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Review Article

What is the clinical value of cancer stem cell markers in gliomas?

Rikke Hedegaard Dahlrot[1,2,3], Simon Kjær Hermansen[2,3], Steinbjørn Hansen[1,3], Bjarne Winther Kristensen[2,3]

[1]Department of Oncology, Odense University Hospital, Odense, Denmark; [2]Department of Pathology, Odense University Hospital, Odense, Denmark; [3]Institute of Clinical Research, University of Southern Denmark, Odense, Denmark

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Abstract: Recent data indicate that cancer stem cells (CSCs) are responsible for resistance of glioblastomas to radiotherapy and chemotherapy, thereby contributing to the poor survival of these patients. In order to identify novel prognostic markers in gliomas, several CSC markers have been investigated. This review summarizes current reports on putative glioma CSC markers and reviews the prognostic value of the individual immunohistochemical markers reported in the literature. Using the Pubmed database, twenty-seven CSC studies looking at membrane markers (CD133, podoplanin, CD15, and A2B5), filament markers (nestin), RNA-binding proteins (Musashi-1) and transcription factors (BMI1, SOX2, Id1 and Oct-4) qualified for this review. The level of CD133 and nestin increased with increasing malignancy grade, and for both markers a prognostic significance was identified in the majority of the studies. Moreover, the co-expression of CD133 and nestin was shown to have an even more powerful prognostic value than just single markers. Regarding podoplanin and Musashi-1, there was a trend towards a prognostic value when summarizing all studies. Especially the co-expression of Musashi-1 and MIB1 seemed promising. For the remaining markers CD15, A2B5, BMI1, SOX2, Id1 and Oct4, no prognostic value was found regarding overall survival in this review. In conclusion we find that CD133, nestin, CD133/nestin, podoplanin, Musashi-1 and Musashi-1/MIB1 are the most promising markers for future investigation. Evaluation in larger cohorts with known clinical data and known status of important biomarkers like MGMT and IDH1 is necessary to reveal their full clinical potential.

Keywords: Glioma, prognosis, cancer stem cell, immunohistochemistry, CD133, nestin

Introduction

The search for new prognostic and predictive biomarkers in gliomas is an area of considerable interest because patients respond differently to treatment and have different prognoses [1]. Recent research suggests that the tumor biology and the resistance to treatment are closely connected to the existence of cancer stem cells (CSCs) [2, 3]. The importance of CSCs for estimating the prognosis of glioma patients has therefore been widely investigated using several markers closely related to the presence of these cells [4-27].

The present CSC hypothesis suggests the existence of a population of tumor cells, the cancer stem cells, having unique self-renewal capabilities thereby sustaining tumor growth, in contrast to the other tumor cells [28, 29]. Moreover, in several studies CSCs have been shown to have tumorigenic potential in addition to enhanced resistance mechanisms [30-32]. Supporting the hypothesis, CSCs have been identified in different cancer types [28, 33-35] including glioblastomas [30, 36]. All markers evaluated in this review; CD133 [6, 13, 14, 17, 21, 26, 27], CD15 [13], A2B5 [4, 19], nestin [7, 9, 13-15, 20, 25, 27], Musashi-1 [12, 14, 20, 21, 23], BMI1 [11, 22], SOX2 [14, 18, 25], Id1 [24], and Oct4 [8], have been suggested to be closely related to CSC properties in glioblastomas.

In the following we summarize current reports on putative glioma CSC markers and review the prognostic value of the individual immunohistochemical markers. A summary will be given for each marker and the prognostic value will be

discussed. An overview of all reviewed markers and the related studies is given in **Table 1**.

Methods

The reviewed articles have been found through a search in PubMed, using the words; brain tumor/glioma/glioblastoma/astrocytoma, prognostic, survival, prognosis, outcome, predictive, immunohistochemistry and the different names of the markers including synonyms (**Table 2**). The results of the PubMed search were manually evaluated looking for prognostic studies.

We evaluate studies using immunohistochemistry (IHC) since prognostic and predictive markers based on IHC and formalin fixed paraffin embedded tissue will work for most brain tumors even small deep tumors, where only needle biopsies can be obtained. Moreover IHC is widely used in pathology.

Results for membrane markers

CD133

CD133 is a 5-TM glycoprotein located in the membrane of human hematopoietic cells and in neural progenitor cells [37, 38]. Singh et al showed that only 100 CD133 positive (CD133+) cells was required to produce a tumor in mice similar to the original patient tumor. In contrast, 10^5 CD133 negative (CD133-) cells were unable to produce tumors [31], suggesting that CD133+ cells have CSC properties [31].

