



HAL
open science

CD133 expression is associated with small round blue cell tumour morphology in human CNS neoplasms .

Jens Schittenhelm, Perikles Simon, Patrick Nikolaus Harter, Cornelia Zachskorn, Holger Schlaszus, Frauke Röttger, Monika Winkels, Michael Weller, Richard Meyermann, Michel Mittelbronn

► **To cite this version:**

Jens Schittenhelm, Perikles Simon, Patrick Nikolaus Harter, Cornelia Zachskorn, Holger Schlaszus, et al.. CD133 expression is associated with small round blue cell tumour morphology in human CNS neoplasms .. Histopathology, 2011, 10.1111/j.1365-2559.2011.03802.x . hal-00626289

HAL Id: hal-00626289

<https://hal.science/hal-00626289>

Submitted on 25 Sep 2011

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



CD133 expression is associated with small round blue cell tumour morphology in human CNS neoplasms .

Journal:	<i>Histopathology</i>
Manuscript ID:	HISTOP-02-10-0075.R1
Wiley - Manuscript type:	Original Article
Date Submitted by the Author:	28-Jun-2010
Complete List of Authors:	Schittenhelm, Jens Simon, Perikles Harter, Patrick Zachskorn, Cornelia Schlaszus, Holger Röttger, Frauke Winkels, Monika Weller, Michael Meyermann, Richard Mittelbronn, Michel; Edinger Institute, Neurological Institutue
Keywords:	CD133, CNS neoplasms, stem cells



CD133 expression is associated with small round blue cell tumour morphology in human CNS neoplasms.

Deleted: in primary and secondary human CNS neoplasms

Deleted: (SRBCT)

Deleted: s

Jens Schittenhelm¹, Perikles Simon², Patrick N. Harter⁶, Cornelia Zachskorn⁶, Holger Schlaszus¹, Frauke Röttger³, Monika Winkels³, Michael Weller^{4,5}, Richard Meyermann¹, Michel Mittelbronn⁶

Deleted: Zysk³

Deleted: ¹,

¹Institute of Brain Research, University of Tuebingen, Germany; ²Department of Sports Medicine, Rehabilitation and Disease Prevention, Johannes Gutenberg University, Mainz, Germany ³Miltenyi Biotec GmbH, Bergisch-Gladbach, Germany ; ⁴Department of Neurology, Hertie-Institute of Clinical Brain Research, University of Tuebingen, Germany; ⁵Department of Neurology, University Hospital of Zurich, Switzerland; ⁶Institute of Neurology (Edinger Institute), Goethe University, Frankfurt, Germany

Deleted: Johann Wolfgang

Address for correspondence:

Michel Mittelbronn, MD

Institute of Neurology (Edinger Institute)

Johann Wolfgang Goethe University

D-60325 Frankfurt

Germany

Tel: +49(0)69-6301-84169

Fax: +49(0)69-6301-84150

Key words: CD133, CNS neoplasms, stem cells

Running title: CD133 in CNS neoplasms

Abstract:

Aims: CD133 is considered as a marker for brain tumour initiating cells. However, most data about CD133 is derived from animal or *in vitro* studies. The aim of our study was to characterize CD133 expression, distribution and morphological features of CD133⁺ cells in primary and secondary human CNS neoplasms.

Methods and results: Tumours were analyzed by realtime RT-PCR, western blot, flow cytometry and immunohistochemistry. Our results show that only small round blue cell tumours (SRBCT) exhibit strong and consistent CD133 expression. Interestingly, glioblastomas, large cell carcinomas or sarcomas were negative for CD133. Only single glioblastomas with focal small cell component exhibited CD133 immunoreactivity in the SRBCT component. In addition, CD133 expression did not correlate with the expression of other markers associated with stem cell differentiation including CD15 or nestin.

Conclusions: This indicates that CD133 expression in human CNS neoplasms may be independent from the grade of malignancy but strongly correlates with SRBCT morphology. Together with recent findings showing that CD133 is regulated by hypoxia and CD133⁺ cells exhibiting also stem cell properties, our data strongly questions the suitability of CD133 as a brain tumour stem cell marker *in vivo*.

Deleted: ¶
¶

Deleted: stem or progenitor

Deleted: M

Deleted: in brain tumour research

Deleted: the current

Deleted: differential

Deleted: in all methods applied and that almost all tumour cells are strongly immunoreactive for CD133 in many SRBCT cases

Deleted: a

Deleted: transition to primitive neuroectodermal tumour

Deleted: s

Deleted: tumour type

Deleted: or malignancy

Deleted: this calls

Deleted:

Deleted: sole

Deleted: into question

Formatted: Font: Italic

Introduction:

Over 20 years ago, first evidence, supported the hypothesis that neoplasms might arise from common stem cells capable to give rise to tumours of various differentiation.¹ Since human stem cells possess the capacity for self-renewal as well as unlimited growth and current treatment strategies mostly fail to eradicate tumour stem cell,² Therefore, the actual aim is not only to treat the tumour bulk, but also to focus on cancer stem cells.³ Cancer stem cells are functionally defined by assays of self-renewal, multilineage differentiation and sphere formation and it was claimed that these cells are could be selected by the five-transmembrane protein CD133 (prominin-1).⁴ After stimulation with either epidermal growth factor (EGF) or basic fibroblast growth factor (bFGF) multipotent, self-renewing neural stem cells proliferate within the spheres and are considered as candidate cells for autologous transplantation after neuronal loss.^{5,6}

The CD133 antigen was detected on germ cells and on progenitor cells of all germ layers, including ectoderm, mesoderm and entoderm as well as neuroectoderm.⁷⁻¹¹

More recently, different splice isoforms of the CD133 antigen were described.^{7,12}

While CD133-1 was stronger expressed in the fetal brain and adult skeletal muscle, CD133-2, (the 9 amino acid shorter variant of CD133-1), showed higher expression levels on hematopoietic stem cells and was inversely correlated with, the terminal differentiation marker involucrin pointing to a spatio-temporal down-regulation of CD133-2 with the grade of cell maturation.¹² The AC133/AC141 epitopes bound by the widely used monoclonal CD133 antibodies are glycosylated and regulated independently of the much more widespread expression of their mRNAs.¹³

While the number of CD133⁺ cells increased with the grade of malignancy in some tumours like melanocytic neoplasms, others such as prostate cancer failed to show a

Deleted: More than

Deleted: s

Deleted: were supplied for

Deleted: these

Deleted: clones, they are now accused being responsible for the fact that cancer is still one of the leading causes of morbidity and mortality

Deleted: functionally through

Deleted: selectable

Deleted: Within the spheres after stimulation

Deleted: Within the recent years

Deleted: more prominent

Deleted: ,

Deleted: ,

Deleted: to

Deleted: o-morphological

correlation of the amount of CD133⁺ cells and tumour grade.^{14,15} Very recently, CD133 expressing tumour cells were shown to be responsible for radio- and chemoresistance in gliomas,^{16,17}

Deleted: aroused suspicion of conferring

Deleted: glioma

Deleted: and being the source of tumour recurrence after radiation as well as being responsible for the resistance to chemotherapy

Deleted: bear

Another study revealed that CD133 positive progenitor cells considerably contribute to tumour neo-vascularization.¹⁸ One way to overcome the long-term propagation of CD133 positive stem cells in neoplasms is the blockade of the Notch signaling pathway finally leading to cell cycle exit, apoptosis and cellular differentiation via its downstream target Hes1.¹⁹ The amount of circulating CD133 positive cells seems to

Deleted: pathway

be regulated by chemotactic factors such as interleukin-8 which on the other hand is significantly upregulated in various neoplasms.²⁰⁻²³ In an epithelial cell model, one feature of both CD133 isoforms s seems to consist in organizing plasma membrane protrusions.²⁴ Since morphological features are potentially mediated by CD133 under

Deleted: 1 and 2

normal conditions, the aim of our study was not to use functional assays which may alter number and expression level of CD133-positive cells but rather to analyze morphological properties and the distribution pattern of the so-called CD133- positive tumour cells within various human neoplasms under conditions that more closely resemble the *in vivo* conditions in the human brain. In the present study we analyze tumours not only *in vitro* but also fresh viable tumour tissue and paraffin embedded specimens, since many cell culture conditions may irrevocably alter the cells.²⁵ This

Formatted: Font: Italic

Formatted: Font: Italic

might be important for further therapeutical approaches focussing on the detection of tumour stem cells. Herein, we demonstrate that CD133⁺ expression is strongly associated with a small round blue cell tumour (SRBCT) phenotype representing neoplasms with a very primitive morphological differentiation. In contrast, other than SRBCTs – even though being very malignant – were almost devoid of CD133⁺ cells.

