were different in most of the studies, making comparisons complicated. In the current study, the mean expression of p21 in GBMs was 21.2% (95% CI = 18.7–23.6), which is close to the mean expression value of 19.8% in GBMs reported by Kirla *et al.* (Kirla *et al.*, 2003). In this study, p21 positivity was significantly higher in GBMs than in DAs: 49.3% (95% CI = 41.3–57.4) versus 15% (95% CI = 5.2–36.0) of cases with high p21 expression (at a cut-off value of 20%), and the mean expression was 21.2% (95% CI = 18.7–23.6) versus 6.9% (95% CI = 2.4–11.4).

Data in this study were in close correlation with data reported by Zolota *et al.*, who also described higher p21 positivity rates in high-grade than in low-grade gliomas – 39.2 % versus 12.5%; however, they used a cut-off value of 10% for p21 positivity (Zolota *et al.*, 2008). Paradoxically, high-grade gliomas – the most malignant and proliferative tumours – had higher p21 expression than low-grade gliomas, which may indicate that p21 expression in gliomas may contribute to its oncoprotein function.

In respect of the prognostic role of p21, the information is limited due to the small number of studies. In this study, no prognostic role of p21 expression was identified, similarly to other studies where such an association between p21 status and survival was not found (Kirla *et al.*, 2003; Zolota *et al.*, 2008). However, other researchers reported p21 as a possible prognostic factor in gliomas (Korkolopoulou *et al.*, 1998; Trabelsi *et al.*, 2016).

In this study, decreased p21 expression was statistically significantly more frequent in GBMs of a larger size (exceeding 4 cm) ($z = -2.460$; $p = 0.014$). This association was also supported by the negative correlation between p21 and the largest tumour diameter ($r_s = -0.181$; $p = 0.045$). These results suggest that decreased p21 expression is involved in processes

controlling tumour growth in line with the function of p21 as a tumour suppressor protein that is capable of cell cycle inhibition (Abbas and Dutta, 2009; Warfel and El-Deiry, 2013).

However, in this study no relationship was found between p21 expression and proliferation rate either in GBMs or in DAs. Also, no correlation was found between p53 and p21 indicating that p21 expression in gliomas may be induced by the p53-independent pathway and regulation of p21 expression is even more complicated than previously thought.

In DAs, in this study, a positive correlation between p21 and MVD was found (r_s = 0.448; p = 0.049), suggesting a possible role of p21 in angiogenesis. There is some evidence in the literature that p21, besides its antiapoptotic effect, can enhance tumour growth by promoting angiogenesis in cancer cells (Kreis *et al.*, 2019; Kuljaca *et al.*, 2009; Lee *et al.*, 2010). Interestingly, p21 had a negative correlation with PDGFRA (r_s = −0.603; p = 0.008) in DAs.

p27 is another cell cycle inhibitor that belongs to the same CIP/Kip family of CDK inhibitors as the p21 protein (Moller, 2000). In contrast to other tumour suppressors, p27 is rarely mutated in human cancer but it is frequently deregulated, showing reduced levels (Slingerland and Pagano, 2000). Recent evidence suggests that this dysregulation and reduced levels of p27 in human cancers may be attributed to post-translation mechanisms, especially the removal of p27 through its degradation in the ubiquitine-proteosome pathway (Bloom and Pagano, 2003; Piva *et al.*, 1999).

In this study, a high expression of p27 (with a cut-off of 70%) was noted in 60.1% (95% CI = 50.9–68.7) of GBMs. The data from this study were very similar to the results reported by Yang *et al.*, who found a high expression of p27 (with a cut-off of 70%) in 57.2% of high-grade gliomas (Yang *et al.*, 2011). However, Faria *et al.* reported a lower frequency of p27 high expression in 40% of GBMs (with the same cut-off of 70%) (Faria *et al.*, 2007). Zolota *et al.* demonstrated an even lower p27 positivity rate of 27% in high-grade gliomas, however they used a different cut-off point of 50% (Zolota *et al.*, 2008).

In this study, p27 expression in DAs was found in 86.9% of cases (95% CI = 67.8–95.4), in contrast to 92.8% and 81.2% in other studies with the same cut-off values (Faria *et al.*, 2007; Yang *et al.*, 2011). In this study, the mean amount of positive cells was significantly higher in DAs than in GBMs – 86.6% (95% CI = 81.6–91.7) versus 69.7% (95% CI = 65.8–73.7) – indicating that a loss of p27 expression is associated with increased malignancy. Most studies supported the decreased p27 expression rates in high-grade compared to low-grade gliomas (Faria *et al.*, 2007; Kirla *et al.*, 2003; Yang *et al.*, 2011; Zolota *et al.*, 2008).

In the current study, no relationship was found between p27 expression and survival either in GBMs or in DAs. In contrast to our results, in other studies the loss of p27 nuclear

expression correlated with a worse prognosis in glioma patients (Kirla *et al.*, 2003; Mizumatsu *et al.*, 1999; Nabika *et al.*, 2010; Tamiya *et al.*, 2001; Yang *et al.*, 2011). However, a tendency towards a negative correlation between p27 and Ki-67 was found in this study in GBMs ($r_s = -0.199$; $p = 0.055$), indicating that the loss of p27 is associated with a more aggressive and proliferative phenotype in GBMs. An inverse correlation between Ki-67 and p27 in gliomas was also supported by other authors (Cavalla *et al.*, 1999; Fuse *et al.*, 2000; Kirla *et al.*, 2003). In addition, lower p27 expression values were found in multifocal gliomas ($z = -2.087$l; $p = 0.037$), confirming the relevance of the correlation between p27 and Ki-67 and supporting the importance of decreased p27 levels in tumour progression and more aggressive behaviour.

Surprisingly, in this study an association between nuclear p27 levels and gender was found. Thus, p27 protein expression was statistically significantly lower in females ($z = -2.174$; $p = 0.030$). This finding does not contradict the results shown by Huang *et al.*, who found that oestrogens promote, but progesterone inhibits, nuclear p27 destruction by regulating ubiquitin-proteasome system activity in endometrial cancer cells (Huang *et al.*, 2012). To the best of our knowledge, gender differences in p27 expression have not been described in gliomas. But we suppose that sex hormones may be implicated in the biological diversity of gliomas and can modify the molecular and immunohistochemical profile of glioma cells. In addition to this finding, the association between Ki-67 and gender has been discussed before.

In this study, a positive correlation between age and p27 expression was also found ($r_s = 0.519$; $p = 0.011$) in DAs but not in GBMs.

CD44 is a transmembrane glycoprotein that serves as a major surface hyaluronic acid receptor and is involved in cell matrix adhesion, cell migration and various cellular signalling pathways (Dzwonek and Wilczynski, 2015; Naor *et al.*, 1997). Its membranous localization may be important in facilitating the invasion of neoplastic cells by CD44-hyaluronan interaction (Bradshaw *et al.*, 2016). CD44 engagement with hyaluronan is also suggested to enhance tumour progression through increased tyrosine kinase activity and resistance to treatment, accentuating this molecular association as a desirable treatment target (Mooney *et al.*, 2016; Shepard, 2015; Thapa and Wilson, 2016). CD44 has multiple isoforms due to splicing and post-translational modification and is characterized by high functional diversity (Prochazka *et al.*, 2014).

It is also involved in cell adhesion, angiogenesis, lymphocyte activation and cytokine release (Afify *et al.*, 2009; Petrey and de la Motte, 2014; Tremmel *et al.*, 2009). CD44 has been described as a cancer stem cell marker in different tumours, including glioblastoma. In the nervous system, CD44 has been identified as a marker of neural stem cells as well as astrocyte and oligodendrocytes precursors (Liu *et al.*, 2004; Naruse *et al.*, 2013). Some authors have also

noted that in glioblastoma CD44 functions as a neural progenitor cell marker being expressed on partially differentiated cells (Bradshaw *et al.*, 2016).

CD44 is involved in mesenchymal transformation of tumour cells and enhances the invasiveness by promoting the adhesion (Xu *et al.*, 2015). In the molecular classification of glioblastoma, CD44 was described as a marker of mesenchymal subtype (Phillips *et al.*, 2006; Verhaak *et al.*, 2010). It is suggested that the immunohistochemical assessment of CD44 should become a mainstay in the subtyping of glioblastomas due to its economically effective surrogate method (Popova *et al.*, 2014) in parallel to the achievements in breast cancer research. However, significant controversies exist in the literature, as is further shown below.

In this study, CD44 expression strongly depended on tumour grade, with the highest expression levels being seen in GBMs. The mean expression value of CD44 in GBMs was 74.1% (95% CI = 69.6–78.7) versus 13.5% (95% CI = 7.7–19.2) in DAs. High CD44 expression (with a cut-off of 50%) was found in 81.5% (95% CI = 74.4–86.9) of GBMs, but no DAs had a CD44 expression above this level. In comparison with this study, Popova *et al.* had found high CD44 expression in 42% of GBMs and 15% of DAs by using the same cut-off point (Popova *et al.*, 2014). However, Ranuncolo *et al.*, by using a higher cut-off point of 70%, had found high CD44 expression in 59% of GBMs and 9.5% of low-grade gliomas (Ranuncolo *et al.*, 2002). Other authors observed a similar relationship between glioma grade and CD44 expression (Popova *et al.*, 2014; Ranuncolo *et al.*, 2002; Yoshida *et al.*, 2012). However, there are studies that found no association between the grade of glioma and CD44 expression (Ylagan and Quinn, 1997). The reason for these conflicting findings regarding CD44 expression is unclear but they may be caused by the multiple splicing variants of CD44 protein (Prochazka *et al.*, 2014) or improvements in the affinity of primary antibodies or general immunohistochemical technologies.

Few studies have specified CD44 staining patterns in glioma tissues. The most prominent and typical is membranous, surface staining of CD44; this staining pattern was intense and has been found in all GBMs in this study. However, such a membranous staining pattern was identified in few DAs and it was patchy and vague. In contrast, all DAs had faint, barely noticeable CD44 immunoreactivity in cytoplasmic processes of astrocytes, creating a branched, delicate network of CD44 immunopositivity. In this study, a weak, diffuse CD44 expression was not counted quantitatively and it did not account for assessment of positive cases of DAs, however it should be noted as a different staining pattern in low-grade gliomas. One study described a similar staining pattern in gliomas: membranous staining was found only in GBMs, and weak staining in the processes of astrocytes characterized DAs (Jijiwa *et al.*, 2011). Interestingly, different authors described the occurrence of CD44 expression within

processes of some fibrillary astrocytes in normal human brain (Kaaijk *et al.*, 1997; Sosunov *et al.*, 2014). Kaaijk *et al.* hypothesized that CD44 expression in normal astrocytes might contribute to the migratory capacity of astrocytes upon inflammation and other injury (Kaaijk *et al.*, 1997). However, Lui *et al.* showed that CD44 expression identifies astrocyte-restricted precursor cells (Liu *et al.*, 2004). In many other recent studies, CD44 has also been identified as a marker of astrocyte precursor cells (Cai *et al.*, 2012; Malik *et al.*, 2014; Naruse *et al.*, 2013; Qemo and Porter, 2019). Thus, we suppose that the weak background of CD44 immunoreactivity in processes may indicate the stemness and precursor state of neoplastic astrocytes in DAs. However, in high-grade gliomas, for the achievement of more aggressive behaviour and increased invasive properties of tumour cells, CD44 is significantly upregulated on the surface of neoplastic cells. In addition, the CD44 molecule may play different roles in the biology of neoplastic and non-neoplastic brain cells and the function of CD44 might be isoform specific.

In regard to the prognostic role of CD44, many authors showed that high CD44 expression is related to a worse prognosis in glioma patients (Anido *et al.*, 2010; Motomura *et al.*, 2012; Pietras *et al.*, 2014; Ranuncolo *et al.*, 2002; Wu *et al.*, 2020; Xu *et al.*, 2010). In this study, no prognostic role of CD44 was found in GBMs and DAs. However, CD44 was used as mesenchymal marker in the subtyping of GBMs, and it was found that GBMs that belong to the mesenchymal subtype (high CD44 expression and low p53, PDGFRA, IDH1 expression) did not respond to radiotherapy. Thus, expression of CD44 can be used as a predictive marker in combination with other markers of proneural signature.

Interestingly, in this study, in GBMs, CD44 expression was higher in females ($z = -2.224$; $p = 0.026$), indicating that the glioma stem cell population may be altered by gender-specific factors. In addition, CD44 showed a weak, statistically significant, negative correlation with GBM size ($r_s = -0.314$; $p < 0.001$). This correlation was also confirmed by the association between the size and CD44 expression: higher CD44 expression values were found more frequently in GBMs of a smaller size (< 4 cm) than in larger-sized tumours (≥ 4 cm) ($z = -2.364$; $p = 0.018$), indicating that expansion and rapid growth of a tumour may lead to depletion of the stem cell population in glioma.

In DAs, CD44 expression had a negative correlation with PDGFRA ($r_s = -0.592$; $p = 0.006$). In addition, Conray *et al.* showed that high CD44 scores were rarely found in gliomas with high PDGFRA expression (Conroy *et al.*, 2014). Cautiously considering that PDGFRA pathway activation in different classifications has been considered as a marker of proneural/proneural-like glioblastoma but CD44 expression is pointing towards the mesenchymal subtype, a negative association seems to be more reasonable.

In DAs, CD44 expression also had a positive correlation with MVD (r_s = 0.490; p = 0.018); this may indicate that expansion of the stem cell population accelerates vascularization of tumour tissues, which enables an increased supply of blood and nutrients essential for tumour growth and progression. It was proved in several studies that glioma stem cells have a critical role in tumour angiogenesis and are capable of secreting several angiogenic substances (Bao *et al.*, 2006; Hardee and Zagzag, 2012; Mihić *et al.*, 2019).

PDGFRA is a cell surface tyrosine kinase receptor for platelet-derived growth factor (PDGF). In the central nervous system, PDGFRA is key molecule involved in the formation of mature oligodendrocytes. PDGFRA is expressed on oligodendrocyte precursor cells and even more multipotent neural stem cells that give rise to mature oligodendrocytes if stimulated by PDGF-A (Fruttiger *et al.*, 1999; Hu *et al.*, 2008).

PDGFRA signalling is also important in the development of the neoplastic process, including gliomagenesis (Calzolari and Malatesta, 2010). Increased PDGF signalling in these immature, precursor cells stimulates their proliferation and blocks their ability to a achieve mature, differentiated cell state, causing them to form tumour-like growths resembling astrocytomas. Continuing proliferation of these precursor cells might lead to the development of astrocytomas (Jackson *et al.*, 2006).

According to Verhaak *et al.*, *PDGFRA* gene amplification together with mutations of *IDH1* and *TP53* characterize the proneural subtype of GBM (Verhaak *et al.*, 2010). However, numerous studies have shown that PDGFRA overexpression frequently does not indicate *PDGFRA* gene abnormalities and thus IHC is not reliable in identifying *PDGFRA* gene amplification (Chen *et al.*, 2013; Szerlip *et al.*, 2012). The levels of PDGFRA protein can be influenced by different molecular loops or microenvironment and can have a significant impact on the tumour cell characteristics. Chen *et al.* pointed out the importance of the surrounding microenvironment and unidentified molecular signalling loops that may cause PDGFRA upregulation in glioma cells, irrespective of the mutational status of the PDGFRA gene (Chen *et al.*, 2013). Consequently, proteomic and genomic investigations for PDGRFA should be considered as different tests yielding different, albeit partially overlapping, subtypes of glioblastoma. Additional studies would be reasonable to identify which test correlated better with the biological potential, treatment response and survival of glioblastoma patients.

In this study, the mean expression of PDGFRA was significantly higher in DAs – 42.3 (95% CI = 25.7–59.0) – than in GBMs – 7.9 (95% CI = 5.0–10.7). With a cut-off level of 50%, high PDGFRA expression was found in 50% (95% CI = 30.1–75.0) of DAs and only in 6.2% (95% CI = 3.2–11.3) of GBMs. The data is comparable with other studies: for example, Popova *et al.*, using the same cut-off value of 50%, reported strong PDGFRA positivity in 23% of DAs

and 4% of GBMs (Popova *et al.*, 2014). In contrast with these results, the highest rate of PDGFRA immunopositivity, which was also the highest rate among all published reports, was described by Le Mercier *et al.*, and this team of researchers reported PDGFRA positivity in as many as of 53% of GBMs (Le Mercier *et al.*, 2012). The difference with the last author may be attributable either to the complex digital computerized approach in evaluating the percentage of immunostained tissue area and global average pixel intensity, or glioblastoma heterogeneity as the digital scoring was targeted towards central tumour areas to avoid data contamination from parameters of non-neoplastic cells (Le Mercier *et al.*, 2012). To include PDGFRA in the subtyping of glioblastoma, scoring guidelines should be elaborated.

Interestingly, it was observed in the present study that higher PDGFRA expression values are more frequent in multifocal GBMs ($z = -1.968$; $p = 0.049$).

In this study, PDGFRA expression correlates with p53 protein expression in both GBMs ($r_s = 0.181$; $p = 0.043$) and DAs ($r_s = 0.544$; $p = 0.013$) . A correlation between p53 and PDGFRA expression in gliomas was also found by other researchers: for example, Popova *et al.* reported such a correlation in DAs and anaplastic astrocytomas but not in GBMs (Popova *et al.*, 2014). As mentioned before, both TP53 and PDGFRA gene mutations are frequently associated with the proneural subtype of GBMs as shown by Verhaak *et al.* (Verhaak *et al.*, 2010). The correlation between p53 and PDGFRA may indicate some functional link: defects in the TP53 gene and PDGFRA overexpression may possibly increase the tumorigenic potential in a subset of gliomas, however the possible mechanism explaining this correlation is not known. Interestingly, Hesselager *et al.* demonstrated that complementary effects of PDGFR signalling and loss of p53 function enhance tumorigenic effects in an experimental glioma model in mice, suggesting such a functional linkage (Hesselager *et al.*, 2003).

Surprisingly, PDGFRA expression also showed a negative correlation with MVD in DAs ($r_s = -0.501$; $p = 0.034$). In contrast, most studies indicate that platelet-derived growth factors (PDGRs) can stimulate angiogenesis by binding with their receptors, including PDGFRA; in addition, the pro-angiogenic effect of PDGFRA signalling is mediated indirectly by the induction of VEGF secretion (Ball *et al.*, 2007; Tsai *et al.*, 1995, 1999). However, the negative correlation between PDGFRA and MVD is contradictory to other studies, which indicate a pro-angiogenic effect of PDGFRA. In this study, another negative correlation between PDGFRA and CD44 ($r_s = -0.592$; $p = 0.006$) may explain this disagreement regarding PDGFRA and MVD. Thus, upregulation of PDGFRA leads to downregulation of CD44 – the potential glioma stem cell marker; however, a decreased population of stem cells suppresses angiogenesis, probably because of the deficiency of angiogenic factors produced by the same stem cells. To support this hypothesis, several reports indicate the critical role of glioma stem

cells in tumour neovascularization; these cells are also capable of producing angiogenic factors such as VEGF promoting angiogenesis (Hardee and Zagzag, 2012; Oka *et al.*, 2007; Wang *et al.*, 2016a; Yao *et al.*, 2008).

Returning to the negative correlation between PDGFRA and CD44 (r_s = - 0.592; p = 0.006), according to Verhaak *et al.*, it indicates the existence of two mutually exclusive molecular signatures in gliomas.

IDH1 R132H. Isocitrate dehydrogenase (IDH) is a metabolic enzyme that has a key role in the citric acid cycle. Three isoforms of IDH are known, encoded by five related genes. IDH3 converts isocitrate to alpha-ketoglutarate and NAD+ to NADH in the Krebs cycle; IDH1 catalyses oxidative decarboxylation of isocitrate to alpha-ketoglutarate and NADP+ to NADPH in the cytoplasm, and IDH2 in the mitochondria (Cohen *et al.*, 2013b; Zhang *et al.*, 2013). IDH1 R132H, the most frequent mutation of the *IDH1* gene in glioma, results in missense replacement of arginine by histidine, leading to production of the mutant enzyme that catalyses the synthesis of oncometabolite 2-hydroxyglutarate (Garber, 2010; Ward *et al.*, 2012). Low levels of IDH also sensitize the cells to oxidative damage due to the lack of reduced glutathione neutralizing reactive oxygen species (Bayley and Devilee, 2010; Yan *et al.*, 2009a). In addition, *IDH1* mutations facilitate promoter hypermethylation and expression of hypoxia-inducible factor HIF-1 alpha (Cohen *et al.*, 2013b).

IDH1 mutations are early events in the development of gliomas, thus the majority of DAs and secondary GBMs bear this signature (Cohen *et al.*, 2013a).

Immunohistochemical detection of IDH1 R132H protein is a routine practice nowadays to distinguish between primary and secondary GBMs that have better prognosis. In the whole group of glioblastomas, secondary GBMs constitute 6–13 % of these tumours (Ohgaki and Kleihues, 2013). A significantly lower frequency (3.4%; 95% CI = 0.5–6.3) of secondary GBMs was observed in our study, probably due to the study design. In the present investigation, recurrent brain tumours were excluded as the previous therapy might be a confounding factor that could affect the molecular characteristics of gliomas (Parsons *et al.*, 2008; Safa *et al.*, 2015; Shankar *et al.*, 2014). Thus, all GBMs in our study clinically resemble primary GBMs, similarly to Le Mercier *et al*., 2012 (Le Mercier *et al.*, 2012). A similar group was assessed by Nobusawa *et al*. who reported a very similar frequency of *IDH1* mutations: namely, 3.7% of cases clinically presenting as primary glioblastomas were identified as secondary GBMs by molecular signature (Nobusawa *et al.*, 2009). The frequency of IDH1 mutation was 3% in the study of Le Mercier *et al*. (Le Mercier *et al.*, 2012). As regards DAs, expression of mutant IDH1 R132H protein was detected in 76.9% (95% CI = 60.7–93.1) of tumours, while the remaining 23.1% (6.9–39.3) were negative for IDH1 R132H mutation. The frequency of IDH1

mutations in low-grade gliomas is 80% and 87% (Christensen *et al.*, 2011; Juratli *et al.*, 2012); however, the most frequent specific mutation, IDH1 R132H, was detected by immunohistochemistry in 57–80 % of low-grade gliomas (Cai *et al.*, 2016; Popova *et al.*, 2014; Wang *et al.*, 2016b), which is also consistent with this study.

Presence of IDH1 gene mutation is one of the known prognostic factors of a more favourable prognosis in patients with high-grade gliomas (Gravendeel *et al.*, 2010; Nobusawa *et al.*, 2009; Parsons *et al.*, 2008; Sanson *et al.*, 2009). For example, the median overall survival rates of mutated and non-mutated IDH 1 patients with GBMs were 3.8 and 1.1 years (Parsons *et al.*, 2008). In this study, patients with secondary GBMs had a significantly better prognosis than those with primary GBMs – the median OS was 18.3 (95% CI = 18.0–18.5) months versus 7.7 (95% CI = 6.3–9.0) months ($p = 0.040$). As for DAs, patients with IDH1 R132H negative and IDH1 R132H positive tumours did not show statistically significant survival differences ($p = 0.336$) in this study.