Four different groups have reported that CD133 is a marker of poor survival in astrocytomas [14, 17, 21, 26]. Pallini et al [17] investigated the expression of CD133 in 44 glioblastoma multiforme (GBMs) and showed that more than 2% CD133+ cells and the presence of CD133/Ki67 co-expression were associated with a poor outcome. In multivariate analysis, patients with less than 2% CD133+ cells had a better progression free survival (PFS) (10 months versus 5 months, p=0.01) and overall survival (OS) (14 months versus 10.5 months, p=0.01). The difference increased when CD133/Ki67 co-expression was evaluated; patients with CD133-/Ki67+ cells had a median OS of 12.3 months compared to 6.8 months in patients with CD133+/Ki67+ cells (p=0.007).

Zeppernick et al [26] found that the presence of CD133 clusters and high amounts of CD133+ cells (>1%) were correlated to shorter PFS (HR 8.13, 95% CI 3.63-18.25, p=0.001) and OS (HR 17.46, 95% CI 5.49-55.52, p<0.001) when adjusting for WHO grade, age and extent of resection.

Ma et al [14] investigated the expression of CD133 using IHC and PCR. The authors showed that the level of CD133+ cells was significantly higher in tumor tissue than in normal brain tissue (15.6% and 2.3% respectively) and that the level of CD133 correlated with malignancy grade.

Thon et al [21] found CD133+ cells in both high-grade and low-grade tumors and a correlation between expression of CD133 and tumor grade was observed (p<0.001).

Two studies have reported that CD133 has no prognostic significance in astrocytic brain tumors. In a study made by our group [6] the localization and distribution of CD133+ cells in astrocytomas grade II-IV was investigated by quantitative stereology. We found that 97% of the GBMs were CD133+, 94% had CD133+ blood vessels and 54% had CD133+ niches. GBMs contained more CD133+ blood vessels per tumor volume than grade II and III astrocytomas. However, when performing Cox regression analysis adjusted for age and gender, the presence of CD133+ niches and single cells had no prognostic value [6]. This result was supported by Kim et al, who reported that no significant differences existed in OS according to the expression of CD133 in a set of 88 GBMs. Surprisingly, they found that the survival of CD133+ patients were longer than in CD133- patients (17.7 months versus 17.0 months) [13].

CD133/nestin

Zhang et al [27] investigated the double-expression of CD133 and nestin in 125 patients with grade II-IV astrocytomas. They found high expression of CD133 and nestin in gliomas compared to normal brain tissues and that an increasing co-expression correlated with increasing tumor grade. The results were significant in multivariate analysis; Wald 24.23, 95% CI 2.17-9.85, p<0.001. No information was provided regarding included variables. A minor drawback is the inclusion of children, as opposed to other studies. Moreover, grade III

Table 1. Table of the reviewed studies, their methods and conclusions. Additional information about anti-bodies and statistical methods are as described in the different articles

Marker	Author	Tissues	Patients	Methods	Antibody	Quantification	Statistics	Conclusion
CD 133	Pallini (2008)	PE	44 grade IV 5 NBT	IHC, In vitro	Anti-CD133/1 (IHC), anti-CD133/2 293C3 (IF) (Miltenyi biotec)	Semiquantitative scoring	Wilcoxon, X^2 test, Fisher´s exact test, Cox	S
	Zeppernick (2008)	FF	24 grade II 24 grade III 47 grade IV	IHC	Anti-CD133/1 AC133 (Miltenyi biotec)	Semiquantitative scoring	Kaplan Meier, Log Rank, Cox	S
	Ma (2008)	FF	18 grade I 12 grade II 17 grade III 25 grade IV 4 NBT	IHC, RT-PCR, CM	Anti-CD133, goat poly-clonal, cu (Santa Cruz)	Not mentioned	Student´s *t*-test, Pearsons correlation coefficients	(S)
	Thon (2010)	FFPE, FF	10 grade II 12 grade III 22 grade IV	IHC, IB, RT-PCR, cc	Anti-CD133/1 AC133 (IHC/WB), anti-CD133/2 293C3 (IHC/WB), anti-CD133/1 W6B3C1 (WB) (Miltenyi Biotec)	Not mentioned	Student´s *t*-test	(S)
	Christensen (2008)	FFPE	24 grade II 18 grade III 72 grade IV	IHC, Tissue array	Anti-CD133/1 W6B3C1 (Miltenyi biotec)	Quantitative stereology	ANOVA, t-test, Kaplan Meier, Cox	NS
	Kim (2011)	FFPE	88 grade IV	IHC	Anti-CD133 cu (Abcam)	Semiquantitative scoring	Fisher´s exact test, X^2-test, Kaplan Meier, Log Rank, Cox	NS
CD133/ nestin	Zhang (2008)	FFPE	56 grade II, 69 grade III/IV 10 NBT	IHC	Monoclonal antibodies to nestin and CD133 (Santa Cruz and Novocastra)	Bin-based scoring	Fisher´s exact test, Pearsons X^2-test, Kaplan Meier, Spear-mans correlation, Cox	S
Podoplanin	Mishima (2006)	FFPE, FF	14 grade III 34 grade IV	IHC, WB, qRT-PCR	Anti-podoplanin/clone YM-1 (Medical Biological Laboratories)	Bin-based scoring	Not mentioned	(S)
	Ernst (2009)	Unknown	41 grade IV	IHC, cc, RNA ext, GE	Unknown	Unknown	Pearsons correlation, Kaplan Meier, Log Rank, Cox	S (all astrocytomas) NS (GBM)
CD15	Kim (2011)	FFPE	88 grade IV	IHC	Anti-CD15/cu (Dako)	Semiquantitative scoring	Fisher´s exact test, X^2-test, Kaplan Meier, Log Rank, Cox	NS
A2B5	Bishop (1989)	PE	9 grade I 8 grade II 7 grade III 14 grade IV	IHC	Anti-A2B5/cu	Semiquantitative scoring	Students t-test, Fisher´s exact test, Pearsons correlation	(S)
	Piepmeier (1993)	PE	20 low grade	IHC	Anti-A2B5/cu (Boehringer Mannheim)	Not mentioned	Kruskal Wallis, Spearman´s cor-relation, multivariate (unspecified)	S