Deleted: stem cell

Deleted: in neuroepithelial tumours

Deleted:

Deleted: ¶

Deleted: ¶

Materials and Methods:

Cell lines

CD34-positive stem cells, burkitt lymphoma cells, the malignant glioma cell lines A172, LNT-229, LN-308, TG98G, U373MG (kind gift of Dr. N. Tribolet, Lausanne), 4 different neuroblastoma cell lines (N-Myk single copy, N-Myk 25, N-Myk 30, N-Myk 100), the retinoblastoma cell line WERI-Rb1, the medulloblastoma cell line DAOY (kind gift of Dr. K. Schilbach, Tübingen) were maintained in DMEM supplemented with 2 mM L-glutamine (Gibco Life Technologies, Paisley, UK), 10% FCS (Biochrom KG) and penicillin (100 IU/ml) / streptomycin (100 µg/ml) (Gibco). CD34+ stem cell were mobilized in healthy donors. Regimen for mobilization of donor stem cells was 10µg/kg/g G-CSF s.c. 5 days prior to leucaphereses, performed on days 5 and 6. CD34+ progenitors were positively selected from leucapheresis products by magnetic-activated cell sorting using Clinimax CD34 reagent and Clinimacs device from Miltenyi Biotec (Bergisch Gladbach, Germany). Purity of CD34+ population was > 99.99%. Progenitor cells were suspended in DMSO and stored in liquid nitrogen until use. Informed consent was obtained from all CD34+ cell donors in accordance with the Declaration of Helsinki. In addition, several primary glioblastoma cells were cultured under different media conditions.

Patients and tissue samples

We investigated a total of 641 paraffin tumour samples obtained from the brain tumour bank of the Institute of Brain Research, University of Tuebingen. These consisted of 295 samples of 213 glioblastoma cases (including 13 cases with known small cell component), 173 samples of 132 astrocytoma cases (including 27 WHO grade I, 44 WHO grade II and 61 WHO grade III tumours), 12 primary cerebral B-cell

Deleted: ¶

Deleted: PNET

lymphomas, 67 medulloblastomas (including 11 desmoplastic variants), 10 CNS-PNET, 19 small cell lung carcinoma metastases, 30 non-small cell carcinoma metastases, 10 melanomas, 10 sarcoma metastases, 6 neuroblastomas (2 peripheral abdominal, 1 central spinal, 3 esthesioneuroblastomas), 5 pineoblastomas and 4 retinoblastomas (Supplementary Table 1). In addition 79 samples of 24 autopsy cases with normal human brain belonging to an established brain bank (Brain Bank Tübingen) and 26 specimens of cases with biopsies of non-neoplastic CNS lesions (Supplementary Table 2) were also included. In all cases mentioned above full mount sections were stained with the exception of astrocytoma samples where additional samples were partially constructed as tissue microarray (Manual Tissue Microarrayer, Beecher Instruments, Sun Prairie, USA; two representative cores measuring 600µm for each tumour; n=78 WHO grade IV, n=75 WHO grade III, n=35 WHO grade II and n=24 WHO grade I). For western blot and RT-PCR frozen tissue of selected CD133-positive and negative cases (n=25) was studied. Utilization of the human specimens was in accordance with the University's ethics commission. Pathological diagnoses were made by at least two experienced neuropathologists according to the WHO criteria.²⁶

Deleted:

Deleted: were also included.

Deleted: available

Preparation of RNA and reverse transcription to cDNA

Total RNA was isolated from the various cryo-preserved tissues and cell lines after homogenisation using Trizol Reagent (Invitrogen, Karlsruhe, Germany). All samples were subjected to treatment with RNase free DNase I (Roche Diagnostics, Mannheim, Germany). Consecutively, 500 µg of each RNA sample was reverse-transcribed with 200 U of Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV-RT, Promega, Mannheim, Germany), using 0.5 µg of Random Primer

(Promega). Glyceraldehyde-3-phosphate-dehydrogenase (gapdh) served as housekeeping-gene.

Quantitative real-time RT-PCR

Quantitative real-time RT-PCR including primer-design and control of the specificity and efficiency of the primer pairs was done as described previously.^{27,28} The sequences of the PCR primers used in this study are listed in table 1. All primers were chosen to span an intron in order to control for and avoid priming of gDNA contaminations. The sense primers were constructed to contain an intron spanning internal to the respective sense primer, which was specific for the respective splice variant of CD133 (see Figure 1 for details). This procedure enabled us to avoid any priming of gDNA, since the sense primer are cDNA specific and to ensure maximum comparability of differential CD133_1 and CD133_2 expression by reducing technical artefacts using the same reverse primer and generating similar amplicon lengths. Quantitative real time RT-PCR was linear over the expression range. For the evaluation of all PCR runs, 'Cycle Threshold' values (CTs) obtained for the different amplicons were processed to Mean Normalized Expression (MNE) as described previously.²⁹ Replicates within one plate were used for statistical analysis. MNE values and the respective 99%-confidence intervals (CI) of the means are reported.

Deleted: .

Immunoblotting

For immunoblotting, 12 tumour samples, 5 established cell lines and 4 primary glioblastoma cell cultures were used. Tissue specimens were homogenized and placed overnight on ice in Tris-HCl-buffer pH 7.6 containing 125mM NaCl, 2.5% Brij 96/98 and a protease inhibitor cocktail (Complete™, Roche). After centrifugation the

Deleted: 8

Deleted: and

Deleted:

remaining pellet was resuspended in 50mM Tris; 150mM NaCl, 1% Triton X100 and 1% NP-40 placed on ice for 6 h. Protein concentration was determined photometrically at OD₂₈₀ against lysis buffer. The protein lysates were stored at -80°C pending immunoblotting. Whole-cell extracts for SDS-PAGE were prepared by solubilizing washed cells in hot SDS-sample buffer and incubating at 95°C for 5 minutes. Proteins were separated by 10% SDS-PAGE and subsequently transferred onto nitrocellulose membranes. MBI Fermentas Prestained Protein Ladder was used as molecular weight standard. Following electrophoresis, the samples were transferred to a polyvinylidene difluoride (PVDF) membrane (Hybond, Amersham) and blocked overnight with 5% bovine serum albumin BSA in Tris-buffered-saline-Tween (TBST; 50 mM Tris-HCl pH 7.6, 150 mM NaCl, 0.1% (v/v) Tween-20). The membranes were incubated with primary antibodies in TBST (1µg/ml) against CD133 clones W6B3C1 (recognizing CD133-1) and 293C3 (recognizing CD133-2, both from Miltenyi, Bergisch-Gladbach, DE), Synaptophysin (Biogenex, San Ramon, USA), pan-cytokeratin (Dako, Glostrup, DK) and glial fibrillary acidic protein GFAP (Neomarkers, Fremont, USA). Exposition times were 3 to 5 mins. A subsequent incubation with an antibody against beta-actin (Santa Cruz) was performed to determine equal protein loading of each lane.

Deleted: D

Flow Cytometry

Immunocytofluorescence staining for CD133 monoclonal antibodies W6B3C1 (recognizing CD133-1) and 293C3 (recognizing CD133-2, both from Miltenyi) was performed on cultured cells. 1×10^6 cells were collected, washed in PBS, and incubated with 100µl 3.7% formaldehyde for 30mins. After addition of 1ml PBS with 2mM EDTA and 0.5% bovine serum albumin (PEB), cells were permeabilized in 100µl 0.5 saponin buffer (20mins) before incubation with the primary antibodies for

15mins (5µg/ml in saponin buffer). After a second washing step, cells were incubated with isotype-matching rat anti-mouse IgG fluorochrome-conjugated secondary antibodies at optimal dilutions for another 15mins (Miltenyi). Finally cells were washed again with PEB. One color analysis was performed on a FACS (FACSCalibur, BD, NJ, USA) using scatter gate with a minimum of 5,000 events counted. Isotype controls were performed for each antibody.

Immunohistochemistry

Immunohistochemistry was performed as described previously.³⁰ Briefly, samples were put in 4% formalin (pH 7.4) after surgery and routinely embedded in paraffin. Four-micron-thick sections were stained routinely with hematoxylin and eosin and in adjacent serial sections, immunohistochemistry was performed with mouse immunoaffinity-purified IgG1 antibodies against CD133 clones W6B3C1 (dilution 1:50) and AC133 (1:10, both from Miltenyi). Deparaffinized and rehydrated sections were immunostained by using the Benchmark Immunohistochemistry System (Ventana, Tucson, AZ, USA). A standard protocol including a cell conditioning pretreatment for 60mins was used. I-View-Inhibitor (Ventana) was applied for 4min and endogenous peroxidase of the tissue sections was blocked with 3% H₂O₂ in methanol for 14min. CD133 antibodies were applied with an incubation time of 32 min. Incubation of amplifier A and B for 8min each were followed by avidin and biotin blockers for 4 mins respectively and I-View-Biotin-Ig for 8mins (all Ventana). Before diaminobenzidine/H₂O₂ visualization (8min), incubation with I-View SA-HRP (8min) was applied. Finally, sections were incubated with a copper enhancer (4min) and counterstained with haematoxylin. Positive control (WERI-Rb1 cell culture slides)

and negative controls (omission of the primary antibody from the diluent) were run with each batch of staining

Results:

Both CD133 splice variants are differentially regulated in vivo and in vitro.

