The prognostic value of clinical factors and cancer stem cell-related markers in gliomas

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Articles included in the thesis

1. INTRODUCTION AND AIMS
Gliomas are the most common primary brain tumours among adults, affecting patients’ physical, emotional, and cognitive status as well as their working ability and quality of life [1]. This has a great social impact on both family life and society as a whole.

High grade glioma patients often have a poor prognosis despite aggressive treatment [2], which in combination with a growing number of patients, more focus on the disease, and low quality of life for patients, and the significant influence on relatives, has increased the need for better treatment. To improve survival further, knowledge about the biological and clinical presentation of gliomas and glioma patients in the entire population is needed in order to be able to offer more individualised and targeted treatment. Identification of patients who would benefit from standard treatment as well as identification of patients who need more aggressive treatment at the time of diagnosis is essential. Even more important is the identification of patients who will not benefit from current standard treatment; patients who should be offered experimental treatment or no treatment at all in order to avoid unnecessary side effects and a long course of treatment with no effect. As the majority of currently published studies include highly selected patients only [3-6], we hypothesised that the prognostic profiles identified in these patients could help clinicians in making treatment-related decisions. The first aim of this thesis was therefore to identify clinical prognostic profiles for patients with high-grade (HGG) and low-grade (LGG) gliomas in a population-based cohort.

It has been shown that somatic stem cells are responsible for self-renewal, proliferation, and differentiation during development of normal tissues. The same characteristics were identified in cancer cells [7, 8], and recently a major part of glioma research has focused on the cancer stem cell (CSC) hypothesis. The CSC hypothesis suggests that only a relative small fraction of the tumour cells, the cancer stem cells, posses the ability of initiating new tumours [7, 9, 10]. CSCs are therefore suggested to be responsible for tumourogenesis in glioma patients including recurrence, which has been explained by the resistance of cancer stem cells towards radiotherapy and chemotherapy [11, 12]. CSC-related markers have been suggested to have a promising prognostic potential by some groups [13-22], whereas other groups have reported that specific CSC-related markers have no prognostic potential [23-29]. Based on a thorough review of the literature [30], we found that the putative CSC-related markers Musashi-1, CD133, and nestin had the most promising prognostic potential. Therefore, we hypothesised that these CSC-related markers would have prognostic value in gliomas.

A common approach for identification of putative prognostic protein markers is immunohistochemistry followed by semi-quantitative scoring of the chromogenic staining reactions by a pathologist [31, 32]. This approach is easy and quickly performed but also biased by inter- and intraobserver variability [33]. To
avoid such observer dependent results, automated quantitative image analyses have been used in some studies [23, 34, 35].

Immunofluorescence is another immunohistochemical approach, in which the secondary antibody is tagged to a fluorescent dye. The combination of quantitative image analysis and fluorescence staining protocol provides an objective, continuous variable when measuring protein expression in defined regions of formalin-fixed paraffin-embedded tissue samples. Moreover, the use of double immunofluorescence makes it possible to determine the location of additional markers in different subcellular compartments and even to identify co-localisation of additional markers within the same cell. We hypothesised that the use of a quantitative immunofluorescence approach would be feasible, robust, reproducible, and provide more information than conventional scoring systems. Based on this, the second aim of this thesis was to investigate the prognostic potential of a set of CSC-related markers including Musashi-1, CD133, and nestin using a novel platform designed for automated quantitative fluorescence-based analysis.

2. BACKGROUND

2.1 Gliomas

Aetiology

Gliomas are the most common class of primary brain tumours in adults. It is estimated that gliomas constitute approximately half of the 1500 new brain tumours diagnosed in Denmark every year [36]. Different subtypes of gliomas exist, each named after the glial cells from which it was previously believed they arose: astrocytomas, oligodendrogliomas, and ependymomas [2]. The cause of gliomas remains unknown; although several possible risk factors have been investigated. In a small percentage of the patients, a connection between hereditary conditions (Li-Fraumeni syndrome and Von Recklinghausen’s neurofibromatosis) and gliomas exists [37].

Classification

Gliomas are graded according to the World Health Organisation (WHO) 2007 classification based on morphology [2]. WHO grade I tumours (pilocytic astrocytoma (PA)) are characterised as slow growing tumours with a bipolar cellular composition and Rosenthal fibres.

WHO grade II tumours (diffuse astrocytoma (DA), oligodendroglioma (O), and mixed oligo-astrocytoma (OA)) consist of diffuse infiltrating cells with cytological atypia. WHO grade I and II tumours are often termed low-grade gliomas (LGG).

WHO grade III tumours (anaplastic astrocytoma (AA), anaplastic oligodendroglioma (AO), and anaplastic mixed oligo-astrocytoma (AOA)) are more aggressive tumours containing cells with anaplasia and clearly increased mitotic activity. A special WHO grade III subtype, gliomatosis cerebri, is characterised by an extensive infiltration of the brain, including at least three brain lobes.

WHO grade IV tumours consist of glioblastoma multiforme (GBM) and gliosarcomas (GS). GBMs, the most common subtype, are characterised by micro-vascular proliferation and necrosis. GS is a GBM containing a mesenchymal or sarcomatous component. This subtype represents approximately 2% of WHO grade IV tumours. WHO grade III and IV tumours are often denoted high-grade gliomas (HGG). HGGs arise “de novo” or from a malignantly transformed LGG [2].

Ependymomas are tumours arising from the brain ventricular wall. They are often seen in children and they are frequently located in the spinal cord. As for astrocytic and oligodendrogial tumours, increasing WHO grade is associated with more aggressive tumour cells, proliferation and necrosis [2]. Patients with this special sub-type of gliomas are not included in this thesis.

Prognosis

Prognosis depends largely on the WHO grade and the histological entities. In general, patients with a WHO grade I glioma have a good prognosis, with more than 96% of the patients being alive 5 years after the primary diagnosis [38]. For patients with WHO grade II tumours, median survival is 6 to 7 years, in general better for patients with oligodendrogial tumours than for pure astrocytomas [38, 39]. For patients with WHO grade III tumours, the prognosis deteriorates, with a median survival ranging from 2 to 3.5 years despite maximal treatment [38]. Patients with a WHO grade IV tumour have the worst prognosis. Despite increased survival after the introduction of the current standard treatment, 2-year survival is 26% [6], and only 10% survive 5 years [40].

Various studies have investigated the prognostic value of clinical parameters in order to identify patients who will benefit from current treatment and, maybe even more importantly, to identify those who will not benefit from standard treatment.

The European Organisation for Research and Treatment of Cancer (EORTC) performed a large prognostic study in patients with LGGs, based on a population selected to fit into phase III studies of post-surgical radiotherapy (RT) [4]. They included 322 patients with LGGs and found that age under 40, oligodendrogial histology, absence of neurologic deficit, largest diameter below 6 cm, and a tumour not crossing the midline were associated with a good prognosis. The results were validated in an additional 288 patients, making this the largest study regarding clinical prognostic variables in LGGs.

The prognostic value of clinical variables has been investigated in HGGs as well, but the results are more scattered. Nevertheless, there seems to be consensus that age, extent of resection, and performance status are clinical parameters with a prognostic value [3, 25, 41].

2.2 Treatment of gliomas

One of the first descriptions of a patient with a glioma occurred in 1874, when Smith et al. [42] published a report on a 38-year-old male patient who experienced seizures. The patient’s condition deteriorated, and an autopsy revealed a tumour in the right temporo-parietal lobe. There were no signs of syphilis, and the tumour was diagnosed as a glioma. In the 19th century no treatment of gliomas existed.
**Surgery**

Today, surgery is considered standard treatment in gliomas [43], but the use of extensive surgery has never been investigated in a prospective controlled trial. In a retrospective study, Lacroix et al. [44] investigated the prognostic significance of the extent of resection in 420 GBM patients using volumetric MRI. Patients who underwent a complete resection had a better outcome than patients who had less aggressive surgery. Median survivals were 13 months and 8.8 months, respectively (p<0.001). This indicated that tumour reduction has a prognostic significance in HGGs.

In LGGs, the prognostic value of resection has been investigated in several studies [45-48], and the majority [46-48] find that a larger resection is associated with a better outcome. Smith et al. [46] retrospectively evaluated 216 LGG patients to assess the influence of resection on survival. Based on volumetric measurements, a significant association between tumour reduction and better survival (HR 0.972, p<0.001) was observed. In Denmark, gross total resection is recommended in patients with both HGGs and LGGs [43].

**Radiation**

Whole brain irradiation became part of standard treatment in the late 1970s. Because whole brain irradiation is associated with considerable side effects, Shapiro et al. [49] investigated the use of whole brain irradiation as compared to whole brain irradiation followed by a coned-down boost towards the glioma. No difference in survival was observed, but due to less pronounced side effects, the more tumour-specific regime was recommended.

The use of hypofractioned RT (45Gy/20 fractions vs. 60Gy/30 fractions) and stereotactic radiosurgery has been investigated [50, 51]. Neither improved survival in comparison with conventional conform RT, which is used as the standard treatment for HGGs in Denmark [43].

Two prospective studies [52, 53] have investigated the use of low-dose vs. high-dose RT in LGGs. No differences in OS or PFS were identified. However, in 2002 Karim et al. [54] published an interim analysis of the EORTC 22845 study, which compared surgery alone vs. surgery plus RT in LGGs. There was no difference in OS (p=0.49), but patients who received RT had a better PFS (p=0.02). The result was confirmed in a long-term follow-up study [55]. Although early RT may provide better tumour control in LGGs, Douw et al. [56] showed that 53% of the patients receiving early RT experienced cognitive deficits as compared to 27% in the group who underwent surgery alone. In Denmark, patients with LGGs do not receive early RT unless they have persisting symptoms or an un-resectable progressive tumour [43].

