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RESEARCH ARTICLE

Association of Stem Cell-Related Markers and Survival in Astrocytic Gliomas

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Abstract

To study the clinical relevance of undifferentiated tumour cells in astrocytic gliomas we employed a large tumour tissue microarray (n = 283) with corresponding clinical data and analyzed the expression of Nestin and Sox-2, which mark undifferentiated stem- and progenitor cells in the normal brain.

Both markers were expressed abundantly and staining of nestin significantly increased with WHO grade. Further, nestin and Sox-2 immunoreactivity was significantly associated with tumour cell proliferation and nestin expression was independently associated with poor patient survival.

Our findings suggest that immature glioma cells are involved in tumour growth and tumour progression and significantly impact on patient prognosis.

Keywords: *Nestin; SOX-2; brain tumour*

Abbreviations: TSC, Tumour stem cells; SLGCs, stem-like glioma cells; TMA, tissue microarray; OS, overall survival; HR, Hazard ratio; CI, confidence interval; p, probability value; rho, spearman rank correlation.

Introduction

The tumour stem cell (TSC) hypothesis has emerged as a challenging concept in the field of cancer research postulating that a variety of cancers contain small subpopulations of highly tumorigenic and therapy-resistant cells capable of repopulating the tumour after treatment (Neuzil et al. 2007, Vescovi et al. 2006, Al-Hajj 2007). TSCs have been successfully isolated from several human cancers such as leukaemia as well as tumours of breast, prostate and brain, using specific stem cell-related surface antigens that facilitate *in vitro* enrichment (Al-Hajj et al. 2007, Collins et al. 2005, Bonnet and Dick 1997). For instance in acute myeloid leukaemia TSCs were identified as a $CD34^+/CD38^-$ cell population (Bonnet and Dick 1997) whereas TSCs in breast cancer were characterized as $CD44^+/CD24^{-/low}/lineage^-$ (Al-Hajj 2007). In glioma, the most common type of brain tumour, stem-like glioma cells (SLGCs) expressing AC133, a glycosylation-dependent epitope of the transmembrane protein CD133, seem to drive tumour formation and to be highly resistant to conventional chemo- and radiotherapy (Singh et al.

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2003, Singh et al. 2004, Bao et al. 2006, Liu et al. 2006). Concordantly, we reported that AC133/CD133 content in gliomas inversely correlates with patient prognosis independent of WHO grade, patient age at diagnosis, and extent of tumour resection corroborating the clinical importance of the AC133/CD133$^+$ SLGC population (Zeppernick et al. 2008). Recent reports suggested that tumorigenicity is not entirely restricted to the AC133/CD133$^+$ tumour compartment (Beier et al. 2007). Wang and co-workers described AC133$^-$/nestin$^+$ cells isolated from glioma tissue that were able to grow tumours in rodents and gave rise to a small fraction of AC133$^+$ cells (Wang et al. 2008). These results highlight the need to better characterize the tumour-driving cell populations in gliomas.

Based on the assumption that normal neural stem cells might be a possible cellular origin of SLGCs we addressed this question by studying the expression of two prominent neural stem cell-associated markers, namely nestin and Sox-2 employing a tissue microarray (TMA) comprising biopsies collected from 283 patients suffering from primary astrocytic gliomas of WHO grade II-IV.

Nestin is an intermediate filament widely used to enrich neural stem and progenitor cells in the adult mammalian brain, where its expression is restricted to specific germinal regions such as the subventricular zone and the dentate gyrus (Dictus et al. 2007). Interestingly, nestin may be re-expressed in the adult organism under certain pathological conditions such as brain injury, ischemia, inflammation or neoplastic transformation (Veselska et al. 2006). Sox-2 is a transcription factor universally marking neural stem and progenitor cells throughout the vertebrate CNS, capable of inhibiting neuronal differentiation and maintaining progenitor characteristics in these cells (Graham et al. 2003).