Nestin	Dahlstrand (1992)	FFPE	7 grade I 18 grade II 12 grade III 20 grade IV 1 neuro-blastoma 10 metastases	IHC, WB, NB	Anti-nestin antisera 129 and 130 produced by author	Not mentioned	Not performed	(S)
	Ehrmann (2005)	FFPE	30 low grade 40 high grade 16 benign naevi 9 malignant melanomas 23 haemangiomas 10 schwannoma 11 phaeochromocytomas 9 carcinoid tumors	IHC	Anti-nestin/clone 5326 (Chemicon)	Semiquantitative scoring	Not men-tioned	(S)
	Maderna (2007)	PE	49 grade II 31 grade III 22 grade IV	IHC, WB	Monoclonal mouse anti-nestin (R&D systems)	Semiquantitative scoring	Log Rank test	S
	Strojnik (2007)	PE	3 grade I 19 grade II 11 grade III 54 grade IV	IHC, RT-PCR, cc, li	Rabbit antihuman poly-clonal^{+}	Bin-based scoring	t-test, Kaplan Meier, Log Rank, Cox	S
	Ma (2008)	FF	18 grade I 12 grade II 17 grade III 25 grade IV 4 NBT	IHC, RT-PCR, CM	Anti-nestin/cu (R&D Systems)	Not mentioned	Student´s *t*-test, Pearsons correlation coefficients	(S)
	Wan (2011)	FFPE, TMA	45 grade II 17 grade III 221 grade IV 2 recurrent tumors	IHC	Anti-nestin/cu (Chemicon)	Semiquantitative scoring	Spearman´s correla-tion, Kaplan Meier, Log Rank, Cox	S
	Arai (2012)	PE	5 grade I 29 grade II 8 grade III 17 grade IV 5 ependymoma 71 meningioma 102 other histology	IHC	Anti-nestin/rabbit poly-clonal (IBL, Gumna, Japan) for TMA Anti-nestin/mouse monoclonal (Chemicon) for whole slides	Semiquantitative scoring	Wilcoxon rank sum test, Fisher`s exact test, Kaplan-Meier	S
	Chinnaiyan (2008)	PE	156 grade IV	IHC, TMA	Anti-nestin/ac 22035 (Abcam)	Computerized quantita-tive image analysis	Kaplan Meier, Log Rank, Cox	NS
	Kanamori (2009)	FFPE	18 grade II 38 grade III	IHC, FISH	Anti-nestin/mouse mono-clonal (Chemicon)	Semiquantitative scoring	Kaplan-Meier, Log rank, Cox	NS
	Kim (2011)	FFPE	88 grade IV	IHC	Anti-nestin/cu (Millipore)	Semiquantitative scoring	Fisher´s exact test, X^2-test, Kaplan Meier, Log Rank, Cox	NS
Musashi-1	Kanemura (2001)	FFPE	28 grade II 22 grade III 23 grade IV	IHC, IB	Anti-Msi1/clone 14H1(ou)	Bin-based scoring	Kruskall Wallis	S
	Toda (2001)	mRNA, FFPE	1 grade III 4 grade IV NBT 16 cell lines	IHC, WB, RT-PCR	Anti-Musashi1/clone14H1 (ou)	Bin-based scoring	Not mentioned	(S)