**Chemotherapy**

The use of adjuvant nitrosoureas was investigated during the 1970s and 1980s. The studies were generally small and no survival effect was observed. Two meta-analyses [57, 58] have subsequently shown that adjuvant chemotherapy is associated with improved survival in HGGs (figure 1).

In the mid 1990s, the alkylating drug temozolomide (TMZ) was developed [59]. TMZ was associated with a good safety profile in both recurrent [60] and newly diagnosed GBMs [61]. In 2005 the EORTC Brain Tumour and Radiotherapy Groups and the National Cancer Institute of Canada Clinical Trials Group published a randomised, multicenter, phase III trial comparing RT with RT and concomitant plus adjuvant TMZ [6] in 573 GBM patients. Only patients younger than 70 years, with a good performance status and normal haematologic, renal, and hepatic function were included. Addition of TMZ increased 2-year survival from 10% to 27%, and the adjusted hazard indicated a 37% reduction in mortality in the RT+TMZ group. When the 5-year survival was published [40], the advantage of receiving TMZ remained significant. Based on
the primary report [6], this regimen (the Stupp regimen) was introduced in 2005 as the standard treatment for GBM patients in Denmark [62].

Previously studies investigating the use of adjuvant chemotherapy included both WHO grade III and IV tumours [58]. When this project was initiated, no studies had been able to demonstrate that patients with WHO grade III tumours benefit from adjuvant or concomitant chemotherapy, and therefore chemotherapy was recommended only at recurrence [62].

No studies have showed that patients with LGGs benefit from post-surgical chemotherapy. Currently, the Eastern Cooperative Oncology Group (ECOG) is performing a phase III study (ECOG E3F05) comparing RT with and without TMZ in LGGs.

Table 1 shows the treatment regimes that have been used at Odense University Hospital during the period 2005–2009.

<table>
<thead>
<tr>
<th>WHO grade</th>
<th>Primary treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Observation</td>
</tr>
<tr>
<td></td>
<td>Surgery (Total resection or diagnostic biopsy)*</td>
</tr>
<tr>
<td>II</td>
<td>Surgery + observation*</td>
</tr>
<tr>
<td></td>
<td>Surgery + RT (45Gy/25).</td>
</tr>
<tr>
<td></td>
<td>Surgery + chemotherapy</td>
</tr>
<tr>
<td>III</td>
<td>Surgery + RT (59.4Gy/33)*</td>
</tr>
<tr>
<td></td>
<td>Surgery + RT (34Gy/10).</td>
</tr>
<tr>
<td>IV</td>
<td>Surgery + RT (59.4Gy/33) + concomitant and adjuvant Temozolomide*</td>
</tr>
<tr>
<td></td>
<td>Surgery + RT (34Gy/10).</td>
</tr>
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</table>

Table 1. Primary treatment for astrocytic and oligodendrogial tumours performed at OUH during the period 2005–2009.

* indicates the primary treatment considered to be most optimal.

Recurrence
Almost all patients will relapse and require further treatment. At recurrence, RT and chemotherapy are treatment modalities which may be used. Re-operation is a possibility in selected patients with a good performance status [63]. Recently the use of metronomic TMZ in heavily pre-treated GBM patients with recurrence was investigated in a prospective phase II trial [64]. The regimen was safe, with a median OS of 7 months, and the authors recommend further investigation of the regimen.

The use of re-irradiation has been investigated in additional studies [65–69] using hypofractionated conformal RT [66], stereotactic radio surgery [67], brachytherapy [68], or conventional fractionated RT [69]. Response rates were 25% to 35%, and it was shown that a cumulative normal dose <100Gy did not induce radio-necrosis in normal brain tissue [65].

Chemotherapy remains the most commonly used treatment at recurrence, although there is no standard regimen. Different chemotherapy regimens have been investigated in recurrent GBMs [70–72]; nitrosoureas had limited effect, whereas retreatment with TMZ was efficient in some prior TMZ responders. In 2007 Vredenburgh et al. [73] investigated the combination of irinotecan and bevacizumab in 167 recurrent HGGs. They showed that this was an effective regimen with limited side effects and the result was later confirmed in two Danish studies [74, 75]. Moreover, in a randomised phase II study conducted by Friedman et al. [76] it was shown that use of bevacizumab alone or in combination with irinotecan increases OS and PFS in comparison with salvage chemotherapy or irinotecan alone. Although not approved as a standard treatment [77], the combination of irinotecan and bevacizumab is often used in recurrent HGGs [43].

2.3 Prognostic and predictive markers - definitions
Various definitions of biomarkers exist. In 2001 “The Biomarkers Definitions Working Group” defined biomarkers as “A characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention”. They perceive biomarkers as having several different properties: as diagnostic tools, as staging tools, as indicators of prognosis and for monitoring the clinical response to an intervention [78].

In cancer treatment, biomarkers are often referred to as prognostic and predictive biomarkers, and they are used as indicators of prognosis, or treatment response. A prognostic biomarker has classically been defined as a marker which provides information about a patient’s outcome in an untreated group. However, as glioma patients usually receive treatment, it is necessary to define prognostic biomarkers by investigating patients treated with standard regimes. In this thesis, a prognostic marker is considered a marker that divides patients into two groups: one with a good prognosis and one with a poor prognosis regardless of treatment. A predictive marker also divides patients into two groups; one group with a good prognosis due to the treatment and one group with a poor survival despite treatment. A biomarker can be both prognostic and predictive simultaneously [79].

2.4 Biomarkers in gliomas
Many studies have dealt with the prognostic impact of biomarkers on survival in gliomas. So far, only O6-methylguanin-DNA methyltransferase (MGMT), isocitrate dehydrogenase 1 (IDH1), and 1p/19q co-deletion (LOH1p/19q) are used as prognostic or predictive markers in a clinical setting. Other markers like epidermal growth factor receptor (EGFR), K167/MB1 and tumour protein 53 (TP53), are currently used as diagnostic tools.

MGMT
MGMT is a gene located on chromosome 10q26 encoding the MGMT protein [80, 81]. Normally, the MGMT protein protects cells from apoptosis by removing alkyl groups from the O6 position of guanine. Unfortunately, the MGMT protein also removes the alkyl groups placed by alkylating chemotherapy like TMZ and carmustine, thereby protecting cancer cells from apoptosis [80, 82]. However, the MGMT gene may be silenced by a methylation of the promoter region, thereby leading to prevention of the product, the MGMT protein, and thus sensitivity to alkylating chemotherapy (figure 2).

The prognostic importance of MGMT methylation in gliomas has been investigated by several groups [80, 82–84]. They agree that patients with a methylated promoter region have a better prog-
nosis, but results are based on the use of different techniques. Esteller et al. [82] and Hegi et al. [80] used a methylation-specific polymerase chain reaction (MSP), whereas Watanabe et al. [84] used immunohistochemistry (IHC). The use of different detection methods provided different results [85], and some studies showed that only MGMT protein expression was associated with survival [86]. Many studies regarding MGMT status using different methods have been performed [85, 87-89]. However, considerations regarding reliability and reproducibility of test results obtained from both frozen and formalin-fixed paraffin embedded tissue remain conflicting [88, 90]. In a recently published study by Lalezari et al. [91] it was suggested that the combined analysis of MGMT protein expression and MGMT methylation status is necessary for an optimized prognostication in GBM patients.

**Figure 2.** An un-methylated MGMT gene promoter allows transcription factor binding and subsequent gene expression whereas hyper-methylation of the CpG islands in the promoter region excludes transcription factor binding and thereby prevents gene expression. Reprint from [107] with permission from Elsevier.

The importance of choosing the “right” detection method has been debated for several years [85-87, 92]. The discussion was ongoing when this project started and although MGMT status was considered important it was decided to await additional studies regarding optimal methodology. Thus MGMT status was not included in this thesis.

**IDH1**

The isocitrate dehydrogenase (IDH) family consists of several proteins; only IDH1 and IDH2 are relevant in gliomas [93]. IDH proteins protect the normal cell from oxidative stress by generation of nicotinamide adenine dinucleotide phosphate hydrogen (NADPH). NADPH is produced when isocitrate is decarboxylated to α-ketoglutarate (α-KG) [94-96].

In 2008 Parson et al. [96] identified a mutation in IDH1 when performing whole exome sequencing of human GBMs. Later a similar mutation in IDH2 was identified, although not as common as mIDH1 [93, 97, 98]. Mutations of IDH1 and IDH2 are considered mutually exclusive. The mutations are somatic point mutations [99-101], that affect codon 132 of IDH1 [94, 95, 99-104]. Several mutations were identified; the most common is the R132H type, although other sub-types exist [94]. If a mutation is present, α-KG is converted into 2-Hydroxylglutarate (2HG) (figure 3), which may cause an alteration in methylation status, a modified differentiation, as well as altered invasive properties of tumours containing the mutation and thereby improving survival [97].

IDH1 mutations were identified in gliomas and in acute myeloid leukaemia, although sporadic reports of the presence in other cancer types exist [97]. Clinical investigations in gliomas have identified the mutation in adult patients only [97] and mIDH1s are associated with younger patients, WHO grade II and III astrocytomas, oligodendrogliomas and oligoastrocytomas, secondary GBMs, and an improved survival [103-108]. Different techniques have been used to assess the IDH1 status, mainly direct sequencing [96, 103-106], but comparison between sequencing and IHC has shown that IHC had high sensitivity regarding detection of the IDH1 R132H mutation [107, 109].

