Stem Cell Marker CD133 Affects Clinical Outcome in Glioma Patients

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Abstract

Purpose: The CD133 antigen has been identified as a putative stem cell marker in normal and malignant brain tissues. In gliomas, it is used to enrich a subpopulation of highly tumorigenic cancer cells. According to the cancer stem cell hypothesis, CD133-positive cells determine long-term tumor growth and, therefore, are suspected to influence clinical outcome. To date, a correlation between CD133 expression in primary tumor tissues and patients' prognosis has not been reported.

Experimental Design: To address this question, we analyzed the expression of the CD133 stem cell antigen in a series of 95 gliomas of various grade and histology by immunohistochemistry on cryostat sections. Staining data were correlated with patient outcome.

Results: By multivariate survival analysis, we found that both the proportion of CD133-positive cells and their topological organization in clusters were significant ($P < 0.001$) prognostic factors for adverse progression-free survival and overall survival independent of tumor grade, extent of resection, or patient age. Furthermore, proportion of CD133-positive cells was an independent risk factor for tumor regrowth and time to malignant progression in WHO grade 2 and 3 tumors.

Conclusions: These findings constitute the first conclusive evidence that CD133 stem cell antigen expression correlates with patient survival in gliomas, lending support to the current cancer stem cell hypothesis.

The cancer stem cell (CSC) model of tumor development suggests that the clinical behavior of a tumor will be largely determined by a subpopulation of cells that are characterized by their ability to initiate new tumors (1). These so-called CSCs have been described to be organized as a hierarchy of stem cells and various types of progenitor cells that are locally restricted to a stem cell niche (2). The size and the degree of organization of the CSC niche in a particular tumor might be an important factor in determining the clinical course of disease.

Using the CD133 stem cell antigen, CSCs have been successfully enriched from brain, prostate, and colorectal tumors (3–7). CD133, formerly known as *PROML-1* or AC133, was originally discovered as the equivalent to mouse prominin, a pentaspan transmembrane glycoprotein of murine neuroepithelial stem cells located in plasma membrane protrusions (8). Although no interacting proteins are known, a role in cell polarity and cell migration was suggested due to its specific localization (9). Whereas CD133 is expressed in a variety of human tissues, the CD133 antigen with the glycosylated epitope AC133 seems to be restricted to stem cells (10–12).

The CD133-positive cell population in brain tumors has been described to be highly tumorigenic after xenotransplantation in nonobese diabetic/severe combined immunodeficient mice (4) and to share many of the characteristics of normal tissue stem cells that could help explain clinical features such as tumor regrowth, metastasis, and therapy resistance (13). Surprisingly, however, apart from the observation that CD133-positive cells are enriched in recurrent gliomas (14), a direct link between CD133 stem cell marker expression and patient outcome thus far has not been established. To address this question, we studied CD133 expression in gliomas, the most frequent primary brain tumors in adults. A grading scheme proposed by the WHO distinguishes four different grades of gliomas, of which glioblastoma multiforme (GBM) WHO grade 4 is the most malignant variant with a median survival time of ~1 year (15). In this study, CD133 analyses were done by immunohistochemistry in a total of 95 gliomas of different WHO grades and results were correlated with patients' survival data.

Materials and Methods

Study sample. Ninety-five glioma samples were obtained from patients with gliomas of different grades. All patients were treated by extensive surgery. Samples were immediately snap frozen and stored at -80°C until processing. Tumors were histopathologically classified

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Table 1. Clinical characteristics of study sample

WHO grade	*n*	Sex (M/F)	Histology/ WHO grade	*n*	Median age (y)	Median PFS (wk)	No tumor regrowth	Median OS (wk)	Alive at LO
2	24	12/12	A/2	7	31.5 (24.8-38.7)	257 (54-381)	2/7	402 (220-695)	6/7
			OA/2	9	48.8 (24.9-63.3)	274 (109-475)	5/9	414 (192-560)	8/9
			O/2	8	51.6 (26.9-65.3)	404 (91-571)	6/8	409 (181-571)	7/8
3	24	15/9	AA/3	9	33 (25.8-43.5)	120 (25-511)	2/9	177 (55-511)	3/9
			AOA/3	7	41.5 (31.7-70.1)	114 (26-303)	4/7	223 (45-303)	3/7*
			AO/3	8	48 (35.4-67.3)	158 (67-418)	5/8	158 (96-418)	5/8
4	47	32/15	pGBM	42	61.6 (32.6-75.4)	23† (3-171)	0/32	53 (3-465)	4/32
			sGBM	5	38.3 (25.8-57.8)	9 (5-31)	0/5	37 (9-113)	1/5