In the present study, we report a WHO grade-dependent expression of nestin and a correlation of nestin and Sox-2 immunoreactivity with tumour cell proliferation. Further, increased nestin expression was significantly associated with worse patient outcome independent of patient age, tumour grade and extent of surgical tumour resection. Altogether, our findings support the hypothesis of distinct immature and clinically relevant tumour cell populations in astrocytic gliomas.

Material and Methods

Tumour material

Formalin-fixed, paraffin-embedded tissue samples from astrocytic gliomas of WHO grade II-IV were used for TMA design. Tumours were gathered from patients, who were operated at the Department of Neurosurgery at Heidelberg University, Germany from 1985 to 2007. The sample consisted of 283 primary tumours and included 52 recurrent tumours, totalling 335 tumour tissues. Informed consent was obtained from each patient according to the research proposals approved by the Institutional Review Board at Heidelberg Medical Faculty and conforming to the provisions of the Declaration of Helsinki in 1995 (as revised in Tokyo 2004). Tumours were graded according to the World Health Organization classification of tumours of the central nervous system.

Generation of the TMA

Representative tumour regions were identified by two experienced pathologists on H&E-stained tissue sections. A maximum of three biopsies were taken from each tumour tissue. Additionally, in 77 cases biopsies of corresponding tumour invasion zones were included. From all selected regions, tissue cylinders with a diameter of 0.6 mm were obtained and arrayed into a recipient block as described earlier (Freier et al. 2003). The recipient block was subsequently cut into 5µm sections on precleaned microscope slides (Superfrost®Plus, Thermo Scientific, Braunschweig, Germany).

Clinical data

Clinical data of patients were assessed in an *MS Access*-based database (Microsoft, Redmond, USA). Clinical information and follow-up data were analyzed by reviewing the medical records on radiographic images, by telephone or written correspondence and by review of death certificate. Mean follow-up time was 11.21 years (±4.26 years). Patients were followed from date of first diagnosis to the end of study (November 30th 2009), whereas dead patients were censored. A patient was considered to have recurrent disease if this was revealed either by MRI imaging or the occurrence of neurological symptoms.

Patient characteristics are shown in table 1. Adjuvant therapy was applied depending on histology and WHO grade as standard therapy in malignant gliomas or optional by considering patient-related parameters in WHO grade II cases. Distribution of treatment strategies including radio- and chemotherapy is summarized in table 1. Patients that could not be followed or experienced a non-tumour-related death and/or had received experimental therapies were excluded from survival analysis and were only considered for descriptive analysis.

Immunohistochemistry

Primary antibodies used were mouse anti-Ki-67 (1:5; B&D Biosciences, Heidelberg, Germany), mouse anti-nestin (1:200; Chemicon, Hofheim, Germany) and mouse anti-Sox-2 (1:200; R&D Systems, Wiesbaden, Germany). Prior to TMA staining specificity of primary antibodies was ensured using respective isotype controls (Acris,

Table 1. Patient characteristics.

Histological diagnosis		**patients**	**recurrent tumors**	**IFZ**
		n = 283 (%)	**n = 52 (%)**	**n = 77 (%)**
Glioblastoma	WHO IV	221 (78.1)	29 (55.8)	55 (71.4)
Astrocytoma	WHO III	17 (6.0)	9 (17.3)	10 (13.0)
Astrocytoma	WHO II	45 (15.9)	14 (26.9)	12 (15.6)
Age		**mean (y) ± SD**		
Glioblastoma	WHO IV	53.8 ± 12.8		
Astrocytoma	WHO III	36.1 ± 14.2		
Astrocytoma	WHO II	33.6 ± 18.7		
Gender		**M:F**		
Glioblastoma	WHO IV	133:88		
Astrocytoma	WHO III	11:6		
Astrocytoma	WHO II	30:15		
Extent of resection		**total**	**subtotal/biopsy**	
		n = (%)	**n = (%)**	
Glioblastoma	WHO IV	151 (68.3)	70 (31.7)	
Astrocytoma	WHO III	11 (64.7)	6 (35.3)	
Astrocytoma	WHO II	33 (73.3)	12 (26.7)	
Non-Surgical treatments*		**radiotherapy**	**chemotherapy**	
		n = (%)	**n = (%)**	
Glioblastoma	WHO IV	186 (84.2)	87 (39.4)	
Astrocytoma	WHO III	16 (94.1)	1 (5.9)	
Astrocytoma	WHO II	14 (31.1)	3 (6.7)	
Tumor localization		**n = (%)**		
Frontal		92 (32.5)		
Temporal		91 (32.1)		
Parietal		35 (12.4)		
Occipital		35 (12.4)		
Others†		30 (10.6)		