1p/19Q

In 1994, Reifenberger et al. [110] performed an allotyping of 37 oligodendrogial tumours. They found that the majority of the tumours contained a deletion of chromosome 1p, 19q, or of both. The combined loss of 1p/19q (LOH1p/9q) was the most common deletion. The deletions usually involve whole chromosome arms, suggesting a t(1;19)(q10;p10) (figure 4) [111, 112]. LOH1p/19q is mainly associated with oligodendrogliomas and mixed oligoastrocytomas [111, 113], where it is considered a marker of chemosensitivity [113].

Cairncross et al. [114] investigated 206 patients with oligodendrogial tumours. Patients were treated with RT alone or RT+PCV. They found that 46% of the patients had a combined LOH1p/19q, and that these patients had a better survival than patients without the deletion (HR 0.31, p<0.001). This applied to both treatment groups, although the largest effect was observed in patients receiving RT+PCV. The results were confirmed in a long-term follow-up [115] and in patients from two prospective EORTC studies who received TMZ [116].

**Figure 3.** Diagram of IDH1-R132H – related gliomagenesis. Reprint from [108] with permission from Elsevier.
**EGFR**

The EGFR gene encodes the EGFR protein, which is a transmembrane glycol-protein also known as HER-1 [117]. When the EGFR protein is activated, an intra-cellular pathway including the Ras-mitogen-activated protein kinase starts [118, 119]. Up-regulation of EGFR protein has been identified in several cancer types including gliomas, where amplification stimulates cancer growth [118-120].

Feldkamp et al. [119] showed that EGFR is up-regulated in GBMs as compared to LGGs and normal brain tissue. In addition, survival in GBM patients with EGFRvIII-mutated tumours was 7 months shorter than survival in patients with wildtype EGFRvIII but the difference was not statistically significant, probably due to small sample size.

Montano et al. [117] recently investigated 73 GBM patients. Patients with a mutated EGFR protein (the EGFRvIII sub-type) had a better survival than patients without the mutation. Moreover, up-regulated EGFR was an independent prognostic factor in a multivariate analysis along with age older than 60 years, Karnofsky score of 70 or above, and a Ki67 index of 20% or less.

**Ki67/MIB1**

Ki67 is a monoclonal antibody recognising a core antigen present in both normal and malignant proliferating cells [121, 122]. The Ki67 antigen is expressed during all active phases in the cell cycles.

In 1993 Key et al. [123] developed an antibody (MIB-1) that could recognise the Ki67 protein in formalin-fixed paraffin-embedded tissue. Various groups have investigated the prognostic potential of Ki67/MIB-1 in gliomas [124-127]. These studies agree that the Ki67 index increases with increasing malignancy grade, and patients with high Ki67 index have poorer prognosis than patients with less proliferation activity. Moreover, in a recent study by Pouleau et al. [128], it was suggested that Ki67 index may be a useful tool to distinguish pseudo-progression from true progression.

**TP53**

The TP53 gene is located on the short arm of chromosome 17. It is a suppressor, often referred to as the “guardian of the genome” [81]. If the function of TP53 is impaired, cells are more susceptible to multiplication of damaged cells and consequently malignant transformation [114, 129]. TP53 is often mutated in gliomas [81, 129-133], but the prognostic and predictive value of TP53 mutations in GBMs is uncertain. Milinkovic et al. [129] investigated TP53 mutations in 30 patients with HGGs, and the authors showed that TP53 mutation is an indicator of a good prognosis in patients with GBMs. This was in accordance with the result obtained by Schmidt et al. [130], who found that TP53 mutation was an independent predictor of improved survival in patients with both primary and secondary GBMs. On the other hand, Ohgaki et al. [132] and Simmons et al. [133] showed that TP53 status had no effect on survival.

Recently Kim et al. [131] showed that the TP53 mutation is significantly associated with shorter survival in patients with LGGs. The result was confirmed in multivariate analysis adjusted for age and treatment (p=0.0005).

### 2.5 Cancer stem cells

Neural stem cells (NSC) are characterised by their ability to self-renew and to give rise to other cells through asymmetric cell division [134]. After neurogenesis, only a limited number of neural stem cells remain in the brain, mainly in the subventricular zone [135].

In 2002 Ignatova et al. [136] showed that tissue from adult cortical glial tumours contained a small population of cells having the same characteristics as NSCs. Further studies [137-140] identified a population of tumour cells, termed the “side-population”. These cells were rare, they seldom divided, and they possessed the ability of self-renewal as well as the ability of performing asymmetric division. Moreover, these cells had the ability to extrude Hoechst 33342 dye as well as mitoxantrone, indicating that these cells would be more resistant towards chemotherapy [140].

Thereof, these results were the basis for the present CSC hypothesis. This hypothesis suggests the existence of a population of tumour cells, the cancer stem cells, having unique self-renewal capabilities thereby sustaining tumour growth, in contrast to the other tumour cells [7, 9]. Furthermore, an association between CSC and the resistance to radiotherapy [141, 142] and chemotherapy [11, 142] has been suggested (figure 5).

Currently, CSCs have been identified in several cancer types, such as acute myeloid leukaemia [8], breast cancer [7], colon cancer [143], head & neck cancer [144], malignant melanoma [145], and prostate cancer [146]. In 2004, CSCs were identified in gliomas by two independent groups [139, 147]. Singh et al. [139] used cell sorting to identify CD133 positive (CD133+) and CD133 negative (CD133-) cells in tissue from five primary brain tumours. Subsequently, CD133+ and CD133- cells were transplanted into the brains of NOD-SCID mice. The authors showed that as little as 100 CD133+ cells were necessary to form new tumours resembling the primary tumour, whereas injection of 100,000 CD133- cells did not produce tumours (figure 6) [148].
2.6 Cancer stem cell-related prognostic biomarkers in gliomas

Based on the supposed crucial role of CSC in gliomas, markers identifying these cells have been of major interest in the recent years. Many biomarkers have been suggested as CSC-related markers in gliomas, and their prognostic potential has been investigated. We reviewed current literature regarding the prognostic value of cancer stem cell-related protein markers identified by IHC [149]. Based on this review, we found that out of 10 different markers, Musashi-1, CD133, nestin, and the co-expression of CD133 and nestin were the markers with most promising prognostic potential. A short review of studies investigating Musashi-1, CD133, and nestin is given in the following. A more detailed description is given in the individual articles.

Musashi-1

Musashi-1 is a RNA-binding protein [17, 20, 26, 28, 150-152] that is important in post-transcriptional gene regulation necessary for proper glial and neuronal development in the human brain [17, 20, 153]. Musashi-1 has been identified in different cancer types [154-158], including gliomas [17, 20, 26, 28, 152]. Hemmati et al. [137] showed that Musashi-1 positive cells from paediatric brain tumours, including gliomas, possess CSC characteristics. This indicated that Musashi-1 may be a prognostic marker.

Four studies [17, 20, 26, 152] reported that the expression of Musashi-1 increased with increasing WHO grades in gliomas, and that Musashi-1 thus is of prognostic value. Furthermore, two of these groups [17, 20] identified a correlation between high expression of Musashi-1 and highly proliferative tumour cells. One study [28] reported that Musashi-1 was not prognostic in univariate analysis, and no multivariate analysis was performed. The lack of multivariate analysis applied to all studies [17, 20, 26, 28, 152]. Musashi-1 was included in this thesis to elucidate its prognostic value in association with known clinical variables.

CD133

CD133 is a 5-trans membrane glycoprotein located in the membrane of human haematopoietic cells [159, 160]. It is expressed in a variety of human tissues including the brain, but its physiological and pathological functions remain unknown [21, 135, 161, 162]. CSCs in gliomas were originally identified by means of CD133+ cells [139], but other groups have shown that CD133-cells also are capable of forming tumours when engrafted intracerebrally into rats or mice [160, 163].

Using IHC, four groups reported that CD133 is a marker of poor survival [19, 21, 26, 152], whereas two studies reported that CD133 has no prognostic significance in astrocytic brain tumours [24, 25]. The inconsistent findings could be due to the use of different CD133 antibody clones, different cut-off points, and inclusion of either frozen or paraffin-embedded brain tumour sections [149, 164]. Furthermore, all studies included a limited number of patients, semi-quantitative scoring was commonly used, and the statistical analysis often included a mix of LGGs and HGGs. Although one of the most investigated CSC-related markers in gliomas, the prognostic value of CD133 was undetermined when this project started. We found that there was a need for...
more quantitative measurements and separate survival analysis for patients with different WHO grades.

**Nestin**

Nestin is a filament marker expressed in neural progenitor cells during development [15, 18, 23, 26]. In the adult human brain, nestin is only expressed in neural stem cells lining the ventricular wall and the central canal [15, 135]. Nestin has been identified in several cancer types [165-167] including gliomas [13-16, 18, 23, 25, 26, 28, 29], where expression of nestin is related to dedifferentiation, improved cell motility, invasive potential, and increased malignancy [135].

The prognostic potential of nestin has been widely investigated [13-16, 18, 23, 25, 26, 28, 29]. There seems to be some agreement about the correlation between high expression of nestin and poor survival in studies using semi-quantitative scoring [13-15, 18, 26, 28, 29]. On the other hand Kanamori et al. [16] and Kim et al. [25] reported that nestin was not prognostic in oligodendroglial tumours or in GBMs. Chinmayan et al. [23] used automated quantitative measurements (Ariol SL-50) for identification of nestin-positive cells. Moreover, patients were stratified into RPA classes (see [168]), which makes this study different from the other prognostic studies. The authors reported that nestin was not a prognostic factor.

**CD133/nestin**

Zhang et al. [22] investigated the double-expression of CD133 and nestin in 125 patients with WHO grade II-IV astrocytomas. They found high expression of both CD133 and nestin in gliomas as compared to normal brain tissues and that co-expression increased with increasing WHO grade. In addition, co-expression of CD133 and nestin had a more powerful prognostic value than just single markers. The explanation may be that the bonafide CSC marker has not yet been found and that combinations of markers may identify an important level of differentiation in the CSC differentiation hierarchy. This was the only study investigating co-expression of multiple CSC-related markers when the current project started, and validation of the results was needed. Furthermore, the co-expression was on the tumour level and not as a co-localisation on a cellular level.

