

Diagnostic Markers for Glioblastoma

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Summary:

Glioblastoma (GBM) is the most malignant form of cerebral gliomas, and despite distinct progress in surgical resection, radiation and chemotherapy, the prognosis of patients with GBM is still very poor. In the past decades knowledge of genomics and proteomics and of diagnostic, prognostic, predictive and pharmacodynamic markers measured in cerebrospinal-fluid (CSF), serum, or tumor tissue biomarkers has improved. This review briefly compiles our concepts on diagnostic markers for GBM, focusing on the latest developments.

Introduction:

Glioblastoma epidemiology, symptoms and life expectancy

Glioblastoma (GBM) accounts for the majority of gliomas and for approximately 17% of all primary brain tumors (Kleihues et al., 1993; Kleihues et al., 2000). The incidence to suffer from a GBM ranges around 3.2/ 100.000 person-years in the United States and in Europe (CBTRUS, Central Brain Tumor Registry of the United States; ABTR, Austrian National Cancer Registry) and increases with higher age (Fritz et al., 2000; Ohgaki et al., 2004; CBTRUS, 2010). Although GBM has been reported in all age groups, it has a peak in prevalence in late adulthood between 65 to 75 years of age. Furthermore, a slight male predilection has been reported (Fritz et al., 2000; Ohgaki et al., 2004). GBM is the most malignant form of cerebral glioma. GBM is a rapid and infiltrative growing tumor, which is histologically reflected by a malignant morphology, tumor necrosis, as well as vascular proliferation. GBM can present as a high-grade lesion from onset (primary or de novo GBM - pGBM) or can evolve from a lower-grade precursor lesion, such as astrocytoma WHO grade II and anaplastic astrocytoma WHO grade III (A III) (secondary or progressive GBM - sGBM) (Kleihues and Ohgaki, 1999; Brat et al., 2002; Louis and International Agency for Research on Cancer., 2007).

Patients with GBM usually show a short medical history. Symptoms can vary from seizures, to headache, to signs of increased intracranial pressure or to focal signs such as palsy or speech disorders depending on the location of the tumor growth.

Using brain imaging, GBM generally appears as a space-occupying, contrast enhancing lesion in the supratentorial white matter. MRI findings demonstrate a heterogeneous mass, which is usually hypo-intense on T1-weighted images and hyper-intense on T2-weighted images.

Furthermore, GBM typically contains central areas of necrosis, and is surrounded by extensive, peritumoral vasogenic edema (Cha et al., 2007; Grunwald et al., 2007). The differential diagnosis of GBM includes metastasis, abscess or even immune-mediated demyelinating mass lesions (Voorhies et al., 1980). In MRI-spectroscopy destruction of brain tissue by tumor growth leads to a loss of N-acetyl-aspartate (NAA). Reactive or malignant cell proliferation cause an increase in choline (Cho) and an increase in choline-creatinine ratio (Cho:Cr). Therefore, GBM usually presents in MRI-spectroscopy with a significant decrease of NAA accompanied by an increased Cho-peak (Grunwald et al., 2007). However, grading of gliomas according to their spectroscopic appearance is limited. Although brain imaging and clinical characteristics may suggest the diagnosis of a GBM, histopathological analysis of the tumor tissue is mandatory for a definite diagnosis.

Despite recent progress in surgical resection, radiation and chemotherapy, the prognosis of GBM is still very poor, with a median survival time of 12.1 to 14.6 months after diagnosis (Stupp and Weber, 2005) and a five-year survival rate of less than 5% (CBTRUS, 2010).

Diagnostic markers

In the past decades, there have been considerable improvements in the way that brain tumors and GBM are characterized. Knowledge of genomics, proteomics and the molecular level has matured and the demand for diagnostic markers and biomarkers in GBM has increased.

Biomarkers are defined as a characteristic that is objectively measured and evaluated as an indicator of normal biologic processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention (Group, 2001). Biomarkers can, therefore, be classified as diagnostic, prognostic, predictive and pharmacodynamic markers, which can be measured in cerebrospinal-fluid (CSF), serum or tumor tissue (histopathology, proteins, RNA/DNA, receptors, antibodies) (Sawyers, 2008).

This review briefly compiles our concepts on diagnostic markers for GBM, focusing on the latest developments.

Histopathology and immunohistochemistry

Histopathological markers

According to the current WHO classification of brain tumors GBM are histologically defined as malignant astrocytomas with necrosis and/or prominent microvascular proliferation. Due to

malignant behavior of GBM it is assigned WHO grade IV (Louis and International Agency for Research on Cancer., 2007).