CD133_1 as well CD133_2 showed the highest mean normalized expression levels in CD34-positive stem cells and in the retinoblastoma cell line WERI-Rb1 (Figure 2).

Deleted: .

While both variants were detectable in four different neuroblastoma cell lines (N-Myk single copy, N-Myk 25, N-Myk 30, N-Myk 100) and in a medulloblastoma cell line (DAOY), the CD133_2 splice variant was not detectable in two malignant glioma cell lines (A172, LN308) or showed levels just above the threshold (LNT-229). In a burkitt lymphoma cell line only CD133_1 mRNA expression was detectable but not CD133_2. Similar levels of both CD133 mRNA splice variants were observed in vivo in small round and blue cell tumours (medulloblastoma, PNET and pineoblastoma

tumour samples). In contrast CD133_1 expression levels in astrocytic neoplasms were either not detectable or displayed levels just above threshold, while CD133_2 expression was always present in higher levels.

In vitro and in vivo expression of CD133 is consistent only in small round blue cell tumours.

Immunoblot analysis using two different CD133 antibodies (W6B3C1 and 293C3) allowed the detection of 120 kDa bands in WERI-Rb1 and DAOY cell-lines (Figure 3a) but were lacking in U373, T98G and LNT-229 glioma cell lines (data not shown).

Presence of 43 kDa beta-actin or 49 kDa GFAP bands in the latter served as control for protein integrity. Flow cytometry revealed membrane-associated CD133 (W6B3C1 and 293C3) in WERI-Rb1 (Figure 3b) and DAOY (Figure 3c) while only a small minority of glioma cell lines could be considered to express membranous CD133 (Figure 3d). Both lineage specific markers (e.g. CD56, Figure 3b) and CD133

were strongly expressed on SBRCT, while non SRBCT entities showed an absence or only very weak frequency of CD133 positive cells compared to isotype and lineage specific positive controls. The results of flow cytometry and immunoblotting nicely correlated with CD133 expression on cultured cells (Supplementary Figure 2).

In vivo results were similar showing strongest CD133 expression patterns in pineoblastomas and medulloblastomas, while all glioblastomas and most primary glioblastoma cell cultures showed no distinct bands for CD133 (Figure 3a and Supplementary Figure 1).

Deleted: lane

Deleted: .

Deleted: lane

Deleted: .

Deleted: .

Deleted: .

Deleted: with

Deleted: immunoblot

Deleted: examined

Deleted: lane

Deleted: .

CD133 immunoreactivity is mainly restricted to small round blue cell tumours in vivo.

Deleted: *in vivo*

The CD133 antibody 293C3, which recognizes the CD133-2 antigen was not suitable for immunohistochemistry. Two different CD133 clones directed against CD133-1

(W6B3C1 and AC133) showed similar results in test rows, with slightly nicer staining

Deleted: but as the signal of

results for AC133. Therefore, AC133 was used for further evaluation. We examined

Deleted: was more unequivocally interpretable (due to absence of unspecific background staining), this

79 different normal brain samples of 24 autopsy cases, including the cerebrum, the

Deleted: antibody was used

cerebellum, and the brainstem. No AC133-immunoreactive cells were found in normal

Deleted: of all immunostainings

control CNS tissue specimens (Supplementary Figure 3). Six out of 56

Deleted: from all four lobes

medulloblastomas (Fig.4a-c) and 2 of 11 desmoplastic medulloblastoma variants

Deleted: , as well as

showed strong expression of AC133 on the majority of tumour cells. In addition 3 out

Deleted: um

of 5 pineoblastomas (Fig. 4d and e), 4 out of 4 retinoblastomas (Fig. 4f), 1 out of 6

Deleted: of 24 autopsy cases

neuroblastomas (an esthesioneuroblastoma) (Fig. 4g) were immunopositive for

Deleted: data not shown

AC133. Further, formalin-fixed and paraffin embedded cell pellets of different cell

lines exhibiting CD133-positive (W6B3C1 and 293C3) phenotype in both FACS and

WB analysis such as DAOY or WERI-Rb1 (Fig. 4i) kept their immunoreactivity also in

AC133 immunocytochemistry (Supplementary Figure 2). All AC133 positive tumour

cells exhibited the typical morphology of small round and blue cells with large nucleus

and small cytoplasm. In addition all 295 samples of 200 classical glioblastomas (Fig.

4j, k), all 173 samples of 27 WHO grade I, 44 WHO grade II and 61 WHO grade III

astrocytomas remained negative for AC133. We further examined non-

Deleted: immuno

neuroepithelial tumours. All 12 primary cerebral B-cell lymphomas, 10 malignant

sarcomas and 10 melanomas were devoid of AC133 expression. 9 out of 19 small

cell lung carcinomas were immunoreactive for AC133 (Fig. 4h) while in the group of

30 non-small cell carcinomas two metastases of breast cancer focally displayed

Deleted: focally

granular immunoreactivity in the cytoplasm of few tumour cells for AC133. None of

the 16 non-small cell lung carcinoma metastases included in this group showed positivity for AC133. CD133 did not at all correlated with the expression of other putative stem cell markers such as CD15 or nestin (Supplementary Figure 4).

Deleted: had positivity

CD133 expression in glioblastomas are restricted to GBM with small cell component.

Deleted: PNET

Since all conventional glioblastomas examined were immunonegative and AC133 expression was restricted mainly to tumour cells with small round and blue phenotype we further examined 13 glioblastomas with more undifferentiated tumour areas – a so called small cell component. These samples only included cases with no known history of prior radiotherapy. In one case we observed immunohistochemical expression of AC133 in a minority of such cells (Fig. 4I). All other normal astrocytic differentiated tumour cells within this glioblastoma remained immunonegative for AC133.

Deleted: additionally

Deleted: known focal

Deleted: parts

Deleted: the

Deleted: primitive neuroectodermal tumour (PNET)

Discussion:

Depending on the tissue of origin, putative cancer stem cells (CSC) have been identified by an array of different markers including CD34 for acute myeloid leukaemia and non small cell lung cancer, CD44 for breast and prostate cancer, while colorectal cancer and brain tumour CSC are considered to express the cell surface marker CD133 (Prominin-1).^{10,15,31-34} CD133 positive brain tumour-initiating cells are capable to proliferate in serum-restricted medium by forming tumourigenic neurospheres and differentiate into mature cells under normal serum-conditions.³⁵

Deleted: further

These cells may exhibit increased radio- and chemoresistance through increased DNA repair mechanisms.^{16,17,36,37} CD133-positive brain tumour cells have also been reported in retinoblastomas and medulloblastomas - tumours known to exhibit a poorly differentiated morphology. Therefore, these tumours are also named small round blue cell tumours.^{34,38} In addition, it has been noted that CD133 positive cells isolated from glioblastomas show absence or very low expression of glial fibrillary acidic protein suggesting that they might not be fully differentiated astrocytic tumour cells.^{37,39}

Deleted: , leading to the classifying term

Deleted: and

Deleted: such isolated

Deleted: consist of

In the current study we aimed to determine the expression levels of CD133 in various brain tumours with emphasis on immunohistochemistry to identify the morphology of CD133-positive tumour cells. Surprisingly, neither immunoblotting nor immunohistochemistry revealed a considerable expression of CD133 in commonly used glioma cell lines and in a large number of glioblastomas, in contrast to high expression levels in many SRBCTs. These results were supported by absent or low-level CD133_1 expression in realtime quantitative PCR and CD133 expression data in flow cytometry being almost similar to isotype controls. This contrasts with data of

Deleted: the

Deleted: any

Deleted: significant

Deleted: studied

Deleted: some

Deleted: of the

other studies that have reported CD133 to be present in gliomas.^{34,40,41} These differences may be in part explained by the fact, that data in other studies were obtained on serum-free cultured neurospheres, while our cell lines were mainly grown under serum conditions. Even the minimal CD133-positive expression in astrocytic cell lines in our flow cytometry analysis could not be detected by immunohistochemistry although we screened a large number of glioblastoma tumour samples. Our observation is further corroborated by an independent flow cytometry study reporting less than 1% viable CD133+ cells in astrocytomas and oligodendrogliomas independent of their WHO grades.⁴² CD133 immunohistochemistry is considered to be difficult, therefore, many groups only use frozen tissue. The use of strong signal amplification techniques results in high numbers of CD133-positive cells also in astrocytomas.⁴³⁻⁴⁵ Since these numbers are higher than flow cytometry results suggests, this indicates a possible overstaining. Our carefully adjusted immunohistochemical protocol permits a strong and unambiguously interpretable staining pattern without background staining on paraffin sections using the AC133 clone.