2.7 Guidelines relevant for prognostic studies

New biomarkers appeared regularly, and there was a need for guidelines on the performance and reporting of prognostic studies. The “REporting recommendations for tumor MARKer prognostic studies” (REMARK) guidelines were published in order to ensure a consistent way of reporting results [169-171]. REMARK provides information in terms of study design, hypotheses, patient and specimen characteristics, assay methods, and statistical analysis methods. In addition, the guidelines suggest relevant presentations of data and important elements to include in the discussion. The aim of these guidelines is to promote clear reporting so that the relevant information will be easily available, and it will be possible to draw meaningful conclusions.

2.8 Immunohistochemistry

Immunohistochemistry is the standard in situ assay to assess protein expression and provides information on the expression level and the localisation of an antigen. The fundamental concept behind IHC is the recognition of antigens by use of specific antibodies. It is a simple and quickly performed method, which is used on a routine basis in most pathological departments [31, 32].

Tissue fixation is a necessity in order to prevent tissue decay. The most commonly used fixative in routine immunohistochemistry is the non-coagulating fixative formaldehyde (4% neutral buffered formalin). The use of fixation preserves and immobilises antigens, but it also changes the tertiary structure of proteins. Before an antibody can recognise a given antigen, the tertiary structure has to be restored by epitope retrieval. Two different techniques are available: protease-induced epitope retrieval and heat-induced epitope retrieval (HIER). The HIER technique as it is known today was developed by Shi et al. [172], who used microwave heating for antigen retrieval. To avoid unspecific staining, endogenous peroxidase activity is blocked before adding the primary antibody.

The formation of an antigen-antibody complex can be visualised in a one-step process in which the primary antibody is labelled with a chromogen or a fluorochrome. This direct method is not very sensitive. A more sensitive method is the indirect method, which can be performed in a two-step process, in which the antigen-antibody complex is visualised using a secondary antibody raised against the primary antibody. This allows for identification of smaller amounts of the antigen, it reduces the amount of primary antibody used, and it increases the intensity of the reaction. A commonly used chromogen is 3,3’-diaminobenzidin (DAB), which is oxidised to a brown non-soluble product, when hydrogen peroxide is applied [31, 173, 174].

The tyramide signal amplification is an enhancement of the peroxidase detection systems. It is based on the deposition of biotinylated tyramide at the location of the antigen-antibody complex. After incubation with peroxidase-linked secondary antibody, the phenol compound tyramide conjugated with biotin is added as well as hydrogen peroxidase. Highly reactive intermediates consisting of a biotin-tyramide complex are formed which subsequently will bind tissue proteins close to the antigen-antibody complex. Biotin can subsequently be visualised using the DAB reaction. The catalysed signal amplification (CSA II) (Dako, Glostrup, Denmark) and the tyramide signal amplification (TSA) (PerkinElmer, Waltham, MA, USA) are both based on the tyramide signal amplification system (figure 7) [173, 174].

**Immunofluorescence**

Immunofluorescence is a specific type of IHC where the secondary antibody is tagged to a fluorescent dye instead of a chromogen. Commonly used fluorescent dyes are fluorescein isothiocyanate (FITC) and tetramethyl rhodamine isothiocyanate (TRITC). The fluorescence can subsequently be visualised using a fluorescence microscopy and quantified by image analysis.
2.8 Automated analysis

During the last decade more and more markers are being used in clinical settings and for a more precise quantitative evaluation of immunohistochemical staining seems to be highly required. One promising approach to obtain this is the use of automated analysis.

In 2002 Rao et al. [175] investigated the expression of BRCA1 using a quantitative fluorescence image analysis (QFIA). They showed that the method was useful on archive material and that the use of fluorescence provided a continuous scoring scale that made it easier to categorise patients. In addition, they showed that the QFIA identified biologically important differences within the patients that were not identified by traditional IHC.

At the same time HistoRX published the development of an automated quantitative analysis (AQUA, HistoRX, Branford, CT, USA), which was specifically developed to provide an objective, continuous variable for measuring protein expression by immunofluorescence signals in defined regions of formalin-fixed paraffin-embedded tissue samples. The calculated AUQA score has recently been used in several studies [34, 35, 176-181], and it has been shown to match or even exceed pathologist-based scoring [34].

3. PATIENTS AND METHODS

3.1 Patients

All patients included in this thesis were diagnosed with a primary glioma from 1 January 2005 to 31 December 2009. Patients resident in the Region of Southern Denmark, aged 16 years or older at the time of diagnosis, were considered for inclusion. All included tissue samples were from the primary surgery, removed before the patients received any kind of radiation or chemotherapy.

Patients were identified using the Danish Cancer Registry (DCR), the Danish Pathology Databank and the local register at Odense University Hospital (OUH). Tables 2 and 3 contain morphology codes (M codes), the histological diagnosis, WHO grades and the ICD-10 codes included in the thesis.

<table>
<thead>
<tr>
<th>M code</th>
<th>Histological diagnosis</th>
<th>WHO</th>
</tr>
</thead>
<tbody>
<tr>
<td>M 94213</td>
<td>Pilocytic astrocytoma</td>
<td>I</td>
</tr>
<tr>
<td>M 94003</td>
<td>Diffuse astrocytoma</td>
<td>II</td>
</tr>
<tr>
<td>M 93823</td>
<td>Oligo-astrocytoma</td>
<td>II</td>
</tr>
<tr>
<td>M 94503</td>
<td>Oligodendroglioma</td>
<td>II</td>
</tr>
<tr>
<td>M 94513</td>
<td>Anaplastic oligodendroglioma</td>
<td>III</td>
</tr>
<tr>
<td>M 93813</td>
<td>Gliomatosis cerebri</td>
<td>III</td>
</tr>
<tr>
<td>M 93853</td>
<td>Anaplastic oligo-astrocytoma</td>
<td>III</td>
</tr>
<tr>
<td>M 94013</td>
<td>Anaplastic astrocytoma</td>
<td>III</td>
</tr>
<tr>
<td>M 94513</td>
<td>Anaplastic oligodendroglioma</td>
<td>III</td>
</tr>
<tr>
<td>M 94403</td>
<td>Glioblastoma multiforme</td>
<td>IV</td>
</tr>
<tr>
<td>M 94423</td>
<td>Glio-sarcoma</td>
<td>IV</td>
</tr>
</tbody>
</table>

Table 2. M codes, histological diagnosis and WHO grade for included gliomas.

<table>
<thead>
<tr>
<th>ICD-10 code</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>D33.0-</td>
<td>Neoplasma benignum cerebri, supratentorial, infratentorial, unspecified</td>
</tr>
<tr>
<td>D33.2</td>
<td>Neoplasma benignum, other specified parts of central nervous system</td>
</tr>
<tr>
<td>D33.7</td>
<td>Central nervous system, unspecified</td>
</tr>
<tr>
<td>D43.0-43.2</td>
<td>Neoplasms of uncertain or unknown behaviour of brain and central nervous system; supratentorial, infratentorial, unspecified</td>
</tr>
<tr>
<td>C71.0-71.9</td>
<td>Malignant neoplasm of brain</td>
</tr>
<tr>
<td>C72.8</td>
<td>Malignant neoplasm of spinal cord, cranial nerves and other parts of central nervous system; overlapping lesion of brain and other parts of central nervous system</td>
</tr>
</tbody>
</table>

Table 3. ICD-10 codes included in the study. For patients with ICD-10 C72.8 only patients with tumour localised in cerebrum or cerebellum were included.

Patients with the ICD-10 codes D42.0 (Neoplasms of uncertain or unknown behaviour of meninges) or C70.0-70.9 (Malignant neoplasm of meninges) were reviewed to ascertain the diagnosis. Patients without a histological diagnosis were included if the clinical diagnosis of a glioma was made by an experienced neurosurgeon or neuro-radiologist and access to patient data including the radiology report was possible.

We identified a total of 433 patients (figure 8); a detailed description of patient’s characteristics is given in manuscripts 1 and 2. Patients included in manuscripts 3 and 4 are briefly described in the respective manuscripts.
3.2 Immunohistochemistry

In manuscripts 1 and 2 a chromogenic staining was performed on the automated BenchMark Ultra from Ventana Medical System. This is a fully automated system performing deparaffinisation, antigen retrieval, and staining. All stains were performed using the same antibody lot in both cohorts. Samples were reviewed using a Leica DM 6000B microscope (Leica, Herlev, Denmark). Previously, Sippaya et al. [182] showed that mIDH1 can be identified in small tissue samples. All available tissue, even very small samples was thus screened for the mutation. As mIDH1 is only present in glioma cells and not in normal brain tissue [107], scoring of mIDH1 was categorised as “present” or “absent”. A more detailed staining protocol is described in manuscripts 1 and 2.

In manuscripts 3 and 4, fluorescence protocols were used on the AutostainerPlus from Dako. In the AutostainerPlus 48 samples can be stained simultaneously. Each time 48 samples were stained, we included one tissue micro array, containing tissue from several different cancer types, as an internal control. All control samples were compared with regard to the distribution and expression level of the markers, ensuring that no differences were observed. In order to minimise photobleaching, all tissue samples were stored in a refrigerator before and after sampling, and sampling was performed in a darkened room. Table 4 shows the antibodies used in this thesis.