The prognostic role of IDH1 mutations in low-grade gliomas is much less clear. Some studies showed that IDH1 mutations correlated with better prognosis (Dubbink *et al.*, 2009; Sanson *et al.*, 2009), but other reports found no prognostic significance in IDH1 mutations in low-grade gliomas (Kim *et al.*, 2010). In addition, Thon *et al.* reported that IDH1 mutations in patients with DAs resulted in shortened progression-free survival and prolonged post-recurrence survival, but there was no impact on overall survival (Thon *et al.*, 2012). Moreover, IDH1 mutations can be predictive markers in low-grade gliomas (Hartmann *et al.*, 2011; Houillier *et al.*, 2010).

IDH gene mutations frequently combine with two other genetic alterations – TP53 mutations and 1p/19q loss. For example, Kim *et al.* showed TP53 gene mutations together with IDH1/2 gene mutations in 32% of low-grade gliomas, but 1p/19q plus IDH1/2 mutations in 37% of cases. Interestingly, some researchers recognized triple-negative low-grade gliomas, which lack all three genetic abnormalities (IDH1/2 mutations, 1p/19q loss, TP53 gene mutations); such tumours had the worst prognosis (Chan *et al.*, 2016; Figarella-Branger *et al.*, 2012; Kim *et al.*, 2010). Among low-grade gliomas, 7 to 11 % were shown to be triple negative (Eckel-Passow *et al.*, 2015; Figarella-Branger *et al.*, 2012). Thus, IDH1 mutation may have more prognostic importance in combined analysis of all three genetic alterations.

In this study, an association between IDH1 and p53 was also found in GBMs ($z = -3.555$; $p = 0.001$), as confirmed by other authors that these two abnormalities frequently coexist.

MVD (by CD34). Sustained angiogenesis is one of six essential hallmarks of neoplastic cells (Hanahan and Weinberg, 2000), and CD34, as an endothelial marker, is useful in studies

of angiogenesis to determine microvascular density (MVD) and vascular patterns of neoplasms (Foote *et al.*, 2005; Weidner, 2008). A simple and easily reproducible quantitative method for determining MVD in tumours was suggested by Weidner *et al.*, and Weidner's approach has been widely adoptedin angiogenesis studies (Weidner *et al.*, 1991).

In this study, GBMs showed statistically significantly higher MVD values than in DAs: the mean number of vessels per high-power field (hpf) (400x) was 40.7 (95% CI = 35.8–45.6) and 18.1 (95% CI = 12.9–23.3). This result is similar to results reported by Zhang *et al.*: 45 ± 6.2 vessels per hpf and 28 ± 7.2 vessels per hpf (Zhang *et al.*, 2014). Assimakopoulou *et al.* reported a mean MVD for GBMs – 50.2 vessels per hpf – and for DAs – 14.5 vessels per hpf (Assimakopoulou *et al.*, 1997).

Sharma *et al.* reported 104 vessels per hpf (200x) in GBMs, however they used a computerized approach in the quantification of MVD at 200x magnification (Sharma *et al.*, 2006). Korkolopoulou *et al.* performed a comprehensive morphometric analysis of glioma vasculature using image analysis software and digital images of microspecimens; the median microvessel count in this study was 13.0 in GBMs and 6.5 in DAs (Korkolopoulou *et al.*, 2002a). The differences above are attributed to the methodology of the counting procedure and also the antibodies used (CD34, CD31, factor VIII). In this study we used Weidner's approach and counted vessels in the areas of highest vascularity (hot spots) because these represent the maximal angiogenic capacity of the tumour.

In this study, no prognostic MVD value was found in either GBMs or DAs. There were have been a few studies regarding the prognostic role of MVD in brain tumours. Some studies indicated the prognostic significance of MVD in gliomas (Abdulrauf *et al.*, 1998; Leon *et al.*, 1996), however others rejected any prognostic role of MVD (Schiffer *et al.*, 1999).

4.4. Molecular and immunohistochemical subtypes of gliomas

For a long time, two GBM subtypes were known, reflecting different pathogenetic pathways: primary GBM, which develops *de novo*, and secondary GBM, which results from progression from lower-grade glial neoplasm (Kleihues and Ohgaki, 1999). These two subtypes of GBMs are morphologically indistinguishable, and only clinical information about pre-existing low-grade glioma may help to differentiate between primary and secondary GBMs carrying different prognoses. Thus, morphologically GBM represented a single entity with almost the same prognostic significance. But the situation dramatically changed when The Cancer Genome Atlas (TCGA) project shed more light on the molecular basis of GBMs. To date, the TCGA project has produced a huge amount of data such as whole genomic sequence, expression and epigenetic analysis of many cancers including GBMs. Verhaak *et al.*, using data

generated by the TCGA project, found that GBMs can be divided into four molecular subtypes based on different molecular alterations and gene expression patterns: classical, mesenchymal, proneural and neural subtypes (Verhaak *et al.*, 2010). In the past several years other studies have been performed, however disconcordance exists regarding the real number of glioma molecular subtypes and their clinical relevance, with most of these studies identifying from two to four glioma subtypes (Brennan *et al.*, 2009; Brown *et al.*, 2015; Freije *et al.*, 2004; Marziali *et al.*, 2016; Nigro *et al.*, 2005; Phillips *et al.*, 2006; Verhaak *et al.*, 2010; Vital *et al.*, 2010). Although the methods and set of molecular signatures used were different among studies, the concordance regarding GBM subtypes with a feature that corresponds to proneural or mesenchymal subtypes was the highest in most of the studies. In 2006, Phillips *et al.* described three GBM subtypes called "proneural", "proliferative" and "mesenchymal" (Phillips *et al.*, 2006). Even in earlier studies, GBMs with a proteomic signatures reminiscent of mesenchymal and proneural subtypes were described (Freije *et al.*, 2004; Tanwar *et al.*, 2002).

Because molecular techniques are complex, time-consuming and expensive they are not practical in routine. Immunohistochemistry (IHC) is an important component of pathology laboratory testing and a good surrogate of more expensive traditional cytogenetic and molecular methods. Based on molecular signatures described by Verhaak *et al.*, several research teams have successfully applied IHC in the subtyping of gliomas (Conroy *et al.*, 2014; Le Mercier *et al.*, 2012; Motomura *et al.*, 2012; Popova *et al.*, 2014). In one study by Le Mercier *et al.*, classical-like and proneural-like subtypes of GBMs were recognized using IHC and a minimal amount of markers (p53, EGFR, PDGFRA); the tumours that did not fit any expression pattern were classified as "Other" (Le Mercier *et al.*, 2012). In this study, a similar approach was used, and based on the study of Verhaak *et al.*, we chose to assess the expression of PDGFRA, IDH1 R132H, p53 and CD44 in order to classify GBM as a proneural or mesenchymal subtype. Our approach comes from the finding that mutations in TP53, IDH1 and PDGFRA genes are commonly associated with the proneural but not the mesenchymal subtype of GBM (Verhaak *et al.*, 2010). In contrast, CD44 is frequently upregulated in the mesenchymal subtype (Verhaak *et al.*, 2010), and CD44 was described as a marker of mesenchymal glioma stem cells (Behnan *et al.*, 2014; Brown *et al.*, 2015). In this study, 50% (95% CI = 42.0–58.0) of GBMs were classified as a proneural subtype, which is lower than in the study by Le Mercier *et al.*, where the proneural subtype was present in 60.2% of cases defined by p53 and PDGFRA immunopositivity (Le Mercier *et al.*, 2012). Le Mercier *et al.* used a different approach, computerized assessment of immunostained cells, while in other studies immunolabelling was assessed manually. The lowest frequency of proneural subtype (4%) was reported by Conroy *et al.* This team of researchers relied only on the detection of IDH1 R132H mutation for

assessment of proneural subtype, thus the proneural subtype was mostly comprised of secondary GBMs. In addition, they suggested PDGFRA and p53 as unreliable immunohistochemical markers of proneural subtype (Conroy *et al.*, 2014). Popova *et al.* reported proneural subtype in 29% of GBMs using p53 and OLIG2 (Popova *et al.*, 2014). Furthermore, according to Verhaak *et al.*, the proneural subtype was found in 29% of GBMs (Verhaak *et al.*, 2010). This large disconcordance is due to the different methodology and IHC markers used among different studies.

In this study, 18.5% (95% CI = 13.0–25.6) of GBMs were of mesenchymal subtype. In other studies, the mesenchymal subtype was reported in 12% to 29% of GBM cases by using different mesenchymal markers such as CD44, MERTK, VIM and YKL40 (Conroy *et al.*, 2014; Popova *et al.*, 2014; Verhaak *et al.*, 2010). In our study, 31.5% (95% CI = 24.5–39.4) of GBM cases remained unclassified and referred to as "Other".

In the current study, no statistically significant associations were found between the glioma subtype and clinical or remaining immunohistochemical parameters (MVD, Ki-67, p21 and p27), which were not used for subtyping. In the current study, no survival differences were found between different subtypes. With regard to clinical outcome, the mesenchymal subtype is described as a subtype with unfavourable prognosis, in contrast to the proneural subtype, which is characterized by better prognosis (Lin *et al.*, 2014; Phillips *et al.*, 2006; Verhaak *et al.*, 2010).

However, the response to adjuvant therapy was different between proneural and mesenchymal subtypes. In the current study, for GBM patients with a proneural subtype, there was a tendency for the addition of chemotherapy to radiotherapy alone to improve overall survival ($p = 0.061$) and radiotherapy alone significantly improved patients' overall survival compared with palliative management and surgery only without adjuvant treatment ($p = 0.008$). However, for GBM patients of mesenchymal subtype, the addition of chemotherapy to radiotherapy alone significantly improved overall survival ($p = 0.002$), but radiotherapy alone showed no beneficial effect when compared with palliative management and surgery only without adjuvant treatment ($p = 0.857$), indicating the radioresistency 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, and all patients with less intensive and more intensive therapy received combined concurent or non-concurent chemo-radiotherapy or chemotherapy of different intensities (Verhaak *et al.*, 2010).

However, Brown *et al.* reported the radioresistency of the mesenchymal subtype while preserving the chemosensitivity of the mesenchymal subtype (Brown *et al.*, 2015).

Several authors have reported that the mesenchymal subtype is associated with the stem cell phenotype, enriched with the presumed stem cell marker CD44 (Cheng *et al.*, 2012). However, glioma mesenchymal stem cells are characterized by extensive radioresistency (Mao *et al.*, 2013; Nakano, 2015), which may explain the failure of the radiotherapeutic effect in the mesenchymal subtype of GBMs in this study.

With regard to glioma stem cells, Brown *et al.* reported two distinct types of glioma stem cells that differed by their surface markers – CD133 positive and CD44 positive glioma stem cells. Interestingly, CD133 glioma stem cells characterized the proneural GBM subtype, whereas the mesenchymal subtype was enriched with CD44 glioma stem cells (Brown *et al.*, 2015). In addition, Brown *et al.* reported that patients with GBM enriched by CD133 positive cells had significantly improved survival from radiotherapy but no benefit from temozolomide; however, patients with GBM enriched by CD44 positive cells had a significant survival benefit from temozolomide treatment, while they had a reduced benefit from radiotherapy (Brown *et al.*, 2015). The data described by Brown *et al.* for mesenchymal stem cells corresponded to the treatment responses of the mesenchymal GBM subtype in the current study. In addition, CD44 positive stem cells had more invasive behaviour than CD133 positive cells (Brown *et al.*, 2015), thus it is possible that the radioresistancy of CD44 positive tumours may be at least partially responsible due to the extensive infiltration of brain tissues by neoplastic cells, and thus it is more problematic to target these infiltrating, diffusely scattered cells with radiotherapy, while infiltrating cells beyond the target field of radiation quickly restore tumour mass.

Surprisingly, the existence of these two different stem cell lines (CD133 + and CD44 +) in gliomas and the association with the same molecular subtypes of GBM have also been reported in another independent study (Lottaz *et al.*, 2010). In another study, Bhat *et al.* also reported that mesenchymal signature and CD44 expression correlated with poor radiation response and shorter overall survival in patients with GBM (Bhat *et al.*, 2013). A more aggressive course, radioresistancy and expression of CD44 on mesenchymal stem cells of GBM were also supported by Mao *et al.* using experimental mouse glioma models and stem cell cultures (Mao *et al.*, 2013).

One interesting observation is that the molecular gene expression signature of glioma is not a static phenotype but can be changed and modified by paracrine factors, microenvironment and previous radiation therapy (Bhat *et al.*, 2013; Nakano, 2015; Natesh *et al.*, 2015). As regards the phenotypic shift of the glioma subtype, recent evidence suggests that a process reminiscent of epithelial-mesenchymal transition (EMT) may occur in gliomas and the number of research papers concerning this issue is continuing to increase (Balasubramaniyan *et al.*, 2015; Bhat *et al.*, 2013; Carro *et al.*, 2010; Iwadate, 2016; Nakano, 2014; Parsa, 2010). EMT is

the transdifferentiation of epithelial cells into motile mesenchymal-like cells that contribute to invasiveness, therapy resistance and cancer progression (Lamouille *et al.*, 2014). Although glioma is not an epithelial cancer, neoplastic glioma cells may undergo transformation into a more invasive mesenchymal phenotype; such a shift was only described as a transition from a proneural to a mesenchymal phenotype, and was thus referred to by some authors as "proneural-mesenchymal transformation" (Nakano, 2014).

The mechanism controlling mesenchymal transformation in gliomas is poorly elucidated, but there is some evidence that this process could be regulated by paracrine factors, and in particular the importance of pro-inflammatory mediators has been shown in some studies, probably released by macrophages and activated microglia (Bhat *et al.*, 2013; Natesh *et al.*, 2015). However, Mao *et al,* reported that a glycolysis-mediated pathway involving aldehyde dehydrogenase 1A3 (ALDH1A3) enhanced mesenchymal transition in high-grade gliomas; furthermore, this group of researchers showed that inhibition of ALDH1A3 inhibited the growth of mesenchymal but not proneural glioma stem cells (Mao *et al.*, 2013). Other researchers have reported that glial stem cells showed susceptibility to minocycline, which can inhibit microglia activation and NF-kB signalling (Bhat *et al.*, 2013; Markovic *et al.*, 2011). Interestingly, Verhaak *et al.* previously reported high expression of genes associated with inflammation in the GBM mesenchymal subtype, indicating the importance of pro-inflammatory pathways in the maintenance of the mesenchymal phenotype, and that was later supported by other studies.

As regards the proneural subtype, it is usually characterized with better prognosis, while some studies showed that more aggressive therapy is less effective in this subtype (Le Mercier *et al.*, 2012; Verhaak *et al.*, 2010). However, there are still a lot of debate over which subtype of glioma has a better or worse prognosis (Mao *et al.*, 2013). In this study, no survival differences were found between mesenchymal and proneural subtypes. Furthermore, the proneural subtype of GBMs is thought to be a mixture of gliomas with several molecular signatures characterized by different prognostic outcomes: for example, Sturm *et al.* reported that a small portion of proneural subtype GBMs had a worse prognosis after removing IDH1 mutant samples (Sturm *et al.*, 2012). In another study, Nourshmer *et al.* reported that GBMs of a proneural subtype were different in terms of genome methylation pattern and had a different prognosis; in addition, this team of researchers described two subpopulations of proneural GBMs, namely G-CIMP positive proneural GBMs with better prognosis and G-CIMP negative proneural GBM with worse prognosis, which was relatively similar to mesenchymal tumours (Noushmehr *et al.*, 2010). All these studies reflect diversity between subtypes and other molecular changes may have an impact on the survival of these subtypes. Thus, more studies

are needed to elucidate which changes are more critical; however, several markers such as IDH1 R132H and CD44 in this study can be successfully used in glioma subtyping.

As regards DAs, the significance of subtyping in low-grade gliomas should be further evaluated. The low-grade gliomas are enriched mainly with markers for the proneural subtype (Cooper *et al.*, 2010; Guan *et al.*, 2014). In addition, a high frequency of IDH1 mutations in low-grade gliomas constitutes a predominance of the proneural subtype (Kim *et al.*, 2010).

In this study, most DAs belong to the proneural subtype – 24/26 (92.3%; 95% CI = 75.8–97.9) – while the remaining 2/26 (7.6%; 95% CI = 2.1–24.1%) DAs were not otherwise classified. Because CD44 expression was very low in DAs with a median value of 8.5% (IQR = 15) and the mesenchymal subtype was not identified in DAs, mesenchymal signature might be of importance only in GBMs, and not in DAs.

More studies are necessary to draw conclusions on the significance of these glioma subtypes, and time will tell whether it would be possible to introduce Verhaak's classification of gliomas in routine clinical practice.

Conclusions

1. The median survival of the studied patients with gliomas is 7.9 months, which is slightly below the survival rates reported in other countries.
2. Patients with secondary GBMs (IDH-mutant) show significantly better prognosis than patients with primary GBMs (IDH-wildtype), thus the presence of IDH1 R132H mutation is the most significant prognostic factor of better survival.
3. In patients with DAs, high PDGFRA expression is associated with significantly better survival.
4. CD44, p21, p27, PDGFRA and proliferation rate (by Ki-67) are grade-specific parameters in gliomas, thus CD44, Ki-67 and p21 are significantly upregulated; however, p27 and PDGFRA are downregulated in GBM. In contrast, p53 expression is grade independent in gliomas.
5. The immunohistochemical profile of gliomas involving expression of p27 and CD44 in GBMs and cellular proliferation (Ki-67) in both GBMs and DAs can be determined by gender-specific factors.
6. In GBMs, decreased expression of both p21 and CD44 is associated with larger tumours. In contrast, multifocal GBMs more frequently experience a loss of p27 and higher expression of PDGFRA.
7. In gliomas, cell cycle proteins, such as p53, p21 and p27, are involved in molecular mechanisms that regulate proliferation and angiogenesis as reflected by Ki-67 and MVD, respectively.
8. PDGFRA expression correlates with p53 expression in both GBMs and DAs, indicating a strong functional link between these proteins. In DAs, PDGFRA correlates inversely with CD44, p21 and MVD.
9. Immunohistochemical subtyping of gliomas is possible by using a limited number of markers – PDGFRA, p53, IDH1 R132H and CD44. Expression of CD44 is a reliable indicator of mesenchymal subtype in GBM, which has a worse response to radiotherapy.

Practical recommendations

1. Immunohistochemical visualization is recommended for all surgically resected glioma material for prognostic and predictive reasons.
2. Detection of IDH1 R132H mutation by IHC is recommended for all GBMs where it allows secondary GBMs to be distinguished from primary GBMs and thus IDH1 R132H is a valuable prognostic marker. The staining protocol developed during the study is recommended for routine use.
3. Detection of IDH1 R132H mutation by IHC is also recommended for DAs as an important and sensitive diagnostic test. Because the majority of DAs are IDH1 mutant tumours, IDH1 R132H immunohistochemistry could be helpful for precisely diagnosing infiltrating low-grade glioma, especially in small, limited tissue material such as stereotactic biopsies.
4. Detection of PDGFRA expression by IHC is recommended for DAs, bearing in mind the significant association with survival.
5. Immunohistochemical subtyping of gliomas is possible and applicable in routine practice. For the prediction of treatment response, assessment of CD44 is recommended to distinguish the mesenchymal subtype of GBM with more pronounced radioresistancy.

Publications and reports on topics of the doctoral thesis

Publications

1. Molecular classification of diffuse gliomas. Jakovlevs, A., Vanags, A., Gardovskis, J., Strumfa, I. Polish Journal of Pathology. (2019). 70(4), 246–258. doi:10.5114/pjp.2019.93126. (ir pieejams Pubmed).
2. Jakovļevs, A., Vanags, A., Gardovskis, J., & Štrumfa, I. (2020). Expression of CD44 and IDH1 R132H in Gliomas and their Prognostic Relevance, Proceedings of the Latvian Academy of Sciences. Section B. Natural, Exact, and Applied Sciences. 74(5).
3. Glioblastoma – Current Concepts, Prognostic Markers and Molecular Classification / Jakovlevs, A., Vanags, A., Balodis, D., Gardovskis, J., Strumfa, I. // Acta Chirurgica Latviensis. - No.13/1 (2013), p. 58–64.
4. Childhood medulloblastoma in Latvia: morphologic and molecular implications for diagnostics and personalised treatment / Franckevica, I., Jakovlevs, A., Abolins, A., Vanags, A., Strumfa, I. ...[et al.] // Acta Chirurgica Latviensis. - No.16/1 (2016), p. 9–15.
5. Solitary and multiple meningiomas : an immunohistochemical comparison / Vikmane, B., Jakovlevs, A., Vanags, A., Strumfa, I. // Acta Chirurgica Latviensis. - No.15/1 (2015), p. 23–28.
6. Prolonged survival after neurosurgical resection of lung cancer metastasis / Jakovlevs, A., Vanags, A., Strumfa, I....[et al.] // Acta Chirurgica Latviensis. - No.14/1 (2014), p. 32–34.
7. Recurrent multiple atypical meningiomas despite neurosurgical resection / Jakovlevs, A., Strumfa, I., Vanags, A. ...[et al.] // Acta Chirurgica Latviensis. - No.14/2 (2014), p. 56–58.
8. Low-grade rhabdoid meningioma : unusual morphological characteristics / Jakovlevs, A., Vanags, A., Gardovskis, J., Strumfa, I. // Acta Chirurgica Latviensis. - No.13/1 (2013), p. 84–86.

Reports and theses at international congresses and conferences

1) Jakovlevs, A., Gardovskis, J., Štrumfa, I. Ki-67 labeling index and CD44 expression in gliomas: does the gender matter? 10th Baltic Morphology Scientific Conference, 24.–25.10.2019, Kaunas, Lithuania. Abstract Book page 262.
2) Jakovlevs, A., Gardovskis, J., Štrumfa, I. Prognostic impact and correlations of Ki-67 labeling index and CD44 expression in gliomas. 10th Baltic Morphology Scientific Conference, 24.–25.10.2019, Kaunas, Lithuania. Abstract Book page 261.
3) Dr. Arvids Jakovlevs, Prof. Ilze Strumfa, Prof. Janis Gardovskis. Prognostic Role of Ki-67 Labeling Index in Diffuse Gliomas // Rīga Stradiņš University International Conference on

Medical and Health Care Sciences Knowledge; 01.04.-03.04. 2019, Riga, Latvia; Tēžu grāmata 730 lpp.