	Ma (2008)	FF	18 grade I 12 grade II 17 grade III 25 grade IV 4 NBT	IHC, RT-PCR, CM	Anti-musashi-1/cu (R&D Systems)	Not mentioned	Student´s *t*-test, Pearsons correlation coefficients	(S)
	Thon (2010)	FFPE, FF	10 grade II 12 grade III 22 grade IV	IHC, IB, RT-PCR, cc	Anti-Musashi-1/cu (Chemicon International)	Not mentioned	Student´s *t*-test	(S)
	Strojnik (2007)	PE	3 grade I 19 grade II 11 grade III 54 grade IV	IHC, RT-PCR, cc, Ii	Anti-musashi-1/cu (Chemicon)	Bin-based scoring	t-test, Kaplan Meier, Log Rank, Cox	NS
BMI1	Häyry (2008)	PE	92 grade II 61 grade III 152 grade IV	IHC	Anti-BMI1/clone 1.T.21 (ou)	Bin-based scoring	X^2-test, Fishers exact test, Kaplan Meier, Log Rank, Cox	S (oligo) NS (astro)
	Tirabosco (2008)	PE	16 grade II 15 grade III 49 grade IV	IHC	Anti-BMI1/clone 229-F (ou)	Not mentioned	Non mentioned	NA
	Cenci (2012)	FFPE	48 grade IV	IHC	Anti-BMI1/clone F6 (Millipore)	Bin-based scoring	Kaplan-Meier, Log rank, Mann-Whitney, X^2-test, Cox	S
SOX-2	Ma (2008)	FF	18 grade I 12 grade II 17 grade III 25 grade IV 4 NBT	IHC, RT-PCR, CM	Anti-SOX2/cu (Santa Cruz)	Not mentioned	Student´s *t*-test, Pearsons correlation coefficients	(NS)
	Phi (2008)	PE (IHC, IF) FF (RT-PCR)	67 grade I 14 grade II 4 grade III 110 grade IV 4 retinoblast 1 lymphoma 1 chondrosarc 3 metastases	IHC, RT-PCR, IF	Anti-SOX2/cu (R&D Systems)	Bin-based scoring	Not mentioned	(NS)
	Wan (2011)	FFPE TMA	45 grade II 17 grade III 221 grade IV 52 recurrent tumors	IHC	Anti-SOX-2/cu (R&D systems)	Semiquantitative scoring	Spearman´s correlation, Kaplan Meier, Log Rank, Cox	NS
ID1	Vanderputte (2002)	FFPE	6 grade I 17 grade II 19 grade III 16 grade IV	IHC, WB	Anti-Id1/cu (Santa Cruz Biotechnology)	Semiquantitative scoring	Kruskall Wallis, Mann-Whitney	S
Oct-4	Du (2009)	PE	14 low-grade 27 high-grade	IHC, RT-PCR, WB, cc, trans	Anti-Oct4/cu (Santa Cruz)	Semiquantitative scoring	ANOVA, Tukey test, X^2-test	(S)

Abbreviations: NBT normal brain tissue, FFPE formalin fixed paraffin-embedded, PE paraffin-embedded, FF fresh frozen, IHC immunohistochemistry, WB western blot, CM confocal microscopy, IF immunofluorescence, RNA ext RNA extraction, IB immunoblotting, cc cell culture, Ii intracerebral implantation, trans transfection, NB northern blot, GE gene expression analysis, cu clone unknown, or origin unknown, Cox cox proportional hazard model, S significant, NS non-significant, (S) significant trend, (NS) non-significant trend. *Ab for nestin (#4350) provided by Urban Lehndal from Karolinska Institute, Stokholm, Sweden.

Table 2. Name and synonyms for each marker, which were used in the PubMed search

Name of the marker	Synonyms
CD133	prominin-1, PROM-1
Podoplanin	gp36, aggrus, PDPN
CD15	Lewis X, IeX, FORSE-1, stage specific embryonic antigen 1 (SSEA-1),
A2B5	
Nestin	
Musashi-1	
BMI1	B-cell-specific Moloney murine leukemia virus insertion site 1 gene
SOX2	SRY, sex determining region Y-box 2
Id1	Inhibitor of Differentiation 1
Oct-4	Oct-3/4 and POU5F1

and IV astrocytomas were pooled in the analysis, which however, seems reasonable since the patient material was collected from 1998 to 2000, a period where all high-grade astrocytomas were treated similarly.

Podoplanin

Podoplanin is a mucin-type transmembrane glycoprotein with a poorly understood biological function [16, 39-41]. Besides a role in motility and invasion in gliomas [42] podoplanin has been suggested to be important for spheroid formation thereby suggesting a role in stemness in gliomas [43].