3.3 Automated analysis

In this thesis fluorescence image analysis and quantitation were carried out using the Visiopharm integrated microscope and software module (Visiopharm, Hørsholm, Denmark). It consists of a Leica DM 6000B microscope (Leica, Herlev, Denmark) equipped with an 8-slide BioPrecision2 stage (Ludl, Hawthorne, USA) and an Olympus DP72 CCD camera. First the region of interest was manually outlined and an image was taken using the Olympus DP72 bright field setting at 1.25 times magnification. This provided an image of the tissue in which sampling should be performed. The region of interest was delineated using the Visiopharm Integrator System. Within the region of interest large vessels, necrosis and regions of normal brain tissue were manually excluded. Software controlled random and systematic sampling in 2% of the vital tumour tissue was subsequently performed. All pictures were recorded at 40 times magnification, and all pictures were reviewed to ensure that no blurring was present. If an image was blurred, recording was repeated using the “re-take image” function, ensuring that the re-recording was at the exact same location as initially. It was pre-defined that at least five useable images should be obtained for each patient. If this was not possible re-recording was performed occasionally using a higher sampling fraction. Images were analysed using an algorithm developed in the Visiomorf module in the Visiopharm Integrator System. The algorithm was based on the RGB three-colour model, and for each pixel, the intensity of three different colours red, green, and blue, was defined in the Visiomorf module. Table 5 show the thresholds used for identification of sub-cellular compartments.

![Figure 8](#). Consort diagram showing inclusion and exclusion of patients.
3.4 Ethics
This study was performed using routinely stored tissue from the evaluated patients. The measurements did not influence treatment decisions in the patients and data management was anonymous. The database is registered at the Danish Data Protection Agency (J.nr. 2009-41-3070) and the Danish National Committee on Biomedical Research Ethics approved the investigation (Project-ID S2DO9Oo8O). No patients were registered in the Danish Tissue Application Register (vaesanvendelsesregistret).

4. RESULTS
4.1 Manuscript 1
Aims: To identify clinical prognostic factors in a population-based cohort of high-grade gliomas. In addition, the prognostic value of mutated isocitrate dehydrogenase 1 (mIDH1) status was determined and correlated with the prognostic clinical variables.

Main findings from manuscript 1
- Median age at time of diagnosis was 66 (range 26–98) years in the entire population. Median ages in patients with WHO grade III, WHO grade IV and in the clinically diagnosed patients were 60, 65, and 82 years.
- In the entire population, the 2-year OS was 18%. For patients with WHO grade III, WHO grade IV, and clinically diagnosed tumours, 2-year OS were 29%, 19% and 2%.
- Patients resembling patients included in the Stupp study (GBM, aged 18–70 years, PS 0–2) treated by the Stupp-regime had a 2-year OS of 34%.
- The number of patients with HGG increased each year from 57 patients in 2005 to 92 patients in 2009, the increase was mainly seen in patients >70 years. At the same time, a significant increase in patients receiving curative intended treatment was observed within all age groups, from 13 patients (23%) in 2005 to 50 patients (54%) in 2009 (p<0.001)
- In patients treated with a curative intent, the 2-year OS was greater in patients <60 years than in patients aged 60–70 years and patients >70 years; 2-year OSs were 47%, 23%, and 17% (p<0.001 and p<0.001).
- Patients selected for curative intended treatment had a better survival than palliative treated patients within all age groups.
- In the best fitting multivariate Cox model young age, a tumour not crossing the midline, absence of neurological deficits, PS 0–1, and receipt of curative intended treatment were associated with better survival.
- A total of 17 out of 277 patients (5%) had mIDH1. These patients had a significantly better 2-year OS than patients with wild type IDH1 (wIDH1): 2-year OSs 59% and 18%, respectively (p=0.011). In multivariate analysis mIDH1 was not prognostic in patients with HGG (HR 0.58, 95% CI 0.32-1.07).

4.2 Manuscript 2
Aims: To identify clinical prognostic factors in a population-based cohort of low-grade gliomas. In addition, the prognostic value of mutated isocitrate dehydrogenase 1 (mIDH1) status was determined and correlated with prognostic clinical variables.

Main findings from manuscript 2
- Median survival and 5-year OS for the entire population were 38 months and 48%, respectively. For patients with Grade I and II tumours, 5-year OSs were 63% and 47%.
- Thirteen patients (20%) received post-surgical treatment at the time of the primary diagnosis, mainly because of persisting symptoms or tumour enhancement on MRI.
- Recurrent disease was observed in 43 patients. Thirty-three patients received further treatment, 19 of whom underwent secondary surgery. In 10 out of the 19 patients malignant transformation into a high-grade tumour had occurred.
- Cox regression analysis was performed for patients with grade II tumours only (n=66); young age, oligodendroglial histology, absence of neurologic deficits, not receiving post-surgical treatment, and PS 0–1 were associated with a good prognosis.
- A total of 30 patients with WHO grade II tumours had mIDH1. These patients had a significantly better survival than patients with wIDH1 (HR 0.24, 95% CI 0.11-0.53).
- In the best fitting multivariate analysis the effect of the classical factors was diminished after adjusting for the effect of mIDH1. Mutated IDH1 was the only parameter with significant prognostic effect on survival (HR 0.40, 95% CI 0.17-0.91).

4.3 Manuscript 3
Aims: To obtain reliable and continuous estimates of the RNA-binding protein Musashi-1 in a population-based setting of glioma patients using automated quantitative analysis based on a fluorescent staining protocol. Furthermore, the independent prognostic value of Musashi-1 was determined in multivariate analysis adjusted for the effect of clinical prognostic factors.

Main findings from manuscript 3
- The Musashi-1 positive area fraction (MA) increased with increasing WHO grade (p=0.0003), whereas the intensity did not differ between WHO grades (p=0.84).
- No statistical significant differences in MA between WHO grades I and II (p=0.58) or between WHO grade III and IV tumours were observed (p=0.80).
- In WHO grade II tumours Musashi-1 was not prognostic in either univariate (HR 0.66, 95% CI 0.22-1.97, p=0.45) or multivariate analyses adjusted for age and performance status (HR 0.74, 95% CI 0.20-2.81, p=0.66).
- In WHO grade III tumours Musashi-1 was not prognostic in either univariate analysis (HR 2.37, 95% CI 0.92-6.09, p=0.07), but in multivariate analysis adjusted for age and performance status, a high MA was significantly associated with poor survival (HR 3.10, 95% CI 1.04-9.18; p=0.042).
- In WHO grade IV, MA was not prognostic when dichotomised at the median in either univariate analysis (HR 0.97, 95% CI 0.71-1.30, p=0.82) or multivariate analysis (HR 0.82, 95% CI 0.60-1.11, p=0.197).
- In an exploratory optimal cut-point analysis in the GBM patients, dichotomising at the 81% level (MA<0.22 vs. MA>0.22) was
identified as the optimal cut-point. This cut-off value was confirmed by a ROC analysis.

- Dichotomising GBM patients at the 81% level, high MA was associated with better OS in univariate analysis (HR 0.65, 95% CI 0.44-0.98, p=0.038). It was not significant in multivariate analysis adjusted for age, performance status, tumour crossing midline, and neurological deficits (HR 0.68, 95% CI 0.45-1.04, p=0.072).
- Stratification by chemo-radiotherapy showed that the high MA only predicted a better survival in GBM patients treated with chemo-radiotherapy.

4.4 Manuscript 4

Aims: To investigate the prognostic value of the CSC-related markers CD133 and nestin in a population-based setting of glioma patients using automated quantitative analysis based on a fluorescent staining protocol. The prognostic value of each marker, their co-expression and co-localisation were subsequently correlated with the effect of clinical prognostic factors.

Main findings from manuscript 4

- The area fraction of CD133 was not associated with OS or PFS in WHO grade II, III or IV tumours in univariate or multivariate analysis.
- Nestin was not associated with OS in WHO grade II, but low levels of nestin were associated with improved PFS (HR 3.42, 95% CI 1.19-9.82 p=0.02).
- High levels of nestin were associated with a poor OS in WHO grade III tumours (HR 4.34, 95% CI 1.61-11.68, p=0.004), although the association was not significant in multivariate analysis adjusted for age and performance status (HR 2.19, 95% CI 0.69-6.93, p=0.18). Nestin was not associated with PFS.
- Nestin was not associated with OS (HR 1.12 95% CI 0.84-1.53 p=0.429) or PFS (HR 1.05 95% CI 0.72-1.55 p=0.788) in WHO grade IV tumours.
- Co-expression was not associated with OS or PFS in multivariate analysis for WHO grade II, III or IV tumours.
- Co-localisation was not associated with OS or PFS, although a trend towards better PFS was observed in patients with WHO grade II tumours and high levels of co-localization (HR 2.58, 95% CI 0.94-7.07, p=0.07). This was significant in multivariate analysis adjusted for age and performance status (HR 3.08, 95% CI 1.05-9.11, p=0.04).

5. GENERAL DISCUSSION

Detailed discussions of the different aspects of this thesis are included in the individual papers. In the following, the most important results are emphasised and the significance of these results in relation to other studies and already existing knowledge is discussed.

5.1 Identification and inclusion of patients

The aim in manuscripts 1 and 2 was to identify clinical prognostic variables in patients with gliomas. Due to a different biological course and hence different treatment regimes, high-grade gliomas and low-grade gliomas were investigated separately. This is an advantage because it provides independent information regarding two clinically different sets of gliomas. The low number of patients with WHO grade I, grade II and grade III tumours is a limitation as is the retrospective data collection, since data were not gathered for the specific purpose of the current studies. One of the problems with retrospective studies is missing data [169, 183, 184]. In our studies, very few clinical data were missing, and furthermore, we chose to exclude patients with missing data from multivariate analysis to avoid misinterpretation of results. Another possibility when handling missing data is the use of multiple imputation. In multiple imputation each missing value is replaced with an estimated value, but in order to use multiple imputation data should be missing at random [185]. In manuscripts 3 and 4, it was pre-defined that at least 15 mm² vital tumour tissue should be available in order to obtain an adequate and reliable staining. We found that patients who did not match this criterion had tumours containing large areas of necrosis or deep-seated tumours that only allowed a diagnostic biopsy. These patients were perceived as a selected group and multiple imputation was not performed.