Various histopathological patterns serve as prognostic markers. Necrosis was identified as one of the most important prognostic factors that allow differentiation between WHO grade III and IV (Nelson et al., 1983; Burger et al., 1985; Burger and Green, 1987; Daumas-Duport et al., 1988; Kim et al., 1991; Alvord, 1992; Revesz et al., 1993). Two variants of necrosis are distinguished: (1) large areas of necrotic tumor tissue and (Barker et al.) small, often irregularly-shaped band-like foci that are surrounded by densely packed, mostly small tumor cells forming a so called 'pseudopalisading' pattern (Fig.1a) (Louis and International Agency for Research on Cancer., 2007). In case of lack of necrosis, prominent microvascular proliferation was identified to have a nearly similar prognostic value in malignant astrocytomas (Fig.1a) (Barker et al., 1996). However, in routine diagnostics microvascular proliferation can sometimes be problematic due to its incoherent definition. It is difficult to distinguish 'multilayered, mitotically active endothelial cells together with smooth muscle cells and pericytes' as found in GBM (Louis and International Agency for Research on Cancer., 2007) from incipient microvascular proliferation as observed in astrocytoma WHO grade III (A III). This might be one of the reasons why the feature 'microvascular proliferation' did not reach the same prognostic quality as 'necrosis' (Barker et al., 1996). Furthermore, different morphological GBM subvariants or 'patterns of differentiation' were found to be associated with outcome and prognosis. Giant cell GBM (gcGBM) are histologically defined by the presence of numerous multinucleated giant cells with an extremely bizarre appearance in a matrix of smaller cells (Louis and International Agency for Research on Cancer., 2007). Different reports indicated that patients with gcGBM have a slightly better prognosis than patients with ordinary GBM (Burger and Vollmer, 1980; Margetts and Kalyan-Raman, 1989; Shinojima et al., 2004). In addition, in the current WHO classification of brain tumors the diagnosis 'anaplastic oligoastrocytoma WHO grade III with necrosis' was removed and replaced by the diagnosis 'glioblastoma with oligodendroglioma component WHO grade IV' (GBMo) (Louis and International Agency for Research on Cancer., 2007), due to the observation that patients with this tumor entity have a worse prognosis than patients with anaplastic oligoastrocytoma WHO grade III without necrosis (Miller et al., 2006; van den Bent et al., 2006). Further studies imply that patients with a GBMo have a slightly better prognosis than patients with ordinary GBM (He et al., 2001; Kraus et al., 2001; Homma et al., 2006). The prognostic value of 'small cell GBM' (scGBM) has not been resolved. This tumor variant can be misdiagnosed as anaplastic

oligodendroglioma WHO grade III and is characterized by an extraordinary high proliferation rate and frequent *EGFR* amplification (Burger et al., 2001; Perry et al., 2004). An early clinicopathological study demonstrated that patients suffering from scGBM have a worse prognosis than those with “ordinary” GBM (Burger and Vollmer, 1980). However, a follow up study by the same author failed to validate the previous observation (Burger and Green, 1987).

Immunohistochemical markers

The availability of poly- or even monoclonal antibodies that selectively bind to epitopes on formalin-fixed paraffin embedded tissue has dramatically improved routine neuropathology. However, a high inter-laboratory and inter-observer variability seriously hampers comparison of results from different studies. Due to this and various other reasons, particularly with regard to frequent inhomogeneous protein expression, the authors of the current WHO classification have tried to avoid defining tumor entity solely by immunohistochemistry (IHC) (Louis and International Agency for Research on Cancer., 2007). On the other hand, IHC is an attractive tool that may allow conclusions with regard to the origin of the tumor tissue and to prognosis by means of protein level / expression without the need for a sophisticated genetic laboratory to test for genetic markers.

One of the most attractive targets for IHC is MGMT protein (for further details regarding the gene *MGMT* see below). Indeed, various studies identified an association between MGMT expression determined by IHC and response to alkylating drugs (Belanich et al., 1996; Jaeckle et al., 1998; Levin et al., 2006; Chinot et al., 2007; Capper et al., 2008). However, various reasons reduce the value of MGMT IHC. Non-neoplastic cells such as reactive astrocytes, microglia, lymphocytes and blood vessel cells within the tumor express MGMT, in that way complicating the evaluation (Fig.1d/e) (Felsberg et al., 2009). In addition, a high inter-laboratory and inter-observer variability resulted in published threshold levels ranging from <10% up to >50% positive cells, to distinguish positive MGMT- GBM from negative MGMT-GBM (Preusser et al., 2008). Furthermore, many studies failed to observe an association between MGMT expression based on IHC and promoter methylation status (Grasbon-Frodl et al., 2007; Lavon et al., 2007; Preusser et al., 2008; Rodriguez et al., 2008). Therefore, evaluation of the MGMT status by IHC appears not to be the appropriate method. However, IHC assays that selectively bind epitopes that only arise by genetic mutations are not so limited. One example is the generation of an antibody selectively binding epidermal growth factor receptor variant III (EGFRvIII or $\Delta 2-7$ EGFR) that results from an in-frame

deletion of exons 2-7 in the *EGFR* gene, as described by various groups (Humphrey et al., 1990; Hills et al., 1995; Wikstrand et al., 1995; Okamoto et al., 1996; Jungbluth et al., 2003). Unfortunately, this antibody which appears to be a promising tool for attaching cytotoxic or radiolabeled adjuncts never became commercially available because the rights to the antibody are patented. Due to this and a certain cross reactivity with wild-type EGFR, an EGFRvIII-specific recombinant antibody has recently been developed, which may overcome the previously noted limitations (Gupta et al., 2010). However, the clear lack of a prognostic or predictive role of an EGFRvIII-specific antibody reduces the value in routine diagnostics (see below).

Recently, the detection of *IDH1* mutations and their prognostic role brought up a new marker in neurooncology (see below). The distribution of *IDH1* mutations with nearly 93% of the R132H variant (Hartmann et al., 2009) renders this mutated protein variant an ideal target and consequent efforts finally led to the generation of monoclonal antibodies (Fig. 1c) (Capper et al., 2009; Kato et al., 2009; Capper et al., 2010). In a large clinicopathological study of patients with A III and GBM it was confirmed that the usage of such an antibody yielded similar results to genetic sequence analysis (Hartmann et al., 2010) (for further details see below). Meanwhile, an antibody was generated which also selectively detects the very rare IDH1 R132S variant (Kaneko et al., 2011).