4) Dr. Arvids Jakovlevs, Prof. Ilze Strumfa, Prof. Janis Gardovskis. Prognostic and Predictive Significance of Immunohistochemically Defined Molecular Subclasses in Glioblastoma // Rīga Stradiņš University International Conference on Medical and Health Care Sciences Knowledge, 01.04.-03.04. 2019, Riga, Latvia; Tēžu grāmata 729 lpp.
5) Prof.Ilze Strumfa, Dr.Dz.Mezale, Prof. Guntis Bahs, Dr.Med. Andrejs Vanags, Dr. Ilze Fridrihsone, Dr. A.Jakovlevs. Digital Pathology in Education: Experience of Rīga Stradiņš University // Rīga Stradiņš University International Conference on Medical and Health Care Sciences Knowledge, 01.04.-03.04.2019, Riga, Latvia; Tēžu grāmata 741 lpp.
6) Assessment of microvascular density in gliomas : diagnostic and prognostic significance / Jakovlevs, A., Vanags, A., Gardovskis, J., Strumfa, I.// Baltic Morphology IX (Tartu, Estonia, Sept. 27–29, 2017) : Conference Programme : Abstracts of Presentations / University of Tartu. - Tartu, 2017. - P. 34.
7) Pleomorphic xanthoastrocytoma - a rare type of cerebral glioma / Jakovlevs, A., Vanags, A., Gardovskis, J., Strumfa, I. // Baltic Morphology IX (Tartu, Estonia, Sept. 27–29, 2017) : Conference Programme : Abstracts of Presentations / University of Tartu. - Tartu, 2017. - P. 35.
8) Prognostic and predictive role of proneural and mesenchymal molecular signatures in glioblastoma / Jakovlevs, A., Vanags, A., Gardovskis, J., Strumfa, I. // 8th Mildred Scheel Cancer Conference (Bonn, Germany, June 14-16, 2017) : Programme and Abstracts. - Bonn, 2017. - P. 148.
9) Expression of aberrant p53 protein in medulloblastoma [Elektroniskais resurss] / G.Kirsakmens, I.Franckevica, L.Kolomencikova, A.Jakovlevs, D.Balodis, I.Strumfa // International Symposium "Targets of immunotherapy of chronic viral infections and cancer" (Riga, Latvia, May 24–26, 2016)[Elektroniskais resurss] : Abstract Book. - Riga, 2016. - 1 p., on CD.
10) p53 protein as a potential target of cancer vaccines in glioblastomas [Elektroniskais resurss] / Jakovlevs, A., Vanags, A., Gardovskis, J., Strumfa, I. // International Symposium "Targets of immunotherapy of chronic viral infections and cancer" (Riga, Latvia, May 24–26, 2016) [Elektroniskais resurss] : Abstract Book. - Riga, 2016. - 1 p., on CD.
11) Jakovļevs, Arvīds. IDH1-R132 immunohistochemistry in human gliomas : a way to improved diagnostics / Jakovlevs, A., Vanags, A., Strumfa, I. // Eesti Arst. - Suppl.2 (2015, Sept.), p. 68. - 8th Congress of the Baltic Association of Surgeons (Tallinn, Estonia, Sept. 10–12, 2015) : [Abstracts].

12) Jakovļevs, Arvīds. Immunohistochemical analysis of platelet-derived growth factor receptor-alpha (PDGFRA) expression in gliomas / Jakovlevs, A., Vanags, A., Strumfa, I. // The 8th Baltic Morphology Scientific Conference "Interdisciplinary nature of contemporary morphology" (Vilnius, Lithuania, Nov.12-14, 2015) : [Abstract Book] / Vilnius University. - Vilnius, 2015. - P. 90.
13) Jakovļevs, Arvīds. The spectrum of brain metastases / Jakovlevs, A., Vanags, A., Strumfa, I. // The 8th Baltic Morphology Scientific Conference "Interdisciplinary nature of contemporary morphology" (Vilnius, Lithuania, Nov.12-14, 2015) : [Abstract Book] / Vilnius University. - Vilnius, 2015. - P. 91.
14) Heterogeneity of Ki-67 and p53 expression in glioblastomas : [abstract] / A.Jakovlevs, I.Strumfa, A.Vanags, J.Gardovskis // Virchows Archiv. - Vol. 465, Suppl. 1 (2014, Aug.), p.S352. - Starptautiski citējamā izdevumā. London. United Kingdom.
15) Lipoastrocytoma of the spinal cord mimicking lipoma : [abstract] / Jakovlevs, A., Strumfa, I., Vanags, A., Gardovskis, J. // Virchows Archiv. - Vol. 465, Suppl.1 (2014, Aug.), p. S353. - Starptautiski citējamā izdevumā. London. United Kingdom.
16) Low-grade rhabdoid meningioma - unusual morphological characteristics / Jakovļevs, A., Feldmane, L., Štrumfa, I., Gardovskis, J. // Baltic Morphology VII Scientific Conference "Morphological sciences in the experimental and clinical medicine" (Rīga, Latvia, Nov. 7–9, 2013) : Abstract Book / Rīga Stradiņš University. - Riga, 2013. - P.85.

Reports and theses at Latvian (local) congresses and conferences

1) Prognostic role of CD44 expression in diffuse gliomas / Jakovļevs, A., Vanags, A., Gardovskis, J., Štrumfa, I. // 2018.gada Zinātniskās konferences tēzes (Rīga, 2018. g. 22.–23. martā) / Rīgas Stradiņa universitāte. - Rīga, 2018. - 165. lpp.
2) Survival analysis of patients with diffuse gliomas in Latvia / Jakovļevs, A., Vanags, A., Gardovskis, J., Štrumfa, I. // 2018.gada Zinātniskās konferences tēzes (Rīga, 2018. g. 22.–23.martā) / Rīgas Stradiņa universitāte. - Rīga, 2018. - 164. lpp.
3) p27 and p21 protein expression in gliomas and their prognostic relevance / Jakovlevs, A., Vanags, A., Gardovskis, J., Strumfa, I. // 2017.gada Zinātniskās konferences tēzes (Rīga, 2017.g. 6.–7.aprīlī) / Rīgas Stradiņa universitāte. - Rīga, 2017. - 210. lpp.
4) Prognostic role of platelet derived growth factor receptor alpha expression in diffuse gliomas / Jakovlevs, A., Vanags, A., Gardovskis, J., Strumfa, I. // 2017.gada Zinātniskās konferences tēzes (Rīga, 2017. g. 6.–7.aprīlī) / Rīgas Stradiņa universitāte. - Rīga, 2017. - 209. lpp.

5) IDH1 R132H mutanta proteīna ekspresijas biežums difūzās astrocitomās / Jakovļevs, A., Vanags, A., Gardovskis, J., Štrumfa, I. // 2016. gada Zinātniskās konferences tēzes (Rīga, 2016. g. 17.–18.martā) / Rīgas Stradiņa universitāte. - Rīga, 2016. - 191. lpp.
6) p21 un p53 proteīna ekspresija gliālos audzējos / Jakovļevs, A., Vanags, A., Štrumfa, I., Gardovskis, J. // 2016.gada Zinātniskās konferences tēzes (Rīga, 2016. g. 17.–18.martā) / Rīgas Stradiņa universitāte. - Rīga, 2016. - 192. lpp.
7) CD44 proteīna ekspresija gliālos audzējos / Jakovļevs, A., Vanags, A., Gardovskis, J., Štrumfa, I. // 2015. gada Zinātniskās konferences tēzes (Rīga, 2015. g. 26.–27.martā) / Rīgas Stradiņa universitāte. - Rīga, 2015. - 281. lpp.
8) Primārās un sekundārās glioblastomas operāciju materiālā pacientiem Latvijā / Jakovļevs, A., Vanags, A., Gardovskis, J., Āboliņš, A., Štrumfa, I. // 2015. gada Zinātniskās konferences tēzes (Rīga, 2015. g. 26.–27. martā) / Rīgas Stradiņa universitāte. - Rīga, 2015. - 280. lpp.
9) Anaplastiska hemangiopericitoma – rets smadzeņu apvalku audzējs / Jakovļevs, A., Vanags, A., Gardovskis, J., Štrumfa, I. ...[u.c.] // 2014. gada Zinātniskās konferences tēzes (Rīga, 2014. g. 10.–11. aprīlī) / Rīgas Stradiņa universitāte. - Rīga, 2014. - 294. lpp.
10) Gliosarkoma – rets gliāls audzējs bērnu vecumā / Jakovļevs, A., Vanags, A., Gardovskis, J., Štrumfa, I. // 2014. gada Zinātniskās konferences tēzes (Rīga, 2014. g. 10.–11.aprīlī) / Rīgas Stradiņa universitāte. - Rīga, 2014. - 308. lpp.
11) Gliosarkomu diagnostiskie un prognostiskie marķieri / Jakovļevs, A., Vanags, A., Gardovskis, J., Štrumfa, I. // 2014.gada Zinātniskās konferences tēzes (Rīga, 2014. g. 10.–11.aprīlī) / Rīgas Stradiņa universitāte. Rīga, 2014. - 293. lpp.
12) Ki-67 proliferācijas indeksa un p53 proteīna ekspresijas heterogenitāte glioblastomās / Jakovļevs, A., Vanags, A., Gardovskis, J., Štrumfa, I. // 2014. gada Zinātniskās konferences tēzes (Rīga, 2014.g. 10.–11. aprīlī) / Rīgas Stradiņa universitāte. - Rīga, 2014. - 309. lpp.
13) CNS audzēju morfoloģiskais spektrs operāciju un stereotaktisku biopsiju materiālā / Jakovļevs, A., Vanags, A., Gardovskis, J., Štrumfa, I. // 2013. gada Zinātniskās konferences tēzes (Rīga, 2013. g. 21.–22.martā) / Rīgas Stradiņa universitāte. - Rīga, 2013. - 267. lpp.

References

1. Abbas, T. and Dutta, A. (2009). p21 in cancer: intricate networks and multiple activities. *Nat Rev Cancer,* 9, 400–414.
2. Abbastabar, M., Kheyrollah, M., Azizian, K., et al. (2018). Multiple functions of p27 in cell cycle, apoptosis, epigenetic modification and transcriptional regulation for the control of cell growth: a double-edged sword protein. *DNA Repair (Amst),* 69, 63–72.
3. Abdulrauf, S. I., Edvardsen, K., Ho, K. L., et al. (1998). Vascular endothelial growth factor expression and vascular density as prognostic markers of survival in patients with low-grade astrocytoma. *J Neurosurg,* 88, 513–520.
4. Afify, A., Pang, L. and Howell, L. (2007). Diagnostic utility of CD44 standard, CD44v6, and CD44v3-10 expression in adenocarcinomas presenting in serous fluids. *Appl Immunohistochem Mol Morphol,* 15, 446–450.
5. Afify, A., Purnell, P. and Nguyen, L. (2009). Role of CD44s and CD44v6 on human breast cancer cell adhesion, migration, and invasion. *Exp Mol Pathol,* 86, 95–100.
6. Ahmadloo, N., Kani, A. A., Mohammadianpanah, M., et al. (2013). Treatment outcome and prognostic factors of adult glioblastoma multiforme. *J Egypt Natl Canc Inst,* 25, 21–30.
7. Al-Hajj, M., Wicha, M. S., Benito-Hernandez, A., et al. (2003). Prospective identification of tumorigenic breast cancer cells. *Proc Natl Acad Sci U S A,* 100, 3983–3988.
8. Ali, T. and Jalal, J. (2013). Immunohistochemical expression of p53 and p21 in gliomas: a clinicopathological study. *Zanco Journal of Medical Sciences,* 17, 435–442.
9. Alkhaibary, A., Alassiri, A. H., AlSufiani, F., et al. (2019). Ki-67 labeling index in glioblastoma: does it really matter? *Hematol Oncol Stem Cell Ther,* 12, 82–88.
10. Amary, M. F., Bacsi, K., Maggiani, F., et al. (2011). IDH1 and IDH2 mutations are frequent events in central chondrosarcoma and central and periosteal chondromas but not in other mesenchymal tumours. *J Pathol,* 224, 334–343.
11. Andrae, J., Gallini, R. and Betsholtz, C. (2008). Role of platelet-derived growth factors in physiology and medicine. *Genes Dev,* 22, 1276–1312.
12. Anido, J., Saez-Borderias, A., Gonzalez-Junca, A., et al. (2010). TGF-beta receptor inhibitors target the CD44(high)/Id1(high) glioma-initiating cell population in human glioblastoma. *Cancer Cell,* 18, 655–668.
13. Ardeleanu, C., Comanescu, M., Comanescu, V., et al. (2005). Uncommon pattern in soft tissues epithelioid sarcoma. *Rom J Morphol Embryol,* 46, 229–233.
14. Arshad, H., Ahmad, Z. and Hasan, S. H. (2010). Gliomas: correlation of histologic grade, Ki67 and p53 expression with patient survival. *Asian Pac J Cancer Prev,* 11, 1637–1640.
15. Ashcroft, M. and Vousden, K. H. (1999). Regulation of p53 stability. *Oncogene,* 18, 7637–7643.
16. Assimakopoulou, M., Sotiropoulou-Bonikou, G., Maraziotis, T., et al. (1997). Microvessel density in brain tumors. *Anticancer Res,* 17, 4747–4753.
17. Back, M. F., Ang, E. L., Ng, W. H., et al. (2007). Improved median survival for glioblastoma multiforme following introduction of adjuvant temozolomide chemotherapy. *Ann Acad Med Singapore,* 36, 338–342.
18. Baeriswyl, V. and Christofori, G. (2009). The angiogenic switch in carcinogenesis. *Semin Cancer Biol,* 19, 329–337.
19. Balasubramaniyan, V., Vaillant, B., Wang, S., et al. (2015). Aberrant mesenchymal differentiation of glioma stem-like cells: implications for therapeutic targeting. *Oncotarget,* 6, 31007–31017.
20. Ball, S. G., Shuttleworth, C. A. and Kielty, C. M. (2007). Vascular endothelial growth factor can signal through platelet-derived growth factor receptors. *The Journal of Cell Biology,* 177, 489–500.

21. Balss, J., Meyer, J., Mueller, W., et al. (2008). Analysis of the IDH1 codon 132 mutation in brain tumors. *Acta Neuropathol,* 116, 597–602.

22. Bandarchi, B., Ma, L., Marginean, C., et al. (2010). D2-40, a novel immunohistochemical marker in differentiating dermatofibroma from dermatofibrosarcoma protuberans. *Mod Pathol,* 23, 434–438.

23. Bao, S., Wu, Q., Sathornsumetee, S., et al. (2006). Stem cell-like glioma cells promote tumor angiogenesis through vascular endothelial growth factor. *Cancer Res,* 66, 7843–7848.

24. Bardin, A., Boulle, N., Lazennec, G., et al. (2004). Loss of ERbeta expression as a common step in estrogen-dependent tumor progression. *Endocr Relat Cancer,* 11, 537–551.

25. Barnard, R. O. and Geddes, J. F. (1987). The incidence of multifocal cerebral gliomas. A histologic study of large hemisphere sections. *Cancer,* 60, 1519–1531.

26. Barnholtz-Sloan, J. S., Williams, V. L., Maldonado, J. L., et al. (2008). Patterns of care and outcomes among elderly individuals with primary malignant astrocytoma. *J Neurosurg,* 108, 642–648.

27. Barth, R. F. and Kaur, B. (2009). Rat brain tumor models in experimental neuro-oncology: the C6, 9L, T9, RG2, F98, BT4C, RT-2 and CNS-1 gliomas. *J Neurooncol,* 94, 299–312.

28. Batistatou, A., Kyzas, P. A., Goussia, A., et al. (2006). Estrogen receptor beta (ERbeta) protein expression correlates with BAG-1 and prognosis in brain glial tumours. *J Neurooncol,* 77, 17–23.

29. Batistatou, A., Stefanou, D., Goussia, A., et al. (2004). Estrogen receptor beta (ERbeta) is expressed in brain astrocytic tumors and declines with dedifferentiation of the neoplasm. *J Cancer Res Clin Oncol,* 130, 405–410.

30. Bauchet, L., Mathieu-Daude, H., Fabbro-Peray, P., et al. (2010). Oncological patterns of care and outcome for 952 patients with newly diagnosed glioblastoma in 2004. *Neuro Oncol,* 12, 725-735.

31. Baumhueter, S., Dybdal, N., Kyle, C., et al. (1994). Global vascular expression of murine CD34, a sialomucin-like endothelial ligand for L-selectin. *Blood,* 84, 2554–2565.

32. Bayley, J. P. and Devilee, P. (2010). Warburg tumours and the mechanisms of mitochondrial tumour suppressor genes. Barking up the right tree? *Curr Opin Genet Dev,* 20, 324–329.

33. Behnan, J., Isakson, P., Joel, M., et al. (2014). Recruited brain tumor-derived mesenchymal stem cells contribute to brain tumor progression. *Stem Cells,* 32, 1110–1123.

34. Benson, E. K., Mungamuri, S. K., Attie, O., et al. (2014). p53-dependent gene repression through p21 is mediated by recruitment of E2F4 repression complexes. *Oncogene,* 33, 3959–3969.

35. Bergmann, N., Delbridge, C., Gempt, J., et al. (2020). The intratumoral heterogeneity reflects the intertumoral subtypes of glioblastoma multiforme: a regional immunohistochemistry analysis. *Front Oncol,* 10, 494–494.

36. Bernstock, J. D., Mooney, J. H., Ilyas, A., et al. (2019). Molecular and cellular intratumoral heterogeneity in primary glioblastoma: clinical and translational implications. *J Neurosurg,* 1–9.

37. Besson, A., Gurian-West, M., Schmidt, A., et al. (2004). p27(Kip1) modulates cell migration through the regulation of RhoA activation. *Genes Dev,* 18, 862–876.

38. Besson, A. and Yong, V. W. (2000). Involvement of p21(Waf1/Cip1) in protein kinase C alpha-induced cell cycle progression. *Molecular and Cellular Biology,* 20, 4580–4590.

39. Bevilacqua, P., Barbareschi, M., Verderio, P., et al. (1995). Prognostic value of intratumoral microvessel density, a measure of tumor angiogenesis, in node-negative breast carcinoma – results of a multiparametric study. *Breast Cancer Res Treat,* 36, 205–217.

40. Bhat, K. P., Balasubramaniyan, V., Vaillant, B., et al. (2013). Mesenchymal differentiation mediated by NF-kappaB promotes radiation resistance in glioblastoma. *Cancer Cell,* 24, 331–346.

41. Bielecka, J. and Markiewicz-Żukowska, R. (2020). The influence of nutritional and lifestyle factors on glioma incidence. *Nutrients,* 12, 1812.

42. Blionas, A., Giakoumettis, D., Klonou, A., et al. (2018). Paediatric gliomas: diagnosis, molecular biology and management. *Ann Transl Med,* 6, 251–251.

43. Bloom, J. and Pagano, M. (2003). Deregulated degradation of the cdk inhibitor p27 and malignant transformation. *Semin Cancer Biol,* 13, 41–47.

44. Blumcke, I. and Wiestler, O. D. (2002). Gangliogliomas: an intriguing tumor entity associated with focal epilepsies. *J Neuropathol Exp Neurol,* 61, 575–584.

45. Borger, D. R., Tanabe, K. K., Fan, K. C., et al. (2012). Frequent mutation of isocitrate dehydrogenase (IDH)1 and IDH2 in cholangiocarcinoma identified through broad-based tumor genotyping. *Oncologist,* 17, 72–79.

46. Bosari, S., Lee, A. K., DeLellis, R. A., et al. (1992). Microvessel quantitation and prognosis in invasive breast carcinoma. *Hum Pathol,* 23, 755–761.

47. Bradshaw, A., Wickremsekera, A., Tan, S. T., et al. (2016). Cancer stem cell hierarchy in glioblastoma multiforme. *Front Surg,* 3, 21.

48. Braganza, M. Z., Kitahara, C. M., Berrington de Gonzalez, A., et al. (2012). Ionizing radiation and the risk of brain and central nervous system tumors: a systematic review. *Neuro Oncol,* 14, 1316–1324.

49. Brat, D. J., Castellano-Sanchez, A. A., Hunter, S. B., et al. (2004). Pseudopalisades in glioblastoma are hypoxic, express extracellular matrix proteases, and are formed by an actively migrating cell population. *Cancer Res,* 64, 920–927.

50. Brennan, C., Momota, H., Hambardzumyan, D., et al. (2009). Glioblastoma subclasses can be defined by activity among signal transduction pathways and associated genomic alterations. *PLoS One,* 4, e7752.

51. Brennan, C. W., Verhaak, R. G. W., McKenna, A., et al. (2013). The somatic genomic landscape of glioblastoma. *Cell,* 155, 462–477.

52. Brenner, A. V., Linet, M. S., Fine, H. A., et al. (2002). History of allergies and autoimmune diseases and risk of brain tumors in adults. *International Journal of Cancer,* 99, 252–259.

53. Brodbelt, A., Greenberg, D., Winters, T., et al. (2015). Glioblastoma in England: 2007-2011. *Eur J Cancer,* 51, 533–542.

54. Brown, D. V., Daniel, P. M., D'Abaco, G. M., et al. (2015). Coexpression analysis of CD133 and CD44 identifies proneural and mesenchymal subtypes of glioblastoma multiforme. *Oncotarget,* 6, 6267–6280.

55. Buckner, J. C. (2003). Factors influencing survival in high-grade gliomas. *Semin Oncol,* 30, 10–14.

56. Bullwinkel, J., Baron-Luhr, B., Ludemann, A., et al. (2006). Ki-67 protein is associated with ribosomal RNA transcription in quiescent and proliferating cells. *J Cell Physiol,* 206, 624–635.

57. Burns, K. A. and Korach, K. S. (2012). Estrogen receptors and human disease: an update. *Arch Toxicol,* 86, 1491–1504.

58. Cai, J., Zhu, P., Zhang, C., et al. (2016). Detection of ATRX and IDH1-R132H immunohistochemistry in the progression of 211 paired gliomas. *Oncotarget,* 7, 16384–16395.

59. Cai, N., Kurachi, M., Shibasaki, K., et al. (2012). CD44-positive cells are candidates for astrocyte precursor cells in developing mouse cerebellum. *Cerebellum,* 11, 181–193.

60. Cairns, R. A. and Mak, T. W. (2013). Oncogenic isocitrate dehydrogenase mutations: mechanisms, models, and clinical opportunities. *Cancer Discov,* 3, 730–741.

61. Calboli, F. C., Cox, D. G., Buring, J. E., et al. (2011). Prediagnostic plasma IgE levels and risk of adult glioma in four prospective cohort studies. *J Natl Cancer Inst,* 103, 1588–1595.

62. Caloglu, M., Yurut-Caloglu, V., Karagol, H., et al. (2009). Prognostic factors other than the performance status and age for glioblastoma multiforme: a single-institution experience. *J buon,* 14, 211–218.