Inclusion of all patients selected for the study is another problem when performing retrospective studies [169, 183, 184]. Therefore three different registers were used for identification of glioma patients in anticipation that this would provide us with the complete glioma population. It was expected that the majority of the patients would be registered in the DCR. However; this was not the case. All patients in the present study are now registered in DCR.

5.2 Clinically diagnosed patients

Only a limited number of population-based glioma studies have been published so far [38, 186], and none of these included clinically diagnosed patients. In this thesis, 76 clinically diagnosed patients were included; 67 (86%) were estimated to have a GBM. The clinically diagnosed GBM patients represented 22% of all GBM patients. Identification of clinically diagnosed patients was expected in a population-based study, but it was surprising that these patients constituted such a large percentage. The majority of these patients (86%) were aged 70 years or older at the time of diagnosis, and the prognosis was dismal. According to the Danish Causes of Death Register, the brain tumour was the cause of death in 66 of the clinically diagnosed patients. This strengthens the glioma diagnosis although it does not definitively exclude other malignant brain tumours or non-malignant diseases.

A total of nine patients (12%) with low-grade gliomas were clinically diagnosed. With the exception of two tumours all were non-contrast enhancing. It has been shown that both HGGs and LGGs may lack contrast enhancement [187, 188], which makes the diagnosis questionable in these patients. However, none of these patients received post-surgical treatment, and with six patients still alive when data were evaluated the diagnosis of HGG seems unlikely. This does not rule out the presence of non-neoplastic lesions, which can also show contrast enhancement [189]. Indeed, the lack of recurrence as well as the long OS may...
indicate that these patients did not have a glioma at all. Similar considerations occurred regarding the two patients with contrast-enhancing tumours in the brain stem. Brain stem tumours are rare in adults, and different sub-types with different prognoses exist. Furthermore, non-neoplastic lesions as well as neoplastic non-glioma lesion have been identified as being contrast-enhancing in the brain stem [190-192]. It was decided to include the clinically diagnosed patients in the thesis because they were perceived as glioma patients and hence followed up with radiological examination every third month.

The use of non-invasive identification of glioma patients is a subject of interest, and additional studies have been published recently [193-197]. All studies are based on identification of 2-HG using magnetic resonance [195, 196] or magnetic resonance spectroscopy [193, 194, 197]. In the future such techniques will probably improve separation of neoplastic and non-neoplastic lesions.

5.3 Histology

This thesis includes a large number of patients from the same treatment era, which is an advantage. In addition, all histological diagnoses were based on the WHO classification from 2007 [198]. All tissue samples were reviewed before inclusion in the project. Thirty-five GBM patients were initially diagnosed as having WHO grade III tumours. Two patients were initially diagnosed with a GBM but were re-diagnosed as having a diffuse astrocytoma and an anaplastic astrocytoma. In the present thesis, the revised histological diagnoses were used, and patients who originally were treated as WHO grade III tumours were considered as patients with GBMs receiving non-curative treatment. It may be argued that the original diagnosis should have been retained, since changing the diagnosis may influence the outcome within the different treatment regimens.

5.4 Endpoints

Overall survival (OS) is often considered as the “gold standard endpoint” to demonstrate a clinical benefit [199, 200] and it was chosen as the primary endpoint in this thesis. OS was measured from date of diagnosis, which was defined as the date of surgery for the patients with histologically verified tumours and date of registration in DCR for the clinically diagnosed patients. For some patients, more than one operation was necessary to obtain sufficient tissue to make the diagnosis, and OS may have been underestimated in these patients. The clinically diagnosed patients were often registered in the DCR when the patients went to the neuro-surgery department for information. This induces bias when comparing OS in histologically verified and clinically diagnosed patients.

Progression-free survival (PFS) is another frequently used endpoint. In general, PFS is an attractive endpoint because it is available earlier than OS and is not biased by subsequent cancer-directed therapies [199, 200]. Nevertheless, PFS as an endpoint has been criticised because the date at which the radiological evaluation confirms progression is in fact proxy for the true progression [200, 201].

In metastatic colorectal cancer, PFS is widely accepted as a valid surrogate of OS [202, 203], whereas no similar perception has been obtained in breast or lung cancer [199, 204]. Only a few studies have investigated the use of PFS as an endpoint in gliomas. Ballmann et al. [205] reported that progression-free survival at 6 months (PFS-6) was a useful endpoint in newly diagnosed as well as recurrent GBM patients. However, all patients were treated before introduction of the Stupp regimen and the result is difficult to apply in a clinical setting today. Lamborn et al. [206] showed that PFS-6 correlated strongly with OS in recurrent GBMs and Polley et al. reported similar findings in newly diagnosed GBM patients [207]. Moreover, Polley et al. showed that PFS-2 and PFS-4 were also correlated with OS.

The use of PFS in gliomas is, however, not without problems. PFS is defined as radiological tumour progression or clinical progression without any sign of radiological progression. After introduction of the Stupp regimen, it has been reported that approximately 20% of GBM patients develop “growth” of the contrast-enhancing region on MRI, which may not represent true progressive disease [208]. This is referred to as “pseudo-progression”.

Pseudo-progression has been described as a sub acute treatment-related reaction with or without clinical deterioration, showing oedema and sometimes contrast enhancement on MRI. The reaction recovers or stabilises spontaneously [208, 209]. It often occurs within the first 2 to 3 months after radiotherapy, which makes the use of PFS-2 and PFS-4 difficult. Currently, no imaging techniques can distinguish between pseudo-progression and true progression [209].

In order to minimise the problem, the Response Assessment in Neuro-Oncology (RANO) working group, recommend that “within 12 weeks of chemo-radiotherapy completion, progression can only be defined via conventional MRI if there is new out-of-field enhancement or if the lesion is histologically confirmed and that clinical decline alone cannot be sufficient to define progressive disease in this time frame” [210]. Patients included in this thesis were evaluated by the McDonald criteria [211] and the Response Evaluation Criteria In Solid Tumor (RECIST) criteria [212, 213], because the RANO criteria was not published until 2012 [210].

5.5 WHO grade III tumours

Compared to others, we observed a poor survival for patients with WHO grade III tumours (median OS 10 months), especially in patients with anaplastic oligodendroglial tumours (median OS 24.5 months). When investigating AAs, AOs and AOAs separately, we found a median OS of 9.8 months, 6.8 months, and 28.2 months, respectively. Two groups [38, 214] have previously investigated survival in WHO grade III tumours. Ohgaki et al. [38] included 987 patients, and the authors reported a median OS of 3.5 years in anaplastic oligodendrogial tumours. Scheie et al. [214] included 95 patients with oligodendrogial tumours; 2-year OSs were 78% as compared to 50% in our study. Our patients were generally in good performance status, and 69% received...
curative intended radiotherapy. They were slightly older, median age at time of diagnosis was 55 years as compared to 49 years in the Ohgaki study and 43 years in the Scheie study. In addition, only 30% of our patients had loss of 1p/19q as compared to 55% in the study made by Scheie and co-workers. In the Ohgaki study, loss of 1p/19q was reported for AOs and AOAs separately, being 69% and 44%, as compared to 25% and 33% in our study. Despite the fact that our patients were older and lacked loss of 1p/19q; survival was disturbingly short. Review of the medical record for each patient revealed that four out of six patients with AO died within the first 6 months after surgery. Three of those patients died of post-surgical complications more than 30 days after surgery. Of the remaining two patients, one patient did not appear at the planned surgery twice. Having four out of six patients with an atypical course of disease may explain the short survival in the AO patients included. However; the short survival in patients with AAs and AOAs cannot be explained in a similar way. In the study published by Scheie et al. 45% of the patients received post-surgical chemotherapy as compared to 12% in our study. Patients with oligodendroglial tumours were usually not offered chemotherapy in our institution based on the initial results from the EORTC26951 randomised trial [215] investigating the value of RT and adjuvant procarbazine, lomustine, and vincristine (PCV) in oligodendroglial tumours. The first report concluded that adjuvant PCV increase PFS but not OS. In June 2012, a long-term follow-up was published as an ASCO abstract. Surprisingly patients receiving PCV had a better survival than patients receiving RT alone (HR 0.75, 95% CI 0.60-0.95) and in patients carrying the 1p/19q co-deletion (LOH1p/19q) the difference seemed even bigger (HR, 0.56; 95% CI, 0.3-1.03). These results were recently published as a full paper [216]. Ongoing studies are trying to validate the results, but based on the EORTC 26951 and RTOG 9402 [115] studies, patients with anaplastic oligodendrogliaomas are now treated with RT and adjuvant PCV in Denmark.