Abbreviations: *n*, case number; M, male; F, female; LO, last observation; A, astrocytoma; OA, oligoastrocytoma; O, oligodendroglioma; AA, anaplastic astrocytoma; AOA, anaplastic oligoastrocytoma; AO, anaplastic oligodendroglioma; pGBM, primary glioblastoma; sGBM, secondary glioblastoma.
*Death of one patient not tumor related.
† PFS of two patients could not be assessed.

according to the WHO classification. Informed consent was obtained from each patient according to the research proposals approved by the Institutional Review Board at the Medical Faculty Heidelberg. Eligibility criteria included written informed consent and availability of frozen tumor tissue and of follow-up data. Clinical information was obtained by reviewing the medical records on radiographic images, by telephone or written correspondence, and by review of death certificate. A patient was considered to have recurrent disease if this was revealed either by magnetic resonance imaging or the occurrence of new neurologic symptoms. Patient characteristics are shown in Table 1. Patient data were analyzed after a mean follow-up period of 86 (±39) months. Extensive surgical resection was done at diagnosis and adjuvant therapy (radiotherapy and/or chemotherapy) was administered in case of high-grade tumors (WHO grade 3 and 4) or tumor recurrence. Additionally, eight GBM patients were treated with an experimental antitumor vaccination and therefore excluded from the survival analysis (16).

Immunohistochemistry. Cryostat sections (5-7 μm) were stained for CD133 using a mouse monoclonal anti-CD133 antibody (clone AC133) originally used to enrich tumorigenic CD133-positive cells from gliomas (4) as well as an isotype IgG2b control antibody (both Miltenyi Biotec), for nestin (mouse monoclonal; R&D Systems), and for the glioma-typical epidermal growth factor receptor (EGFR) type III deletion variant (mouse monoclonal; LOXO). Fixation and staining were carried out as described (17). CD133 staining data were obtained from at least two sections per tissue. Immunohistochemically stained slides were reviewed by two investigators independent from one another and blinded to all clinical data. CD133 staining of the whole tissue section was semiquantitatively graded for percentage of cells stained in n.d. (not detectable), ≤1%, 1% to 5%, 5% to 10%, 10% to 25%, and 25% to 50% CD133-positive cells per section. Additionally, topology of CD133-positive cells was categorized in single cell and cluster staining. Cluster was defined as dense aggregation of more than five neighboring cells. Sections containing at least one CD133-positive cluster besides positive single cells were assigned to the cluster-positive group.

Statistical analysis. Progression-free survival (PFS) was calculated from the date of surgery until the date of documented tumor recurrence or further growth of residual tumor and defined as "tumor regrowth," whereas an increase in WHO grade was defined as "malignant progression" and time to malignant progression (TtMP) was calculated from the date of surgery until the date of increase in WHO grade. For patients who had not experienced recurrence or death at the time of last follow-up, PFS and overall survival (OS) were censored at the date of last follow-up. In case of impossible patient contact, the last date of visit was taken as provisional end point to allow statistical analysis. The association between PFS or OS and CD133 expression was calculated using log-rank tests and presented as Kaplan-Meier plots. Furthermore, a multivariate analysis was done by using Cox proportional hazards regression to determine the prognostic effect of CD133 expression, CD133 topology, and potential clinical variables (age, WHO grade, and extent of resection) on OS, PFS, and TtMP. Backward selection applying a stopping rule based on the Akaike information criterion was used to exclude redundant or unnecessary variables. Hazard ratios (HR) and their corresponding 95% confidence intervals (95% CI) were computed to provide quantitative information about the relevance of results of the statistical analysis. All calculations were done using the statistical software environment R, version 2.4.1.[4] Statistical significance was set at the level of $P < 0.05$.