We were able to demonstrate that CD133 in medulloblastomas is not only expressed *in vitro*³⁶ but also *in vitro* and that other tumours with a small round blue cell phenotypes such as pineoblastoma, neuroblastoma, retinoblastoma and and small cell lung cancer specimens often consist of completely CD133-positive tumour cells.

To our knowledge, CD133 expression in some of the entities in our study (e.g. pineoblastomas) has not been reported so far. The role of the the CD133_2 splice variant which was present in all tumours examined still needs further investigation. Interestingly in epithelial tumours both isoforms seem to be equally distributed at low levels.⁴⁶ CD133 expression was also observed in a significant number of cases of

Deleted: which likely more represents the conditions in human brain

Deleted: in vivo results for

Deleted: astrocytomas

Deleted: FACS

Deleted: was

Deleted: seen in

Deleted: stains

Deleted: s

Deleted: are

Deleted: FACS

Deleted: is

Deleted: is the reason why

Deleted: only

Deleted: ,

Deleted: and used

Deleted: different antibody clones in addition to a

Deleted: resulting

Deleted: in almost half of

Deleted: astrocytomas tumour cells to be positive for

Deleted: FACS

Deleted: In addition, in our experience often high background rates are present in native tissue stains. Using a

Deleted:

Deleted: , we are able to achieve a

Deleted: signal

Deleted: with

Deleted: and

Deleted: fully

Deleted: ,

Deleted: - although expression levels were rather low, still needs further investigation

small cell lung cancer but was not fully restricted to a SRBCT phenotype in epithelial neoplasms in contrast to all neuroepithelial tumours. However, both CD133-positive cases were metastases of breast cancer in which CD133 expression has been reported and for which CD44 (and not CD133) is considered to be a putative stem cell marker.^{33,47}

- Deleted:** exception to
- Deleted:** not restricted to SRBCT phenotype in epithelial neoplasms
- Deleted:** the two
- Deleted:** positive for CD133
- Deleted:** both
- Deleted:** where
- Deleted:** as the

It is noteworthy that multipotent neural stem cells present in the adult brain (located within the subgranular zone of the hippocampal dentate gyrus and the subventricular zone of the lateral ventricles) morphologically resemble undifferentiated small round blue cells and not astrocytes.⁴⁸ Furthermore CD133⁺ cells in neuroepithelial SRBCTs frequently show a perivascular distribution pattern with signs of clonal expansion suggesting that there might be the possibility that these cells are integrated hematopoietic stem cells rather than primary tumour intrinsic cells. The recruitment of hematopoietic progenitor cells by VEGF expression of tumour cells has been already demonstrated.⁵⁴ A close relationship between CD133 positive cells and adjacent endothelial cells was noted in in vitro experiments showing that these cells are able to secrete VEGF.^{16,49} CD133-positive cells not closely related to tumour vessels have been reported to reside in pseudopalisading areas of necroses.⁴⁴ These areas are mainly subjected to low oxygen concentrations. From cell experiments it is known that cultured glioma cells are capable to upregulate CD133 on protein and mRNA level when kept under hypoxic conditions.^{13,50,51}

- Deleted:** morphologically
- Deleted:** and
- Deleted:** a
- Deleted:** Such
- Deleted:** mobilization
- Deleted:** through induction
- Deleted:** tumour produced
- Deleted:** for CD34
- Deleted:** Similar, a
- Formatted:** Font: Italic
- Deleted:** and
- Deleted:** i

Very recent studies demonstrated that primary glioma cell cultures analysed by flow cytometry did also consist of CD133-negative tumour cells that fulfilled stem cell criteria. These cells were tumourigenic in nude mice. Furthermore, CD133-positive cells could also be obtained from CD133-negative tumourigenic cells.^{41,52,53} Even though the CD133 portion represents undifferentiated cells, only a minority of these

- Deleted:** were able to
- Deleted:** in
- Deleted:** FACS
- Deleted:** ,
- Deleted:** not only CD133-positive but
- Deleted:** ,
- Deleted:** ,
- Deleted:** ,
- Deleted:** and that
- Deleted:** an

cells shows the ability of self-renewal and tumorigenesis.^{45,53} Furthermore cell populations depleted of CD133-positive cells are also capable to form glioblastomas.⁵⁵ These data led to the notion that CD133 expression in freshly isolated tumour cells, is considerably different, and therefore might not be an useful approach to detect all tumour stem cells.⁴²

In conclusion, our data shows that CD133 expression in human CNS neoplasms is independent from the blastodermic layer, malignancy or prognosis, but strongly correlates with SRBCT morphology.

Deleted:

Deleted: some authors

Deleted: s

Deleted: freshly isolated is significantly different

Deleted: ay

Deleted: strongly

Deleted: Our data also shows that fresh viable glioma tumour specimens and serum-cultured glioma cells lack a distinct CD133 expression suggesting that presence of CD133 in gliomas may be externally influenced.

Acknowledgements

The authors would like to thank Katrin Trautmann, Ines Kienzler, Janina Drynski, Ulrich Drott and Peter Baumgarten for excellent technical assistance.

References:

1. Terrin BN, Studer EJ, Wood N, Mohanakumar T, Russell EC. Childhood leukemia with simultaneously expressed myeloid and lymphoid markers suggesting stem cell origin. *Am J Hematol* 1985; **20**:175-181.
2. Schulenburg A, Ulrich-Pur H, Thurnher D, *et al.* Neoplastic stem cells: A novel therapeutic target in clinical oncology. *Cancer* 2006; **107**: 2512-2520.
3. Burkert J, Wright NA, Alison MR. Stem cells and cancer: an intimate relationship. *J Pathol* 2006; **209**: 287-297.
4. Uchida N, Buck DW, He D, *et al.* Direct isolation of human central nervous system stem cells. *Proc Natl Acad Sci* 2000; **97**:14720-14725
5. Gritti A, Frolichstahl-Schoeller P, Galli R, *et al.* Epidermal and fibroblast growth factors behave as mitogenic regulators for a single multipotent stem cell-like population from the subventricular region of the adult mouse forebrain. *J Neurosci* 1999; **19**: 3287-3297.
6. Richardson RM, Fillmore HL, Holloway KL, Broaddus WC (2004) Progress in cerebral transplantation of expanded neuronal stem cells. *J Neurosurg* 2004; **100**: 659-671.
7. Fargeas CA, Joester A, Missol-Kolka E, *et al.* Identification of novel Prominin-1/CD133 splice variants with alternative C-termini and their expression in epididymis and testis. *J Cell Sci* 2004; **117**: 4301-4311.
8. Frank NY, Margaryan A, Huang Y, *et al.* ABCB5-mediated doxorubicin transport and chemoresistance in human malignant melanoma. *Cancer Res* 2005; **65**: 4320-4333.

9. Padovan CS, Jahn K, Birnbaum T, *et al.* Expression of neuronal markers in differentiated marrow stromal cells and CD133+ stem-like cells. *Cell Transplant* 2003; **12**: 839-848.
10. Ricci-Vitiani L, Lombardi DG, Pilozzi E, *et al.* Identification and expansion of human colon-cancer-initiating cells. *Nature* 2007; **445**: 111-115.
11. Lee A, Kessler JD, Read TA, *et al.* Isolation of neural stem cells from the postnatal cerebellum. *Nat Neurosci* 2005; **8**: 723-729.
12. Yu Y, Flint A, Dvorin EL, Bischoff J. AC133-2, a novel isoform of human AC133 stem cell antigen. *J Biol Chem* 2002; **277**: 20711-20716.
13. Bidlungmeier S, Zhu X, Liu B. The utility and limitations of glycosylated human CD133 epitopes in defining cancer stem cells. *J Mol Med* 2008; **86**: 1025-1032.
14. Klein WM, Wu BP, Zhao S, Wu H, Klein-Szanto AJ, Tahan SR. Increased expression of stem cell markers in malignant melanoma. *Mod Pathol* 2007; **20**: 102-107.
15. Collins AT, Berry PA, Hyde C, Stower MJ, Maitland NJ. Prospective identification of tumorigenic prostate cancer stem cells. *Cancer Res* 2005; **65**: 10946-10951.
16. Bao S, Wu Q, McLendon RE *et al.* Glioma stem cells promote radioresistance by preferential activation of the DNA damage response. *Nature* 2006; **444**: 756-760.
17. Liu G, Yuan X, Zeng Z *et al.* Analysis of gene expression and chemoresistance of CD133+ cancer stem cells in glioblastoma. *Mol Cancer* 2006; **5**: 6.