63. Calzolari, F. and Malatesta, P. (2010). Recent insights into PDGF-induced gliomagenesis. *Brain Pathol,* 20, 527–538.

64. Camelo-Piragua, S., Jansen, M., Ganguly, A., et al. (2011). A sensitive and specific diagnostic panel to distinguish diffuse astrocytoma from astrocytosis: chromosome 7 gain with mutant isocitrate dehydrogenase 1 and p53. *J Neuropathol Exp Neurol,* 70, 110–115.

65. Camelo-Piragua, S., Jansen, M., Ganguly, A., et al. (2010). Mutant IDH1-specific immunohistochemistry distinguishes diffuse astrocytoma from astrocytosis. *Acta Neuropathol,* 119, 509–511.

66. Cancer Genome Atlas Research, N. (2008). Comprehensive genomic characterization defines human glioblastoma genes and core pathways. *Nature,* 455, 1061–1068.

67. Capelle, L., Fontaine, D., Mandonnet, E., et al. (2013). Spontaneous and therapeutic prognostic factors in adult hemispheric World Health Organization Grade II gliomas: a series of 1097 cases: clinical article. *J Neurosurg,* 118, 1157–1168.

68. Capper, D., Weissert, S., Balss, J., et al. (2010). Characterization of R132H mutation-specific IDH1 antibody binding in brain tumors. *Brain Pathol,* 20, 245–254.

69. Carro, M. S., Lim, W. K., Alvarez, M. J., et al. (2010). The transcriptional network for mesenchymal transformation of brain tumours. *Nature,* 463, 318–325.

70. Castelli, J., Feuvret, L., Haoming, Q. C., et al. (2016). Prognostic and therapeutic factors of gliosarcoma from a multi-institutional series. *J Neurooncol,* 129, 85–92.

71. Catzavelos, C., Bhattacharya, N., Ung, Y. C., et al. (1997). Decreased levels of the cell-cycle inhibitor p27Kip1 protein: prognostic implications in primary breast cancer. *Nat Med,* 3, 227–230.

72. Cavalla, P., Piva, R., Bortolotto, S., et al. (1999). p27/kip1 expression in oligodendrogliomas and its possible prognostic role. *Acta Neuropathol,* 98, 629–634.

73. Cazzalini, O., Scovassi, A. I., Savio, M., et al. (2010). Multiple roles of the cell cycle inhibitor p21(CDKN1A) in the DNA damage response. *Mutat Res,* 704, 12–20.

74. Chai, L., Liu, H., Zhang, Z., et al. (2014). CD44 expression is predictive of poor prognosis in pharyngolaryngeal cancer: systematic review and meta-analysis. *Tohoku J Exp Med,* 232, 9–19.

75. Chaichana, K. L., Chaichana, K. K., Olivi, A., et al. (2011). Surgical outcomes for older patients with glioblastoma multiforme: preoperative factors associated with decreased survival. Clinical article. *J Neurosurg,* 114, 587–594.

76. Chaichana, K. L., Martinez-Gutierrez, J. C., De la Garza-Ramos, R., et al. (2013). Factors associated with survival for patients with glioblastoma with poor pre-operative functional status. *J Clin Neurosci,* 20, 818–823.

77. Chaloob, M. K., Ali, H. H., Qasim, B. J., et al. (2012). Immunohistochemical expression of Ki-67, PCNA and CD34 in astrocytomas: a clinicopathological study. *Oman Medical Journal,* 27, 368–374.

78. Chan, A. K., Mao, Y. and Ng, H. K. (2016). TP53 and histone H3.3 mutations in triple-negative lower-grade gliomas. *N Engl J Med,* 375, 2206–2208.

79. Chehab, N. H., Malikzay, A., Appel, M., et al. (2000). Chk2/hCds1 functions as a DNA damage checkpoint in G(1) by stabilizing p53. *Genes Dev,* 14, 278–288.

80. Chen, D., Li, D., Xu, X.-B., et al. (2019). Galangin inhibits epithelial-mesenchymal transition and angiogenesis by downregulating CD44 in glioma. *Journal of Cancer,* 10, 4499–4508.

81. Chen, D., Persson, A., Sun, Y., et al. (2013). Better prognosis of patients with glioma expressing FGF2-dependent PDGFRA irrespective of morphological diagnosis. *PLoS One,* 8, e61556.

82. Chen, G., Cheng, Y., Zhang, Z., et al. (2011). Prognostic significance of cytoplasmic p27 expression in human melanoma. *Cancer Epidemiol Biomarkers Prev,* 20, 2212–2221.

83. Chen, J., Willingham, T., Shuford, M., et al. (1996). Tumor suppression and inhibition of aneuploid cell accumulation in human brain tumor cells by ectopic overexpression of the cyclin-dependent kinase inhibitor p27KIP1. *J Clin Invest,* 97, 1983–1988.

84. Cheng, T., Rodrigues, N., Shen, H., et al. (2000). Hematopoietic stem cell quiescence maintained by p21cip1/waf1. *Science,* 287, 1804–1808.

85. Cheng, W. Y., Kandel, J. J., Yamashiro, D. J., et al. (2012). A multi-cancer mesenchymal transition gene expression signature is associated with prolonged time to recurrence in glioblastoma. *PLoS One,* 7, e34705.

86. Chetty, R. (2003). p27 Protein and cancers of the gastrointestinal tract and liver: an overview. *J Clin Gastroenterol,* 37, 23–27.

87. Chowdhury, R., Yeoh, K. K., Tian, Y. M., et al. (2011). The oncometabolite 2-hydroxyglutarate inhibits histone lysine demethylases. *EMBO Rep,* 12, 463–469.

88. Christensen, B. C., Smith, A. A., Zheng, S., et al. (2011). DNA methylation, isocitrate dehydrogenase mutation, and survival in glioma. *J Natl Cancer Inst,* 103, 143–153.

89. Claes, A., Idema, A. J. and Wesseling, P. (2007). Diffuse glioma growth: a guerilla war. *Acta Neuropathol,* 114, 443–458.

90. Claus, E. B., Walsh, K. M., Wiencke, J., et al. (2015). Survival and low grade glioma: the emergence of genetic information. *Neurosurg Focus,* 38, E6-E6.

91. Cmielova, J. and Rezacova, M. (2011). p21Cip1/Waf1 protein and its function based on a subcellular localization [corrected]. *J Cell Biochem,* 112, 3502–3506.

92. Cohen, A., Holmen, S. and Colman, H. (2013a). IDH1 and IDH2 Mutations in gliomas. *Curr Neurol Neurosci Rep,* 13, 345–345.

93. Cohen, A. L., Holmen, S. L. and Colman, H. (2013b). IDH1 and IDH2 mutations in gliomas. *Curr Neurol Neurosci Rep,* 13, 345.

94. Conroy, S., Kruyt, F. A., Joseph, J. V., et al. (2014). Subclassification of newly diagnosed glioblastomas through an immunohistochemical approach. *PLoS One,* 9, e115687.

95. Cooper, L. A., Gutman, D. A., Long, Q., et al. (2010). The proneural molecular signature is enriched in oligodendrogliomas and predicts improved survival among diffuse gliomas. *PLoS One,* 5, e12548.

96. Coqueret, O. (2003). New roles for p21 and p27 cell-cycle inhibitors: a function for each cell compartment? *Trends Cell Biol,* 13, 65–70.

97. Cummings, T. J., Burchette, J. L. and McLendon, R. E. (2001). CD34 and dural fibroblasts: the relationship to solitary fibrous tumor and meningioma. *Acta Neuropathol,* 102, 349–354.

98. Currier, A. W., Kolb, E. A., Gorlick, R. G., et al. (2019). p27/Kip1 functions as a tumor suppressor and oncoprotein in osteosarcoma. *Sci Rep,* 9, 6161.

99. Dalerba, P., Dylla, S. J., Park, I. K., et al. (2007). Phenotypic characterization of human colorectal cancer stem cells. *Proc Natl Acad Sci U S A,* 104, 10158–10163.

100. Dang, L., White, D. W., Gross, S., et al. (2010). Cancer-associated IDH1 mutations produce 2-hydroxyglutarate. *Nature,* 465, 966.

101. Daumas-Duport, C., Scheithauer, B., O'Fallon, J., et al. (1988). Grading of astrocytomas. A simple and reproducible method. *Cancer,* 62, 2152–2165.

102. Davis, F., Il'yasova, D., Rankin, K., et al. (2011). Medical diagnostic radiation exposures and risk of gliomas. *Radiat Res,* 175, 790–796.

103. De la Cueva, E., Garcia-Cao, I., Herranz, M., et al. (2006). Tumorigenic activity of p21Waf1//Cip1 in thymic lymphoma. *Oncogene,* 25, 4128–4132.

104. Delgado-Lopez, P. D. and Corrales-Garcia, E. M. (2016). Survival in glioblastoma: a review on the impact of treatment modalities. *Clin Transl Oncol,* 18, 1062–1071.

105. Deltour, I., Auvinen, A., Feychting, M., et al. (2012). Mobile phone use and incidence of glioma in the Nordic countries 1979-2008: consistency check. *Epidemiology,* 23, 301–307.

106. Denicourt, C., Saenz, C. C., Datnow, B., et al. (2007). Relocalized p27Kip1 tumor suppressor functions as a cytoplasmic metastatic oncogene in melanoma. *Cancer Res,* 67, 9238-9243.

107. Di, L., Heath, R. N., Shah, A. H., et al. (2020). Resection versus biopsy in the treatment of multifocal glioblastoma: a weighted survival analysis. *J Neurooncol,* 148, 155-164.

108. Di, X., Nishizaki, T., Harada, K., et al. (1997). Proliferative potentials of glioma cells and vascular components determined with monoclonal antibody MIB-1. *J Exp Clin Cancer Res,* 16, 389-394.

109. Djalilian, H. R., Shah, M. V. and Hall, W. A. (1999). Radiographic incidence of multicentric malignant gliomas. *Surg Neurol,* 51, 554-557; discussion 557-558.

110. Dobes, M., Shadbolt, B., Khurana, V. G., et al. (2011). A multicenter study of primary brain tumor incidence in Australia (2000-2008). *Neuro Oncol,* 13, 783-790.

111. Dolecek, T. A., Propp, J. M., Stroup, N. E., et al. (2012). CBTRUS Statistical Report: Primary Brain and Central Nervous System Tumors Diagnosed in the United States in 2005–2009. *Neuro Oncol,* 14, v1-v49.

112. Dong, Q., Li, Q., Wang, M., et al. (2019). Elevated CD44 expression predicts poor prognosis in patients with low-grade glioma. *Oncol Lett,* 18, 3698-3704.

113. Dubbink, H. J., Taal, W., van Marion, R., et al. (2009). IDH1 mutations in low-grade astrocytomas predict survival but not response to temozolomide. *Neurology,* 73, 1792-1795.

114. Dubrow, R. and Darefsky, A. S. (2011). Demographic variation in incidence of adult glioma by subtype, United States, 1992-2007. *BMC Cancer,* 11, 325-325.

115. Duensing, A., Ghanem, L., Steinman, R. A., et al. (2006). p21(Waf1/Cip1) deficiency stimulates centriole overduplication. *Cell Cycle,* 5, 2899-2902.

116. Duffau, H. and Capelle, L. (2004). Preferential brain locations of low-grade gliomas. *Cancer,* 100, 2622-2626.

117. Duncan, T. J., Al-Attar, A., Rolland, P., et al. (2010). Cytoplasmic p27 expression is an independent prognostic factor in ovarian cancer. *Int J Gynecol Pathol,* 29, 8-18.

118. Dunn, K. L., Espino, P. S., Drobic, B., et al. (2005). The Ras-MAPK signal transduction pathway, cancer and chromatin remodeling. *Biochem Cell Biol,* 83, 1-14.

119. Duregon, E., Bertero, L., Pittaro, A., et al. (2016). Ki-67 proliferation index but not mitotic thresholds integrates the molecular prognostic stratification of lower grade gliomas. *Oncotarget,* 7, 21190-21198.

120. Dziurzynski, K., Blas-Boria, D., Suki, D., et al. (2012). Butterfly glioblastomas: a retrospective review and qualitative assessment of outcomes. *J Neurooncol,* 109, 555-563.

121. Dzwonek, J. and Wilczynski, G. M. (2015). CD44: molecular interactions, signaling and functions in the nervous system. *Front Cell Neurosci,* 9, 175.

122. Eckel-Passow, J. E., Lachance, D. H., Molinaro, A. M., et al. (2015). Glioma groups based on 1p/19q, IDH, and TERT promoter mutations in tumors. *N Engl J Med,* 372, 2499-2508.

123. Eckner, R. (2012). p53-dependent growth arrest and induction of p21: a critical role for PCAF-mediated histone acetylation. *Cell Cycle,* 11, 2591-2591.

124. Ewelt, C., Goeppert, M., Rapp, M., et al. (2011). Glioblastoma multiforme of the elderly: the prognostic effect of resection on survival. *J Neurooncol,* 103, 611-618.

125. Fan, C., Zhang, J., Liu, Z., et al. (2019). Prognostic role of microvessel density in patients with glioma. *Medicine,* 98, e14695-e14695.

126. Fan, K. J. and Pezeshkpour, G. H. (1992). Ethnic distribution of primary central nervous system tumors in Washington, DC, 1971 to 1985. *Journal of the National Medical Association,* 84, 858-863.

127. Faria, M. H., Patrocinio, R. M., Moraes Filho, M. O., et al. (2007). Immunoexpression of tumor suppressor genes p53, p21 WAF1/CIP1 and p27 KIP1 in humam astrocystic tumors. *Arq Neuropsiquiatr,* 65, 1114-1122.

128. Feng, Y., Wang, J., Tan, D., et al. (2019). Relationship between circulating inflammatory factors and glioma risk and prognosis: a meta-analysis. 8, 7454-7468.

129. Figarella-Branger, D., Bouvier, C., de Paula, A. M., et al. (2012). Molecular genetics of adult grade II gliomas: towards a comprehensive tumor classification system. *J Neurooncol,* 110, 205-213.

130. Filippini, G., Falcone, C., Boiardi, A., et al. (2008). Prognostic factors for survival in 676 consecutive patients with newly diagnosed primary glioblastoma. *Neuro Oncol,* 10, 79-87.

131. Fina, L., Molgaard, H., Robertson, D., et al. (1990). Expression of the CD34 gene in vascular endothelial cells. *Blood,* 75, 2417-2426.

132. Fischer, M., Steiner, L. and Engeland, K. (2014). The transcription factor p53: not a repressor, solely an activator. *Cell Cycle,* 13, 3037-3058.

133. Fischer, N. W., Prodeus, A. and Gariépy, J. (2018). Survival in males with glioma and gastric adenocarcinoma correlates with mutant p53 residual transcriptional activity. *JCI Insight,* 3.

134. Fleming, T. P., Saxena, A., Clark, W. C., et al. (1992). Amplification and/or overexpression of platelet-derived growth factor receptors and epidermal growth factor receptor in human glial tumors. *Cancer Res,* 52, 4550-4553.

135. Foote, R. L., Weidner, N., Harris, J., et al. (2005). Evaluation of tumor angiogenesis measured with microvessel density (MVD) as a prognostic indicator in nasopharyngeal carcinoma: Results of RTOG 9505. *International Journal of Radiation Oncology*Biology*Physics,* 61, 745-753.

136. Forman, D., Bray, F., Brewster, D. H., et al. (2012). *Cancer Incidence in Five Continents*.

137. Forte, I. M., Indovina, P., Iannuzzi, C. A., et al. (2019). Targeted therapy based on p53 reactivation reduces both glioblastoma cell growth and resistance to temozolomide. *Int J Oncol,* 54, 2189-2199.

138. Freije, W. A., Castro-Vargas, F. E., Fang, Z., et al. (2004). Gene expression profiling of gliomas strongly predicts survival. *Cancer Res,* 64, 6503-6510.

139. Fruttiger, M., Karlsson, L., Hall, A. C., et al. (1999). Defective oligodendrocyte development and severe hypomyelination in PDGF-A knockout mice. *Development,* 126, 457-467.

140. Fuse, T., Tanikawa, M., Nakanishi, M., et al. (2000). p27Kip1 expression by contact inhibition as a prognostic index of human glioma. *J Neurochem,* 74, 1393-1399.

141. Gaiser, T., Becker, M. R., Meyer, J., et al. (2009). p53-mediated inhibition of angiogenesis in diffuse low-grade astrocytomas. *Neurochem Int,* 54, 458-463.

142. Garber, K. (2010). Oncometabolite? IDH1 discoveries raise possibility of new metabolism targets in brain cancers and leukemia. *J Natl Cancer Inst,* 102, 926-928.

143. Gartel, A. L. (2005). The conflicting roles of the cdk inhibitor p21(CIP1/WAF1) in apoptosis. *Leuk Res,* 29, 1237-1238.

144. Gartel, A. L. (2006). Is p21 an oncogene? *Mol Cancer Ther,* 5, 1385-1386.

145. Gartel, A. L. (2009). p21(WAF1/CIP1) and cancer: a shifting paradigm? *Biofactors,* 35, 161-164.

146. Gartel, A. L. and Tyner, A. L. (2002). The role of the cyclin-dependent kinase inhibitor p21 in apoptosis. *Mol Cancer Ther,* 1, 639-649.

147. Gerdes, J., Schwab, U., Lemke, H., et al. (1983). Production of a mouse monoclonal antibody reactive with a human nuclear antigen associated with cell proliferation. *Int J Cancer,* 31, 13-20.

148. Gesbert, F., Sellers, W. R., Signoretti, S., et al. (2000). BCR/ABL regulates expression of the cyclin-dependent kinase inhibitor p27Kip1 through the phosphatidylinositol 3-Kinase/AKT pathway. *J Biol Chem,* 275, 39223-39230.

149. Giannopoulos, S. and Kyritsis, A. P. (2010). Diagnosis and management of multifocal gliomas. *Oncology,* 79, 306-312.

150. Gillet, E., Alentorn, A., Doukoure, B., et al. (2014). TP53 and p53 statuses and their clinical impact in diffuse low grade gliomas. *J Neurooncol,* 118, 131-139.

151. Gittleman, H., Kromer, C., Ostrom, Q. T., et al. (2017). Is mortality due to primary malignant brain and other central nervous system tumors decreasing? 133, 265-275.

152. Gondim, D. D., Gener, M. A., Curless, K. L., et al. (2019). Determining IDH-mutational status in gliomas using IDH1-R132H antibody and polymerase chain reaction. *Applied Immunohistochemistry & Molecular Morphology,* 27, 722-725.

153. Gravendeel, L. A., Kloosterhof, N. K., Bralten, L. B., et al. (2010). Segregation of non-p.R132H mutations in IDH1 in distinct molecular subtypes of glioma. *Hum Mutat,* 31, E1186-1199.

154. Grimmler, M., Wang, Y., Mund, T., et al. (2007). Cdk-inhibitory activity and stability of p27Kip1 are directly regulated by oncogenic tyrosine kinases. *Cell,* 128, 269-280.

155. Gross, M. W., Kraus, A., Nashwan, K., et al. (2005). Expression of p53 and p21 in primary glioblastomas. *Strahlenther Onkol,* 181, 164-171.

156. Gu, W. and Roeder, R. G. (1997). Activation of p53 sequence-specific DNA binding by acetylation of the p53 C-terminal domain. *Cell,* 90, 595-606.

157. Guan, X., Vengoechea, J., Zheng, S., et al. (2014). Molecular subtypes of glioblastoma are relevant to lower grade glioma. *PLoS One,* 9, e91216.

158. Gunthert, U., Stauder, R., Mayer, B., et al. (1995). Are CD44 variant isoforms involved in human tumour progression? *Cancer Surv,* 24, 19-42.

159. Guo, C., Pirozzi, C. J., Lopez, G. Y., et al. (2011). Isocitrate dehydrogenase mutations in gliomas: mechanisms, biomarkers and therapeutic target. *Curr Opin Neurol,* 24, 648-652.

160. Guo, C. F., Zhuang, Y., Chen, Y., et al. (2020). Significance of tumor protein p53 mutation in cellular process and drug selection in brain lower grade (WHO grades II and III) glioma. 14, 1139-1150.

161. Gupta, M., Djalilvand, A. and Brat, D. J. (2005). Clarifying the diffuse gliomas: an update on the morphologic features and markers that discriminate oligodendroglioma from astrocytoma. *American Journal of Clinical Pathology,* 124, 755-768.

162. Hall, P. A. and Lane, D. P. (1994). p53 in tumour pathology: can we trust immunohistochemistry?--Revisited! *J Pathol,* 172, 1-4.

163. Hamilton, S. R., Liu, B., Parsons, R. E., et al. (1995). The molecular basis of Turcot's syndrome. *N Engl J Med,* 332, 839-847.

164. Hanahan, D. and Weinberg, R. A. (2000). The hallmarks of cancer. *Cell,* 100, 57-70.

165. Hardee, M. E. and Zagzag, D. (2012). Mechanisms of glioma-associated neovascularization. *Am J Pathol,* 181, 1126-1141.

166. Hardell, L. and Carlberg, M. (2015). Mobile phone and cordless phone use and the risk for glioma: analysis of pooled case-control studies in Sweden, 1997–2003 and 2007–2009. *Pathophysiology,* 22, 1-13.

167. Hartman, J., Edvardsson, K., Lindberg, K., et al. (2009). Tumor repressive functions of estrogen receptor beta in SW480 colon cancer cells. *Cancer Res,* 69, 6100-6106.

168. Hartmann, C., Hentschel, B., Tatagiba, M., et al. (2011). Molecular markers in low-grade gliomas: predictive or prognostic? *Clin Cancer Res,* 17, 4588-4599.

169. Hartmann, C., Hentschel, B., Wick, W., et al. (2010). Patients with IDH1 wild type anaplastic astrocytomas exhibit worse prognosis than IDH1-mutated glioblastomas, and IDH1 mutation status accounts for the unfavorable prognostic effect of higher age: implications for classification of gliomas. *Acta Neuropathol,* 120, 707-718.

170. He, S. M., Zhao, Z. W., Wang, Y., et al. (2012). Potential role of Jun activation domain-binding protein 1 and phosphorylated p27 expression in prognosis of glioma. *Brain Tumor Pathol,* 29, 3-9.

171. Hegi, M. E., Diserens, A. C., Gorlia, T., et al. (2005). MGMT gene silencing and benefit from temozolomide in glioblastoma. *N Engl J Med,* 352, 997-1003.

172. Heinke, T., Espirito Santo, K. S., Longatto Filho, A., et al. (2013). Vascular endothelial growth factor and KIT expression in relation with microvascular density and tumor grade in supratentorial astrocytic tumors. *Acta Cir Bras,* 28, 48-54.

173. Hengst, L. and Reed, S. I. (1996). Translational control of p27Kip1 accumulation during the cell cycle. *Science,* 271, 1861-1864.