Two groups [10, 16] agree that podoplanin may be a prognostic marker in high-grade astrocytomas. Using tissue micro arrays (TMA), Mishima et al [16] found high expression of podoplanin in high-grade astrocytomas (n=48), but not in low-grade astrocytomas (n=8). A significant difference in the expression was observed between WHO grade III and IV astrocytomas ($p<0.001$) and the authors conclude that higher expression is correlated to higher malignancy grade and thereby to survival. A comparison between expression of podoplanin in TMAs and in surgical resection samples was performed. There was a clear tendency towards higher expression in whole slides compared to TMAs; no statistical comparison of this was made.

Ernst et al [10] showed that high expression of podoplanin was a prognostic factor (HR 1.94, 95% CI 1.00-3.74, p=0.049) in the entire group of astrocytomas grade II-IV (n=52), but in GBMs the significance disappeared in both univariate analysis (HR 0.989, 95% CI 0.445-2.20, p=0.980) and in multivariate analysis (HR 0.381, 95% CI 0.137-1.06, p=0.065).

CD15

CD15 is a cluster of differentiation antigen [44], which is identified in various normal tissues and in different cancer types including gliomas [44-46]. In addition; implantations of CD15 positive glioma cells into mouse brains produce new tumors thereby suggesting CD15 to be a CSC marker [47].

Recently, Kim et al [13] evaluated the prognostic potential of CD15 in 88 GBMs, using a classification method where tumors were divided as having low (<50% positive) or high (>50% positive) expression. No statistical difference was identified in OS (OS 18.2 and 17.1 months, respectively, p=0.79).

A2B5

A2B5 is a surface glycoside that marks O-2A neural progenitor cells [19, 48]. Transplantation studies have showed that both A2B5+/CD133+ and A2B5+/CD133- cell populations were capable of generating tumors in transplantation models [49], suggesting that A2B5+ cells have CSC properties.

Piepmeier et al [19] and Bishop et al [4] investigated the prognostic potential of A2B5 in gliomas. Two different antibody clones were used and only 20 and 38 patients were included. Both studies suggested that A2B5 is a marker of poor prognosis, although the amount of statistical evaluation was sparse.

Results for filament markers

Nestin

Nestin is a filament marker expressed in neural progenitor cells during development [5, 9, 14, 15, 50-53]. The expression of nestin in gliomas has been suggested to be related to dedifferentiated status, improved cell motility, invasive potential and increased malignancy [54].

Dahlstrand et al [7] found that nestin was expressed in 46% of primary CNS tumors (n=57), but not in carcinoma metastases (n=10). Moreover, the expression of nestin increased with increasing malignancy grade in astrocytomas [7].

Maderna et al [15] found nestin in both low-grade and high-grade astrocytomas. In WHO grade II tumors, nestin was mostly found in recently developed vessels and not in tumor cells. Univariate analysis showed no correlation between the expression of nestin and survival (n=49). Expression was higher in WHO grade IV tumors than in WHO grade III tumors, and the expression of nestin was associated with PFS (p=0.001) and OS (p=0.0005). The highly significant values were not tested for confounders in a multivariate analysis.

Strojnik et al [20] found a higher percentage of nestin positive tumor cells in high-grade gliomas (n=55) as compared to low-grade gliomas (n=22) (75.4% and 4.5% respectively). Furthermore, using IHC and RT-PCR, the authors showed that nestin expression within the tumor cells increased with increasing malignancy. This was confirmed in both univariate and multivariate analyses (p=0.002 and p<0.001, respectively). All material was collected before 1999, meaning that WHO classification and treatment differ from the ones used today, which makes comparison with results obtained today difficult.

Ma et al [14] found higher levels of nestin in 72 astrocytomas grade I-IV compared to normal brain tissue. Moreover, the level of both mRNA and protein was highest in WHO grade IV tumors.

Wan et al [25] included a total of 382 tumors; 221 of them being GBMs. Nestin expression increased with malignancy grade, and high expression of nestin was associated with poor survival in both univariate (HR 1.40, 95% CI 1.27-1.56, p<0.001) and multivariate analysis (HR 1.12, 95% CI 1.00-1.26, p=0.042). Inclusion of WHO grade, patient age and extent of resection in the multivariate analysis, make this study one of the most powerful in this review.

Arai et al [55] investigated the use of nestin as a diagnostic marker in 257 primary brain tumors, including 79 gliomas. Using TMAs the expression was identified using an immunohistochemical score, defined as the product of the percentage of positively stained cells and the staining intensity of positively stained cells. The prognostic value of nestin was evaluated in 64 high-grade gliomas using whole slides. Patients were dichotomized based on their immunohistochemical score; 0-2 versus 3-9. Patients with a high score had a significantly poor survival compared to patients with low scores; median survival was 1.0 year and 5.5 years, respectively, p<0.005.