18. Bruno S, Bussolati B, Grange C, *et al.* CD133+ Renal Progenitor Cells Contribute to Tumour Angiogenesis. *Am J Pathol* 2006; **169**: 2223-2235.
19. Fan X, Matsui W, Khaki L. *et al.* Notch pathway inhibition depletes stem-like cells and blocks engraftment in embryonal brain tumours. *Cancer Res* 2006; **66**: 7445-7452.
20. Schomig K, Busch G, Steppich B *et al.* Interleukin-8 is associated with circulating CD133+ progenitor cells in acute myocardial infarction. *Eur Heart J* 2006; **27**: 1032-1037.
21. Brat DJ, Bellail AC, Van Meir EG. The role of interleukin-8 and its receptors in gliomagenesis and tumoural angiogenesis. *Neuro-oncol* 2005; **7**:122-133.
22. Melnikova VO, Bar-Eli M. Bioimmunotherapy for melanoma using fully human antibodies targeting MCAM/MUC18 and IL-8. *Pigment Cell Res* 2006; **19**: 395-405.
23. Tas F, Duranyildiz D, Oguz H, Camlica H, Yasasever V, Topuz E. Serum vascular endothelial growth factor (VEGF) and interleukin-8 (IL-8) levels in small cell lung cancer. *Cancer Invest* 2006; **24**: 492-496.
24. Florek M, Bauer N, Janich P *et al.* Prominin-2 is a cholesterol-binding protein associated with apical and basolateral plasmalemmal protrusions in polarized epithelial cells and released into urine. *Cell Tissue Res* 2007; **328**: 31-47.
25. Lee CC, Ye F, Tarantal AF. Comparison of growth and differentiation of fetal and adult rhesus monkey mesenchymal stem cells. *Stem Cells Dev.* 2006; **15**: 209-20.
26. DN Louis, H Ohgaki, ODWiestler, WK Cavenee (eds), Pathology and Genetics of Tumours of Central Nervous System. International Agency for Research on Cancer: Lyon 2007

27. Simon P, Schott K, Williams RW, Schaeffel F. Posttranscriptional regulation of the immediate-early gene EGR1 by light in the mouse retina. *Eur J Neurosci* 2004; **20**: 3371-3377.
28. Simon P, Feldkaemper M, Bitzer M, Ohngemach S, Schaeffel F. Early transcriptional changes of retinal and choroidal TGFbeta-2, RALDH-2, and ZENK following imposed positive and negative defocus in chickens. *Mol Vis* 2004; **10**: 588-597.
29. Simon P. Q-Gene: processing quantitative real-time RT-PCR data. *Bioinformatics* 2003; **19**: 1439-1440.
30. Mittelbronn M, Capper D, Bunz B *et al.* De novo erythropoietin receptor (EPO-R) expression in human neoplastic glial cells decreases with grade of malignancy but is favourably associated with patient survival. *Neuropathol Appl Neurobiol* 2007; **33**: 299-307.
31. Lapidot T, Sirard C, Vormoor *et al.* A cell initiating human acute myeloid leukaemia after transplantation into SCID mice. *Nature* 1994; **367**: 645-648.
32. Kim CF, Jackson EL, Woolfenden AE *et al.* Identification of bronchioalveolar stem cells in normal lung and lung cancer. *Cell* 2005; **121**: 823-835.
33. Al-Hajj M, Wicha MS, Benito-Hernandez A, Morrison SJ, Clarke MF. Prospective identification of tumourigenic breast cancer cells. *Proc Natl Acad Sci USA* 2003; **100**: 3983-3988.
34. Singh SK, Hawkins C, Clarke ID, *et al.* Identification of human brain tumour initiating cells. *Nature* 2004; **432**: 396-401.
35. Piccirillo SG, Reynolds BA, Zanetti N *et al.* Bone morphogenetic proteins inhibit the tumourigenic potential of human brain tumour-initiating cells. *Nature* 2007; **444**: 761-765.

36. Blazek ER, Foutch JL, Maki G. Daoy medulloblastoma cells that express CD133 are radioresistant relative to CD133⁻ cells, and the CD133⁺ sector is enlarged by hypoxia. *Int J Radiat Oncol Biol Phys* 2007; **67**: 1-5.
37. Salmaggi A, Boiardi A, Gelati M, *et al.* Glioblastoma-derived tumourspheres identify a population of tumour stem-like cells with angiogenic potential and enhanced multidrug resistance phenotype. *Glia*. 2006; **54**: 850-860.
38. Seigel GM, Hackam AS, Ganguly A, Mandell LM, Gonzalez-Fernandez F. Human embryonic and neuronal stem cell markers in retinoblastoma. *Mol Vis* 2007; **13**: 823-832.
39. Qiang L, Yang Y, Ma YJ, *et al.* Isolation and characterization of cancer stem like cells in human glioblastoma cell lines 2009; **279**: 13-21.
40. Beier D, Hau P, Proescholdt M, *et al.* (CD133⁺) and CD133⁻) glioblastoma-derived cancer stem cells show differential growth characteristics and molecular profiles. *Cancer Res* 2007; **67**:4010-4015.
41. Liu Q, Nguyen DH, Dong Q, *et al.* Molecular properties of CD133⁺ glioblastoma stem cells derived from treatment-refractory recurrent brain tumors. *J Neurooncol*. 2009; **94**:1-19.
42. Clément V, Dutoit V, Marino D, Dietrich PY, Radovanovic I. Limits of CD133 as a marker of glioma self-renewing cells. *Int J Cancer* 2009; **125**: 244-248.
43. Christensen K, Schrøder HD, Kristensen BW. CD133 identifies perivascular niches in grade II-IV astrocytomas. *J Neurooncol* 2008; **90**: 157-170.
44. Ma YH, Mentlein R, Knerlich F, Kruse ML, Mehdorn HM, Held-Feindt J. Expression of stem cell markers in human astrocytomas of different WHO grades. *Neurooncol* 2008; **86**: 31-45

45. Zeppernick F, Ahmadi R, Campos B *et al.* Stem cell marker CD133 affects clinical outcome in glioma patients. *Clin Cancer Res* 2008; **14**: 123-129
46. Ferrandina G, Bonanno G, Pierelli L *et al.* Expression of CD133-1 and CD133-2 in ovarian cancer. *Int J Gynecol Cancer* 2008; **18**: 506-514.
47. Wright MH, Calcagno AM, Salcido CD, Carlson MD, Ambudkar SV, Varticovski L. Brca1 breast tumors contain distinct CD44+/CD24- and CD133+ cells with cancer stem cell characteristics. *Breast Cancer Res.* 2008; **10**: R10.
48. Ramalho-Santos M, Yoon S, Matsuzaki Y, Mulligan RC, Melton DA. Stemness: transcriptional profiling of embryonic and adult stem cells. *Science* 2002; **298**: 597-600
49. Calabrese C, Poppleton H, Kocak M *et al.* A perivascular niche for brain tumour stem cells. *Cancer Cell* 2008; **11**: 69-82.
50. McCord AM, Jamal M, Shankavarum UT, Lang FF, Camphausen K, Tofilon PJ. Physiologic oxygen concentration enhances the stem-like properties of CD133+ human glioblastoma cells in vitro. *Mol Cancer Res* 2009; **7**: 489-497
51. Platet N, Liu SY, Atifi ME, Oliver L, Vallette FM, Berger F, Wion D. Influence of oxygen tension on CD133 phenotype in human glioma cell cultures. *Cancer Lett* 2007; **258**: 286-290.
52. Günther HS, Schmidt NO, Phillips HS, *et al.* Glioblastoma-derived stem cell-enriched cultures form distinct subgroups according to molecular and phenotypic criteria. *Oncogene* 2008; **27**: 2897-2909.
53. Taylor MD, Poppleton H, Fuller C *et al.* Radial glia cells are candidate stem cells of ependymoma. *Cancer Cell* 2005; **8**: 323-335.

54. Lyden D, Hattori K, Dias S, *et al.* Impaired recruitment of bone-marrow-derived endothelial and hematopoietic precursor cells blocks tumor angiogenesis and growth. *Nat Med* 2001; **7**: 1194-201.
55. Nishide K, Nakatani Y, Kiyonari H, Kondo T. Glioblastoma formation from cell population depleted of Prominin1-expressing cells. *PLoS One* 2009; **4**: e6869.

For Peer Review

Figure legends:

Figure 1: Position of sense and antisense primers on the different positions for the CD133_1 and CD133_2 splice variants generating similar amplicon lengths. The pairs were constructed spanning at least one intron to avoid priming possible genomic DNA contaminations.