174. Hermansen, S. K., Christensen, K. G., Jensen, S. S., et al. (2011). Inconsistent immunohistochemical expression patterns of four different CD133 antibody clones in glioblastoma. *Journal of Histochemistry and Cytochemistry,* 59, 391-407.

175. Hesselager, G., Uhrbom, L., Westermark, B., et al. (2003). Complementary effects of platelet-derived growth factor autocrine stimulation and p53 or Ink4a-Arf deletion in a mouse glioma model. *Cancer Res,* 63, 4305-4309.

176. Hilmani, S., Abidi, O., Benrahma, H., et al. (2013). Clinicopathological features and molecular analysis of primary glioblastomas in Moroccan patients. *J Mol Neurosci,* 49, 567-573.

177. Hilton, D. A., Love, S., Barber, R., et al. (1998). Accumulation of p53 and Ki-67 expression do not predict survival in patients with fibrillary astrocytomas or the response of these tumors to radiotherapy. *Neurosurgery,* 42, 724-729.

178. Hilton, D. A., Penney, M., Evans, B., et al. (2002). Evaluation of molecular markers in low-grade diffuse astrocytomas: loss of p16 and retinoblastoma protein expression is associated with short survival. *Am J Surg Pathol,* 26, 472-478.

179. Ho, D. M., Hsu, C. Y., Ting, L. T., et al. (2003). MIB-1 and DNA topoisomerase II alpha could be helpful for predicting long-term survival of patients with glioblastoma. *Am J Clin Pathol,* 119, 715-722.

180. Houillier, C., Lejeune, J., Benouaich-Amiel, A., et al. (2006). Prognostic impact of molecular markers in a series of 220 primary glioblastomas. *Cancer,* 106, 2218-2223.

181. Houillier, C., Wang, X., Kaloshi, G., et al. (2010). IDH1 or IDH2 mutations predict longer survival and response to temozolomide in low-grade gliomas. *Neurology,* 75, 1560-1566.

182. Hsu, D. W., Louis, D. N., Efird, J. T., et al. (1997). Use of MIB-1 (Ki-67) immunoreactivity in differentiating grade II and grade III gliomas. *J Neuropathol Exp Neurol,* 56, 857-865.

183. Hu, J. G., Fu, S. L., Wang, Y. X., et al. (2008). Platelet-derived growth factor-AA mediates oligodendrocyte lineage differentiation through activation of extracellular signal-regulated kinase signaling pathway. *Neuroscience,* 151, 138-147.

184. Hu, X., Miao, W. E. I., Zou, Y., et al. (2013). Expression of p53, epidermal growth factor receptor, Ki-67 and O(6)-methylguanine-DNA methyltransferase in human gliomas. *Oncol Lett,* 6, 130-134.

185. Huang, J., Yu, J., Tu, L., et al. (2019). Isocitrate dehydrogenase mutations in glioma: from basic discovery to therapeutics development. *Front Oncol,* 9, 506.

186. Huang, K.-T., Pavlides, S. C., Lecanda, J., et al. (2012). Estrogen and progesterone regulate p27kip1 levels via the ubiquitin-proteasome system: pathogenic and therapeutic implications for endometrial cancer. *PLoS One,* 7, e46072.

187. Hussein, M. R., El-Ghorori, R. M. H. and El-Rahman, Y. G. A. (2006). Alterations of p53, BCL-2, and hMSH2 protein expression in the normal brain tissues, gliosis, and gliomas. *International Journal of Experimental Pathology,* 87, 297-306.

188. Iacob, G. and Dinca, E. B. (2009). Current data and strategy in glioblastoma multiforme. *Journal of Medicine and Life,* 2, 386-393.

189. Iwadate, Y. (2016). Epithelial-mesenchymal transition in glioblastoma progression. *Oncol Lett,* 11, 1615-1620.

190. Iwamoto, F. M., Reiner, A. S., Nayak, L., et al. (2009). Prognosis and patterns of care in elderly patients with glioma. *Cancer,* 115, 5534-5540.

191. Jackson, E. L., Garcia-Verdugo, J. M., Gil-Perotin, S., et al. (2006). PDGFR alpha-positive B cells are neural stem cells in the adult SVZ that form glioma-like growths in response to increased PDGF signaling. *Neuron,* 51, 187-199.

192. Jakel, H., Weinl, C. and Hengst, L. (2011). Phosphorylation of p27Kip1 by JAK2 directly links cytokine receptor signaling to cell cycle control. *Oncogene,* 30, 3502-3512.

193. Jeremic, B., Grujicic, D., Antunovic, V., et al. (1994). Influence of extent of surgery and tumor location on treatment outcome of patients with glioblastoma multiforme treated with combined modality approach. *J Neurooncol,* 21, 177-185.

194. Jijiwa, M., Demir, H., Gupta, S., et al. (2011). CD44v6 regulates growth of brain tumor stem cells partially through the AKT-mediated pathway. *PLoS One,* 6, e24217.

195. Jin, M. C., Wu, A., Xiang, M., et al. (2019). Prognostic factors and treatment patterns in the management of giant cell glioblastoma. *World Neurosurg,* 128, e217-e224.

196. Jin, Q., Zhang, W., Qiu, X. G., et al. (2011). Gene expression profiling reveals Ki-67 associated proliferation signature in human glioblastoma. *Chin Med J (Engl),* 124, 2584-2588.

197. Johannessen, A. L. and Torp, S. H. (2006). The clinical value of Ki-67/MIB-1 labeling index in human astrocytomas. *Pathol Oncol Res,* 12, 143-147.

198. Johnson, D. R. and O'Neill, B. P. (2012). Glioblastoma survival in the United States before and during the temozolomide era. *J Neurooncol,* 107, 359-364.

199. Jooma, R., Waqas, M. and Khan, I. (2019). Diffuse low-grade glioma: changing concepts in diagnosis and management: a review. *Asian J Neurosurg,* 14, 356-363.

200. Jordan, R. C., Bradley, G. and Slingerland, J. (1998). Reduced levels of the cell-cycle inhibitor p27Kip1 in epithelial dysplasia and carcinoma of the oral cavity. *Am J Pathol,* 152, 585-590.

201. Jung, J. M., Bruner, J. M., Ruan, S., et al. (1995). Increased levels of p21WAF1/Cip1 in human brain tumors. *Oncogene,* 11, 2021-2028.

202. Juratli, T. A., Engellandt, K., Lautenschlaeger, T., et al. (2013). Is there Pseudoprogression in secondary glioblastomas? *Int J Radiat Oncol Biol Phys,* 87, 1094-1099.

203. Juratli, T. A., Kirsch, M., Robel, K., et al. (2012). IDH mutations as an early and consistent marker in low-grade astrocytomas WHO grade II and their consecutive secondary high-grade gliomas. *J Neurooncol,* 108, 403-410.

204. Juuti, A., Nordling, S., Louhimo, J., et al. (2003). Loss of p27 expression is associated with poor prognosis in stage I-II pancreatic cancer. *Oncology,* 65, 371-377.

205. Kaaijk, P., Pals, S. T., Morsink, F., et al. (1997). Differential expression of CD44 splice variants in the normal human central nervous system. *J Neuroimmunol,* 73, 70-76.

206. Kaminska, B., Czapski, B., Guzik, R., et al. (2019). Consequences of IDH1/2 mutations in Gliomas and an assessment of inhibitors targeting mutated IDH proteins. *Molecules (Basel, Switzerland),* 24, 968.

207. Kang, M. R., Kim, M. S., Oh, J. E., et al. (2009). Mutational analysis of IDH1 codon 132 in glioblastomas and other common cancers. *Int J Cancer,* 125, 353-355.

208. Karimian, A., Ahmadi, Y. and Yousefi, B. (2016). Multiple functions of p21 in cell cycle, apoptosis and transcriptional regulation after DNA damage. *DNA Repair (Amst),* 42, 63-71.

209. Karlsson, P., Holmberg, E., Lundell, M., et al. (1998). Intracranial tumors after exposure to ionizing radiation during infancy: a pooled analysis of two Swedish cohorts of 28,008 infants with skin hemangioma. *Radiat Res,* 150, 357-364.

210. Karsy, M., Gelbman, M., Shah, P., et al. (2012). Established and emerging variants of glioblastoma multiforme: review of morphological and molecular features. *Folia Neuropathologica,* 4, 301-321.

211. Kashi, A. S., Rakhsha, A. and Houshyari, M. (2015). Overall survival in adult patients with low-grade, supratentorial glioma: ten years' follow up at a single institution. *Electron Physician,* 7, 1114-1120.

212. Katz, A. M., Amankulor, N. M., Pitter, K., et al. (2012). Astrocyte-specific expression patterns associated with the PDGF-induced glioma microenvironment. *PLoS One,* 7, e32453.

213. Katz, M., Amit, I. and Yarden, Y. (2007). Regulation of MAPKs by growth factors and receptor tyrosine kinases. *Biochim Biophys Acta,* 1773, 1161-1176.

214. Kaufman, D. K., Kimmel, D. W., Parisi, J. E., et al. (1993). A familial syndrome with cutaneous malignant melanoma and cerebral astrocytoma. *Neurology,* 43, 1728-1731.

215. Kaur, H., Lachance, D. H., Ryan, C. S., et al. (2019). Asthma and risk of glioma: a population-based case–control study. *BMJ Open,* 9, e025746.

216. Kawasoe, T., Takeshima, H., Yamashita, S., et al. (2015). Detection of p53 mutations in proliferating vascular cells in glioblastoma multiforme. *J Neurosurg,* 122, 317-323.

217. Kenney, B., Deng, Y. and Mitchell, K. (2013). Expression of p27, COX-2, MLH1, and MSH2 in young patients with colon carcinoma and correlation with morphologic findings. *Hum Pathol,* 44, 591-597.

218. Kernohan, J. W., Mabon, R. F. and et al. (1949). A simplified classification of the gliomas. *Proc Staff Meet Mayo Clin,* 24, 71-75.

219. Khalid, M. H., Yagi, N., Hiura, T., et al. (1998). Immunohistochemical analysis of p53 and p21 in human primary glioblastomas in relation to proliferative potential and apoptosis. *Brain Tumor Pathol,* 15, 89-94.

220. Kim, H. S., Lee, H. S., Nam, K. H., et al. (2014). p27 Loss is associated with poor prognosis in gastroenteropancreatic neuroendocrine tumors. *Cancer Res Treat,* 46, 383-392.

221. Kim, W. and Liau, L. M. (2012). IDH mutations in human glioma. *Neurosurg Clin N Am,* 23, 471-480.

222. Kim, Y.-H., Nobusawa, S., Mittelbronn, M., et al. (2010). Molecular classification of low-grade diffuse gliomas. *Am J Pathol,* 177, 2708-2714.

223. Kippin, T. E., Martens, D. J. and van der Kooy, D. (2005). p21 loss compromises the relative quiescence of forebrain stem cell proliferation leading to exhaustion of their proliferation capacity. *Genes Dev,* 19, 756-767.

224. Kirkegaard, L. J., DeRose, P. B., Yao, B., et al. (1998). Image cytometric measurement of nuclear proliferation markers (MIB-1, PCNA) in astrocytomas. Prognostic significance. *Am J Clin Pathol,* 109, 69-74.

225. Kirla, R. M., Haapasalo, H. K., Kalimo, H., et al. (2003). Low expression of p27 indicates a poor prognosis in patients with high-grade astrocytomas. *Cancer,* 97, 644-648.

226. Kita, D., Ciernik, I. F., Vaccarella, S., et al. (2009). Age as a predictive factor in glioblastomas: population-based study. *Neuroepidemiology,* 33, 17-22.

227. Kleihues, P. and Ohgaki, H. (1999). Primary and secondary glioblastomas: from concept to clinical diagnosis. *Neuro Oncol,* 1, 44-51.

228. Kleinschmidt-DeMasters, B. K., Lillehei, K. O. and Varella-Garcia, M. (2005). Glioblastomas in the older old. *Arch Pathol Lab Med,* 129, 624-631.

229. Kloosterhof, N. K., Bralten, L. B. C., Dubbink, H. J., et al. (2011). Isocitrate dehydrogenase-1 mutations: a fundamentally new understanding of diffuse glioma? *The Lancet Oncology,* 12, 83-91.

230. Ko, E.-A., Lee, H., Sanders, K. M., et al. (2020). Expression of alpha-type platelet-derived growth factor receptor–influenced genes predicts clinical outcome in glioma. *Translational Oncology,* 13, 233-240.

231. Koev, I. G., Feodorova, Y. N., Kazakova, M. H., et al. (2014). Glioblastoma multiforme classified as mesenchymal subtype. *Folia Med (Plovdiv),* 56, 215-219.

232. Korkolopoulou, P., Kouzelis, K., Christodoulou, P., et al. (1998). Expression of retinoblastoma gene product and p21 (WAF1/Cip 1) protein in gliomas: correlations with proliferation markers, p53 expression and survival. *Acta Neuropathol,* 95, 617-624.

233. Korkolopoulou, P., Patsouris, E., Kavantzas, N., et al. (2002a). Prognostic implications of microvessel morphometry in diffuse astrocytic neoplasms. *Neuropathol Appl Neurobiol,* 28, 57-66.

234. Korkolopoulou, P., Vassilopoulos, I., Konstantinidou, A. E., et al. (2002b). The combined evaluation of p27Kip1 and Ki-67 expression provides independent information on overall survival of ovarian carcinoma patients. *Gynecologic Oncology,* 85, 404-414.

235. Korshunov, A. and Golanov, A. (2001). The prognostic significance of DNA topoisomerase II-alpha (Ki-S1), p21/Cip-1, and p27/Kip-1 protein immunoexpression in oligodendrogliomas. *Arch Pathol Lab Med,* 125, 892-898.

236. Kozak, K. R., Mahadevan, A. and Moody, J. S. (2009). Adult gliosarcoma: epidemiology, natural history, and factors associated with outcome. *Neuro Oncol,* 11, 183-191.

237. Kozak, K. R. and Moody, J. S. (2009). Giant cell glioblastoma: a glioblastoma subtype with distinct epidemiology and superior prognosis. *Neuro Oncol,* 11, 833-841.

238. Kreis, N. N., Louwen, F. and Yuan, J. (2015). Less understood issues: p21(Cip1) in mitosis and its therapeutic potential. *Oncogene,* 34, 1758-1767.

239. Kreis, N. N., Louwen, F. and Yuan, J. (2019). The multifaceted p21 (Cip1/Waf1/CDKN1A) in cell differentiation, migration and cancer therapy. *Cancers (Basel),* 11.

240. Krell, D., Assoku, M., Galloway, M., et al. (2011). Screen for IDH1, IDH2, IDH3, D2HGDH and L2HGDH mmutations in glioblastoma. *PLoS One,* 6, e19868.

241. Krex, D., Klink, B., Hartmann, C., et al. (2007). Long-term survival with glioblastoma multiforme. *Brain,* 130, 2596-2606.

242. Kruck, S., Merseburger, A. S., Hennenlotter, J., et al. (2012). High cytoplasmic expression of p27(Kip1) is associated with a worse cancer-specific survival in clear cell renal cell carcinoma. *BJU Int,* 109, 1565-1570.

243. Kuan, A. S., Green, J., Kitahara, C. M., et al. (2019). Diet and risk of glioma: combined analysis of 3 large prospective studies in the UK and USA. *Neuro Oncol,* 21, 944-952.

244. Kuljaca, S., Liu, T., Dwarte, T., et al. (2009). The cyclin-dependent kinase inhibitor, p21(WAF1), promotes angiogenesis by repressing gene transcription of thioredoxin-binding protein 2 in cancer cells. *Carcinogenesis,* 30, 1865-1871.

245. Kumar, N., Kumar, P., Angurana, S. L., et al. (2013). Evaluation of outcome and prognostic factors in patients of glioblastoma multiforme: a single institution experience. *Journal of Neurosciences in Rural Practice,* 4, S46-S55.

246. Kuppner, M. C., Van Meir, E., Gauthier, T., et al. (1992). Differential expression of the CD44 molecule in human brain tumours. *Int J Cancer,* 50, 572-577.

247. Kuratsu, J., Takeshima, H. and Ushio, Y. (2001). Trends in the incidence of primary intracranial tumors in Kumamoto, Japan. *Int J Clin Oncol,* 6, 183-191.

248. Kushnir, I. and Tzuk-Shina, T. (2011). Efficacy of treatment for glioblastoma multiforme in elderly patients (65+): a retrospective analysis. *Isr Med Assoc J,* 13, 290-294.

249. Ladomersky, E., Scholtens, D. M., Kocherginsky, M., et al. (2019). The coincidence between increasing age, immunosuppression, and the incidence of patients with glioblastoma. *Front Pharmacol,* 10, 200.

250. Ladomersky, E. and Zhai, L. (2020). Advanced age increases immunosuppression in the brain and decreases immunotherapeutic efficacy in subjects with glioblastoma. 26, 5232-5245.

251. Lakomy, R., Kazda, T., Selingerova, I., et al. (2020). Real-world evidence in glioblastoma: stupp's regimen after a decade. *Front Oncol,* 10, 840.

252. Lamborn, K. R., Chang, S. M. and Prados, M. D. (2004). Prognostic factors for survival of patients with glioblastoma: recursive partitioning analysis. *Neuro Oncol,* 6, 227-235.

253. Lamouille, S., Xu, J. and Derynck, R. (2014). Molecular mechanisms of epithelial–mesenchymal transition. *Nat Rev Mol Cell Biol,* 15, 178-196.

254. Lane, D. P. (1992). Cancer. p53, guardian of the genome. *Nature,* 358, 15-16.

255. Larjavaara, S., Mäntylä, R., Salminen, T., et al. (2007). Incidence of gliomas by anatomic location. *Neuro Oncol,* 9, 319-325.

256. Larjavaara, S., Schuz, J., Swerdlow, A., et al. (2011). Location of gliomas in relation to mobile telephone use: a case-case and case-specular analysis. *Am J Epidemiol,* 174, 2-11.

257. Lasota, J. and Miettinen, M. (2006). KIT and PDGFRA mutations in gastrointestinal stromal tumors (GISTs). *Semin Diagn Pathol,* 23, 91-102.

258. Le Mercier, M., Hastir, D., Moles Lopez, X., et al. (2012). A simplified approach for the molecular classification of glioblastomas. *PLoS One,* 7, e45475.

259. Lee, J. and Kim, S. S. (2009). The function of p27 KIP1 during tumor development. *Exp Mol Med,* 41, 765-771.

260. Lee, S., Bui Nguyen, T. M., Kovalenko, D., et al. (2010). Sprouty1 inhibits angiogenesis in association with up-regulation of p21 and p27. *Mol Cell Biochem,* 338, 255-261.

261. Lee, S. and Helfman, D. M. (2004). Cytoplasmic p21Cip1 is involved in Ras-induced inhibition of the ROCK/LIMK/cofilin pathway. *J Biol Chem,* 279, 1885-1891.

262. Lee, S. M., Koh, H. J., Park, D. C., et al. (2002). Cytosolic NADP(+)-dependent isocitrate dehydrogenase status modulates oxidative damage to cells. *Free Radic Biol Med,* 32, 1185-1196.

263. Leighton, C., Fisher, B., Bauman, G., et al. (1997). Supratentorial low-grade glioma in adults: an analysis of prognostic factors and timing of radiation. *J Clin Oncol,* 15, 1294-1301.

264. Leon, S. P., Folkerth, R. D. and Black, P. M. (1996). Microvessel density is a prognostic indicator for patients with astroglial brain tumors. *Cancer,* 77, 362-372.

265. Lessel, D., Wu, D., Trujillo, C., et al. (2017). Dysfunction of the MDM2/p53 axis is linked to premature aging. *J Clin Invest,* 127, 3598-3608.

266. Li, H. Y., Sun, C. R., He, M., et al. (2018). Correlation between tumor location and clinical properties of glioblastomas in frontal and temporal lobes. *World Neurosurg,* 112, e407-e414.

267. Li, L., Quang, T. S., Gracely, E. J., et al. (2010). A Phase II study of anti-epidermal growth factor receptor radioimmunotherapy in the treatment of glioblastoma multiforme. *J Neurosurg,* 113, 192-198.

268. Li, R., Li, H., Yan, W., et al. (2015). Genetic and clinical characteristics of primary and secondary glioblastoma is associated with differential molecular subtype distribution. *Oncotarget,* 6, 7318-7324.

269. Li, W., Winters, A., Poteet, E., et al. (2013). Involvement of estrogen receptor beta5 in the progression of glioma. *Brain Res,* 1503, 97-107.

270. Li, Y., Zhang, Z. X., Huang, G. H., et al. (2020). A systematic review of multifocal and multicentric glioblastoma. *J Clin Neurosci*.

271. Li, Z., Jiao, X., Wang, C., et al. (2006). Cyclin D1 induction of cellular migration requires p27(KIP1). *Cancer Res,* 66, 9986-9994.

272. Liang, J., Lv, X., Lu, C., et al. (2020). Prognostic factors of patients with gliomas: an analysis on 335 patients with glioblastoma and other forms of gliomas. *BMC Cancer,* 20, 35-35.

273. Liang, Y., Diehn, M., Watson, N., et al. (2005). Gene expression profiling reveals molecularly and clinically distinct subtypes of glioblastoma multiforme. *Proc Natl Acad Sci U S A,* 102, 5814-5819.

274. Liesche-Starnecker, F., Mayer, K., Kofler, F., et al. (2020). Immunohistochemically characterized intratumoral heterogeneity is a prognostic marker in human glioblastoma. *Cancers (Basel),* 12, 2964.

275. Lin, N., Yan, W., Gao, K., et al. (2014). Prevalence and clinicopathologic characteristics of the molecular subtypes in malignant glioma: a multi-institutional analysis of 941 cases. *PLoS One,* 9, e94871.

276. Lin, T., Wang, M., Liang, H. S., et al. (2015). The expression of p53, mgmt and egfr in brain glioma and clinical significance. *J Biol Regul Homeost Agents,* 29, 143-149.

277. Lin, Z., Yang, R., Li, K., et al. (2020). Establishment of age group classification for risk stratification in glioma patients. 20, 310.

278. Lindboe, C. F. and Torp, S. H. (2002). Comparison of Ki-67 equivalent antibodies. *J Clin Pathol,* 55, 467-471.

279. Liouta, E., Stranjalis, G., Kalyvas, A. V., et al. (2018). Parietal association deficits in patients harboring parietal lobe gliomas: a prospective study. *J Neurosurg,* 130, 773-779.

280. Little, M. P., Rajaraman, P., Curtis, R. E., et al. (2012). Mobile phone use and glioma risk: comparison of epidemiological study results with incidence trends in the United States. *Bmj,* 344, e1147.