CSF-marker

Cerebrospinal fluid surrounds the brain and the spinal cord and circulates within the ventricles. Small molecules, salts, peptides and proteins form the physiological compounds of CSF (Yuan and Desiderio, 2005) e.g. protein concentration in normal CSF ranges between 0.2-0.8mg/mL (Zheng et al., 2003). Changes in CSF composition can indicate pathophysiological conditions such as infection, neurodegenerative diseases or tumor growth (Khwaja et al., 2006; Zougman et al., 2008). Because of the proximity of CSF to the brain and to brain-tumors, GBM-related proteins might reach the CSF via direct secretion into the CSF, diffusion or a disruption of the blood-brain-CSF-barrier (Papadopoulos et al., 2001; Schneider et al., 2004). However, currently there is no diagnostic, prognostic or predictive CSF marker in clinical use for GBM.

A limiting factor for CSF markers could be the way to access and withdraw CSF in GBM patients. Possible approaches are collection during surgery, which is unsuitable for repetitive examinations, as well as via an external drainage or lumbar puncture, all being quite invasive. Furthermore, the majority of GBM patients are diagnosed with a space occupying lesion

which can contraindicate lumbar puncture. Therefore, the need for repeated CSF examinations for prognostic or continuous monitoring disease marker may limit the widespread use of CSF GBM markers.

IHC and in situ hybridization studies showed high concentrations and up-regulation of VEGF mRNA activity of angiogenic factors such as e.g. basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF) in GBM (Brem et al., 1992; Plate and Risau, 1995). VEGF and bFGF levels of tumor extracts showed not only a significant correlation with microvessel density, which was higher in GBM than in other glial tumors (Takano et al., 1996), but also correlated with survival of patients with astrocytic tumors (Fukui et al., 2003) and indicated glioma grades (Takahashi et al., 1992). Angiogenic factors were also analyzed in CSF in relatively small patient groups of 15 and 27 patients (Peles et al., 2004). VEGF and bFGF levels in CSF correlated with the degree of tumor vascularity and were adversely associated with patient survival (Peles et al., 2004; Sampath et al., 2004). They were significantly higher in GBM than in non-astrocytic tumors and were not detectable in normal, a pathological CSF samples (Peles et al., 2004; Sampath et al., 2004). Therefore, determination of bFGF and VEGF in CSF may serve as a marker for vascularity in GBM, may predict survival and could prove useful for future antiangiogenic treatment methods. However, caution must be exercised with interpreting elevated bFGF and VEGF levels, as non-tumoral conditions such as bacterial meningitis can also lead to elevated CSF levels (van der Flier et al., 2001) and studies with large numbers of cases, as well as follow-up studies, are lacking. A number of other markers have been found in the CSF of GBM patients, including MIC-1/GDF15 (growth differentiation factor 15), deoxythymidine-kinase, NSE (neuron-specific enolase) and tenascin (Gronowitz et al., 1984; Cochran and Wen, 1985; Taomoto et al., 1987; Yoshida et al., 1993; Shnaper et al., 2009). MIC-1/GDF15 (growth differentiation factor 15) is a secreted protein of the TGF-beta superfamily and exhibits increasing mRNA expression during malignant progression of glioma (Godard et al., 2003). In a study including 94 patients with intracranial glioma, meningioma and metastasis, MIC-1/GDF15 showed significantly increased CSF concentrations but no elevated serum levels in GBM patients compared with a non-neoplastic control group. Since only 5 brain metastasis patients with a trend toward elevated CSF MIC-1/GDF15 levels were evaluated by Shnaper et al, a larger cohort should be tested to validate MIC-1/GDF15 as a diagnostic marker. However, elevated and high CSF MIC-1/GDF15 levels were correlated with a decreased overall survival, suggesting MIC-1/GDF15 in CSF as a prognostic GBM marker (Shnaper et al., 2009).

In addition to soluble CSF-markers, Huttner et al. found membrane particles containing the neural stem cell marker prominin-1/CD133 in human CSF (Huttner et al., 2008). CD133 antigen has been identified as a putative stem cell marker and is expressed in GBM cells (Bao et al., 2006). Furthermore, Zeppernick et al. reported in a series of 95 glioma patients that after multivariate survival analysis, CD133-positive stained cells and their topological organization in clusters were significant prognostic factors for adverse progression-free survival and overall survival independent of tumor grade, extent of resection or patient age. The proportion of CD133-positive cells was also an independent risk factor for tumor regrowth and time to malignant progression in WHO grade II and III tumors (Zeppernick et al., 2008). In CSF levels of membrane particle-associated prominin-1/CD133 declined during childhood and remained constant in adulthood, with a narrow range of 7.4 ± 3.8 ng in healthy adults. In contrast, GBM patients with a disease duration of <30 months showed elevated levels of prominin-1/CD133, which decreased in the final stage of the disease (>30 months). Although these observations were obtained from a pilot investigation with a rather heterogeneous GBM group, demanding a longitudinal examination to detect possible relationships between prominin-1/CD133 particle in CSF and clinical, neuroradiological or histopathological parameters, analysis of CSF for membrane particles carrying the somatic stem cell marker prominin-1/CD133 may offer a novel approach for studying GBM disease (Huttner et al., 2008).