Figure 2: Realtime RT-PCR results. Course of expression for the different CD_133 splice variants in cultured cells and excised tumour samples. Means and 99% CIs for three measurement repetitions are given.

Figure 3: a) In vivo and in vitro immunoblotting results (CD133, clone W3B6C1: 120kDa band, control: beta-actin: 43 kDa band) b) FACS analysis of retinoblastoma cell line WERI-Rb1 (left: unstained, middle: CD56 as positive control and right: CD133, clone W6B3C1), c) FACS data of medulloblastoma cell line DAOY (left: unstained, middle: CD133 - 293C3 and right: CD133 – W6B3C1) d) FACS data for glioblastoma cell lines T98G, U373, LN 229, left: unstained, middle: GFAP, right: CD133 – W6B3C1).

Deleted: lane

Deleted: lane

Figure 4: Immunohistochemical results (CD133, clone AC133): Strong membranous staining of small round blue cell tumours: a-c): medulloblastomas; d-e) pineoblastomas; e) ; f) retinoblastoma; g) neuroblastoma (esthesioneuroblastoma); h) metastasis of small cell lung carcinoma; i) retinoblastoma cells (WERI-Rb1). Within the CD133-positive small round blue cell tumours, endothelial cells or perivascular non-neoplastic cells remain immunonegative. j-k) classical

glioblastomas without small round blue cell features were completely immunonegative for CD133; l) In contrast only small round and blue cells of a glioblastoma partially exhibiting small cell differentiation show a rather diffuse pattern.

Deleted: partially PNET differentiated

Supplementary Figure 1: Immunoblotting results (CD133, clone W3B6C1: 120kDa band, control: beta-actin: 43 kDa band). WERI-Rb1 cells were used as positive control. In addition, fresh frozen tissue of 4 different primary glioblastomas and primary cell cultures of 3 different primary glioblastomas under different growth conditions were investigated.

Supplementary Figure 2: Immunocytochemical analysis of CD133 expression in cell lines. Cells were fixed in 4%-buffered formalin (pH 7.4) and centrifuged for 5 min at 1700 U/min. This fixed cell pellet was embedded in paraffin and stained for CD133 expression (AC133): a) WERI-Rb1 retinoblastoma cell line strongly expressed CD133 on most cells; b) DAOY medulloblastoma cell line exhibited several CD133-positive cells (arrows) while glioma cell lines such as T98G (c) remained negative.

Supplementary Figure 3: CD133 expression in biopsies of non-neoplastic CNS diseases. In contrast to small round blue cell neoplasms such as medulloblastomas (a), non neoplastic CNS diseases including epilepsies (b: Wyler grade 3; c: Wyler grade 4) as well as surrounding tissue of arterio-venous malformations (d), epilepsy brains without gross morphological changes (e) or cerebellar bleeding (f) remained negative for CD133.

Supplementary Figure 4: Comparison of CD133, CD15 and nestin expression in CNS neoplasms (n=30). (A) WERI-Rb1 for CD133, (B) a brain infarction for CD15, (C) and a fetal brain of the for nestin were used as positive controls. Small round blue cell neoplasms including pineoblastoma (D-F) and medulloblastoma (G-I) were labelled for CD133 (D, G), CD15 (E, H) and nestin (F, I). CD133-negative normal brain (J) and astrocytoma ((K) pilocytic astrocytoma WHO grade I, (L) diffuse astrocytoma WHO grade II, (M) anaplastic astrocytoma WHO grade III and (N, O) glioblastoma WHO grade IV) cases were further investigated for nestin (J-N) and CD15 (N).

For Peer Review

Tables:

Table 1: Primer sequences used in this study for real-time RT-PCR. All primers were chosen to span at least one intron to control for and exclude the possibility of contamination with genomic DNA.

Formatted: fulltext-text
fulltext-indent, Justified

Formatted: English U.S.

mRNA	Sense Primer	Antisense Primer	Product Size
<i>Cd133_1</i>	5'TTG ATT ATG ACA AGC CAG AAA CTG3'	5'ACC CCA CCA GAG GCA TCA G3'	120 bp
<i>Cd133_2</i>	5'GAC AAG ATT GTC TAC TAT GAA GCA GG3'	5'ACC CCA CCA GAG GCA TCA G3'	85 bp
<i>GAPDH</i>	5'GCC TGG AGA AAC CTG CCA AG3'	5'TGG TCC TCA GTG TAG CCC AAG3'	96 bp

Deleted: Table 1: Primer sequences used in this study for real-time RT-PCR. All primers were chosen to span at least one intron to control for and exclude the possibility of

Supplementary Table 1: Epidemiological data. For tumour specimens received from other pathological institutes, no epidemiological data were obtained (e.g. neuroblastomas, retinoblastomas).

Deleted: contamination with genomic DNA.

Diagnosis	mean age	sex (m/f)
Glioblastoma	58.4	54/35
Astrocytoma WHO III	46.0	32/20
Astrocytoma WHO II	43.7	20/16
Astrocytoma WHO I	21.1	16/9
Medulloblastoma	19.7	41/25
PNET	44.1	9/1
Neuroblastoma	N/A	N/A
Carcinoma	63.4	24/9

For Peer Review

Supplementary Table 2: Epidemiological data of cases with biopsies of non-neoplastic CNS lesions.

Deleted: 1

Diagnosis	Age	Sex
pineal cyst + adjacent CNS	40	M
pineal cyst + adjacent CNS	28	M
pineal gland + adjacent CNS	58	F
mesial temporal sclerosis Wyler 3	35	F
mesial temporal sclerosis Wyler 4	28	M
mesial temporal sclerosis Wyler 3	37	M
mesial temporal sclerosis Wyler 3	35	F
pineal cyst + adjacent CNS	7	M
pineal gland + adjacent CNS	27	M
CNS + Stroke	54	M
CNS + CAA	81	M
CNS + hematoma	55	M
Cerebellum	84	F
CNS + AVM	20	M
PML	64	F
CNS + cavernoma	52	M
CNS + AVM	60	M
CNS + AVM	27	M
normal CNS	55	F
Cerebellum + bleeding	70	F
CNS + cavernoma	21	M
CNS + cavernoma	19	M
CAA	83	M
Stroke	36	F
mesial temporal sclerosis Wyler 4	36	M
CNS + cavernoma	38	M

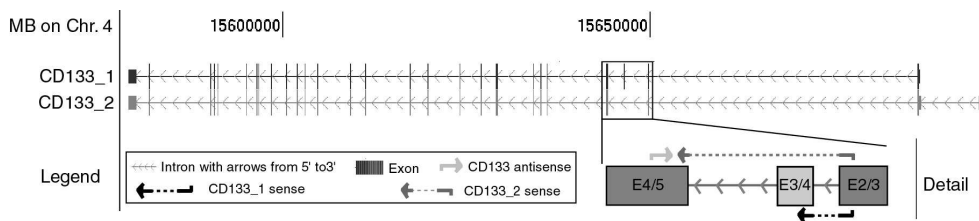


Figure 1: Position of sense and antisense primers on the different positions for the CD133_1 and CD133_2 splice variants generating similar amplicon lengths. The pairs were constructed spanning at least one intron to avoid priming possible genomic DNA contaminations.
157x33mm (300 x 300 DPI)

For Peer Review

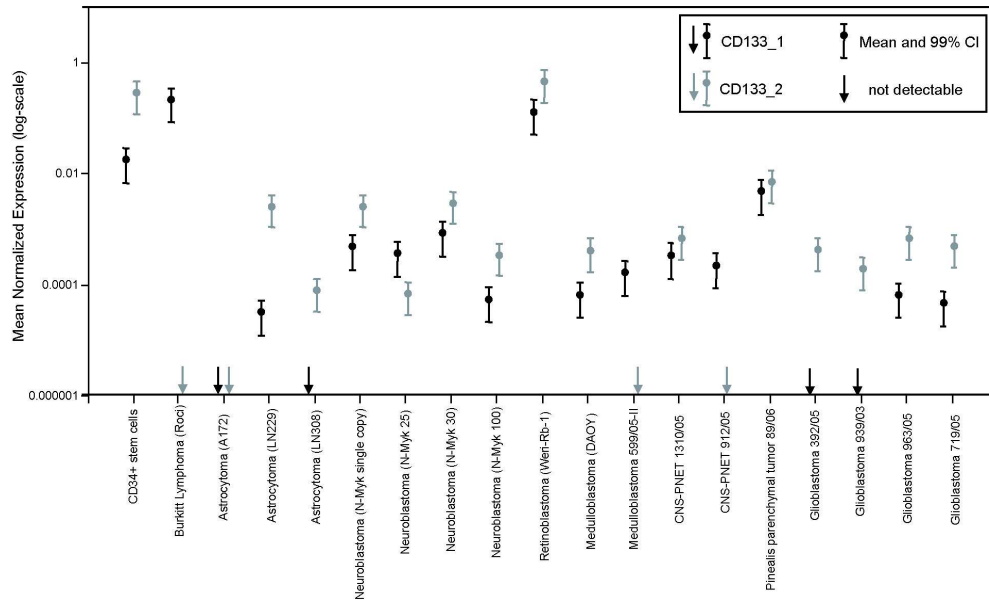


Figure 2: Realtime RT-PCR results. Course of expression for the different CD_133 splice variants in cultured cells and excised tumour samples. Means and 99% CIs for three measurement repetitions are given.