Results

Degree and topology of CD133 expression in glioma tissues. Expression of the CD133 antigen was assessed by immunohistochemistry in cryostat sections in a panel of 95 gliomas of different WHO grades and histologies (Table 1). Both the proportion of CD133-positive cells and their topological organization showed considerable variability among tumors ranging from complete lack of immunoreactivity (Fig. 1A) to expression in single cells (Fig. 1B and C) or staining of cell clusters (Fig. 1D). Substantial differences in CD133 expression were observed between tumors of different WHO grades (Fig. 2A). In the majority of low-grade tumors (WHO grade 2), CD133 was either not detectable (14 of 24, 58%) or expressed only in up to 1% of cells (7 of 24, 29%); higher percentages of CD133-positive cells were found in a small fraction (3 of 24, 13%) of tumors only. With progression to anaplastic gliomas (WHO grade 3) and GBM (WHO grade 4), the percentage of CD133-negative tumors was only 38% (9 of 24) and 2% (1 of 47), whereas the proportion of tumors in which CD133 was detectable in >1% of cells was 38% (9 of 24, WHO grade 3) and 96% (45 of 47, WHO grade 4). Moreover, with increasing malignancy, CD133-positive cell clusters (Fig. 1D) were found more frequently, occurring in 33% (8 of 24) of WHO grade 3 and 87% (41 of 47) of WHO grade 4 tumors while being entirely absent in grade 2 tumors (Fig. 2B). This staining pattern occurred in all tumors with higher proportions of CD133-positive cells (>5%) and only rarely in tumors with a lower