During the last years, further screening studies for GBM marker detection were performed using proteomic and peptidomic techniques. Ohnishi et al. performed a CSF proteomic screening study with 2D gel electrophoresis mass spectroscopy and final confirmation by IHC, recognizing Gelsolin, Rho GDP-dissociation inhibitor alpha, anti-thrombin III variant, α -1B-glycoprotein and IGHG1 as differing proteins in CSF of 2 GBM and 2 astrocytoma grade II patients, reported to be related to tumors (Ohnishi et al., 2009). Gelsolin, an actin-binding protein has been reported to affect cellular configuration, differentiation, adhesiveness, invasiveness, and apoptosis (Winston et al., 2001). In GBM patients Gelsolin expression in CSF and IHC was significantly lower (2.01 fold) than in grade II astrocytomas and therefore proposed as a prognostic marker in astrocytoma. However, a significant correlation between survival rate and low (<30%) or high (>30%) Gelsolin expression could not be detected in the examined cohort, as two long-term survivors were found among the low Gelosin expression group (Ohnishi et al., 2009). Schuhmann et al. screened CSF with a mass spectroscopy based peptidomics technology for possible peptide markers of GBM (Schuhmann et al., 2010). Four CSF peptides, which are constituents of

normal CSF (C-terminal fragments of osteopontin, alpha-1-antichymotrypsin, transthyretin and N-terminal residue of albumin) were significantly elevated and distinguished CSF of control patients from CSF of GBM patients (Schuhmann et al., 2010). Osteopontin, a phosphorylated non-collagenous matrix protein is found in all body fluids and is over-expressed by the majority of human cancer, as well as human malignant glioma cells (Rittling and Denhardt, 1999; Coppola et al., 2004). Alpha-1-antichymotrypsin is expressed in the brain by astrocytes and is part of the acute phase response of inflammation, malignancy and trauma. Furthermore, alpha-1-antichymotrypsin IHC staining of high-grade gliomas, as well as of other brain tumors, was reported (Abraham et al., 1990). Transthyretin is synthesized in high quantity in the choroid plexus, but was not found within the brain parenchyma or GBM (Herbert et al., 1986; Lignelid et al., 1997). Therefore, Schuhmann et al. suspected an elevated protease activity at constant transthyretin levels, causing the observed increase in transthyretin fragments (Schuhmann et al., 2010). However, it remains unclear if any of these peptides mentioned above, which were identified by modern screening methods, might have the capacity to act as a potential GBM marker. Further trials are needed to validate their usefulness as a marker for differential diagnosis or as a prognostic marker in monitoring the response to surgery, radiotherapy or chemotherapy.

Serum marker

Serum is easier to collect than CSF, therefore, a GBM serum marker might be easier to quantify, reproduce and use in clinical practice.

A few angiogenesis-related growth factors, such as vascular endothelial growth factor and the glycoprotein YKL-40, have been proposed as glial tumor markers in serum or cerebrospinal fluid (Tanwar et al., 2002; Peles et al., 2004; Nutt et al., 2005). In contrast to measurements of bFGF and VEGF in CSF, bFGF and VEGF analysis in serum do not serve as a marker for tumor grading, vascularity or predictor of survival (Peles et al., 2004). This difference in efficacy of angiogenic factors in CSF and serum might be explained by efficient function of the blood-brain-barrier. Furthermore, lack of specificity has unfortunately invalidated the clinical application of these putative markers until now.

In the serum of GBM patients a few potential markers have been detected, including cathepsin D, low-molecular weight caldesmon (l-CaD), recoverin and GFAP (Huber, 1997).

Cathepsin D is an aspartyl protease involved in protein catabolism and tissue remodelling which can be secreted from cancer cells. Fukuda et al reported that Cathepsin D tissue gene expression levels and Cathepsin D serum levels were significantly higher in GBM patients

than in low-grade astrocytoma patients. Multivariate analysis further confirmed that the cathepsin D expression level was an independent predictor for short survival, suggesting that cathepsin D could be used as a potential serum marker for the aggressive nature of GBM and as a predictor of short survival (Fukuda et al., 2005).

Caldesmon (1-CaD) is a calmodulin binding protein and cytoskeleton-associated protein which is crucially involved in the assembly and stabilization of the microfilament network in non-muscle cells. Furthermore, 1-CaD is an important regulator of cell motility and other various cell functions (Huber, 1997). Zheng et al (2004) discovered that the differential expression of 1-*CaD* in blood vessels of gliomas and normal brain tissue was mainly a sequel of abnormal splicing of the caldesmon gene (*CALDI*) in glioma vasculature, which functionally resulted in up-regulation of 1-CaD, and that this overexpression of 1-CaD in glioma vasculature was connected to the activation of endothelial cell motility, which is an essential step in neovascularization-dependent glioma progression (Zheng et al., 2004). Subsequently, they evaluated 1-CaD as a GBM serum marker (Zheng et al., 2005): No serum 1-CaD level was observed in 7% of the glioma patients, in 59% of patients with other intracranial tumors and in 53% of healthy controls. However, 1-CaD was also detectable in 41% of serum samples of patients with non-glioma intracranial tumors, leading in this study to a calculated cutoff score of 45 for 1-CaD, by which 1-CaD reached a high sensitivity of 91% and a specificity of 82%. One reason for this moderate specificity might be the source of serum 1-CaD. 1-CaD is produced by the tumor vasculature. As GBM as well as non-glioma tumors, such as carcinomas, can be strongly vascularized, they can both express high levels of 1-CaD.