Kanamori et al [56] investigated nestin in 56 patients with oligodendroglial tumors. The expression of nestin was measured as no/focal staining or diffuse staining. Nineteen patients (34%) had a diffuse staining pattern and these patients had a significant poorer survival than patients with a focal staining pattern; median OS not reached and 38 months, respectively. A multivariate analysis was performed and only 1p/19q status and p53 index were independent prognostic factors. However; patients with a diffuse nestin staining pattern had a poorer PFS; HR 3.8, 95% CI 1.5-9.6.

Chinnayan et al [5] showed that nestin is not a prognostic factor, when investigating 143 GBMs. Patients were divided into three groups with low, intermediate and high expression. No differences in OS or PFS were seen between the three groups in neither univariate nor multivariate analysis (intermediate/low: HR 1.66, 95% CI 0.94-2.93, p=0.98, high/low HR 1.47, 95% CI 0.83-2.60, p=0.18).

A study from Kim et al [13] supports the negative results obtained by Chinnayan et al. No correlation was seen between the expression of nestin and survival in multivariate analysis (HR 1.01, 95% CI 0.57-1.80, p=0.97) and OS was 18 months in patients with nestin+ tumors

compared to 17 months in patients with nestin- tumors. The authors state that only gross total resection and combined radiotherapy and chemotherapy are prognostic factors.

Results for RNA-binding protein

Musashi-1

Musashi-1 belongs to a family of evolutionary well conserved neural RNA-binding proteins [12, 14, 20, 21, 57, 58]. Hemmati et al [59] showed that Musashi-1 positive dissociated tumor cells were capable of forming neurospheres, and that these neurospheres were able to self-renew and differentiate into different cell types, thereby suggesting a CSC function of this protein.

Four studies [12, 14, 21, 23] report that high expression of Musashi-1 correlates with malignancy grade and thereby with OS. Kanemura et al [12] also found a correlation between Musashi-1 expression and the proliferation marker MIB1 in a set of anaplastic astrocytomas (n=22) ($p<0.05$), but not in GBMs (n=23). Noteworthy, the Musashi-1 and MIB1 correlation was later supported by Toda et al [23].

Ma et al [14] demonstrated that the percentage of Musashi-1 labeled cells correlated with malignancy grade and they found high mRNA and protein expression in astrocytomas compared to normal brain tissue.

Thon et al [21] found lower expression of Musashi-1 in WHO grade II tumors (n=10) than in WHO grade IV tumors (n=22) ($p<0.001$) as well as a difference between WHO grade II (n=10) and grade III tumors (n=12) ($p=0.07$). No difference between WHO grade III and grade IV tumors was seen ($p>0.05$).

Strojnik et al [20] found a correlation between the amount of Musashi-1 positive cells and malignancy grade in WHO grade I-IV tumors, but in multivariate analysis (n=87) no correlation with survival was observed. As the only group, they report that Musashi-1 did not have any prognostic potential.

Results for transcriptional markers

BMI1

BMI1 is a member of the Polycom-Group and a known regulator of two major tumor suppressor pathways [11, 22]. Moreover, knockdown of BMI1 in a CD133+ glioma cell population led to a reduced number of secondary spheres and thus decreased self-renewal. In CD133- glioma cells knockdown of BMI1 did not affect the formation of secondary spheres, indicating that BMI1 may regulate tumor initiation in CD133+ glioma cells and maintain tumor growth in CD133- glioma cells [60, 61].

Häyry et al [11] investigated the expression of BMI1 in 62 oligodendroglial and 243 astrocytic tumors WHO grade II-IV. In oligodendroglial tumors both univariate and multivariate analyses showed that high expression of BMI1 is a prognostic factor of poor survival compared to low expression (univariate: $p=0.007$, multivariate: HR 8.41, 95% CI 1.08-7.78, $p=0.035$). However, the effect of 1p/19q co-deletion, a known prognostic factor in oligodendroglial tumors [62-66] was not taken into account. Regarding astrocytomas, only three GBMs (2%) was not BMI1 positive and BMI1 was found not to be a prognostic factor.

Tirabosco et al [22] found that all tumors (n=80), regardless of malignancy grade, had a diffuse nuclear staining and a variable staining intensity. The authors abandoned scoring of BMI1 expression and based on the observation that there was no difference between the expression of BMI1 in high-grade and low-grade tumors, they concluded that BMI1 is not a prognostic marker. The tissue samples were collected between 1980 and 2006 and it could be speculated that treatment as well as other variables introduce some survival bias during this 26-year long period. Cenci et al [67] investigated the prognostic potential of BMI1 and c-myc in 48 patients with glioblastoma. High levels of BMI1 was asociated with improved survival ($p=0.0009$). In multivariate analysis adjusted for age, c-myc expression. Ki-67, performance status, MGMT status and gender, BMI1 was no longer prognostic ($p=0.17$).

SOX2

SOX2 is essential for normal pluripotent cell development and maintenance. SOX2 downregulation after embryogenesis is correlated with loss of pluripotency and self-renewal [18] and knock-out of SOX2 in gliomas causes loss of tumorigenicity [68].