[4] http://www.r-project.org

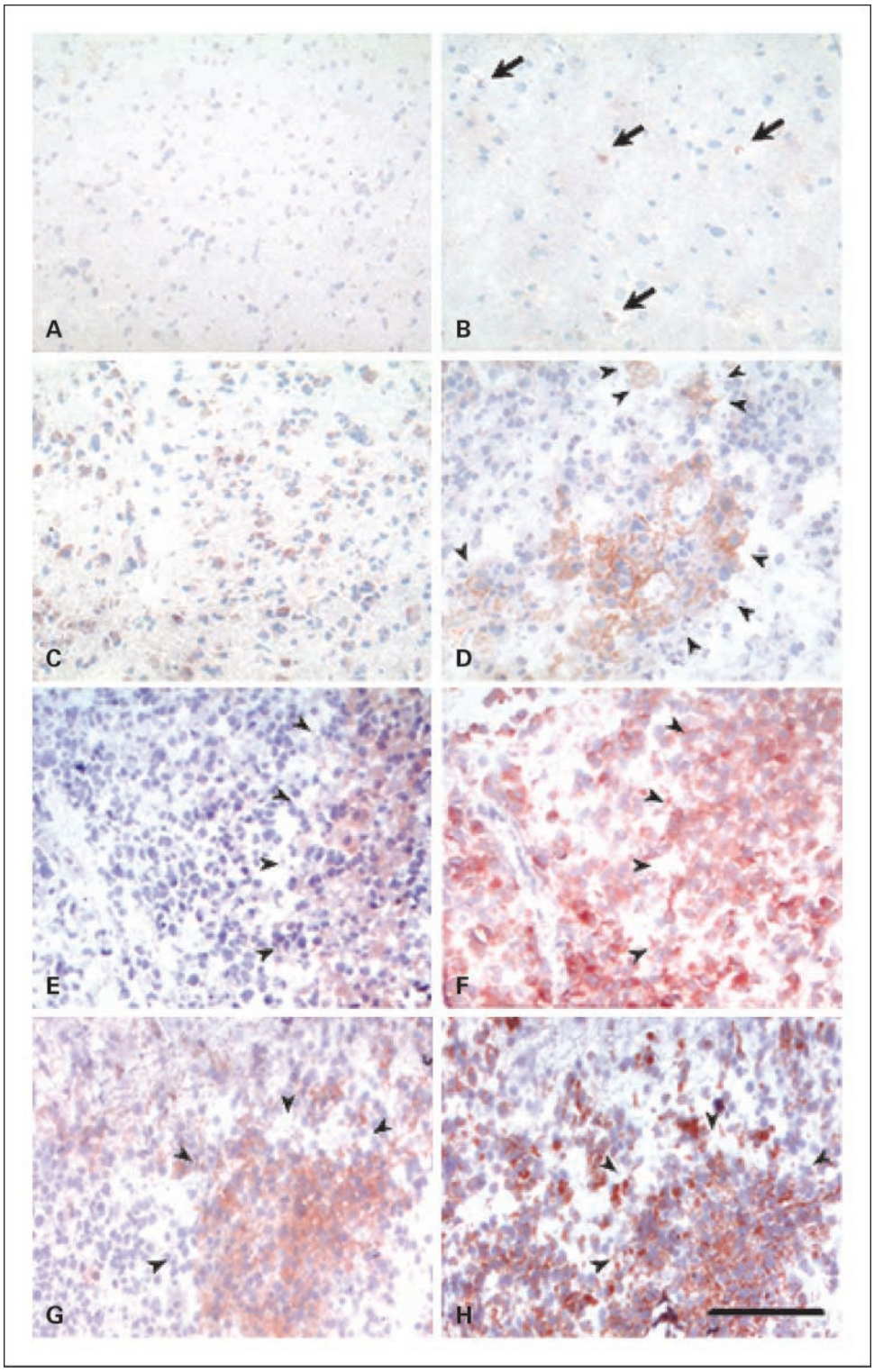

Fig. 1. Cellular distribution of CD133 expression in glial tumor tissues compared with EGFRvIII tumor antigen and nestin expression. Immunohistochemical analysis of CD133-negative astrocytoma WHO grade 2 (*A*), oligoastrocytoma WHO grade 2 with few CD133-positive cells (*B, arrows*), anaplastic astrocytoma WHO grade 3 with an increased percentage of CD133-positive single cells (*C*), and GBM WHO grade 4 with CD133-expressing cells organized in clusters (*D, arrowheads*). *E* to *H,* colocalization of CD133 (*E*) and EGFRvIII (*F*) as well as CD133 (*G*) and nestin (*H*) in GBM WHO grade 4 tissues. Arrowheads, CD133-positive cell clusters. Bar, 100 μm.

amount of CD133-positive cells (2 of 9 cases in tissues with 1-5% CD133-positive cells).

To prove the tumor cell origin of CD133-positive cells, expression of a deletion variant of the EGFR (EGFRvIII) known as a possible tumor antigen of gliomas but not of normal cells (18) was analyzed on serial sections confirming that CD133-positive cells (Fig. 1E) also expressed this tumor antigen (Fig. 1F). Further, we did immunohistochemical stainings for nestin, a marker well known to be expressed on normal neural stem and progenitor cells (19) and on glioma cells (20), revealing that CD133-positive cells (Fig. 1G) were a subpopulation of nestin-positive cells (Fig. 1H).

CD133 expression and patient prognosis. To investigate the effect of proportion of CD133 positive cells on patient

outcome, corresponding PFS and OS data were assessed from the study sample. Eight glioblastoma patients were excluded from survival analysis to avoid an unwanted bias because they had received experimental immunotherapy that was beneficial for patient outcome and increased the median survival time of the respective patients to ~2 years (16). TtMP and PFS could not be assessed in three and two patients, respectively. Univariate analysis documents significant correlation of shorter PFS and OS with both increasing numbers of CD133-positive cells (Fig. 2C) and presence of CD133-positive cell clusters (Fig. 2D). Particularly large differences were observed between survival estimates of patients with tumors containing ≤1% and >1% CD133-positive cells (Fig. 2C). Multivariate analysis confirmed CD133 expression >1% (HR, 17.46; 95% CI, 5.49-55.52; $P < 0.001$) and topological organization of CD133-positive cells in clusters (HR, 5.62; 95% CI, 2.04-15.51; $P < 0.001$) as significant prognostic factors for shorter OS, independent of WHO grade, age, and extent of resection; similar results were obtained for PFS and CD133-positive cells (HR, 8.13; 95% CI, 3.63-18.25; $P < 0.001$) and cluster formation (HR, 4.67; 95% CI, 1.94-11.23), respectively (Table 2).