Recoverin is an intracellular signal transduction protein, whose physiologic role in gliomas remains unknown. Recoverin was primarily detected in serum of patients suffering from paraneoplastic syndrome. Sampath et al evaluated recoverin levels in glioma patients and discovered a 10-fold increase in patients with recurrent GBM compared to healthy controls. In patients with stable GBM disease, serum levels were similar to those of low-grade and anaplastic glioma (Sampath et al., 2004). Serum 1-CaD and recoverin levels showed no significant difference between patients with low-grade and patients with high-grade gliomas (Sampath et al., 2004; Zheng et al., 2005). Therefore, both markers seem not suitable as a differential GBM marker. However, the preliminary recoverin data is intriguing, as recoverin levels were particularly elevated in patients with recurrent GBM, which suggests the value of recoverin as prognostic follow-up GBM marker.

Glial fibrillary acidic protein (GFAP) is a member of the cytoskeletal protein family and is widely expressed in astroglial cells, in neural stem cells and in astroglial tumors, such as astrocytoma and GBM (Jacque et al., 1978; Hamaya et al., 1985; Abaza et al., 1998). The majority of astrocytic tumors express GFAP. However, GBM tissues show a strong variability in GFAP expression ranging from < 25% to 100 % in others (Fig. 1b) (Royds et al., 1986). GFAP has a relatively high molecular weight of 52 kDa (Yen et al., 1976), which limits its transit through the blood brain barrier (BBB) under physiological conditions. However, under conditions such as acute head trauma, intracerebral hemorrhage or brain infarction, in which the BBB is disrupted, elevated serum GFAP concentrations were detected (Herrmann and Ehrenreich, 2003; Pelinka et al., 2004; Foerch et al., 2006). Furthermore, GFAP is detectable in the serum of patients with GBM. In a study comparing GFAP serum levels of 50 GBM patients with those of 31 astrocytoma grade II and III, 17 single brain metastases and 50 healthy controls, serum GFAP levels of GBM patients were significantly higher than those of patients with astrocytoma or with brain metastasis. Furthermore, serum GFAP levels were correlated with the GBM volume and tumor necrosis volume (Jung et al., 2007). As tumor necrosis is absent in low-grade glioma and present in GBM, this might explain elevated GFAP serum levels in patients with voluminous GBM. Interestingly, the product of GFAP expression in tissue samples and tumor necrosis, as a measure for necrotic GFAP positive cells in GBM patients, was strongly correlated with GFAP serum levels, emphasizing the direct and/or indirect influence of these two factors on GFAP detectability in serum. In this study, a ROC analysis cut-off point of 0.05 µg/l of serum GFAP afforded a sensitivity of 76% and a specificity of 100% for the differentiation of GBM patients from non-GBM tumor patients or healthy controls. The positive and negative predictive values were 1.0 and 0.89, respectively (Jung et al., 2007). Serum GFAP therefore appears to be a promising (preoperative) diagnostic biomarker. Extending the scope to monitor clinical follow-up with serum GFAP is a different matter, since it is known that elevated GFAP levels occur after head trauma, intracerebral hemorrhage, ischemic stroke as well as in reactive gliosis, (Herrmann and Ehrenreich, 2003; Pelinka et al., 2004; Foerch et al., 2006) as expected after cranial surgery. Furthermore, recurrent GBM should be detected while still small. This is difficult with a marker which is correlated with tumor volume. This is in accordance with an extracted set of peri- and post-operative (unpublished, CJ) data (Table 1). Recent observations of *IDH1* and *IDH2* mutations in gliomas and the excessive production of 2-hydroxyglutarate (2HG) in tumor cells that carry such alteration (see below) prompted the examination of 2HG in serum. In acute myeloid leukemia (AML), the other tumor entity that

frequently exhibits *IDH1* and *IDH2* mutations, a correlation between this alteration and elevated 2HG levels in serum has been demonstrated (Gross et al., 2010; Sellner et al., 2010). Currently, no published data are available that show a similar association between *IDH1* and *IDH2* mutations and elevated 2HG levels in serum of GBM patients. However, our own preliminary results do not indicate that 2HG can serve as a GBM marker (unpublished data, CH). This might be due to only marginal excretion of 2HG from the tumor or to serum detoxication by the liver or kidney.

Molecular and genetic marker

A large number of genetic alterations have been described in GBM and several of them were found to be associated with shorter or longer survival. However, most of them are still lacking confirmation by an independent study or the independent study has already demonstrated that the suggested prognostic or predictive value of this particular marker cannot be validated. Currently, only two markers, *MGMT* promoter methylation and *IDH1* mutations, are commonly accepted genetic biomarkers for patients with GBM.