281. Liu, C., Zhang, Y., Zhang, K., et al. (2014). Expression of estrogen receptors, androgen receptor and steroid receptor coactivator-3 is negatively correlated to the differentiation of astrocytic tumors. *Cancer Epidemiol,* 38, 291-297.

282. Liu, Y., Han, S. S., Wu, Y., et al. (2004). CD44 expression identifies astrocyte-restricted precursor cells. *Dev Biol,* 276, 31-46.

283. Lobbous, M., Bernstock, J. D., Coffee, E., et al. (2020). An update on neurofibromatosis type 1-associated gliomas. *Cancers (Basel),* 12, 114.

284. Loda, M., Cukor, B., Tam, S. W., et al. (1997). Increased proteasome-dependent degradation of the cyclin-dependent kinase inhibitor p27 in aggressive colorectal carcinomas. *Nat Med,* 3, 231-234.

285. Lopez, G. Y., Reitman, Z. J., Solomon, D., et al. (2010). IDH1(R132) mutation identified in one human melanoma metastasis, but not correlated with metastases to the brain. *Biochem Biophys Res Commun,* 398, 585-587.

286. Lottaz, C., Beier, D., Meyer, K., et al. (2010). Transcriptional profiles of CD133+ and CD133- glioblastoma-derived cancer stem cell lines suggest different cells of origin. *Cancer Res,* 70, 2030-2040.

287. Louis, D. N., Ohgaki, H., Wiestler, O., et al. (2007). *WHO Classification of Tumours of the Central Nervous System*.

288. Louis, D. N., Ohgaki, H., Wiestler, O. D., et al. (2016). *WHO Classification of Tumours of the Central Nervous System*.

289. Loussouarn, D., Le Loupp, A. G., Frenel, J. S., et al. (2012). Comparison of immunohistochemistry, DNA sequencing and allele-specific PCR for the detection of IDH1 mutations in gliomas. *Int J Oncol,* 40, 2058-2062.

290. Lu, C., Ward, P. S., Kapoor, G. S., et al. (2012). IDH mutation impairs histone demethylation and results in a block to cell differentiation. *Nature,* 483, 474-478.

291. Lu, Z. and Hunter, T. (2010). Ubiquitylation and proteasomal degradation of the p21(Cip1), p27(Kip1) and p57(Kip2) CDK inhibitors. *Cell Cycle,* 9, 2342-2352.

292. Lutterbach, J., Bartelt, S., Momm, F., et al. (2005). Is older age associated with a worse prognosis due to different patterns of care? A long-term study of 1346 patients with glioblastomas or brain metastases. *Cancer,* 103, 1234-1244.

293. Ma, X., Lv, Y., Liu, J., et al. (2009). Survival analysis of 205 patients with glioblastoma multiforme: clinical characteristics, treatment and prognosis in China. *J Clin Neurosci,* 16, 1595-1598.

294. MacKenzie, D. J. (1926). A classification of the tumours of the glioma group on a histogenetic basis with a correlated study of prognosis. *Canadian Medical Association Journal,* 16, 872-872.

295. Malik, N., Wang, X., Shah, S., et al. (2014). Comparison of the gene expression profiles of human fetal cortical astrocytes with pluripotent stem cell derived neural stem cells identifies human astrocyte markers and signaling pathways and transcription factors active in human astrocytes. *PLoS One,* 9, e96139.

296. Mallya, V., Siraj, F., Singh, A., et al. (2015). Giant cell glioblastoma with calcification and long-term survival. *Indian J Cancer,* 52, 704-705.

297. Mandelzweig, L., Novikov, I. and Sadetzki, S. (2009). Smoking and risk of glioma: a meta-analysis. *Cancer Causes Control,* 20, 1927-1938.

298. Manfredi, J. J. (2020). Inactivation of Wild-Type p53 by asparagine endopeptidase in glioblastoma: an opportunity to target the "undruggable". *J Natl Cancer Inst,* 112, 327-329.

299. Mao, P., Joshi, K., Li, J., et al. (2013). Mesenchymal glioma stem cells are maintained by activated glycolytic metabolism involving aldehyde dehydrogenase 1A3. *Proc Natl Acad Sci U S A,* 110, 8644-8649.

300. Markovic, D. S., Vinnakota, K., van Rooijen, N., et al. (2011). Minocycline reduces glioma expansion and invasion by attenuating microglial MT1-MMP expression. *Brain Behav Immun,* 25, 624-628.

301. Marques-Torrejon, M. A., Porlan, E., Banito, A., et al. (2013). Cyclin-dependent kinase inhibitor p21 controls adult neural stem cell expansion by regulating Sox2 gene expression. *Cell Stem Cell,* 12, 88-100.

302. Martinho, O., Longatto-Filho, A., Lambros, M. B., et al. (2009). Expression, mutation and copy number analysis of platelet-derived growth factor receptor A (PDGFRA) and its ligand PDGFA in gliomas. *Br J Cancer,* 101, 973-982.

303. Marziali, G., Signore, M., Buccarelli, M., et al. (2016). Metabolic/proteomic signature defines two glioblastoma subtypes with different clinical outcome. *Sci Rep,* 6, 21557.

304. Matarredona, E. R. and Pastor, A. M. (2019). Neural stem cells of the subventricular zone as the origin of human glioblastoma stem cells. therapeutic implications. *Front Oncol,* 9, 779-779.

305. Mathews, J. D., Forsythe, A. V., Brady, Z., et al. (2013). Cancer risk in 680 000 people exposed to computed tomography scans in childhood or adolescence: data linkage study of 11 million Australians. *BMJ : British Medical Journal,* 346, f2360.

306. McKeever, P. E., Strawderman, M. S., Yamini, B., et al. (1998). MIB-1 proliferation index predicts survival among patients with grade II astrocytoma. *J Neuropathol Exp Neurol,* 57, 931-936.

307. Meek, D. W. (1999). Mechanisms of switching on p53: a role for covalent modification? *Oncogene,* 18, 7666-7675.

308. Meis, J. M., Martz, K. L. and Nelson, J. S. (1991). Mixed glioblastoma multiforme and sarcoma. A clinicopathologic study of 26 radiation therapy oncology group cases. *Cancer,* 67, 2342-2349.

309. Michaud, D. S., Holick, C. N., Batchelor, T. T., et al. (2009). Prospective study of meat intake and dietary nitrates, nitrites, and nitrosamines and risk of adult glioma. *The American Journal of Clinical Nutrition,* 90, 570-577.

310. Miettinen, M., Sobin, L. H. and Sarlomo-Rikala, M. (2000). Immunohistochemical spectrum of GISTs at different sites and their differential diagnosis with a reference to CD117 (KIT). *Mod Pathol,* 13, 1134-1142.

311. Mihić, J., Rotim, K., Vučić, M., et al. (2019). Prognostic role of CD44 expression and neovascularization determined by endoglin (CD105) in glioblastoma patients. *Acta Clin Croat,* 58, 455-462.

312. Miklja, Z., Yadav, V. N., Cartaxo, R. T., et al. (2020). Everolimus improves the efficacy of dasatinib in PDGFRα-driven glioma. *J Clin Invest,* 130, 5313-5325.

313. Miller, C. R. and Perry, A. (2007). Glioblastoma. *Arch Pathol Lab Med,* 131, 397-406.

314. Mischel, P. S., Shai, R., Shi, T., et al. (2003). Identification of molecular subtypes of glioblastoma by gene expression profiling. *Oncogene,* 22, 2361-2373.

315. Mishra, A., Godavarthi, S. K. and Jana, N. R. (2009). UBE3A/E6-AP regulates cell proliferation by promoting proteasomal degradation of p27. *Neurobiol Dis,* 36, 26-34.

316. Mizumatsu, S., Tamiya, T., Ono, Y., et al. (1999). Expression of cell cycle regulator p27Kip1 is correlated with survival of patients with astrocytoma. *Clin Cancer Res,* 5, 551-557.

317. Modrek, A. S., Bayin, N. S. and Placantonakis, D. G. (2014). Brain stem cells as the cell of origin in glioma. *World J Stem Cells,* 6, 43-52.

318. Moller, M. B. (2000). P27 in cell cycle control and cancer. *Leuk Lymphoma,* 39, 19-27.

319. Mooney, K. L., Choy, W., Sidhu, S., et al. (2016). The role of CD44 in glioblastoma multiforme. *J Clin Neurosci,* 34, 1-5.

320. Morantz, R. A., Feigin, I. and Ransohoff, J., 3rd. (1976). Clinical and pathological study of 24 cases of gliosarcoma. *J Neurosurg,* 45, 398-408.

321. Morris-Hanon, O., Furmento, V. A., Rodríguez-Varela, M. S., et al. (2017). The cell cycle inhibitors p21(Cip1) and p27(Kip1) control proliferation but enhance DNA damage resistance of glioma stem cells. *Neoplasia,* 19, 519-529.

322. Moskowitz, S. I., Jin, T. and Prayson, R. A. (2006). Role of MIB1 in predicting survival in patients with glioblastomas. *J Neurooncol,* 76, 193-200.

323. Motomura, K., Natsume, A., Watanabe, R., et al. (2012). Immunohistochemical analysis-based proteomic subclassification of newly diagnosed glioblastomas. *Cancer Sci,* 103, 1871-1879.

324. Muller, Patricia A. and Vousden, Karen H. (2014). Mutant p53 in cancer: new functions and therapeutic opportunities. *Cancer Cell,* 25, 304-317.

325. Nabika, S., Kiya, K., Satoh, H., et al. (2010). Prognostic significance of expression patterns of EGFR family, p21 and p27 in high-grade astrocytoma. *Hiroshima J Med Sci,* 59, 65-70.

326. Nakamura, I., Kariya, Y., Okada, E., et al. (2015). A novel Chromosomal translocation associated with COL1A2-PDGFB gene fusion in dermatofibrosarcoma protuberans: PDGF expression as a new diagnostic tool. *JAMA Dermatol,* 151, 1330-1337.

327. Nakano, I. (2014). Proneural-mesenchymal transformation of glioma stem cells: do therapies cause evolution of target in glioblastoma? *Future Oncol,* 10, 1527-1530.

328. Nakano, I. (2015). Stem cell signature in glioblastoma: therapeutic development for a moving target. *J Neurosurg,* 122, 324-330.

329. Naor, D., Nedvetzki, S., Golan, I., et al. (2002). CD44 in cancer. *Crit Rev Clin Lab Sci,* 39, 527-579.

330. Naor, D., Sionov, R. V. and Ish-Shalom, D. (1997). CD44: structure, function, and association with the malignant process. *Adv Cancer Res,* 71, 241-319.

331. Naruse, M., Shibasaki, K., Yokoyama, S., et al. (2013). Dynamic changes of CD44 expression from progenitors to subpopulations of astrocytes and neurons in developing cerebellum. *PLoS One,* 8, e53109.

332. Natesh, K., Bhosale, D., Desai, A., et al. (2015). Oncostatin-M differentially regulates mesenchymal and proneural signature genes in gliomas via STAT3 signaling. *Neoplasia,* 17, 225-237.

333. Nayak, A., Ralte, A. M., Sharma, M. C., et al. (2004). p53 protein alterations in adult astrocytic tumors and oligodendrogliomas. *Neurol India,* 52, 228-232.

334. Naydenov, E., Bussarsky, V., Nachev, S., et al. (2009). Long-term survival of a patient with giant cell glioblastoma: case report and review of the literature. *Case Rep Oncol,* 2, 103-110.

335. Naydenov, E., Tzekov, C., Minkin, K., et al. (2011). Long-term survival with primary glioblastoma multiforme: a clinical study in bulgarian patients. *Case Rep Oncol,* 4, 1-11.

336. Neder, L., Colli, B. O., Machado, H. R., et al. (2004). MIB-1 labeling index in astrocytic tumors--a clinicopathologic study. *Clin Neuropathol,* 23, 262-270.

337. Neglia, J. P., Meadows, A. T., Robison, L. L., et al. (1991). Second neoplasms after acute lymphoblastic leukemia in childhood. *N Engl J Med,* 325, 1330-1336.

338. Neglia, J. P., Robison, L. L., Stovall, M., et al. (2006). New primary neoplasms of the central nervous system in survivors of childhood cancer: a report from the Childhood Cancer Survivor Study. *J Natl Cancer Inst,* 98, 1528-1537.

339. Newcomb, E. W., Cohen, H., Lee, S. R., et al. (1998). Survival of patients with glioblastoma multiforme is not influenced by altered expression of p16, p53, EGFR, MDM2 or Bcl-2 genes. *Brain Pathol,* 8, 655-667.

340. Nicholas, M. K., Lukas, R. V., Chmura, S., et al. (2011). Molecular heterogeneity in glioblastoma: therapeutic opportunities and challenges. *Semin Oncol,* 38, 243-253.

341. Nielsen, J. S. and McNagny, K. M. (2008). Novel functions of the CD34 family. *Journal of Cell Science,* 121, 3683-3692.

342. Nigro, J. M., Misra, A., Zhang, L., et al. (2005). Integrated array-comparative genomic hybridization and expression array profiles identify clinically relevant molecular subtypes of glioblastoma. *Cancer Res,* 65, 1678-1686.

343. Nishimori, H., Shiratsuchi, T., Urano, T., et al. (1997). A novel brain-specific p53-target gene, BAI1, containing thrombospondin type 1 repeats inhibits experimental angiogenesis. *Oncogene,* 15, 2145-2150.

344. Nobusawa, S., Watanabe, T., Kleihues, P., et al. (2009). IDH1 mutations as molecular signature and predictive factor of secondary glioblastomas. *Clin Cancer Res,* 15, 6002-6007.

345. Noushmehr, H., Weisenberger, D. J., Diefes, K., et al. (2010). Identification of a CpG island methylator phenotype that defines a distinct subgroup of glioma. *Cancer Cell,* 17, 510-522.

346. Nutt, C. L., Mani, D. R., Betensky, R. A., et al. (2003). Gene expression-based classification of malignant gliomas correlates better with survival than histological classification. *Cancer Res,* 63, 1602-1607.

347. Obermair, A., Kurz, C., Czerwenka, K., et al. (1995). Microvessel density and vessel invasion in lymph-node-negative breast cancer: effect on recurrence-free survival. *International Journal of Cancer,* 62, 126-131.

348. Ogawa, K., Kurose, A., Kamataki, A., et al. (2020). Giant cell glioblastoma is a distinctive subtype of glioma characterized by vulnerability to DNA damage. *Brain Tumor Pathol,* 37, 5-13.

349. Ogura, R., Tsukamoto, Y., Natsumeda, M., et al. (2015). Immunohistochemical profiles of IDH1, MGMT and P53: practical significance for prognostication of patients with diffuse gliomas. *Neuropathology,* 35, 324-335.

350. Ohgaki, H., Dessen, P., Jourde, B., et al. (2004). Genetic pathways to glioblastoma: a population-based study. *Cancer Res,* 64, 6892-6899.

351. Ohgaki, H. and Kleihues, P. (2005a). Epidemiology and etiology of gliomas. *Acta Neuropathol,* 109, 93-108.

352. Ohgaki, H. and Kleihues, P. (2005b). Population-based studies on incidence, survival rates, and genetic alterations in astrocytic and oligodendroglial gliomas. *J Neuropathol Exp Neurol,* 64, 479-489.

353. Ohgaki, H. and Kleihues, P. (2007). Genetic pathways to primary and secondary glioblastoma. *Am J Pathol,* 170, 1445-1453.

354. Ohgaki, H. and Kleihues, P. (2013). The definition of primary and secondary glioblastoma. *Clin Cancer Res,* 19, 764-772.

355. Oka, N., Soeda, A., Inagaki, A., et al. (2007). VEGF promotes tumorigenesis and angiogenesis of human glioblastoma stem cells. *Biochem Biophys Res Commun,* 360, 553-559.

356. Okamoto, Y., Di Patre, P. L., Burkhard, C., et al. (2004). Population-based study on incidence, survival rates, and genetic alterations of low-grade diffuse astrocytomas and oligodendrogliomas. *Acta Neuropathol,* 108, 49-56.

357. Olafson, L. R., Gunawardena, M., Nixdorf, S., et al. (2020). The role of TP53 gain-of-function mutation in multifocal glioblastoma. *J Neurooncol,* 147, 37-47.

358. Olivier, M., Goldgar, D. E., Sodha, N., et al. (2003). Li-Fraumeni and related syndromes: correlation between tumor type, family structure, and TP53 genotype. *Cancer Res,* 63, 6643-6650.

359. Ortensi, B., Setti, M., Osti, D., et al. (2013). Cancer stem cell contribution to glioblastoma invasiveness. *Stem Cell Research & Therapy,* 4, 18-18.

360. Orzan, F. and Pagani, F. (2020). A simplified integrated molecular and immunohistochemistry-based algorithm allows high accuracy prediction of glioblastoma transcriptional subtypes. 100, 1330-1344.

361. Ostman, A. and Heldin, C. H. (2007). PDGF receptors as targets in tumor treatment. *Adv Cancer Res,* 97, 247-274.

362. Ostrom, Q. T., Adel Fahmideh, M., Cote, D. J., et al. (2019). Risk factors for childhood and adult primary brain tumors. *Neuro Oncol,* 21, 1357-1375.

363. Ostrom, Q. T., Cote, D. J., Ascha, M., et al. (2018). Adult glioma incidence and survival by race or ethnicity in the United States from 2000 to 2014. *JAMA Oncol,* 4, 1254-1262.

364. Ostrom, Q. T., Gittleman, H., Farah, P., et al. (2013). CBTRUS statistical report: Primary brain and central nervous system tumors diagnosed in the United States in 2006–2010. *Neuro Oncol,* 15 Suppl 2, ii1-56.

365. Oszvald, A., Guresir, E., Setzer, M., et al. (2012). Glioblastoma therapy in the elderly and the importance of the extent of resection regardless of age. *J Neurosurg,* 116, 357-364.

366. Ozawa, T., Brennan, C. W., Wang, L., et al. (2010). PDGFRA gene rearrangements are frequent genetic events in PDGFRA-amplified glioblastomas. *Genes Dev,* 24, 2205-2218.

367. Paldor, I., Pearce, F. C., Drummond, K. J., et al. (2016). Frontal glioblastoma multiforme may be biologically distinct from non-frontal and multilobar tumors. *J Clin Neurosci,* 34, 128-132.

368. Pardo, F. S., Hsu, D. W., Zeheb, R., et al. (2004). Mutant, wild type, or overall p53 expression: freedom from clinical progression in tumours of astrocytic lineage. *Br J Cancer,* 91, 1678-1686.

369. Park, K. H., Lee, J., Yoo, C. G., et al. (2004). Application of p27 gene therapy for human malignant glioma potentiated by using mutant p27. *J Neurosurg,* 101, 505-510.

370. Parsa, A. T. (2010). A newly identified transcriptional network for mesenchymal transformation of brain tumors: potential targets for therapeutic intervention. *World Neurosurg,* 73, 424-424.

371. Parsons, D. W., Jones, S., Zhang, X., et al. (2008). An integrated genomic analysis of human glioblastoma multiforme. *Science,* 321, 1807-1812.

372. Paruthiyil, S., Parmar, H., Kerekatte, V., et al. (2004). Estrogen receptor beta inhibits human breast cancer cell proliferation and tumor formation by causing a G2 cell cycle arrest. *Cancer Res,* 64, 423-428.

373. Parveen, A., Akash, M. S., Rehman, K., et al. (2016). Dual role of p21 in the progression of cancer and its treatment. *Crit Rev Eukaryot Gene Expr,* 26, 49-62.

374. Paschka, P., Schlenk, R. F., Gaidzik, V. I., et al. (2010). IDH1 and IDH2 mutations are frequent genetic alterations in acute myeloid leukemia and confer adverse prognosis in cytogenetically normal acute myeloid leukemia with NPM1 mutation without FLT3 internal tandem duplication. *J Clin Oncol,* 28, 3636-3643.

375. Paszat, L., Laperriere, N., Groome, P., et al. (2001). A population-based study of glioblastoma multiforme. *Int J Radiat Oncol Biol Phys,* 51, 100-107.

376. Paterni, I., Bertini, S., Granchi, C., et al. (2015). Highly selective salicylketoxime-based estrogen receptor beta agonists display antiproliferative activities in a glioma model. *J Med Chem,* 58, 1184-1194.

377. Patil, C. G., Yi, A., Elramsisy, A., et al. (2012). Prognosis of patients with multifocal glioblastoma: a case-control study. *J Neurosurg,* 117, 705-711.

378. Pearce, M. S., Salotti, J. A., Little, M. P., et al. (2012). Radiation exposure from CT scans in childhood and subsequent risk of leukaemia and brain tumours: a retrospective cohort study. *Lancet,* 380, 499-505.

379. Pechnick, R. N., Zonis, S., Wawrowsky, K., et al. (2008). p21Cip1 restricts neuronal proliferation in the subgranular zone of the dentate gyrus of the hippocampus. *Proc Natl Acad Sci U S A,* 105, 1358-1363.

380. Peraud, A., Kreth, F. W., Wiestler, O. D., et al. (2002). Prognostic impact of TP53 mutations and P53 protein overexpression in supratentorial WHO grade II astrocytomas and oligoastrocytomas. *Clin Cancer Res,* 8, 1117-1124.

381. Perry, A. (2003). Pathology of low-grade gliomas: an update of emerging concepts. *Neuro Oncol,* 5, 168-178.

382. Perry, A., Aldape, K. D., George, D. H., et al. (2004). Small cell astrocytoma: an aggressive variant that is clinicopathologically and genetically distinct from anaplastic oligodendroglioma. *Cancer,* 101, 2318-2326.

383. Petrey, A. C. and de la Motte, C. A. (2014). Hyaluronan, a crucial regulator of inflammation. *Front Immunol,* 5, 101.

384. Phillips, H. S., Kharbanda, S., Chen, R., et al. (2006). Molecular subclasses of high-grade glioma predict prognosis, delineate a pattern of disease progression, and resemble stages in neurogenesis. *Cancer Cell,* 9, 157-173.

385. Pietras, A., Katz, A. M., Ekstrom, E. J., et al. (2014). Osteopontin-CD44 signaling in the glioma perivascular niche enhances cancer stem cell phenotypes and promotes aggressive tumor growth. *Cell Stem Cell,* 14, 357-369.

386. Piroth, M. D., Gagel, B., Pinkawa, M., et al. (2007). Postoperative radiotherapy of glioblastoma multiforme: analysis and critical assessment of different treatment strategies and predictive factors. *Strahlenther Onkol,* 183, 695-702.

387. Piva, R., Cancelli, I., Cavalla, P., et al. (1999). Proteasome-dependent degradation of p27/kip1 in gliomas. *J Neuropathol Exp Neurol,* 58, 691-696.

388. Plotkin, S. R., O'Donnell, C. C., Curry, W. T., et al. (2011). Spinal ependymomas in neurofibromatosis Type 2: a retrospective analysis of 55 patients. *J Neurosurg Spine*, 14, 543-547.