CD133 expression and malignant progression. Finally, we tested whether CD133 expression might serve as a marker for tumor recurrence and/or a predictor for malignant progression to a higher tumor grade in gliomas of WHO grades 2 and 3

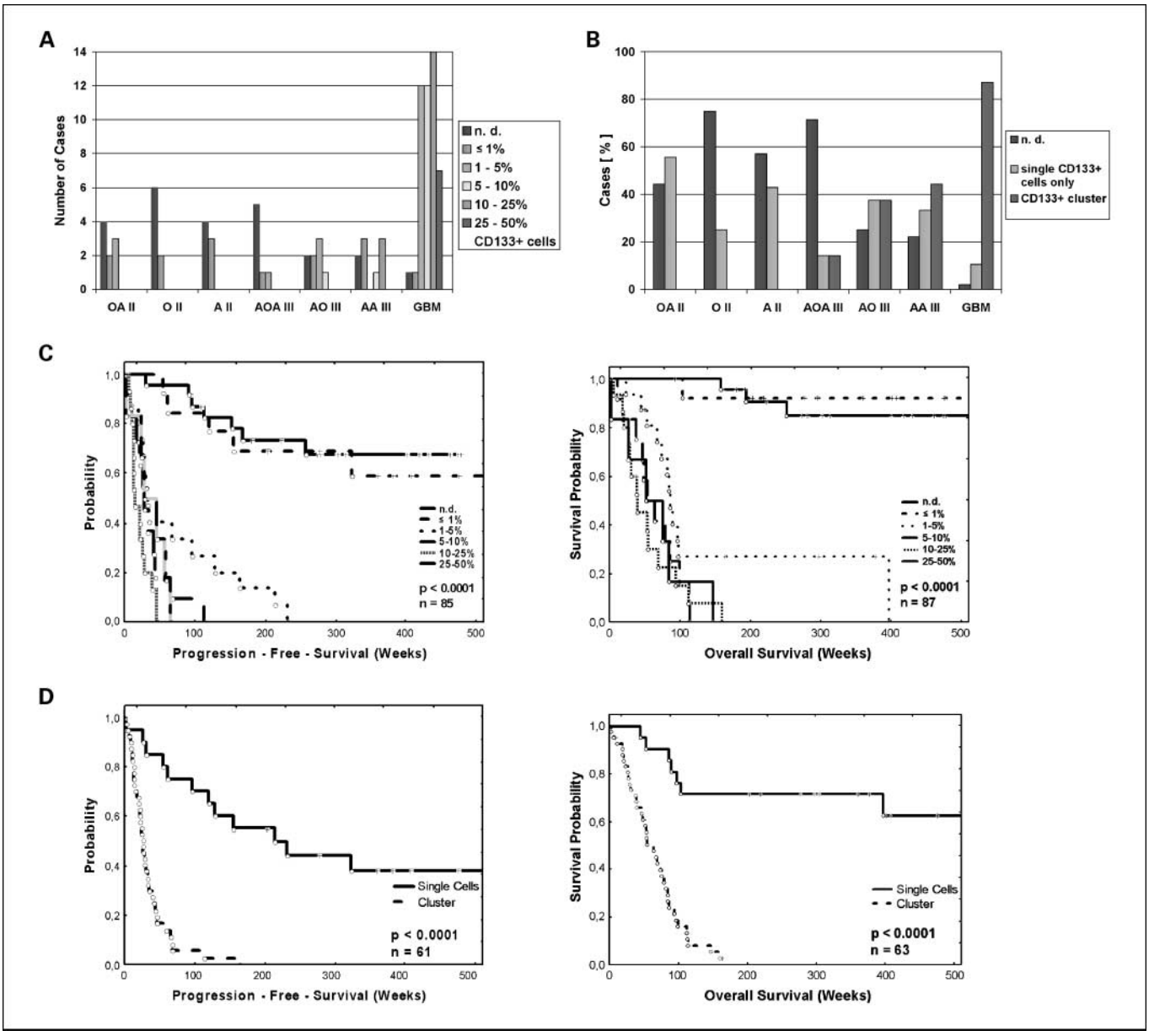

Fig. 2. CD133 expression affects survival. *A,* percentage of CD133-positive cells in 95 glial tumors differing in histology and WHO grade. *B,* topological organization of CD133-positive cells dependent on histology and WHO grade. Kaplan-Meier plots showing a correlation of percentage of CD133-positive cells with PFS and OS (*C*) and cluster formation with PFS and OS (*D*). n.d., not detectable; OA, oligoastrocytoma; O, oligodendroglioma; A, astrocytoma; AOA, anaplastic oligoastrocytoma; AO, anaplastic oligodendroglioma; AA, anaplastic astrocytoma.