MGMT

O⁶-methylguanine-DNA methyltransferase (MGMT) is a DNA repair protein that catalyzes the transfer of a methyl group from the O⁶-position of guanine to a cysteine at position 145. The alkylation of MGMT is a one way process that ends up with a degradation of MGMT (Olsson and Lindahl, 1980; Pegg et al., 1983; Gerson, 2004). Application of alkylating drugs like temozolomide (TMZ) causes, among other things, the binding of an alkyl group to the O⁶-position of guanine, thereby, impairing DNA replication and triggering cell death. MGMT protein lessens the chemotherapeutical effect by repairing the desired DNA damage. The *MGMT* gene on 10q26 has five exons and a large CpG island of 763 bp with 98 CpG sites covering the first exon and large parts of the promoter. In normal brain the CpG sites are typically unmethylated. However, in tumors the cytosine in CpG sites often carry methyl groups, thereby increasing the affinity of Methyl-CpG-binding proteins like methyl-CpG-binding protein 2 and methyl-CpG-binding domain protein 2 to the DNA. These proteins alter the chromatin structure and prevent binding of transcription factors, thereby silencing expression of MGMT (Nakagawachi et al., 2003). A certain number of patients with a hypermethylated *MGMT* promoter in GBM cells lack the corresponding DNA repair protein MGMT and, therefore, the cytotoxic effect of alkylating drugs becomes amplified. Thus, *MGMT* hypermethylation is a predictor for response to chemotherapy (Hegi et al., 2005). This

widely accepted concept to understand the beneficial role of a hypermethylated *MGMT* promoter recently became challenged by the observation in patients with anaplastic gliomas which showed that this alteration is associated with a better clinical course even if patients became treated by radiotherapy alone (van den Bent et al., 2009a; Wick et al., 2009).

Since the first description of *MGMT* hypermethylation in GBM (Esteller et al., 2000) various studies confirmed *MGMT* hypermethylation in GBM and reported frequencies between 35% and 73% (Hegi et al., 2004; Hegi et al., 2005; Herrlinger et al., 2006; Criniere et al., 2007; Wick et al., 2008; Brandes et al., 2009a; Brandes et al., 2009b; Clarke et al., 2009; Dunn et al., 2009; Prados et al., 2009; van den Bent et al., 2009b; Weller et al., 2009; Zawlik et al., 2009; Weiler et al., 2010). This substantial variation of reported frequencies is presumably not only a consequence of different tumor sampling but also due to technical reasons (von Deimling et al., 2011).

The predominant interest in *MGMT* promoter hypermethylation is based on the predictive role of this biomarker for TMZ chemotherapy: GBM patients with hypermethylated *MGMT* promoter exhibited survival rates of 49% after 2 years and 14% after 5 years when treated with concomitant and adjuvant TMZ and radiotherapy. However, only 24% of GBM patients with hypermethylated *MGMT* promoter survived after 2 years, and 5% after 5 years when initially treated with radiotherapy only. GBM patients without hypermethylated *MGMT* promoter demonstrated survival rates of 15% and 8% after 2 and 5 years while receiving radiochemotherapy, as well as 2% and 0% after 2 and 5 years when treated with radiotherapy alone (Hegi et al., 2005; Stupp et al., 2005; Stupp et al., 2009). In the following years multiple studies confirmed that *MGMT* promoter hypermethylation is one of the strongest prognostic factors for patients with newly diagnosed GBM and that this alteration is a predictor for response to treatment with alkylating drugs (Esteller et al., 2000; Hegi et al., 2004; Herrlinger et al., 2006; Gorlia et al., 2008; Brandes et al., 2009b; Weller et al., 2009).

Due to this important prognostic role detection of *MGMT* promoter hypermethylation is nowadays more or less essentially required for every trial that is evaluating a new therapeutic treatment. Although in daily routine practice the determination of the *MGMT* promoter status might be helpful for counseling of patients suffering from GBM by neurooncologists, the lack of different therapeutical options for patients without hypermethylated *MGMT* promoter results in a similar therapy for both patient groups and, therefore, determination of the *MGMT* status has nowadays no direct clinical implications or therapeutical influence.

IDH1 and IDH2

By sequencing of more than 20,000 genes, mutations in the gene encoding the cytosolic NADP⁺ dependent isocitrate dehydrogenase 1 (*IDH1*) were identified in 12% of the analyzed GBM. Most of the tumors carrying *IDH1* mutations were sGBM. All mutations were identified on only one allele and all mutations affected exclusively codon 132 (Parsons et al., 2008). Subsequent studies identified *IDH1* mutations in 60 to 80% of diffusely infiltrating astrocytomas and oligodendrogliomas of the WHO grade II and III and in sGBM. In contrast, *IDH1* mutations were identified only in approximately 5 to 10% of pGBM (Balss et al., 2008; Yan et al., 2009). Nearly 93% of *IDH1* mutations are of the R132H variant, while the other mutational variants that affect codon 132 occur in frequencies below 5% (Hartmann et al., 2009). Glioma without *IDH1* mutations revealed in a few cases mutations in codon 172 of the gene encoding mitochondrial NADP⁺ dependent isocitrate dehydrogenase (*IDH2*). Codon 172 in *IDH2* is homolog to codon 132 in *IDH1*, both encoding the active site of the protein (Yan et al., 2009). Due to the observation that there is no difference in the frequencies of *IDH1* mutation between diffuse glioma of various WHO grades it is very likely that this alteration is a tumor inducing early event (Balss et al., 2008). *IDH1* mutations occur nearly exclusively in diffusely infiltrating glioma and in acute myeloid leukemia, but not in other tumor entities (Balss et al., 2008; Bleeker et al., 2009; Kang et al., 2009; Mardis et al., 2009). In GBM *IDH1* mutations are inversely associated with *EGFR* amplification, a genetic marker which is typically altered in pGBM (Sanson et al., 2009).