IFZ: Infiltration zone, *therapy at primary tumor diagnosis, †Other localizations such as midline, intraventricular, insular, etc.

Hiddenhausen, Germany). Preceding staining deparaffination was carried out by immersing slides in 100% xylol (3x3min), followed by 90%, 80%, 70% and 50% ethanol (2x3min each). Finally, slides were washed in distilled water (2x3min). Antigen retrieval was performed in a water bath at 100°C for 20min using antigen retrieval buffers (DAKO, Hamburg, Germany) with antigen-specific pH values (Ki-67 = pH6.1; nestin and Sox-2 = 9.9). Incubation with primary and secondary antibodies as well as detection with Vectastain ELITE ABC Kit (Vector Laboratories, Burlingame, USA) was carried out as described (Karcher et al. 2006). Additional antibodies used for double immunofluorescence staining were: mouse monoclonal anti-human CD31 (1:100; BD Pharmingen, Hamburg, Germany) and rabbit polyclonal anti-human nestin (1:100; Chemicon, Hofheim, Germany), as well as secondary antibodies anti-mouse ALEXA488 and anti-rabbit ALEXA555 (1:500; both Invitrogen, Karlsruhe, Germany).

Evaluation of staining

Antigen expression was pre-tested in a set of glioma tissues of WHO grade II-IV (n = 20-30) to establish suitable antigen evaluation categories based on antigen expression variability. Grading scores with uniform distribution of antigen expression levels among individual grading categories were chosen for the final TMA evaluation. Each tumour biopsy was evaluated at 20x magnification. Staining of TMA biopsies was semiquantitatively graded in an antigen-dependent manner according to the estimated percentage of Ki-67/Sox-2-/nestin-positive cells covering the whole tissue spot as indicated in figure1-3. In cases where staining frequencies were difficult to asses frequencies were counted manually. Staining of endothelial cells was distinguished from that of tumour cells. Average staining results from all biopsies of each individual patient were taken as final statistical parameters.

Statistical analysis

Overall survival (OS) was censored for patients alive at the end of study. In case of impossible patient contact (n = 11), the date of last visit was also taken as censored end-point. Association between OS and staining frequency was calculated using a log-rank test and represented as Kaplan-Meier plots. To evaluate the contribution of established

prognostic factors to survival, WHO grade, patient age at diagnosis, and extent of resection were included in a multivariate Cox proportional hazards regression. The relationship between expression and WHO grade was quantified by Spearman's rank correlation rho. Calculations were performed using the statistical software environment R, version 2.4.1 (http://www.r-project.org). In the results section, probability values smaller than 0.05 are referred to as 'statistically significant.'

Results

Validation of the TMA using Ki-67

In order to test reliability of the TMA to determine prognostically relevant molecules, we analyzed expression of the proliferation marker Ki-67, which is known to be strongly correlated with WHO grade in gliomas (Mao et al. 2007). A clear nuclear staining pattern was observed for Ki-67 with low frequency in WHO grade II, intermediate frequency in WHO grade III and high frequency in WHO grade IV (figure 1A-D): In WHO grade II and WHO grade III most of the tumours were devoid of Ki-67 immunoreactivity or showed marginal Ki-67 staining not exceeding 1% of total cells (64.7% / 47.6%). In WHO grade IV, however, most tumours contained at least 1% (86.9%) of proliferating cells and a fraction of biopsies featured 5-10% of proliferating cells (25.1%). In agreement with previous findings (Mao et al. 2007), univariate analysis revealed a significant correlation of Ki-67 expression with tumour grade (rho = 0.42, $p < 0.001$, figure 1E) and a significant association of poorer OS with increased Ki-67 levels (HR = 1.47, 95%CI [1.25 to 1.73]; $p < 0.001$, figure 1F).