Three groups have investigated the prognostic potential of SOX2. Allthough different methods and tumors with different histology are used, all groups agree that SOX2 is not a prognostic marker. Ma et al [14] reported high expression of SOX2 in high-grade gliomas using IHC and RT-PCR. They found that the expression of SOX2 was up-regulated in tumor cells compared to normal brain tissue, but this was not statistically significant. It was not reported whether the expression detected with IHC was in accordance with the expression detected with RT-PCR.

Wan et al [25] examined 283 astrocytomas WHO grade II-IV and 52 recurrent tumors. Nearly half of the tumor cells were positive for SOX2 regardless of WHO grade. No association between SOX2 and survival was found in univariate or multivariate analyses. TMAs were used and this might be problematic when it comes to heterogeneous tumors as GBMs [6, 11].

Phi et al [18] investigated the expression of SOX2 in 23 different kinds of brain tumors including 3 metastases. They found that SOX2 was expressed in tumors of glial lineages and they concluded that the expression of SOX2 did not correlate with malignancy grade in astrocytic tumors although a univariate analysis was not carried out.

Id1

Id1 is over-expressed in several cancer types, where it is involved in proliferation, anaplasia, invasiveness, metastasis and neo-angiogenesis [69, 70]. Anido et al [71] showed that knock-out of the TGF-β pathway led to decreased expression of Id1 in gliomas. This prevented tumor growth, suggesting that Id1 is important for maintaining tumor growth in gliomas.

Vanderputte et al [24] found that high expression of Id1 correlated with high malignancy grade in gliomas. In this study Id1 expression was variable in both astrocytomas WHO grade I-IV and in oligodendroglial tumors WHO grade II-III. High expression was seen in tumor vessels, whereas no expression was seen in normal brain.

Oct-4

Oct-4 is expressed in pluripotent embryonic stem and germ cells, where it is a regulator of self-renewal and differentiation [8, 72, 73]. It is expressed in several cancer types [72-74] including lung cancer where knock-out of Oct4 enhanced sensitivity towards chemotherapy and radiotherapy and increased apoptotic activity [73].

One group [8] investigated the prognostic value of Oct-4 in gliomas. A limited number of patients (n=41) were included but Oct-4 was found in both high-grade and low-grade astrocytomas. The protein level was highest in high-grade tumors ($p<0.01$) and no expression was found in the normal control tissue.

Discussions

We reviewed present reports on the prognostic value of putative glioma CSC markers and found that CD133 and nestin had prognostic significance. A trend towards a prognostic potential was identified for podoplanin and Musashi-1. For the remaining markers no prognostic value was identified.

Prognostic value of CD133

CD133 is the most frequently investigated CSC marker in gliomas, and despite the use of different designs, different antibodies and different statistical analyses, most studies agree that CD133 is a prognostic marker in gliomas.

Pallini et al [17] and Zeppernick et al [26] both reported that high expression of CD133 is associated with poor survival, although they did not use the same cut-off point (2% and 1%, respectively). However, in both studies the AC133 clone was used; as the only clone, it has been used to identify a prognostic significance of CD133 in both frozen [21, 26] and paraffin embedded brain tumor sections [17]. Several antibodies against CD133 have been developed but besides AC133 [17, 21, 26], only 293C3 (Miltenyi) [75] and an anti-CD133 antibody from Santa Cruz [14], have been used to identify a prognostic potential of CD133. In a study from our group [76] the use of four different antibodies against CD133 were investigated. All antibodies recognized CD133+ cells, but the distribution rarely corresponded. We assume that the use of different CD133 antibody clones possibly recognizing different CD133 splice variants with frail epitopes of different glycosylation status may explain the different findings. It is therefore most likely the

distribution of certain CD133 antigens and not the distribution of the CD133 protein itself that is prognostic [76].

Prognostic value of nestin

Many studies report that nestin is a prognostic marker, and there seems to be some kind of agreement about the correlation between high expression of nestin and poor survival in studies using semi-quantitative scoring [7, 9, 14, 15, 20, 55] although inclusion of different histological sub types, different scorings systems, evaluation methods and antibody clones have been used. As the only group, Chinnayan et al [5] divided the patients into RPA classes (see [77]), and the authors showed that nestin was not a prognostic factor, whereas clinical parameters gathered in RPA classes were. Another difference between this study and the other studies evaluating nestin, is the use of automated quantitative measurements (Ariol SL-50) and that patients were stratified in low, intermediate and high expression. The results showed no significant difference in survival between low and intermediate or between low and high expression. No correlation between intermediate and high expression was performed. The use of TMAs may be a drawback of this study. Häyry et al [11] used TMAs when investigating BMI1; if the TMA was BMI1 negative, a whole section was stained to validate the result. Surprisingly, of the 22 GBMs that did not express BMI1 in the TMA, 19 GBMs were positive when the whole tissue section was stained. We also investigated the use of TMAs compared to whole sections focusing on CD133. The results showed that the CD133 expression often was underestimated in TMAs [6]. This in combination with the results obtained by Häyry et al [11] suggests that TMAs should be used with caution, when investigating GBMs and it may be speculated that the use of whole sections would change the conclusion made by Chinnayan et al.