685x407mm (72 x 72 DPI)

Review

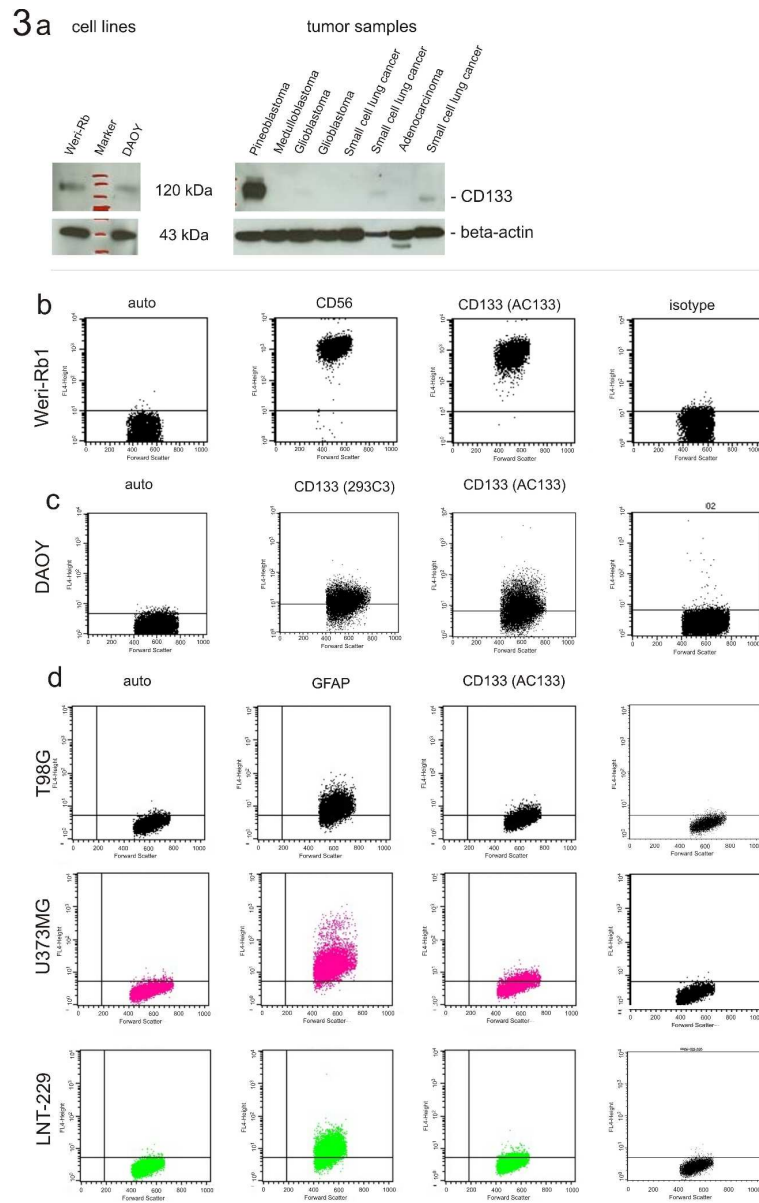


Figure 3: a) In vivo and in vitro immunoblotting results (CD133, clone W3B6C1: 120kDa band, control: beta-actin: 43 kDa band) b) FACS analysis of retinoblastoma cell line WERI-Rb1 (left: unstained, middle: CD56 as positive control and right: CD133, clone W6B3C1), c) FACS data of medulloblastoma cell line DAOY (left: unstained, middle: CD133 - 293C3 and right: CD133 - W6B3C1) d) FACS data for glioblastoma cell lines T98G, U373, LN 229, left: unstained, middle: GFAP, right: CD133 - W6B3C1).
226x351mm (300 x 300 DPI)

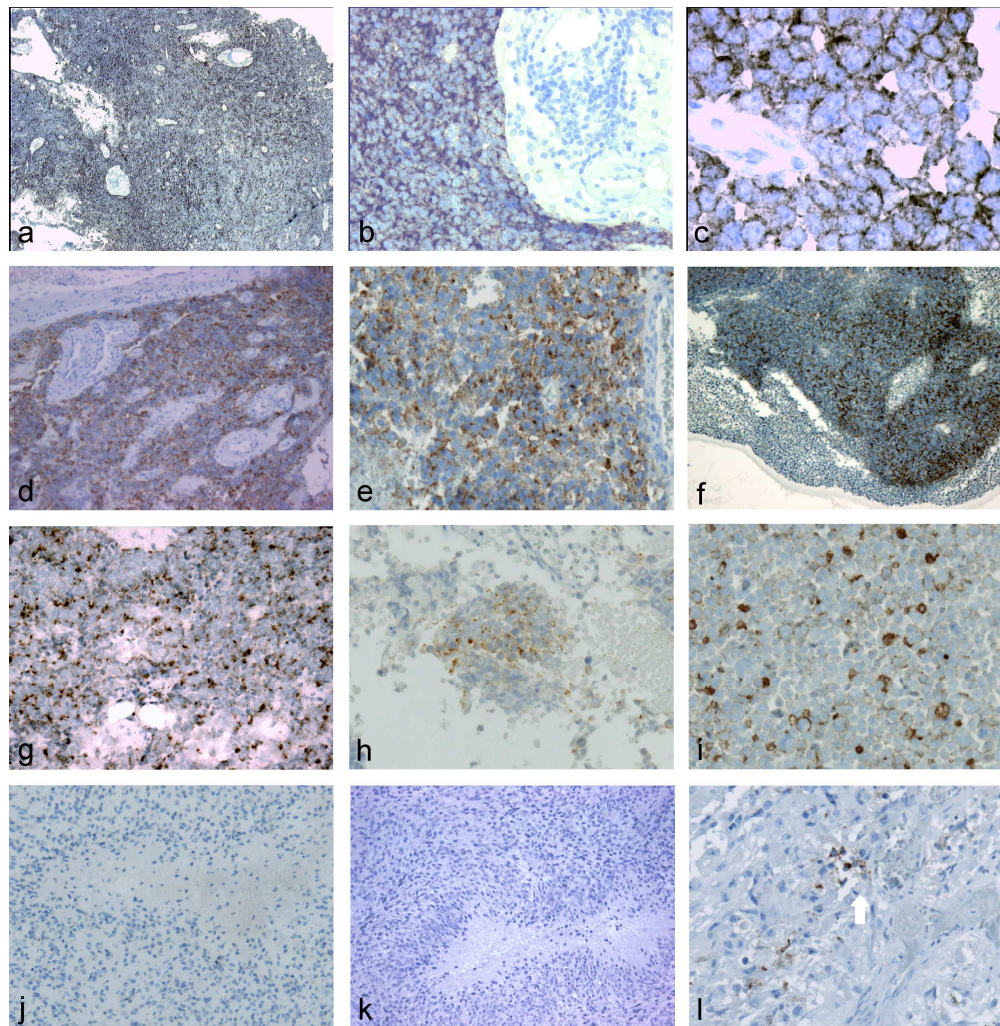
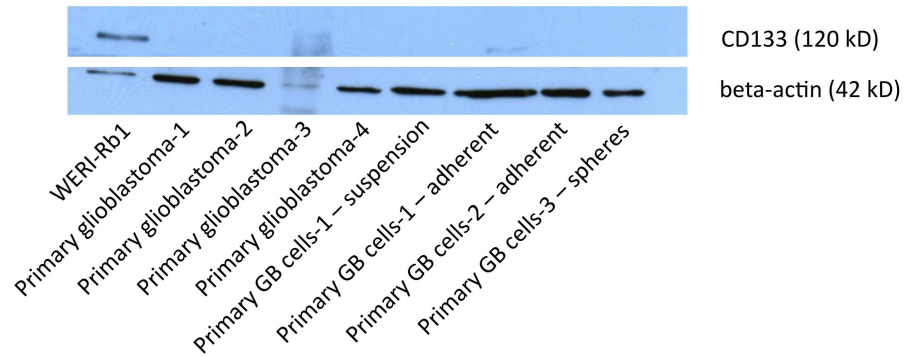
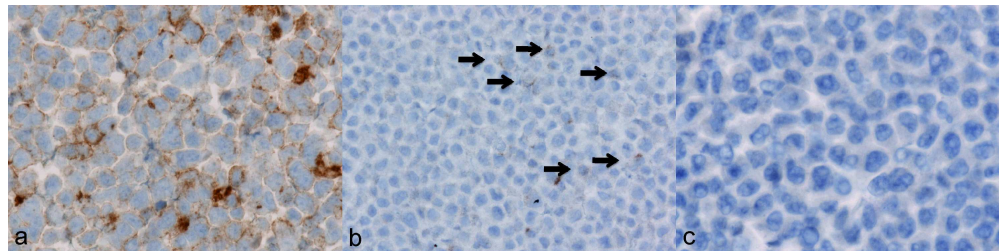