5.6 Older patients

A total of 145 patients were aged 70 years or older at the time of diagnosis, and 138 had HGGs. As the number of geriatric glioma patients increases, there has been an increased awareness regarding treatment of these patients. In 2007, Keime-Guibert et al. [217] showed that median OS was better in patients aged 70 years given RT than patients who received best supportive care. Other groups have investigated the use of hypo-fractioned RT vs. long-term RT [218], TMZ vs. hypo-fractioned RT vs. long-term RT [219], and TMZ vs. long-term RT [220]. However no prospective studies have investigated the use of the Stupp regimen in elderly patients. Two groups [221, 222] retrospectively investigated the use of RT plus concomitant and adjuvant TMZ in patients aged 65 or older. Both groups reported acceptable tolerability of this regimen. This is in accordance with our study, which showed that older patients with a good clinical profile do benefit from more aggressive treatment. This, however, needs further validation in prospective studies, and currently the EORTC 26062 is investigating the use of TMZ and short-course radiation versus short-course radiation alone in the treatment of newly diagnosed glioblastoma multiforme in elderly patients (aged 65 years and older) [223]. Although Wick et al. [220] showed that patients aged 65 years or older with an unmethylated MGMT did not benefit from TMZ whereas MGMT status could not predict response in patients receiving RT alone an association between MGMT status and OS has not been performed in older patients receiving the Stupp regimen yet. Determination of MGMT status is included in the EORTC 26062, which is still recruiting patients and the ideal treatment of older GBM patients is still uncertain.

5.7 IDH1 status

The prognostic value of IDH1 was reported in the total population of high-grade gliomas. A multivariate analysis adjusted for the effect of relevant clinical factors in patients with WHO grade III and IV tumours was subsequently performed. This analysis showed that IDH1 is prognostic in WHO grade III tumours with regard to OS (HR 0.24, 95% CI 0.09-0.61) but not in patients with GBMs (HR 0.97, 95% CI 0.39-2.38). The analysis included a small number of patients with WHO grade III tumours, which is a limitation in the study. The limited number of patients also applies to paper 2. Especially the numbers of patients with oligodendroglial tumours were limited in both manuscripts, and it was not possible to perform a separate analysis of the oligodendroglial tumours that took the prognostic effect of IDH1 and LOH1p/19q into account. Enlargement of the patient cohort, including more WHO grade II and III tumour patients, is considered a future project.

So far, the prognostic value of IDH1 status has not been correlated with the prognostic value of cancer stem cell markers in gliomas [224]. However, a correlation between mIDH1 and the prognostic variables LOH1p/19q [5, 104, 131, 225], MGMT status [104, 226], and TP53 mutations [224, 225, 227] has been established. In a study by Hartmann et al. [228], it was shown that stratifying patients based on IDH1 and 1p/19q status induced a better separation of patients with different prognoses. This was confirmed in a recently published study by Leu et al. [229], who included four different markers: IDH1, MGMT, LOH1p/19q, and TP53. The authors report that the combined molecular model induced a better separation of patients with different prognoses than did the histological analysis. No similar classification has been made for patients with WHO grade III tumours.

Using the combined molecular model, Leu et al. [229] showed that patients with mIDH1/methylated MGMT/TP53 positive tumours had an increased risk of malignant transformation. This is in contrast to a study done by Ahmadi et al. [230], who did not find an association between IDH1 status and time to malignant progression in 100 pure astrocytomas. Leu et al. [229] included both astrocytomas and oligodendroglial tumours, and it may be speculated that the opposing results may be due to inclusion of different histological tumours. However, studies investigating the association between IDH1 status and time to malignant transformation are limited [108, 227, 229, 230]. Olar et al. [108] investigated nine patients with non-enhancing WHO grade II and grade III tumours, and the authors showed that patients with mIDH1 had
an increased risk of malignant transformation into a GBM. Since non-enhancing HGG tumours are rare and only patients aged 50 or older were included, use of this result in a clinical setting is difficult.

5.8 Intratumoural heterogeneity and the use of whole slides

Intratumoural heterogeneity is known in many cancer types [231-233] including gliomas [234-236]. The majority of tumours are presumed to originate from a single cell, but multiple cell divisions are required to produce macroscopic tumours. As genomic instability is expected to constantly produce new mutations, development of different sub-clones occurs. These sub-clones differ in many biological features, e.g. morphology, gene expression, expression of cell surface markers, metabolism, proliferation rate, invasive behaviour, angiogenic and metastatic potential, and drug resistance [233, 234, 236]. This heterogeneity should be kept in mind when a study is designed.

A commonly used technology in cancer biomarker research is tissue micro arrays (TMAs), which have the ability to assay hundreds of tumour samples arrayed on a single slide, and the technique is therefore timesaving [34, 237-240]. The reliability of TMAs in gliomas has been investigated by several groups including our group [24, 241, 242], and the conclusion is that TMAs provide unreliable results in gliomas. Investigation of whole slides was used in this thesis, which was considered an advantage in our study.

For some glioma patients, only a small biopsy can be taken. Small samples make a histological diagnosis difficult and may often prevent further immunohistochemical staining. In the present study, large differences in the amount of viable tumour tissue were observed, although we excluded small biopsies (area < 15 mm²) in manuscripts 3 and 4. This minimised the bias of heterogeneity but we do not know whether we underestimated or overestimated the expression of the marker studied. However, since biopsy size and tumour size are expected to be correlated to some degree, excluding small sized biopsies may itself introduce bias.

5.9 The use of automated quantitative analysis

Immunohistochemistry is the standard in situ assay to assess protein expression. However, IHC involves additional steps, all of which provide a risk of inducing bias. Some of the major problems in IHC are fixation [243], antigen-retrieval methods [31], sensitivity and quality of the different antibodies, as well as the use of different clones and lots [32, 244]. Moreover, oversaturation of chromogen-based immunohistochemical reactions, bin-based scoring systems (0, 1+, 2+, and 3+), as well as inter- and intraobserver variability may introduce biases [33, 244].

Inadequate fixation is, in particular, known from large tissue samples in which slow diffusion of formalin leaves the central part of the tissue under-fixated. This is not a problem in gliomas due to the small tissue samples [243]. Over-fixation can produce false negative results [173], but introduction of HIER has reduced this problem [245]. Moreover, the use of HIER is an advantage in comparison with protease-induced epitope retrieval, which may destroy the epitopes if not carefully monitored [173, 245]. However, the use of HIER may not reveal all epitopes adequately, and other retrieval methods may be needed [245]. In the present study HIER was chosen as the retrieval method only after testing additional methods.

The use of automated analysis does not avoid all the pitfalls known to interpretation of immunohistochemistry, but it increases the reproducibility and diminishes interobserver and intraobserver variability [34]. In addition, the use of fluorescence has been shown to reveal that small biological differences which cannot be revealed by conventional scoring methods are important in terms of survival [175]. This makes the combination of fluorescent staining and automated analysis an attractive approach in the clinical setting.

In this thesis, automated quantitative analysis using the Vi siopharm Integrator System (Visiopharm, Hørsholm, Denmark) was used. An essential difference between our study and previously published studies using the AQUA system [34, 179, 246-248] is the use of a tumour-specific tag. We investigated additional markers as potential markers of glioma tissue, but all markers labelled only part of the cells. So far no markers, with the exception of mIDH1, are considered tumour specific in gliomas, but mIDH1 is not present in all gliomas. However, epithelial-specific antigens are present and widely distributed in many carcinomas, making it easy to identify the tumour cells in patients with these cancers [174]. Lack of tumour-specific tags complicates the recognition of tumour tissue in glioma samples as compared to other tumour types. To avoid this problem, the total area of each frame was used instead of the total area of the cytoplasm, which due to differences in cellularity between tumours may induce some bias. The total area of nuclei could easily be identified with the Dapi staining.

The use of fluorescence provided continuous measurement in manuscripts 3 and 4. Optimally, continuous measurements should not be dichotomised due to loss of information and statistical power [169]. However, it is difficult to interpret the prognostic value of continuous measurements, and in a clinical setting an optimal cut-point is needed. Therefore, the expression of Musashi-1, CD133, and nestin was investigated as a continuous variable and as dichotomised at the median. Moreover, patients with WHO grade IV tumours were also separated based on quartiles. The use of the median and the quartiles was decided upon principally.

As the initial results suggested that high levels of Musashi-1 were associated with superior survival, an exploratory optimal cut point-analysis of Musashi-1 including a receiver operating characteristic (ROC) analysis was performed. This was considered reasonable since this is the first study investigating the prognostic value of Musashi-1 within the different WHO grades, and no cutoffs have previously been reported. Using this approach, we identified a sub group of GBM patients with high levels of Musashi-1 and a superior prognosis.
Nestin has been suggested as a prognostic marker in gliomas [14, 15, 18, 26, 28, 29], although multivariate analysis was performed in one study only [28]. Moreover, two groups reported that nestin was not prognostic and that only clinical variables were significantly associated with OS in multivariate analysis [23, 25]. This may partly be due to the presence of nestin in endothelial cells as it has been shown that nestin is expressed in proliferating cells during active angiogenesis [14, 260]. Although larger vessels were excluded in the present thesis, it is possible that detection of nestin-positive endothelial cells may influence the evaluation of the prognostic potential.

Musashi-1 is a RNA-binding protein [261] associated with a complex network of cancer-related genes [262, 263]. It has been identified in several types of cancer including GBMs [17, 20, 26, 28, 151, 155, 264-269]. Musashi-1 has been widely investigated in breast cancer, where it has been reported that approximately 60% of primary breast cancers and 100% of lymph node metastasis are Musashi-1 positive [267]. Knockdown of Musashi-1 positive cells inhibited growth of breast cancer as well as growths of colon cancer, medulloblastomas, and GBMs [267, 270, 271]. Moreover colon cancer co-expressing CD133 and Musashi-1 demonstrated resistance towards oxaliplatin and 5-fluoro-2,4(1H,3H)pyrimidinedione [272]. With all these data in mind, we expected that high levels of Musashi-1 would be a predictor of poor survival. In the present study, a high level of Musashi-1 was associated with poor survival in patients with WHO grade III tumours and in GBM patients who underwent surgery only. However, we identified GBM patients with a high expression of Musashi-1 and an improved prognosis as compared to patients with low expression of Musashi-1. No difference in the clinical data could explain the different outcome. At the present time, we cannot explain this result. As an association between high levels of Musashi-1 and absence of MGMT protein has been suggested in colon cancer [273], it may be speculated whether these patients have a methylated MGMT promoter, which hence might contribute to the improved prognosis.