Zhang et al [27] showed that co-expression of CD133 and nestin had a more powerful prognostic value than just single markers. The explanation for this may be that the bonafide CSC marker has not yet been found but that combinations of markers may identify an important level of differentiation in the CSC differentiation hierarchy.

Prognostic value of podoplanin, musashi-1 and other markers

Ernst et al [10] concluded that podoplanin has no prognostic potential, but the p-value in the multivariate analysis was just above 0.05 and it was based on a limited number of patients. Combining this information with the prognostic value obtained by Mishima et al [16], podoplanin may be a prognostic marker in primary gliomas.

Looking at all studies investigating Musashi-1 there seemed to be a trend towards a prognostic value of Musashi-1. In the studies by Thon et al [21], Ma et al [14], and Strojnik et al [20] the expression of Musashi-1 was correlated to malignancy grade. In addition; Strojnik et al reported a strong trend suggesting that Musashi-1 is a prognostic marker, although the significance disappeared in the multivariate analysis. Moreover; Kanemura et al [12] and Toda et al [23] reported that the co-expression of Musashi-1 and MIB1 are of prognostic importance.

For the remaining markers, CD15, A2B5, BMI1, SOX2, Id1 and Oct4, no prognostic value were found in this review, suggesting a low clinical potential by these markers, at least in terms of protein detection by IHC. We are aware that this statement is based on a limited number of patients for some of the markers and that future studies containing more patients may reveal a prognostic potential.

Performing and reporting prognostic studies

Performing and especially reporting prognostic studies is not easy [78] and several guidelines have been published [78-81]. These guidelines were developed after the publication of most of the reviewed studies and for use in a different setting. Therefore; it was not surprising that the reviewed studies did not match all the criteria from the different guidelines.

An important item that has reached a kind of consensus over the years is the classification of studies as pilot, exploratory and confirmatory studies. So far, all studies cited in this review should be considered pilot studies, which are essential for the discovery of new biomarkers as they probe the potential of the individual markers.

Other issues mentioned in several of the guidelines are the use of small sample sizes, the use of different variables, different cut-off-points, sub-analysis of different patient groups, inclusion of different tumor types, different preparation of tissue (frozen or paraffin embedded), different clones and assays and use of stepwise variable selection methods. The reviewed studies have missing information on several of these topics: information about inclusion and exclusion criteria [5, 13], information of follow-up [11, 13, 15, 20], and information of treatment (standardized or randomized) [6, 21, 27]. However, on the other hand many informations are included: half of the reviewed studies [5, 6, 9, 11, 15, 16, 18, 25, 27] report data for more than 100 patients and most of the reviewed studies [7, 8, 11, 12, 14-16, 20, 21, 23, 25-27] compare the prognostic value of the investigated marker with tumor grade. In 7 studies [6, 11, 13, 17, 20, 25, 26] clinical parameters like performance status, extend of resection, age, gender or treatment with radiotherapy and chemotherapy are included in multivariate analysis.

Recently presence of 1p/19q co-deletion [62], methylation of the O6-methylguanine methyltransferase (MGMT) promoter region [1] or mutation of isocitrate dehydrogenase (IDH) 1 [81] have been shown to have clinical significance suggesting these biomarkers for use in clinical decision making. Therefore future prognostic glioma studies would greatly benefit from including these molecular markers in data analysis.

Conclusion

For pathologists and oncologists, the questions of which marker(s) should be investigated in future studies are of major importance. Further exploring the significance of these markers for prognosis and prediction of treatment response would help development of individual treatment strategies as well as help clarifying the clinical importance of cancer stem cell biology. To do so additional investigations with large cohorts as well as careful considerations about antibody clones, staining protocols, scoring methods, statistical methods and strategies of validation is mandatory. We conclude that all the reviewed studies contribute substantially to our understanding of the prognostic value of the different stem cells markers and provide new ideas for further studies. Based on this review, we find that CD133, nestin, CD133/nestin, podoplanin, Musashi-1 and Musashi-1/MIB1 are the most promising CSC markers for future investigation.

Address correspondence to: Dr. Bjarne W Kristensen, Department of Pathology, Odense University Hospital, Winsløwparken 15, 5000 Odense C, Denmark. E-mail: bjarne.winther.kristensen@ouh.regionsyddanmark.dk

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