389. Poon, M. T. C., Sudlow, C. L. M., Figueroa, J. D., et al. (2020). Longer-term (≥ 2 years) survival in patients with glioblastoma in population-based studies pre- and post-2005: a systematic review and meta-analysis. *Sci Rep*, 10, 11622.

390. Popova, S. N., Bergqvist, M., Dimberg, A., et al. (2014). Subtyping of gliomas of various WHO grades by the application of immunohistochemistry. *Histopathology*, 64, 365-379.

391. Porlan, E., Morante-Redolat, J. M., Marques-Torrejon, M. A., et al. (2013). Transcriptional repression of Bmp2 by p21(Waf1/Cip1) links quiescence to neural stem cell maintenance. *Nat Neurosci*, 16, 1567-1575.

392. Prager, B. C., Bhargava, S., Mahadev, V., et al. (2020). Glioblastoma stem cells: driving resilience through chaos. *Trends in Cancer*, 6, 223-235.

393. Preston, D. L., Ron, E., Tokuoka, S., et al. (2007). Solid cancer incidence in atomic bomb survivors: 1958–1998. *Radiat Res*, 168, 1-64.

394. Preusser, M., Heinzl, H., Gelpi, E., et al. (2006). Histopathologic assessment of hot-spot microvessel density and vascular patterns in glioblastoma: poor observer agreement limits clinical utility as prognostic factors: a translational research project of the European Organization for Research and Treatment of Cancer Brain Tumor Group. *Cancer*, 107, 162-170.

395. Prochazka, L., Tesarik, R. and Turanek, J. (2014). Regulation of alternative splicing of CD44 in cancer. *Cell Signal*, 26, 2234-2239.

396. Puputti, M., Tynninen, O., Sihto, H., et al. (2006). Amplification of KIT, PDGFRA, VEGFR2, and EGFR in gliomas. *Mol Cancer Res*, 4, 927-934.

397. Qemo, I. and Porter, L. A. (2019). Cell cycle dynamics in glioma cancer stem cells. *Methods Mol Biol*, 1869, 117-126.

398. Rahmanzadeh, R., Huttmann, G., Gerdes, J., et al. (2007). Chromophore-assisted light inactivation of pKi-67 leads to inhibition of ribosomal RNA synthesis. *Cell Prolif*, 40, 422-430.

399. Ralte, A. M., Sharma, M. C., Karak, A. K., et al. (2001). Clinicopathological features, MIB-1 labeling index and apoptotic index in recurrent astrocytic tumors. *Pathol Oncol Res*, 7, 267-278.

400. Ranuncolo, S. M., Ladeda, V., Specterman, S., et al. (2002). CD44 expression in human gliomas. *J Surg Oncol*, 79, 30-35; discussion 35-36.

401. Ravi, R., Mookerjee, B., Bhujwalla, Z. M., et al. (2000). Regulation of tumor angiogenesis by p53-induced degradation of hypoxia-inducible factor 1α. *Genes Dev*, 14, 34-44.

402. Raysi Dehcordi, S., De Paulis, D., Marzi, S., et al. (2012). Survival prognostic factors in patients with glioblastoma: our experience. *J Neurosurg Sci*, 56, 239-245.

403. Reavey-Cantwell, J. F., Haroun, R. I., Zahurak, M., et al. (2001). The prognostic value of tumor markers in patients with glioblastoma multiforme: analysis of 32 patients and review of the literature. *J Neurooncol*, 55, 195-204.

404. Reifenberger, G., Kaulich, K., Wiestler, O. D., et al. (2003). Expression of the CD34 antigen in pleomorphic xanthoastrocytomas. *Acta Neuropathol*, 105, 358-364.

405. Reis, R. M., Hara, A., Kleihues, P., et al. (2001). Genetic evidence of the neoplastic nature of gemistocytes in astrocytomas. *Acta Neuropathol*, 102, 422-425.

406. Reitman, Z. J. and Yan, H. (2010). Isocitrate dehydrogenase 1 and 2 mutations in cancer: alterations at a crossroads of cellular metabolism. *J Natl Cancer Inst*, 102, 932-941.

407. Ringertz, N. (1950). Grading of gliomas. *Acta Pathol Microbiol Scand*, 27, 51-64.

408. Rodriguez-Pereira, C., Suarez-Penaranda, J. M., Vazquez-Salvado, M., et al. (2000). Value of MIB-1 labelling index (LI) in gliomas and its correlation with other prognostic factors. A clinicopathologic study. *J Neurosurg Sci*, 44, 203-209; discussion 209-210.

409. Rodriguez, F. J., Perry, A., Gutmann, D. H., et al. (2008). Gliomas in neurofibromatosis type 1: a clinicopathologic study of 100 patients. *J Neuropathol Exp Neurol,* 67, 240-249.

410. Rønning, P. A., Helseth, E., Meling, T. R., et al. (2012). A population-based study on the effect of temozolomide in the treatment of glioblastoma multiforme. *Neuro Oncol,* 14, 1178-1184.

411. Rossi, G., Valli, R., Bertolini, F., et al. (2005). PDGFR expression in differential diagnosis between KIT-negative gastrointestinal stromal tumours and other primary soft-tissue tumours of the gastrointestinal tract. *Histopathology,* 46, 522-531.

412. Roy, S., Lahiri, D., Maji, T., et al. (2015). Recurrent glioblastoma: where we stand. *South Asian J Cancer,* 4, 163-173.

413. Ruano, Y., Ribalta, T., de Lope, A. R., et al. (2009). Worse outcome in primary glioblastoma multiforme with concurrent epidermal growth factor receptor and p53 alteration. *Am J Clin Pathol,* 131, 257-263.

414. Ryan, K. M., Phillips, A. C. and Vousden, K. H. (2001). Regulation and function of the p53 tumor suppressor protein. *Curr Opin Cell Biol,* 13, 332-337.

415. Ryu, M. S., Park, H. J., Moon, C. M., et al. (2018). Expression of CD44 according to clinicopathologic characteristics of gastric cancer. *emj,* 41, 63-74.

416. Sadetzki, S., Chetrit, A., Freedman, L., et al. (2005). Long-term follow-up for brain tumor development after childhood exposure to ionizing radiation for tinea capitis. *Radiat Res,* 163, 424-432.

417. Safa, A. R., Saadatzadeh, M. R., Cohen-Gadol, A. A., et al. (2015). Glioblastoma stem cells (GSCs) epigenetic plasticity and interconversion between differentiated non-GSCs and GSCs. *Genes Dis,* 2, 152-163.

418. Saha, T., Guha, D., Manna, A., et al. (2016). G-actin guides p53 nuclear transport: potential contribution of monomeric actin in altered localization of mutant p53. *Sci Rep,* 6, 32626.

419. Sahlberg, S. H., Spiegelberg, D., Glimelius, B., et al. (2014). Evaluation of cancer stem cell markers CD133, CD44, CD24: association with AKT isoforms and radiation resistance in colon cancer cells. *PLoS One,* 9, e94621.

420. Saneei, P., Willett, W. and Esmaillzadeh, A. (2015). Red and processed meat consumption and risk of glioma in adults: a systematic review and meta-analysis of observational studies. *Journal of Research in Medical Sciences : The Official Journal of Isfahan University of Medical Sciences,* 20, 602-612.

421. Sang, Y., Hou, Y., Cheng, R., et al. (2019). Targeting PDGFRα-activated glioblastoma through specific inhibition of SHP-2-mediated signaling. *Neuro Oncol,* 21, 1423-1435.

422. Sanson, M., Marie, Y., Paris, S., et al. (2009). Isocitrate dehydrogenase 1 codon 132 mutation is an important prognostic biomarker in gliomas. *J Clin Oncol,* 27, 4150-4154.

423. Sareddy, G. R., Li, X., Liu, J., et al. (2016). Selective estrogen receptor beta agonist LY500307 as a novel therapeutic agent for glioblastoma. *Sci Rep,* 6, 24185.

424. Sareddy, G. R., Nair, B. C., Gonugunta, V. K., et al. (2012). Therapeutic significance of estrogen receptor beta agonists in gliomas. *Mol Cancer Ther,* 11, 1174-1182.

425. Sarica, F. B., Cekinmez, M., Tufan, K., et al. (2012). Five-year follow-up results for patients diagnosed with anaplastic astrocytoma and effectiveness of concomitant therapy with temozolomide for recurrent anaplastic astrocytoma. *Asian Journal of Neurosurgery,* 7, 181-190.

426. Sasaki, M., Knobbe, C. B., Itsumi, M., et al. (2012). D-2-hydroxyglutarate produced by mutant IDH1 perturbs collagen maturation and basement membrane function. *Genes Dev,* 26, 2038-2049.

427. Schiffer, D., Bosone, I., Dutto, A., et al. (1999). The prognostic role of vessel productive changes and vessel density in oligodendroglioma. *J Neurooncol,* 44, 99-107.

428. Schiffer, D., Cavalla, P., Chio, A., et al. (1997). Proliferative activity and prognosis of low-grade astrocytomas. *J Neurooncol,* 34, 31-35.

429. Schittenhelm, J. and Psaras, T. (2010). Glioblastoma with granular cell astrocytoma features: a case report and literature review. *Clin Neuropathol,* 29, 323-329.

430. Schmidt, M. C., Antweiler, S., Urban, N., et al. (2002). Impact of genotype and morphology on the prognosis of glioblastoma. *J Neuropathol Exp Neurol,* 61, 321-328.

431. Schoemaker, M. J., Swerdlow, A. J., Hepworth, S. J., et al. (2006). History of allergies and risk of glioma in adults. *International Journal of Cancer,* 119, 2165-2172.

432. Scholzen, T. and Gerdes, J. (2000). The Ki-67 protein: from the known and the unknown. *J Cell Physiol,* 182, 311-322.

433. Schomas, D. A., Laack, N. N., Rao, R. D., et al. (2009). Intracranial low-grade gliomas in adults: 30-year experience with long-term follow-up at Mayo Clinic. *Neuro Oncol,* 11, 437-445.

434. Schwartzbaum, J., Ding, B., Johannesen, T. B., et al. (2012). Association between prediagnostic IgE levels and risk of glioma. *J Natl Cancer Inst,* 104, 1251-1259.

435. Schwartzbaum, J. A., Fisher, J. L., Aldape, K. D., et al. (2006). Epidemiology and molecular pathology of glioma. *Nat Clin Pract Neurol,* 2, 494-503; quiz 491 p following 516.

436. Scoccianti, S., Magrini, S. M., Ricardi, U., et al. (2010). Patterns of care and survival in a retrospective analysis of 1059 patients with glioblastoma multiforme treated between 2002 and 2007: a multicenter study by the Central Nervous System Study Group of Airo (italian Association of Radiation Oncology). *Neurosurgery,* 67, 446-458.

437. Scott, J. G., Bauchet, L., Fraum, T. J., et al. (2012). Recursive partitioning analysis of prognostic factors for glioblastoma patients aged 70 years or older. *Cancer,* 118, 5595-5600.

438. Seger, R. and Krebs, E. G. (1995). The MAPK signaling cascade. *Faseb j,* 9, 726-735.

439. Seigneurin, D. and Guillaud, P. (1991). [Ki-67 antigen, a cell cycle and tumor growth marker]. *Pathol Biol (Paris),* 39, 1020-1028.

440. Senger, D., Cairncross, J. G. and Forsyth, P. A. (2003). Long-term survivors of glioblastoma: statistical aberration or important unrecognized molecular subtype? *Cancer J,* 9, 214-221.

441. Sengupta, S., Chatterjee, U., Banerjee, U., et al. (2012). A study of histopathological spectrum and expression of Ki-67, TP53 in primary brain tumors of pediatric age group. *Indian Journal of Medical and Paediatric Oncology : Official Journal of Indian Society of Medical & Paediatric Oncology,* 33, 25-31.

442. Servant, M. J., Coulombe, P., Turgeon, B., et al. (2000). Differential regulation of P27(Kip1) expression by mitogenic and hypertrophic factors: involvement of transcriptional and posttranscriptional mechanisms. *The Journal of Cell Biology,* 148, 543-556.

443. Shah, A. H., Mahavadi, A., Di, L., et al. (2020). Survival benefit of lobectomy for glioblastoma: moving towards radical supramaximal resection. *J Neurooncol,* 148, 501-508.

444. Shamma, A., Doki, Y., Tsujinaka, T., et al. (2000). Loss of p27(KIP1) expression predicts poor prognosis in patients with esophageal squamous cell carcinoma. *Oncology,* 58, 152-158.

445. Shankar, A., Kumar, S., Iskander, A., et al. (2014). Subcurative radiation significantly increases cell proliferation, invasion, and migration of primary glioblastoma multiforme in vivo. *Chin J Cancer,* 33, 148-158.

446. Sharma, S., Sharma, M. C., Gupta, D. K., et al. (2006). Angiogenic patterns and their quantitation in high grade astrocytic tumors. *J Neurooncol,* 79, 19-30.

447. Shepard, H. M. (2015). Breaching the castle walls: hyaluronan depletion as a therapeutic approach to cancer therapy. *Front Oncol,* 5, 192.

448. Shinojima, N., Kochi, M., Hamada, J., et al. (2004). The influence of sex and the presence of giant cells on postoperative long-term survival in adult patients with supratentorial glioblastoma multiforme. *J Neurosurg,* 101, 219-226.

449. Shiraishi, S., Tada, K., Nakamura, H., et al. (2002). Influence of p53 mutations on prognosis of patients with glioblastoma. *Cancer,* 95, 249-257.

450. Shivaprasad, N. V., Satish, S., Ravishankar, S., et al. (2016). Ki-67 immunostaining in astrocytomas: association with histopathological grade – a South Indian study. *Journal of Neurosciences in Rural Practice,* 7, 510-514.

451. Si, D., Yin, F., Peng, J., et al. (2020). High expression of CD44 predicts a poor prognosis in glioblastomas. *Cancer Manag Res,* 12, 769-775.

452. Simmons, M. L., Lamborn, K. R., Takahashi, M., et al. (2001). Analysis of complex relationships between age, p53, epidermal growth factor receptor, and survival in glioblastoma patients. *Cancer Res,* 61, 1122-1128.

453. Simpson, J. R., Horton, J., Scott, C., et al. (1993). Influence of location and extent of surgical resection on survival of patients with glioblastoma multiforme: results of three consecutive Radiation Therapy Oncology Group (RTOG) clinical trials. *Int J Radiat Oncol Biol Phys,* 26, 239-244.

454. Skjulsvik, A. J., Mørk, J. N., Torp, M. O., et al. (2014). Ki-67/MIB-1 immunostaining in a cohort of human gliomas. *International Journal of Clinical and Experimental Pathology,* 7, 8905-8910.

455. Slingerland, J. and Pagano, M. (2000). Regulation of the cdk inhibitor p27 and its deregulation in cancer. *J Cell Physiol,* 183, 10-17.

456. Smrdel, U., Vidmar, M. S. and Smrdel, A. (2018). Glioblastoma in patients over 70 years of age. *Radiol Oncol,* 52, 167-172.

457. Sneath, R. J. and Mangham, D. C. (1998). The normal structure and function of CD44 and its role in neoplasia. *Molecular Pathology,* 51, 191-200.

458. Song, K., Yuan, Y., Lin, Y., et al. (2018). ERBB3, IGF1R, and TGFBR2 expression correlate with PDGFR expression in glioblastoma and participate in PDGFR inhibitor resistance of glioblastoma cells. *Am J Cancer Res,* 8, 792-809.

459. Song, X., Andrew Allen, R., Terence Dunn, S., et al. (2011). Glioblastoma with PNET-like components has a higher frequency of isocitrate dehydrogenase 1 (IDH1) mutation and likely a better prognosis than primary glioblastoma. *International Journal of Clinical and Experimental Pathology,* 4, 651-660.

460. Sonoda, Y. and Tominaga, T. (2010). 2-hydroxyglutarate accumulation caused by IDH mutation is involved in the formation of malignant gliomas. *Expert Rev Neurother,* 10, 487-489.

461. Soriano, P. (1997). The PDGF alpha receptor is required for neural crest cell development and for normal patterning of the somites. *Development,* 124, 2691-2700.

462. Sosunov, A. A., Wu, X., Tsankova, N. M., et al. (2014). Phenotypic heterogeneity and plasticity of isocortical and hippocampal astrocytes in the human brain. *The Journal of Neuroscience,* 34, 2285-2298.

463. Soussi, T. and Wiman, K. G. (2007). Shaping genetic alterations in human cancer: the p53 mutation paradigm. *Cancer Cell,* 12, 303-312.

464. SPKC. (2017a). Iedzīvotāju mirstības cēloņi 2008. – 2017. gadā Vol. 2017.

465. SPKC. (2017b). Pirmreizēji reģistrēto gadījumu skaits ar ļaundabīga audzēja diagnozi 2010.-2017. gadā.

466. Stander, M., Peraud, A., Leroch, B., et al. (2004). Prognostic impact of TP53 mutation status for adult patients with supratentorial World Health Organization Grade II astrocytoma or oligoastrocytoma: a long-term analysis. *Cancer,* 101, 1028-1035.

467. Stark, A. M., Hugo, H. H., Witzel, P., et al. (2003). Age-related expression of p53, Mdm2, EGFR and Msh2 in glioblastoma multiforme. *Zentralbl Neurochir,* 64, 30-36.

468. Stark, A. M., Nabavi, A., Mehdorn, H. M., et al. (2005). Glioblastoma multiforme-report of 267 cases treated at a single institution. *Surg Neurol,* 63, 162-169; discussion 169.

469. Stivala, L. A., Cazzalini, O. and Prosperi, E. (2012). The cyclin-dependent kinase inhibitor p21CDKN1A as a target of anti-cancer drugs. *Curr Cancer Drug Targets,* 12, 85-96.

470. Stupp, R., Brada, M., van den Bent, M. J., et al. (2014). High-grade glioma: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. *Ann Oncol,* 25 Suppl 3, iii93-101.

471. Stupp, R., Mason, W. P., van den Bent, M. J., et al. (2005). Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma. *N Engl J Med,* 352, 987-996.

472. Sturm, D., Witt, H., Hovestadt, V., et al. (2012). Hotspot mutations in H3F3A and IDH1 define distinct epigenetic and biological subgroups of glioblastoma. *Cancer Cell,* 22, 425-437.

473. Sun, T., Plutynski, A., Ward, S., et al. (2015). An integrative view on sex differences in brain tumors. *Cellular and Molecular Life Sciences,* 72, 3323-3342.

474. Sun, T., Warrington, N. M. and Rubin, J. B. (2012). Why does Jack, and not Jill, break his crown? Sex disparity in brain tumors. *Biology of Sex Differences,* 3, 3-3.

475. Syed, M., Liermann, J., Verma, V., et al. (2018). Survival and recurrence patterns of multifocal glioblastoma after radiation therapy. *Cancer Manag Res,* 10, 4229-4235.

476. Szerlip, N. J., Pedraza, A., Chakravarty, D., et al. (2012). Intratumoral heterogeneity of receptor tyrosine kinases EGFR and PDGFRA amplification in glioblastoma defines subpopulations with distinct growth factor response. *Proc Natl Acad Sci U S A,* 109, 3041-3046.

477. Takahashi, K., Tsuda, M., Kanno, H., et al. (2014). Differential diagnosis of small cell glioblastoma and anaplastic oligodendroglioma: a case report of an elderly man. *Brain Tumor Pathol,* 31, 118-123.

478. Takano, S., Kato, Y., Yamamoto, T., et al. (2012). Immunohistochemical detection of IDH1 mutation, p53, and internexin as prognostic factors of glial tumors. *J Neurooncol,* 108, 361-373.

479. Takeuchi, H., Kitai, R., Hosoda, T., et al. (2016). Clinicopathologic features of small cell glioblastomas. *J Neurooncol,* 127, 337-344.

480. Tamiya, T., Mizumatsu, S., Ono, Y., et al. (2001). High cyclin E/low p27Kip1 expression is associated with poor prognosis in astrocytomas. *Acta Neuropathol,* 101, 334-340.

481. Tang, Q., Lian, Y., Yu, J., et al. (2017). Anatomic mapping of molecular subtypes in diffuse glioma. *BMC Neurol,* 17, 183.

482. Tanwar, M. K., Gilbert, M. R. and Holland, E. C. (2002). Gene expression microarray analysis reveals YKL-40 to be a potential serum marker for malignant character in human glioma. *Cancer Res,* 62, 4364-4368.

483. Tardio, J. C. (2008). CD34-reactive tumors of the skin. An updated review of an ever-growing list of lesions. *J Cutan Pathol,* 35, 1079-1092.

484. Tehrani, M., Friedman, T. M., Olson, J. J., et al. (2008). Intravascular thrombosis in central nervous system malignancies: a potential role in astrocytoma progression to glioblastoma. *Brain Pathol,* 18, 164-171.

485. Teo, W. Y., Sekar, K., Seshachalam, P., et al. (2019). Relevance of a TCGA-derived glioblastoma subtype gene-classifier among patient populations. 9, 7442.

486. Teodoro, J. G., Evans, S. K. and Green, M. R. (2007). Inhibition of tumor angiogenesis by p53: a new role for the guardian of the genome. *J Mol Med (Berl),* 85, 1175-1186.

487. Tesileanu, C. M. S., Dirven, L., Wijnenga, M. M. J., et al. (2020). Survival of diffuse astrocytic glioma, IDH1/2 wildtype, with molecular features of glioblastoma, WHO grade IV: a confirmation of the cIMPACT-NOW criteria. *Neuro Oncol,* 22, 515-523.

488. Thakkar, J. P., Dolecek, T. A., Horbinski, C., et al. (2014). Epidemiologic and molecular prognostic review of glioblastoma. *Cancer Epidemiol Biomarkers Prev,* 23, 1985-1996.

489. Thapa, R. and Wilson, G. D. (2016). The importance of CD44 as a stem cell biomarker and therapeutic target in cancer. *Stem Cells Int,* 2016, 2087204.

490. Thon, N., Eigenbrod, S., Kreth, S., et al. (2012). IDH1 mutations in grade II astrocytomas are associated with unfavorable progression-free survival and prolonged postrecurrence survival. *Cancer,* 118, 452-460.

491. Thota, B., Shukla, S. K., Srividya, M. R., et al. (2012). IDH1 mutations in diffusely infiltrating astrocytomas: grade specificity, association with protein expression, and clinical relevance. *Am J Clin Pathol,* 138, 177-184.

492. Thotakura, M., Tirumalasetti, N. and Krishna, R. (2014). Role of Ki-67 labeling index as an adjunct to the histopathological diagnosis and grading of astrocytomas. *J Cancer Res Ther,* 10, 641-645.