It has often been suggested that CSCs represent a small part of the tumour cells only. However, more recent reviews have proposed that the CSC hypothesis does not require a stem cell cell-of-origin, that CSCs are better described as tumour-propagating cells and that CSCs therefore do not need to be rare [274]. This may still explain the poor response towards RT and chemotherapy in glioma patients, but at the same time it may also explain why some studies report a large percentage of CD133+ cells [21, 138, 163]. In line with this, it may also be argued that a high percentage of glioma cells are CSCs and therefore are resistant to RT and chemotherapy [275], suggesting that markers for these cell are so prevalent that their prognostic potential is limited.

Another perspective is that the use of a single CSC-related marker for prediction of prognosis is insufficient in GBM, taking into account that GBM is known as a very heterogenic tumour, and the prognostic significance of different sub-classes of GBMs has been investigated [276, 277]. Verhaak et al. [276] used a transcriptomic approaches for identification of four different sub-
types, proneural, neural, classical, and mesenchymal, based on the expression of EGFR, NF1, and PDGFRα/IDH1. It was shown that patients with the proneural sub-type did not benefit from concurrent chemo-radiotherapy, whereas aggressive treatment reduced mortality in patients with the classical or mesenchymal sub-type. However, Vo et al. identified genes which were closely associated with expression of Musashi-1, and these genes were subsequently compared to the genes defining Verhaak’s four subtypes of GBMs. Genes associated with expression of Musashi-1 were identified within all four subtypes [262], suggesting that there is no association with the level of Musashi-1 in the present study and the four subtypes.

As the transcriptomic approaches is not easily performed in most pathological departments, Motomura et al. [277] tried to identify an immunohistochemical analysis-based GBM subclassification based on the 16 proteins identified by Verhaak and colleagues. Only a partial overlap between the groups identified by Verhaak [276] and Motomura [277] was observed, indicating that sub-classification of GBMs is very complicated and should include additional markers and mutations. The novel possibilities coming up regarding targeted sequencing of a number of genes at low cost may give much better possibilities in future prognostic studies and reveal the full potential of novel protein biomarkers combined with more precise knowledge about the mutational status of a given tumour.

6. CONCLUSION AND PERSPECTIVES

To improve OS, knowledge about the biological and clinical presentation of gliomas and glioma patients in the entire population is needed. Identification of population-based clinical prognostic variables can help predicting outcome and may help clinicians in making treatment-related decisions. Such knowledge does not however, give information on the potential benefit of treatment. The majority of currently published studies include highly selected patients only, and the prognostic profiles do not fit the true population. This thesis reports clinical prognostic variables for patients with high-grade and low-grade gliomas. Prognostic variables for HGG patients consist of age, performance status, tumour crossing midline, neurological deficits, and post-surgical treatment, whereas younger age, a non-astrocytic histology, absence of neurological deficits, and performance status 0-1 were associated with a better prognosis in LGG patients. The prognostic potential of different clinical variables in HGGs and LGGs emphasises the differences between these groups. All clinical variables can, however, be easily obtained during the primary consultation and used in prognostic counselling of the patients and to guide the clinicians’ choice of treatment.

Until recently, treatment decisions in gliomas were based on clinical data, but in order to provide more individualised treatment, additional prognostic biomarkers like MGMT, LOH1p/19q, and IDH1status are now measured routinely [43]. Patients with WHO grade II or grade III tumours and wild type IDH1 had a significantly poorer survival than patients with miDH1. Presently, IDH1 status is considered prognostic in gliomas and may be used to identify a poor prognosis in patients with WHO grade III tumours and which patients should be candidates for a more aggressive treatment or a closer follow-up in order to initiate early recurrence treatment due to a more aggressive biology.

High levels of Musashi-1 are a predictor of poor survival in patients with WHO grade III tumours and a predictor of superior survival in a sub-group of GBM patients treated with radiochemotherapy. These conclusions need validation in a study with a larger number of patients.

CD133 and nestin did not provide further information regarding outcome as compared to the use of clinical variables when analysed separately or in combination. Several antibodies against CD133 have been developed but the clone W6B3C1, used in this thesis, has never been associated with OS in gliomas. Use of the W6B3C1 clone is not recommended in future studies. Identification of nestin-positive glioma cells may be biased by nestin-positive endothelial cells. Further investigation of nestin therefore requires identification and exclusion of the endothelial cells using an endothelial marker. This can be investigated by the fluorescence-based approach established and used in the present thesis. Co-localisation of CD133 and nestin was identified, but did not have a prognostic significance, probably due to the biases mentioned. However, use of double fluorescence has promising potential, and it may be used in future studies regarding other marker combinations.

Although the cancer stem cell-related markers in the present thesis were without promising potential, a better understanding of the complex field of GBM cancer stem cell biology may reveal the existence of critical biomarkers and therapeutic targets.

The introduction of clinically important protein biomarkers requires standardised methods and interpretations. Based on results in this thesis the use of automated quantitative analysis is shown to be a feasible, robust, and reproducible method having a clinical potential in terms of biomarker quantification and stratification.

Although there has been focus on developing new and better treatment strategies for glioma patients, their survival is still poor. In other cancer types however, the use of biomarkers [278, 279] has lead to identification of different sub-groups of patients with distinct biological behaviour and hence development of targeted therapies. Similar differentiation of glioma patients provides the opportunity of differentiated treatment; such an approach is expected to be introduced for future glioma patients.

7. SUMMARY

Gliomas are the most frequent brain tumours among adults, and it is estimated that gliomas constitute half of the about 1500 new brain tumours diagnosed in Denmark every year. Existing treatment strategies include neurosurgery, radiation, and chemotherapy. Therapy selection is based on experiences from clinical trials, with the risk that the results obtained are restricted to highly selected patients only. Moreover, these studies provided only little knowledge of the clinical behaviour of the tumours. For some time, it has been believed that somatic stem cells are re-
sponsible for self-renewal, proliferation, and differentiation during development of different (normal) tissues. The same characteristics were identified in cancer cells, and recently a major part of the glioma research has focused on the cancer stem cell (CSC) hypothesis, suggesting that only CSCs possess the ability of initiating new tumours. Moreover, CSCs have been suggested as the cause of resistance towards radiotherapy and chemotherapy. In gliomas, CSCs were originally identified by means of the expression of CD133, but other proteins have subsequently been suggested as CSC related.

To improve patients survival, further knowledge about the biological but also about the clinical presentation of gliomas and of glioma patients in an entire population was needed. Identification of patients who would benefit from standard treatment as well as identification of patients who need more aggressive treatment at the time of diagnosis is essential. Equally important is the identification of patients who will not benefit from current standard treatment. Moreover, as common exclusion criteria in clinical trials are age, performance status, and a histologically verified diagnosis, knowledge regarding clinical characteristics in the total population was highly needed.

In manuscripts 1 and 2, sampling from national registries was performed and clinical data were collected in order to indentify a clinical prognostic profile for patients with WHO grade I–II tumours (LGG) and WHO grade III–IV tumours (HGG). By using a population-based setup, we identified 433 patients who were diagnosed with a primary glioma in the period 1 January 2005 to 31 December 2009, and of these 76 patients were clinically diagnosed and 357 had a histologically verified diagnosis. We found that younger age, a non-astrocytic histology, having performance status (PS) 0–1, and the absence of neurological deficits were associated with a better prognosis in patients with LGGs. In patients with HGGs younger age, having PS 0–1, absence of neurological deficits, having a tumour that does not cross the midline, and receiving curatively intended post-surgical treatment were associated with a superior prognosis. Older patients also benefitted from curatively intended treatment, although their survival was inferior as compared to younger patients receiving similar treatment. In addition, the prognostic value of having somatic mutation affecting the protein isocitrate dehydrogenase 1 (IDH1) was evaluated. Presence of a mutated IDH1 was associated with a better prognosis in patients with WHO grade II and III tumours, whereas no prognostic potential was identified in the group of GBMs.

In manuscript 3, the independent prognostic value of the RNA-binding protein Musashi-1 was evaluated using fluorescence-based automated quantitative image acquisition. The prognostic significance was subsequently investigated in relation to the observed clinical prognostic variables. We found that Musashi-1 was not prognostic in WHO grade II tumours, but in WHO grade III high levels of Musashi-1 were associated with poor survival, although the conclusion is based on very few patients. The opposite effect was identified in a sub-group of postsurgical treated GBM patients expressing high levels of Musashi-1 and a superior prognosis. It may be speculated that Musashi-1 status has a predictive value to the effect of chemo radiotherapy in GBM patients, but the study was not designed to explore a potential predictive potential, and this should be investigated in further material.

In manuscript 4, a double staining of CD133 and nestin was performed. The use of fluorescence made it possible to identify expression of CD133 and nestin in the same cell, which has never been done before. However, neither co-localisation nor expression of CD133 or nestin was associated with survival.

In conclusion: Clinical variables associated with better survival were identified for patients with both LGGs and HGGs. All variables are already used in clinical decision making, and they can be used in prognostic counselling of the patients and to guide clinicians regarding the potential benefit from standard treatment in specific patients. Musashi-1 was a predictor of poor survival in WHO grade III tumours, but in patients with GBMs, high levels of Musashi-1 were associated with improved survival. No prognostic value was identified regarding CD133, nestin, or co-localisation of these markers in multivariate analysis adjusted for clinical variables. None of the investigated CSC markers can be used in a clinical setting at the present time. Quantitative automated image acquisition and processing was demonstrated to be a feasible, robust, and reproducible method that will be used in future projects investigating other potential prognostic factors.

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