Figure 4: Immunohistochemical results (CD133, clone AC133): Strong membranous staining of small round blue cell tumours: a-c): medulloblastomas; d-e) pineoblastomas; e) ; f) retinoblastoma; g) neuroblastoma (esthesioneuroblastoma); h) metastasis of small cell lung carcinoma; i) retinoblastoma cells (WERI-Rb1). Within the CD133-positive small round blue cell tumours, endothelial cells or perivascular non-neoplastic cells remain immunonegative. j-k) classical glioblastomas without small round blue cell features were completely immunonegative for CD133; l) In contrast only small round and blue cells of a glioblastoma partially exhibiting small cell differentiation show a rather diffuse pattern.

874x893mm (72 x 72 DPI)



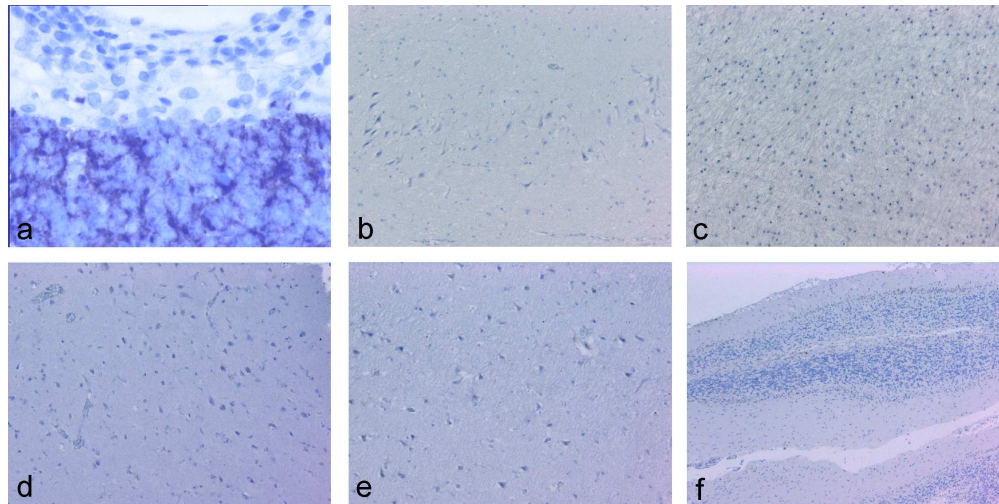
Supplementary Figure 1: Immunoblotting results (CD133, clone W3B6C1: 120kDa band, control: beta-actin: 43 kDa band). WERI-Rb1 cells were used as positive control. In addition, fresh frozen tissue of 4 different primary glioblastomas and primary cell cultures of 3 different primary glioblastomas under different growth conditions were investigated.
1057x793mm (72 x 72 DPI)



Supplementary Figure 2: Immunocytochemical analysis of CD133 expression in cell lines. Cells were fixed in 4%-buffered formalin (pH 7.4) and centrifuged for 5 min at 1700 U/min. This fixed cell pellet was embedded in paraffin and stained for CD133 expression (AC133): a) WERI-Rb1 retinoblastoma cell line strongly expressed CD133 on most cells; b) DAOY medulloblastoma cell line exhibited several CD133-positive cells (arrows) while glioma cell lines such as T98G (c) remained negative.

1064x265mm (72 x 72 DPI)

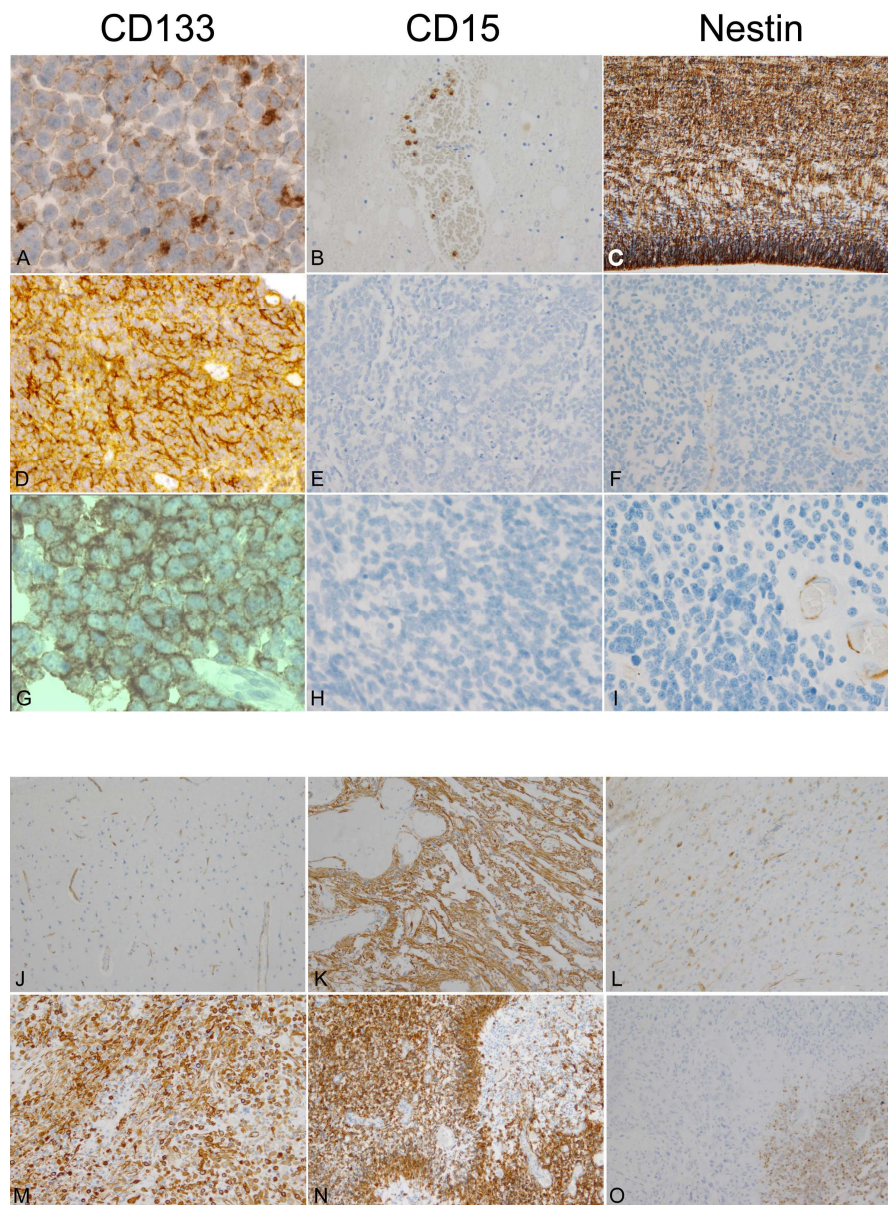
Peer Review



Supplementary Figure 3: CD133 expression in biopsies of non-neoplastic CNS diseases. In contrast to small round blue cell neoplasms such as medulloblastomas (a), non neoplastic CNS diseases including epilepsies (b: Wyler grade 3; c: Wyler grade 4) as well as surrounding tissue of arteriovenous malformations (d), epilepsy brains without gross morphological changes (e) or cerebellar bleeding (f) remained negative for CD133.

874x442mm (72 x 72 DPI)

Review



Supplementary Figure 4: Comparison of CD133, CD15 and nestin expression in CNS neoplasms (n=30). (A) WERI-Rb1 for CD133, (B) a brain infarction for CD15, (C) and a fetal brain of the for nestin were used as positive controls. Small round blue cell neoplasms including pineoblastoma (D-F) and medulloblastoma (G-I) were labelled for CD133 (D, G), CD15 (E, H) and nestin (F, I). CD133-negative normal brain (J) and astrocytoma ((K) pilocytic astrocytoma WHO grade I, (L) diffuse astrocytoma WHO grade II, (M) anaplastic astrocytoma WHO grade III and (N, O) glioblastoma WHO grade IV) cases were further investigated for nestin (J-N) and CD15 (N). 202x273mm (300 x 300 DPI)