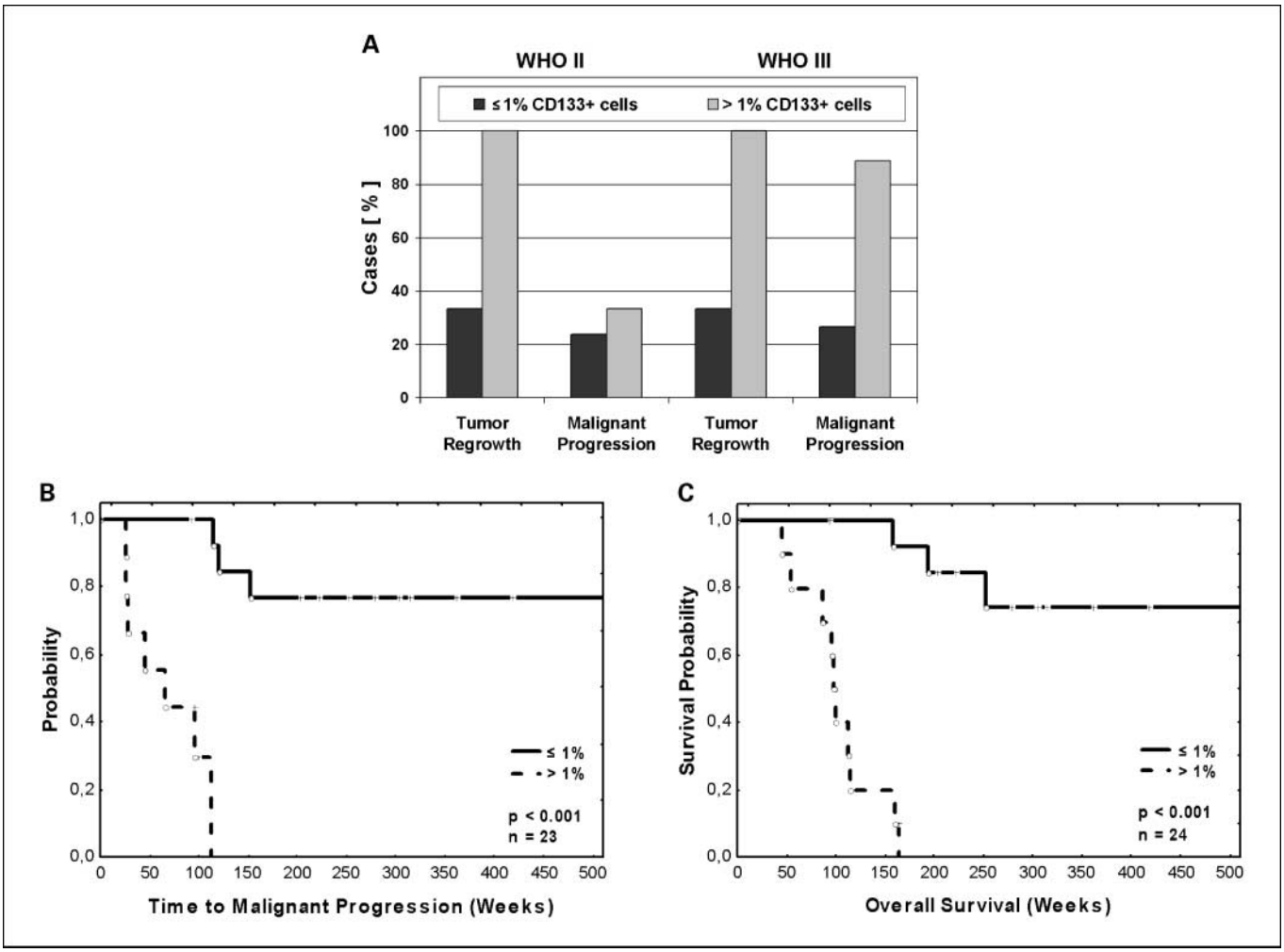

Fig. 3. CD133 expression affects regrowth and malignant progression in WHO grade 2 and 3 gliomas. *A,* subgroup analysis of WHO grade 2 and 3 gliomas for occurrence of tumor regrowth and progression. Correlation of time to progression (*B*) and OS dependent on CD133 expression in WHO grade 3 gliomas (*C*).