493. Thuy, M. N., Kam, J. K., Lee, G. C., et al. (2015). A novel literature-based approach to identify genetic and molecular predictors of survival in glioblastoma multiforme: analysis of 14,678 patients using systematic review and meta-analytical tools. *J Clin Neurosci,* 22, 785-799.

494. Tian, M., Ma, W., Chen, Y., et al. (2018). Impact of gender on the survival of patients with glioblastoma. *Biosci Rep,* 38.

495. Toffalini, F. and Demoulin, J. B. (2010). New insights into the mechanisms of hematopoietic cell transformation by activated receptor tyrosine kinases. *Blood,* 116, 2429-2437.

496. Topley, G. I., Okuyama, R., Gonzales, J. G., et al. (1999). p21(WAF1/Cip1) functions as a suppressor of malignant skin tumor formation and a determinant of keratinocyte stem-cell potential. *Proc Natl Acad Sci U S A,* 96, 9089-9094.

497. Torp, S. H. (2002). Diagnostic and prognostic role of Ki67 immunostaining in human astrocytomas using four different antibodies. *Clin Neuropathol,* 21, 252-257.

498. Torp, S. H. and Alsaker, M. (2002). Ki-67 immunoreactivity, basic fibroblastic growth factor (bFGF) expression, and microvessel density as supplementary prognostic tools in low-grade astrocytomas. An immunohistochemical study with special reference to the reliability of different Ki-67 antibodies. *Pathol Res Pract,* 198, 261-265.

499. Tove, L.-L., Andreas Hanssøn, H., Stein, S., et al. (2012). Prognostic value of histological features in diffuse astrocytomas WHO grade II. *International Journal of Clinical and Experimental Pathology,* 5, 152-158.

500. Trabelsi, S., Chabchoub, I., Ksira, I., et al. (2016). Molecular diagnostic and prognostic subtyping of gliomas in Tunisian population. *Mol Neurobiol*.

501. Trembath, D., Miller, C. R. and Perry, A. (2008). Gray zones in brain tumor classification: evolving concepts. *Adv Anat Pathol,* 15, 287-297.

502. Tremmel, M., Matzke, A., Albrecht, I., et al. (2009). A CD44v6 peptide reveals a role of CD44 in VEGFR-2 signaling and angiogenesis. *Blood,* 114, 5236-5244.

503. Tsai, J. C., Goldman, C. K. and Gillespie, G. Y. (1995). Vascular endothelial growth factor in human glioma cell lines: induced secretion by EGF, PDGF-BB, and bFGF. *J Neurosurg,* 82, 864-873.

504. Tsai, J. C., Hsiao, Y. Y., Teng, L. J., et al. (1999). Regulation of vascular endothelial growth factor secretion in human meningioma cells. *J Formos Med Assoc,* 98, 111-117.

505. Tsidulko, A. Y., Kazanskaya, G. M., Kostromskaya, D. V., et al. (2017). Prognostic relevance of NG2/CSPG4, CD44 and Ki-67 in patients with glioblastoma. *Tumour Biol,* 39, 1010428317724282.

506. Tugcu, B., Postalci, L. S., Gunaldi, O., et al. (2010). Efficacy of clinical prognostic factors on survival in patients with glioblastoma. *Turk Neurosurg,* 20, 117-125.

507. Tunthanathip, T., Ratanalert, S., Sae-Heng, S., et al. (2017). Butterfly tumor of the Corpus callosum: clinical characteristics, diagnosis, and survival analysis. *J Neurosci Rural Pract,* 8, S57-s65.

508. Tural, S., Gercek, A., Konya, D., et al. (2009). Microvessel density and vascular endothelial growth factor expression as predictors of childrens' survival from cerebellar medulloblastoma. *J Clin Neurosci,* 16, 1199-1202.

509. Turcan, S., Rohle, D., Goenka, A., et al. (2012). IDH1 mutation is sufficient to establish the glioma hypermethylator phenotype. *Nature,* 483, 479-483.

510. Ulutin, C., Fayda, M., Aksu, G., et al. (2006). Primary glioblastoma multiforme in younger patients: a single-institution experience. *Tumori,* 92, 407-411.

511. Upadhyay, N. and Waldman, A. D. (2011). Conventional MRI evaluation of gliomas. *Br J Radiol,* 84, S107-S111.

512. Valle-Folgueral, J. M., Mascarenhas, L., Costa, J. A., et al. (2008). Giant cell glioblastoma: review of the literature and illustrated case. *Neurocirugia (Astur),* 19, 343-349.

513. van Diest, P. J., van Dam, P., Henzen-Logmans, S. C., et al. (1997). A scoring system for immunohistochemical staining: consensus report of the task force for basic research of the EORTC-GCCG. European Organization for Research and Treatment of Cancer-Gynaecological Cancer Cooperative Group. *J Clin Pathol,* 50, 801-804.

514. Verger, E., Valduvieco, I., Caral, L., et al. (2011). Does gender matter in glioblastoma? *Clin Transl Oncol,* 13, 737-741.

515. Verhaak, R. G., Hoadley, K. A., Purdom, E., et al. (2010). Integrated genomic analysis identifies clinically relevant subtypes of glioblastoma characterized by abnormalities in PDGFRA, IDH1, EGFR, and NF1. *Cancer Cell,* 17, 98-110.

516. Viegas, C., Moritz-Gasser, S., Rigau, V., et al. (2011). Occipital WHO grade II gliomas: oncological, surgical and functional considerations. *Acta Neurochir (Wien),* 153, 1907-1917; discussion 1917.

517. Vienne-Jumeau, A., Tafani, C. and Ricard, D. (2019). Environmental risk factors of primary brain tumors: A review. *Rev Neurol (Paris),* 175, 664-678.

518. Vital, A. L., Tabernero, M. D., Castrillo, A., et al. (2010). Gene expression profiles of human glioblastomas are associated with both tumor cytogenetics and histopathology. *Neuro Oncol,* 12, 991-1003.

519. Vizcaino, M. A., Palsgrove, D. N., Yuan, M., et al. (2019). Granular cell astrocytoma: an aggressive IDH-wildtype diffuse glioma with molecular genetic features of primary glioblastoma. 29, 193-204.

520. Vousden, K. H. and Woude, G. F. V. (2000). The ins and outs of p53. *Nat Cell Biol,* 2, E178-E180.

521. Wakimoto, H., Aoyagi, M., Nakayama, T., et al. (1996). Prognostic significance of Ki-67 labeling indices obtained using MIB-1 monoclonal antibody in patients with supratentorial astrocytomas. *Cancer,* 77, 373-380.

522. Walker, C., Baborie, A., Crooks, D., et al. (2011). Biology, genetics and imaging of glial cell tumours. *Br J Radiol,* 84, S090-S106.

523. Wang, C., Wang, Z., Chen, C., et al. (2020). A small-molecule CD44 dimerizing inhibitor for glioblastoma treatment. *Br J Pharmacol*.

524. Wang, J., Hu, G. and Quan, X. (2019). Analysis of the factors affecting the prognosis of glioma patients. *Open Medicine (Warsaw, Poland),* 14, 331-335.

525. Wang, L. E. I., Zhang, L., Shen, W., et al. (2016a). High expression of VEGF and PI3K in glioma stem cells provides new criteria for the grading of gliomas. *Exp Ther Med,* 11, 571-576.

526. Wang, P.-f., Liu, N., Song, H.-w., et al. (2016b). IDH-1(R132H) mutation status in diffuse glioma patients: implications for classification. *Oncotarget,* 7, 31393-31400.

527. Wang, X., Chen, J. X., Liu, J. P., et al. (2014). Gain of function of mutant TP53 in glioblastoma: prognosis and response to temozolomide. *Ann Surg Oncol,* 21, 1337-1344.

528. Wang, X. Q., Lui, E. L., Cai, Q., et al. (2008). p27Kip1 promotes migration of metastatic hepatocellular carcinoma cells. *Tumour Biol,* 29, 217-223.

529. Wang, Z., Jensen, M. A. and Zenklusen, J. C. (2016c). A practical guide to the cancer genome atlas (TCGA). *Methods Mol Biol,* 1418, 111-141.

530. Ward, P. S., Cross, J. R., Lu, C., et al. (2012). Identification of additional IDH mutations associated with oncometabolite R(-)-2-hydroxyglutarate production. *Oncogene,* 31, 2491-2498.

531. Warfel, N. A. and El-Deiry, W. S. (2013). p21WAF1 and tumourigenesis: 20 years after. *Curr Opin Oncol,* 25, 52-58.

532. Watanabe, K., Tachibana, O., Sata, K., et al. (1996). Overexpression of the EGF receptor and p53 mutations are mutually exclusive in the evolution of primary and secondary glioblastomas. *Brain Pathol,* 6, 217-223; discussion 223-214.

533. Watanabe, K., Tachibana, O., Yonekawa, Y., et al. (1997). Role of gemistocytes in astrocytoma progression. *Lab Invest,* 76, 277-284.

534. Watanabe, T., Nobusawa, S., Kleihues, P., et al. (2009). IDH1 mutations are early events in the development of astrocytomas and oligodendrogliomas. *Am J Pathol,* 174, 1149-1153.

535. Wei, C. L., Wu, Q., Vega, V. B., et al. (2006). A global map of p53 transcription-factor binding sites in the human genome. *Cell,* 124, 207-219.

536. Weidner, N. (2008). Chapter 14. Measuring intratumoral microvessel density. *Methods Enzymol,* 444, 305-323.

537. Weidner, N., Semple, J. P., Welch, W. R., et al. (1991). Tumor angiogenesis and metastasis--correlation in invasive breast carcinoma. *N Engl J Med,* 324, 1-8.

538. Wesseling, P., Schlingemann, R. O., Rietveld, F. J., et al. (1995). Early and extensive contribution of pericytes/vascular smooth muscle cells to microvascular proliferation in glioblastoma multiforme: an immuno-light and immuno-electron microscopic study. *J Neuropathol Exp Neurol,* 54, 304-310.

539. Wong, E., Nahar, N., Hau, E., et al. (2019). Cut-point for Ki-67 proliferation index as a prognostic marker for glioblastoma. 15, 5-9.

540. Woods, D. B. and Vousden, K. H. (2001). Regulation of p53 function. *Exp Cell Res,* 264, 56-66.

541. Wu, G., Song, X., Liu, J., et al. (2020). Expression of CD44 and the survival in glioma: a meta-analysis. *Biosci Rep,* 40.

542. Xiao, B. D., Zhao, Y. J., Jia, X. Y., et al. (2020). Multifaceted p21 in carcinogenesis, stemness of tumor and tumor therapy. *World J Stem Cells,* 12, 481-487.

543. Xu, H., Niu, M., Yuan, X., et al. (2020). CD44 as a tumor biomarker and therapeutic target. *Experimental Hematology & Oncology,* 9, 36.

544. Xu, H., Tian, Y., Yuan, X., et al. (2015). The role of CD44 in epithelial–mesenchymal transition and cancer development. *OncoTargets and therapy,* 8, 3783-3792.

545. Xu, W., Yang, H., Liu, Y., et al. (2011). Oncometabolite 2-hydroxyglutarate is a competitive inhibitor of alpha-ketoglutarate-dependent dioxygenases. *Cancer Cell,* 19, 17-30.

546. Xu, Y., Stamenkovic, I. and Yu, Q. (2010). CD44 attenuates activation of the Hippo signaling pathway and is a prime therapeutic target for glioblastoma. *Cancer Res,* 70, 2455-2464.

547. Yadav, A. K. and Madan, R. (2020). Small cell glioblastoma multiforme: a case series and clinicopathological update. 9, Cns63.

548. Yamamichi, K., Uehara, Y., Kitamura, N., et al. (1998). Increased expression of CD44v6 mRNA significantly correlates with distant metastasis and poor prognosis in gastric cancer. *Int J Cancer,* 79, 256-262.

549. Yan, H., Bigner, D. D., Velculescu, V., et al. (2009a). Mutant metabolic enzymes are at the origin of gliomas. *Cancer Res,* 69, 9157-9159.

550. Yan, H., Parsons, D. W., Jin, G., et al. (2009b). IDH1 and IDH2 mutations in gliomas. *N Engl J Med,* 360, 765-773.

551. Yang, J., Liao, D., Wang, Z., et al. (2011). Mammalian target of rapamycin signaling pathway contributes to glioma progression and patients' prognosis. *J Surg Res,* 168, 97-102.

552. Yang, P., Wang, Y., Peng, X., et al. (2013). Management and survival rates in patients with glioma in China (2004-2010): a retrospective study from a single-institution. *J Neurooncol,* 113, 259-266.

553. Yang, W., Warrington, N. M. and Taylor, S. J. (2019). Sex differences in GBM revealed by analysis of patient imaging, transcriptome, and survival data. 11.

554. Yao, X. H., Ping, Y. F., Chen, J. H., et al. (2008). Glioblastoma stem cells produce vascular endothelial growth factor by activation of a G-protein coupled formylpeptide receptor FPR. *J Pathol,* 215, 369-376.

555. Yemelyanova, A., Vang, R., Kshirsagar, M., et al. (2011). Immunohistochemical staining patterns of p53 can serve as a surrogate marker for TP53 mutations in ovarian carcinoma: an immunohistochemical and nucleotide sequencing analysis. *Mod Pathol,* 24, 1248-1253.

556. Ylagan, L. R. and Quinn, B. (1997). CD44 expression in astrocytic tumors. *Mod Pathol,* 10, 1239-1246.

557. Yogosawa, S. and Yoshida, K. (2018). Tumor suppressive role for kinases phosphorylating p53 in DNA damage-induced apoptosis. 109, 3376-3382.

558. Yoshida, T., Matsuda, Y., Naito, Z., et al. (2012). CD44 in human glioma correlates with histopathological grade and cell migration. *Pathol Int,* 62, 463-470.

559. Young, P., Baumhueter, S. and Lasky, L. (1995). The sialomucin CD34 is expressed on hematopoietic cells and blood vessels during murine development. *Blood,* 85, 96-105.

560. Yu, C. P., Ho, J. Y., Huang, Y. T., et al. (2013). Estrogen inhibits renal cell carcinoma cell progression through estrogen receptor-beta activation. *PLoS One,* 8, e56667.

561. Zhang, C., Moore, L. M., Li, X., et al. (2013). IDH1/2 mutations target a key hallmark of cancer by deregulating cellular metabolism in glioma. *Neuro Oncol,* 15, 1114-1126.

562. Zhang, C. and Zhu, Q. X. (2017). Allergy is associated with reduced risk of glioma: a meta-analysis. *Allergol Immunopathol (Madr),* 45, 553-559.

563. Zhang, H. and Sun, X. F. (2001). Loss of p27 expression predicts poor prognosis in patients with Dukes' B stage or proximal colorectal cancer. *Int J Oncol,* 19, 49-52.

564. Zhang, J., Yang, W. E. I., Zhao, D., et al. (2014). Correlation between TSP-1, TGF-β and PPAR-γ expression levels and glioma microvascular density. *Oncol Lett,* 7, 95-100.

565. Zhang, Y., Dube, C., Gibert, M., Jr., et al. (2018). The p53 pathway in glioblastoma. *Cancers (Basel),* 10.

566. Zheng, K., Wang, C., Yang, J., et al. (2018). Molecular and genetic evidence for the PDGFRα-independent population of oligodendrocyte progenitor cells in the developing mouse brain. 38, 9505-9513.

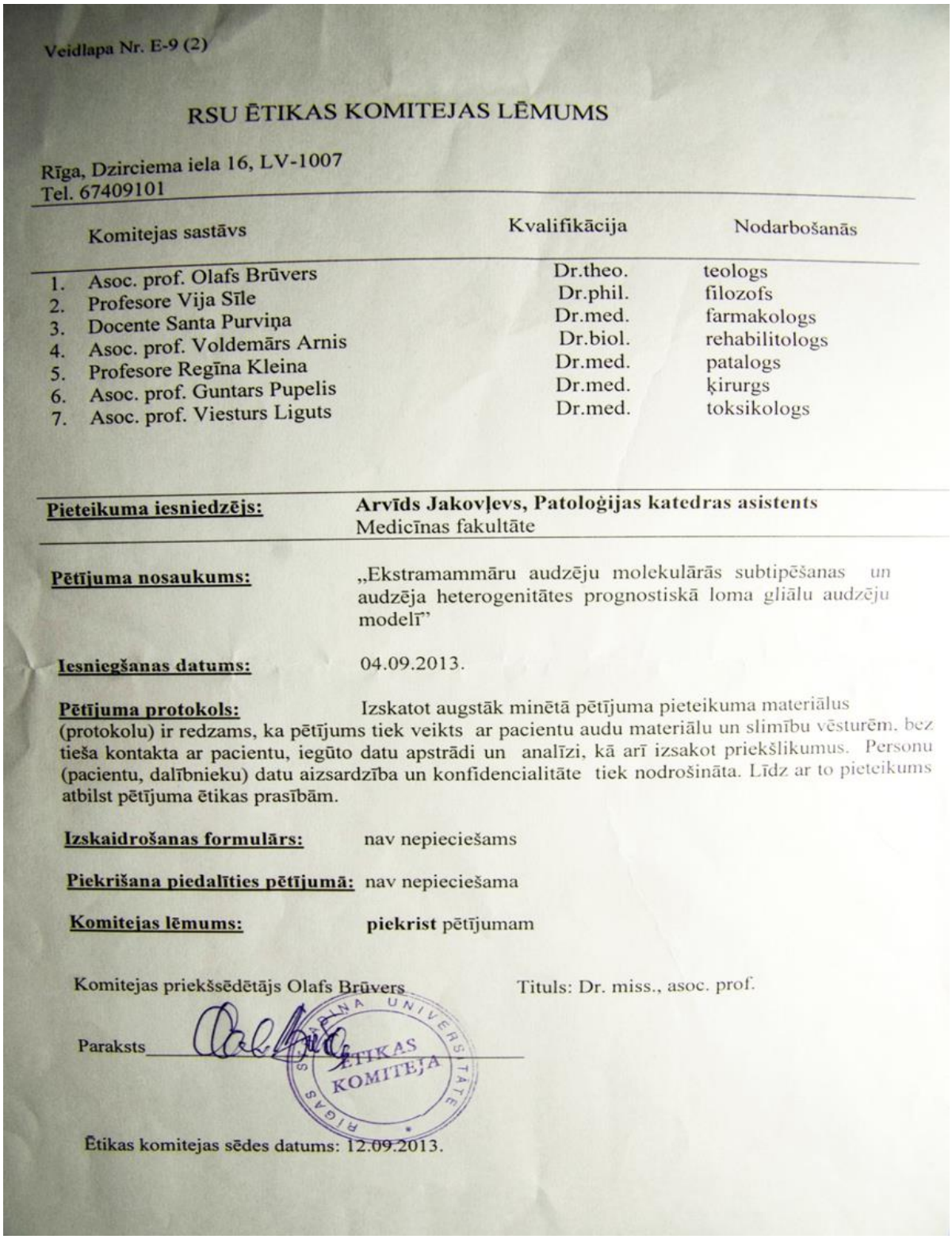
567. Zhu, Q., Zhao, X., Zheng, K., et al. (2014). Genetic evidence that Nkx2.2 and Pdgfra are major determinants of the timing of oligodendrocyte differentiation in the developing CNS. *Development,* 141, 548-555.

568. Zoller, M. (2011). CD44: can a cancer-initiating cell profit from an abundantly expressed molecule? *Nat Rev Cancer,* 11, 254-267.

569. Zolota, V., Tsamandas, A. C., Aroukatos, P., et al. (2008). Expression of cell cycle inhibitors p21, p27, p14 and p16 in gliomas. Correlation with classic prognostic factors and patients' outcome. *Neuropathology,* 28, 35-42.

Supplements

Ethical permission



Veidlapa Nr. E-9 (2)

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Komitejas sastāvs	Kvalifikācija	Nodarbošanās
1. Asoc. prof. Olafs Brūvers	Dr.theo.	teologs
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3. Docente Santa Purviņa	Dr.med.	farmakologs
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5. Profesore Regīna Kleina	Dr.med.	patalogs
6. Asoc. prof. Guntars Pupelis	Dr.med.	ķirurgs
7. Asoc. prof. Viesturs Liguts	Dr.med.	toksikologs

Pieteikuma iesniedzējs: **Arvīds Jakovļevs, Patoloģijas katedras asistents**
Medicīnas fakultāte

Pētījuma nosaukums: „Ekstramammāru audzēju molekulārās subtipēšanas un audzēja heterogenitātes prognostiskā loma gliālu audzēju modelī"

Iesniegšanas datums: 04.09.2013.

Pētījuma protokols: Izskatot augstāk minētā pētījuma pieteikuma materiālus (protokolu) ir redzams, ka pētījums tiek veikts ar pacientu audu materiālu un slimību vēsturēm, bez tieša kontakta ar pacientu, iegūto datu apstrādi un analīzi, kā arī izsakot priekšlikumus. Personu (pacientu, dalībnieku) datu aizsardzība un konfidencialitāte tiek nodrošināta. Līdz ar to pieteikums atbilst pētījuma ētikas prasībām.

Izskaidrošanas formulārs: nav nepieciešams

Piekrišana piedalīties pētījumā: nav nepieciešama

Komitejas lēmums: **piekrist** pētījumam

Komitejas priekšsēdētājs Olafs Brūvers Tituls: Dr. miss., asoc. prof.

Paraksts

RIGAS STRADIŅA UNIVERSITĀTES ĒTIKAS KOMITEJA

Ētikas komitejas sēdes datums: 12.09.2013.

IDH1 R132H staining protocol

1) Dewax and rehydrate sections: Xylene: 2x5 min / Ethanol: 99%, 2x95%, 1x70%, 1xTris-buffered saline (pH 7.6) (TBS); 3min each
2) Perform heat-induced antigen retrieval using Tris-EGTA-buffer (TEG buffer) at pH 9.0 in a microwave oven 3 x 5 min
3) Cool slides for 20 min
4) Wash in TBS for 5 min
5) Blocking endogenous peroxidases: Place slides in peroxidase-blocking solution for 10 min at room temperature
6) Wash with 3 changes of TBS buffer, 3 min incubation per step
7) Cover tissue with primary antibody anti-IDH1 R132H/clone H09
   Dilute 1:50 in PBS with antibody diluent at room temperature for 60 min
8) Wash with 3 changes of TBS buffer, 3 min incubation per step
9) Secondary antibody: Cover tissue with anti-rabbit polymer horseradish peroxidase (HRP) for 30 min at RT
10) Wash with 3 changes of TBS buffer, 3 min incubation per step
11) Prepare diaminobenzidine (DAB) by adding 2 drops of DAB-chromogen per 1 ml DAB-substrate buffer and mix
12) Staining reaction: Cover tissue with prepared DAB-chromogen solution, incubate approximately for 10 min to allow for proper brown colour development
13) Wash slides in TBS
14) Counterstain with haematoxylin for 2 min
15) Wash slides in water
16) Coverslip with mounting medium