The functional consequences of *IDH1* and *IDH2* mutations remain in many aspects unresolved. However, recent publications allow first conclusions which imply that metabolic alterations play an important role in induction and progression of those gliomas that carry *IDH1* or *IDH2* mutations. The wild-type isocitrate dehydrogenases catalyze by reducing NADP to NADPH isocitrate to α -ketoglutarate (α KG) (Geisbrecht and Gould, 1999). Initial studies indicated a loss of function mechanism driven by mutations in *IDH1*, with reduced catalytic activity and lower concentration of α KG, as demonstrated in cell culture assays (Ichimura et al., 2009; Yan et al., 2009). However, a more appropriate alternative concept demonstrating a gain of function mechanism has emerged to explain the function of *IDH1* mutations in tumor cells. Codon 132 mutations in *IDH1* and codon 172 mutations in *IDH2* affect the catalytic pocket of the enzymes. The mutated enzyme reduces by consuming NADPH α KG to 2-hydroxyglutarate (2HG) which is found in excessive concentrations in tumors cells (Dang et al., 2009). This gain of function concept permits a sufficient explanation for the heterozygous character of *IDH1* or *IDH2* mutations: if 2HG is an important onco-metabolite for tumor cells, then α KG generated by wild-type *IDH1* is essentially required for

this catalytic cascade. Indeed, it was demonstrated that mutated IDH1 protein does not affect the catalytic activity of wild-type IDH1 (Jin et al., 2011). Subsequently, it was shown that 2HG in excessive concentrations is a competitive inhibitor of various α KG-dependent dioxygenases like histone demethylases and the TET family of 5-methylcytosine hydroxylases, thereby epigenetically affecting tumor cells (Xu et al., 2011). Excessive 2HG levels have various other metabolic consequences for tumor cells. By profiling more than 200 metabolites it was found that concentrations of different amino acids, glutathione metabolites, choline derivatives and tricarboxylic acid cycle intermediates in part dramatically varied in cells expressing mutant IDH1 or IDH2 compared to cells which only expressed wild-type protein (Reitman et al., 2011).

Meanwhile it became obvious that *IDH1* is one of the most important prognostic markers for diffusely infiltrating gliomas. The number of patients with pGBM that carry *IDH1* or *IDH2* mutation is rather low (see above). However, those few patients exhibit a much better clinical course than patients with pGBM without mutations. The fact that patients with *IDH1* or *IDH2* mutations are younger than those with wild-type status may partially explain the prognostic effect of this marker (Parsons et al., 2008; Nobusawa et al., 2009; Sanson et al., 2009; Weller et al., 2009; Yan et al., 2009; Hartmann et al., 2010). In anaplastic gliomas *IDH1* mutations were identified as a prognostic marker that even outperforms established markers like *MGMT* promoter methylation or combined 1p/19q losses (Wick et al., 2009).

The low frequency of approximately 5 to 10% of *IDH1* or *IDH2* mutations in patients with pGBM in comparison to patients suffering from sGBM or diffusely infiltrating gliomas of the WHO grade II and III raised the question of whether pGBM can be defined by the absence of those mutations. In contrast to this hypothesis, pGBM with *IDH1* mutations may have progressed rapidly from a less malignant precursor lesion that escaped clinical diagnosis and were thus misclassified as de-novo GBM (Balss et al., 2008; Nobusawa et al., 2009). By comparing a large series of patients with A III and pGBM with and without *IDH1* or *IDH2* mutations it was demonstrated that the overall survival was longer for patients with pGBM and *IDH1* mutation than for patients with A III with *IDH1* wild-type status. The Kaplan-Meier plots for progression free survival basically showed only two lines: patients with pGBM and A III with *IDH1* mutations and patients with pGBM and A III with *IDH1* wild-type status. The *IDH1* status was found to be a better prognostic marker than the established histological criteria, like necrosis or microvascular proliferation, for grading of malignant astrocytomas (Hartmann et al., 2010). These findings may have different consequences: 1) Currently, pGBM are interpreted as a variant of diffusely infiltrating gliomas essentially

related to grade II and III astrocytomas and oligodendrogliomas and sGBM (Louis and International Agency for Research on Cancer., 2007). The *IDH1* findings now show that pGBM is a rather different tumor with morphological similarities but distinctive clinical course and pathogenetic background. 2) It needs to be proven in upcoming clinical trials that patients with A III and *IDH1* wild-type status may benefit from a more aggressive first-line combined radiochemotherapy treatment corresponding to the current standard of care for patients with GBM. In turn, those few patients with pGBM and *IDH1* mutation may receive either radio- or chemotherapy but not both treatments combined to avoid treatment-associated side effects. 3) These findings may influence the next WHO classification of brain tumors. It will be an issue of debate to either add the parameter ‘absence of *IDH1* mutation’ to the classical histological parameters ‘necrosis’ and ‘microvascular proliferation’ for grading of malignant astrocytomas or to reduce the grading criteria only on *IDH1* status alone. The fact that the most frequent *IDH1* mutational variant R132H can be easily and reliably detected by immunohistochemistry alone (Capper et al., 2009; Capper et al., 2010) should allow such modification of the upcoming WHO classification because no sophisticated molecular analysis is required.