(Fig. 3A). Due to the obvious survival differences illustrated in Fig. 2C, we assigned staining results to groups with "up to 1%" or ">1%" CD133-positive cells. In both grade 2 and 3 gliomas, elevated CD133 expression was associated with a higher incidence of tumor regrowth. All patients with >1% CD133-positive cells relapsed in both groups (3 of 3 in WHO grade 2 and 9 of 9 in WHO grade 3), in contrast to only one third of the patients with <1% CD133-positive cells (7 of 21 in WHO grade

Table 2. Cox proportional hazards regressions for PFS and OS in WHO grades 2, 3, and 4

Variable*	PFS		OS	
	HR (95% CI)	*P*	HR (95% CI)	*P*
CD133 frequency	*n* = 85 †		*n* = 87	
CD133-positive cells	8.13 (3.63-18.25)	<0.001	17.46 (5.49-55.52)	<0.001
WHO grade	18.37 (6.15-54.86)	<0.001	6.22 (2.59-14.93)	<0.001
Patient age	0.98 (0.96-1.00)	0.059	1.02 (1.00-1.05)	0.073
Extent of resection	1.47 (0.80-2.69)	0.220	1.40 (0.71-2.76)	0.330
CD133 topology	*n* = 61 †		*n* = 63	
CD133 cluster formation	4.67 (1.94-11.23)	<0.001	5.62 (2.04-15.51)	<0.001
WHO grade	12.63 (4.07-39.14)	<0.001	5.07 (1.96-13.10)	<0.001
Patient age	0.98 (0.95-1.01)	0.140	1.03 (1.00-1.06)	0.081
Extent of resection	1.71 (0.90-3.22)	0.100	1.82 (0.88-3.76)	0.110

*The variables were compared in the following ways: CD133-positive cells, >1% versus ≤1%; WHO grade, >2 versus 2; patient age, x + 10 versus x; extent of resection, subtotal versus total; CD133 cluster formation, cluster versus single cells.
† PFS could not be assessed in two cases.

Table 3. Cox proportional hazards regressions for PFS, TtMP, and OS in WHO grades 2 and 3 and in WHO grade 3

Variable*	PFS		TtMP		OS	
	HR (95% CI)	P	HR (95% CI)	P	HR (95% CI)	P
WHO grades 2 and 3						
CD133 frequency	n = 48		n = 47 †		n = 48	
CD133-positive cells	10.79 (4.17-27.96)	<0.001	7.189 (2.34-22.13)	<0.001	44.72 (5.17-386.68)	<0.001
WHO grade	2.68 (1.07-6.70)	0.036	4.24 (1.22-14.76)	0.023	52.04 (3.46-781.89)	0.004
Patient age	0.96 (0.93-1.00)	0.062	0.93 (0.87-0.99)	0.015	1.01 (0.96-1.06)	0.680
Extent of resection	0.72 (0.30-1.77)	0.480	0.84 (0.28-2.55)	0.760	0.67 (0.21-2.11)	0.490
CD133 topology	n = 25		n = 24 †		n = 25	
CD133 cluster formation	6.81 (1.56-29.76)	0.011	4.56 (0.83-25.06)	0.081	5.37 (0.94-30.76)	0.059
WHO grade	0.95 (0.23-3.85)	0.940	1.97 (0.39-9.88)	0.410	6.10 (0.48-77.16)	0.160
Patient age	0.98 (0.94-1.02)	0.280	0.94 (0.88-1.00)	0.066	1.00 (0.94-1.06)	0.980
Extent of resection	1.13 (0.40-3.20)	0.820	1.00 (0.29-3.46)	1.00	0.94 (0.27-3.34)	0.930
WHO grade 3						
CD133 frequency	n = 24		n = 23 †		n = 24	
CD133-positive cells	24.59 (5.21-116.08)	<0.001	n.d.	1.00	27.88 (3.36-231.13)	0.002
Patient age	0.95 (0.90-1.01)	0.130	0.94 (0.88-1.02)	0.130	1.00 (0.95-1.06)	0.930
Extent of resection	0.50 (0.14-1.74)	0.280	0.72 (0.18-2.92)	0.640	0.99 (0.28-2.91)	0.860
CD133 topology	n = 15		n = 14 †		n = 15	
CD133 cluster formation	4.77 (1.12-20.28)	0.034	3.74 (0.66-21.15)	0.140	0.21 (0.04-1.15)	0.073
Patient age	0.98 (0.92-1.04)	0.570	0.97 (0.90-1.04)	0.370	1.02 (0.95-1.09)	0.590
Extent of resection	0.99 (0.26-3.78)	0.990	1.17 (0.26-5.23)	0.840	1.30 (0.34-4.91)	0.700

Abbreviation: n.d., not determinable.
*The variables were compared in the following ways: CD133-positive cells, >1% versus ≤1%; WHO grade, 3 versus 2; patient age, x + 10 versus x; extent of resection, subtotal versus total; CD133 cluster formation, cluster versus single cells.
† TtMP could not be assessed in one case.

2 and 5 of 15 in WHO grade 3). In addition, patients suffering from grade 3 gliomas more frequently experienced malignant progression to a WHO grade 4 glioma when their tumors expressed higher proportions of CD133-positive cells. Remarkably, in these cases, malignant progression to a higher tumor grade occurred significantly earlier (Fig. 3B) and OS was significantly worse (Fig. 3C). These observations could be confirmed in multivariate analysis for OS in grade 3 tumors and TtMP when analyzing grade 2 and 3 gliomas together (Table 3).

Discussion

Despite its central role in the identification and isolation of brain tumor stem cells, the expression of the CD133 stem cell marker in tumor tissues thus far has not been systematically analyzed. To our knowledge, this study is the first to address this issue. In a large panel of glioma samples, we found frequencies of CD133-positive cells to increase with tumor grade, with many glioblastomas containing >25% positive cells. In contrast, tissue sections of many WHO grade 2 tumors were devoid of immunoreactive cells, probably indicative of a low frequency of CSCs in these less malignant tumors. These observations are in good agreement with frequencies of 19% to 29% positive cells reported for a set of four glioblastoma tissues as well as significantly increased CD133 expression in melanomas compared with banal nevi (4, 21). Additionally, we confirmed the tumor cell origin of CD133-positive cells by colocalization with the EGFRvIII tumor antigen. In addition, we showed that the AC133 epitope of CD133 is only expressed in a subset of nestin-positive cells, suggesting that CD133 expression occurs in a more restricted and possibly less differentiated subpopulation of tumor cells compared with the stem cell and progenitor marker nestin.

Interestingly, in tumors of higher grade, we observed the formation of cell clusters characterized by strong CD133-specific staining and tighter packing of cells compared with the surrounding tissue. These properties suggest that the cell clusters are not random aggregations of CD133-positive cells; we assume that they rather might be due to a shift in the balance of symmetrical and asymmetrical cell division in the CD133-positive cells. Loss of asymmetrical cell division recently was shown to induce tumorigenesis in *Drosophila* neuroblasts (22).

Increased frequencies of CD133-positive cells as well as the presence of clusters of positive cells were significant prognostic factors in gliomas, independent of tumor grade, extent of resection, and patient age. In clinical practice, these findings might be particularly relevant in the case of patients with WHO grade 2 and 3 tumors for whom the further course of disease can vary substantially. We were able to show that expression of CD133 could serve as a prognostic factor for tumor regrowth, malignant progression, and patient survival.

However, we think that the importance of this study goes beyond showing the putative clinical benefit of CD133 as a marker. Currently, little data exist on the clinical relevance of CSCs. Previous studies described properties of CSCs that could well explain many clinical features of cancer, such as recurrence, metastasis, and therapy resistance (4–7, 23, 24). One of these studies also tested whether the prevalence of putative breast CSCs in the tumor was relevant to patient outcome, but no correlation was found (23). In light of the

results reported here, this may seem surprising; however, this apparent discrepancy very well might be due to the fact that in breast cancer a different combination of surface markers ($CD44^{+/}CD24^{low}$) is used to define the putative CSCs. These markers might be less specific than CD133, as indicated by the finding that normal breast tissue contains up to 40% of $CD44^{+/}CD24^{low}$ cells, making it very difficult to isolate the effect of the CSC subpopulation on survival (23). Here, for the first time, we presented a direct link between the expression of a CSC antigen and patients' outcome. These data provide strong supportive evidence for the CSC model and the clinical relevance of the CD133-positive cell population in gliomas.

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