Combined losses of 1p and 19q in GBM

Combined loss of the whole chromosomal arms 1p and 19q is associated with oligodendroglioma morphology and is an established prognostic marker for diffusely infiltrating gliomas WHO grade II and III (Hartmann and von Deimling, 2009). The combined losses are mediated by a t(1;19)(q10,p10) translocation (Griffin et al., 2006; Jenkins et al., 2006). In the current WHO classification of brain tumors the anaplastic oligoastrocytomas WHO grade III with necrosis was removed and renamed to a new subvariant termed ‘glioblastoma with oligodendroglioma component’ (GBMo) (Louis and International Agency for Research on Cancer., 2007). Up to 9% of patients with GBM exhibit combined losses of 1p/19q (Houillier *et al.* 2006), but around one third of GBMo showed combined 1p/19q losses by using loss of heterozygosity (LOH) markers (He et al., 2001). However, three studies imply that combined loss of 1p/19q is prognostically not relevant for patients with GBM (Idbaih et al., 2005; Houillier et al., 2006; Krex et al., 2007). This discrepancy might be due to technical reasons: malignant astrocytomas exhibit in a small fraction telomeric deletions of 1p36 (Ichimura et al., 2007) and interstitial deletions of 19q13.3 (Hartmann et al., 2002). Telomeric 1p losses are even associated with a worse prognosis (Idbaih et al., 2005). However, assays like LOH markers that focus on these chromosomal regions do not allow

differentiation between complete and partial losses (He et al., 2001; Houillier et al., 2006). In summary, it remains unclear if combined losses of the whole chromosomal arms 1p and 19q might be prognostically relevant. All currently published studies focusing on this issue do not sufficiently differentiate between complete and partial losses.

Predictive marker for drug response

The availability of small molecule drugs and antibodies specifically targeting tumor-relevant epitopes may influence treatment of GBM in the near future. The detection of genetic alterations or overexpression/loss of expression of the drug targets may predict response to such new substances. However, a validated proof of principle is still missing for GBM apart from *MGMT* testing. For example, first promising results were demonstrated in a study with GBM patients treated with the EGFR kinase inhibitors erlotinib or gefitinib: responsiveness to those kinase inhibitors was strongly associated with coexpression of EGFRvIII, a constitutively active mutant variant of EGFR, and PTEN (Mellinghoff et al., 2005). These results imply that successful treatment was only feasible if 1) the drug-targeted protein is mutated (EGFRvIII expression) and 2) the downstream signaling pathway is intact (PTEN expression). In turn this denotes that the oncogenetic process in GBM is driven by several signaling pathways that are differentially activated or silenced with both parallel and converging complex interactions, and that targeted therapy will be successful only if analysis of these pathways shows a positive read-out (Omuro et al., 2007). However, despite the promising results of the initial report, following studies unfortunately failed to validate the predictive value of these markers in the context of EGFR kinase inhibitor therapy (Brown et al., 2008; Prados et al., 2009; van den Bent et al., 2009a).

Conclusions:

Although significant progress has been made in the development and evaluation of diagnostic, prognostic and predictive markers for GBM, no established clinical CSF or serum marker exists. However, GFAP in serum seems to be a promising preoperative diagnostic marker. In CSF, MIC-1/GDF15 and prominin-1/CD133 particle might be worth evaluating in trials to further validate their potential as prognostic GBM markers. Distinct improvements have been made concerning genetic GBM marker. *MGMT* promoter hypermethylation has been established as an important prognostic marker and has strong influence nowadays in every trial evaluating new therapeutic treatments. Furthermore, *IDH1* became one of the most important prognostic markers for diffusely infiltrating gliomas. Those patients who exhibit

IDH1 mutations have a better clinical course than patients with pGBM without mutations. The most frequent *IDH1* mutational variant R132H can be easily and reliably detected by immunohistochemistry, which might facilitate the diagnostic procedure and may influence the upcoming WHO classification.

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Tables & Figures**Table 1**

GFAP [$\mu\text{g/l}$]	GBM 1	GBM 2	non GBM 1	non GBM 2	Recurr GBM	Recurr GBM
pre-op	0.02	0.11	0	0	0	0
1d post op	0.49	1.15	0	0.5		
3-7d post op	0.13	0	1.07	0.16		

Follow-up peri-operative GFAP serum levels in patients with and without GBM, but with an intracranial lesion (non GBM) as well as in 2 patients with recurrent GBM. GBM: glioblastoma; Recurr: recurrent.

Figure 1

Histological appearance of a glioblastoma from a patient without known low grade precursor lesion; a: H&E staining, star: small band-like necrotic areas, arrow: multilayered proliferating microvessels, arrow head: densely packed, mostly small tumor cells forming a so called 'pseudopalisading' pattern; b: GFAP immunohistochemistry – nearly all tumor cells show loss of GFAP expression. Just around the vessels single GFAP positive cells remain; c: immunohistochemistry using an IDH1 R132H specific antibody demonstrating ubiquitous expression of this mutated protein variant; d: MGMT immunohistochemistry of a glioblastoma, arrow: strongly positive nuclei of tumor cells, arrow head: positive nuclei of endothelial cells serving as internal control; e: MGMT immunohistochemistry of a different glioblastoma, star: positive infiltrating lymphocytes, arrow: negative nuclei of tumor cells.