Expression of nestin increases with tumour grade and tumour cell proliferation

TMA staining of nestin revealed a broad cytoplasmatic immunoreactivity in all WHO grades (figure 2A-E). Altogether, the occurrence of nestin-positive tumours was lowest in WHO grade II (66.7%), increased in WHO grade III (73.1%) and was highest in WHO grade IV (96.0%). Concordantly, frequencies of nestin-positive cells were found to increase with WHO grade, with the largest increase occurring from WHO grade III to WHO grade IV (figure 2E). In WHO grade IV more than half of the tumours (58.7%) showed immunoreactivity in over 50% of cells, whereas the same frequency of nestin-positive cells could only be found in a minor fraction of WHO grade III (11.5%) and WHO grade II (4.4%) tumours. In some biopsies nestin-positive cells could also be found in the tumour infiltration zone (figure 2D). In most of these cases frequency of nestin-immunoreactive cells in the infiltration zone was 10% or less. However, nestin expression was not confined exclusively to the tumour tissue but could also be frequently detected in the cytoplasm of tumour-supplying endothelial cells (figure 2B-D). The amount of nestin-positive tumour cells and the amount of nestin-positive endothelial cells depended on tumour grade (rho = 0.51, $p < 0.001$, and rho = 0.36, $p < 0.001$, respectively; figure 2E, G). To substantiate our results on nestin expression on blood vessels we performed an additional double immunfluorescence staining of nestin and the established vessel marker CD31 on our TMA and re-evaluated staining frequencies of double-positive cells. Indeed, co-expression of nestin and CD31 was clearly discernable on blood-vessels (suppl. fig.1A). Interestingly, however, the relative frequency of nestin$^+$/CD31$^+$ endothelial cells was unrelated to tumour grade (rho = 0.01, p = 0.93, suppl. fig. 1B) although the absolute number of nestin$^+$/CD31$^+$ cells showed a clear trend towards an increased expression in high-grade tumours (rho = 0.18, p = 0.08, suppl. fig. 1C).

In 16 individuals that underwent malignant transformation, nestin immunoreactivity increased with tumour progression (n = 10) or showed stable immunoreactivity (n = 6), but did not decrease in any patient. In 31 patients where the recurrent tumour did not progress to a higher tumour grade, nestin immunoreactivity decreased in 15 patients, remained stable in 7 patients and increased in 9 patients. The difference between the distribution of patients according to nestin immunoreactivity in the two previous categories was marginally significant (p = 0.06).

When comparing nestin and Ki-67 expression, tumour cells and endothelial cells showed a positive correlation with tumour proliferation (rho = 0.38, $p < 0.001$ and rho = 0.28, p = 0.003, respectively). Univariate statistic analysis revealed a significantly shorter overall survival with increasing frequencies of nestin-positive cells, both in the tumour tissue (HR 1.40 95%CI [1.27 to 1.56]; $p < 0.001$, figure 2F) and in blood vessels (HR 1.40 95%CI [1.15 to 1.70]; p = 0.008, figure 2H). Finally, multivariate Cox regression resulted in a significant association between nestin-positive cells and OS (HR 1.12 95%CI [1.00-1.26]; p = 0.042) indicating that elevated nestin immunoreactivity in tumour cells was associated with worse clinical outcome independent of known prognostic confounders. In contrast, we were unable to identify a significant association between nestin-positive blood vessels or CD31$^+$/nestin$^+$ cells and OS (HR 1.22 95%CI [0.99-1.51]; p = 0.07, and HR 1.27; 95%CI [0.95-1.71]; p = 0.11, respectively).

Abundant expression of Sox-2 is correlated with tumour cell proliferation

Sox-2 was found to be abundantly expressed in all three WHO grades and to a lesser extent in tumour infiltration

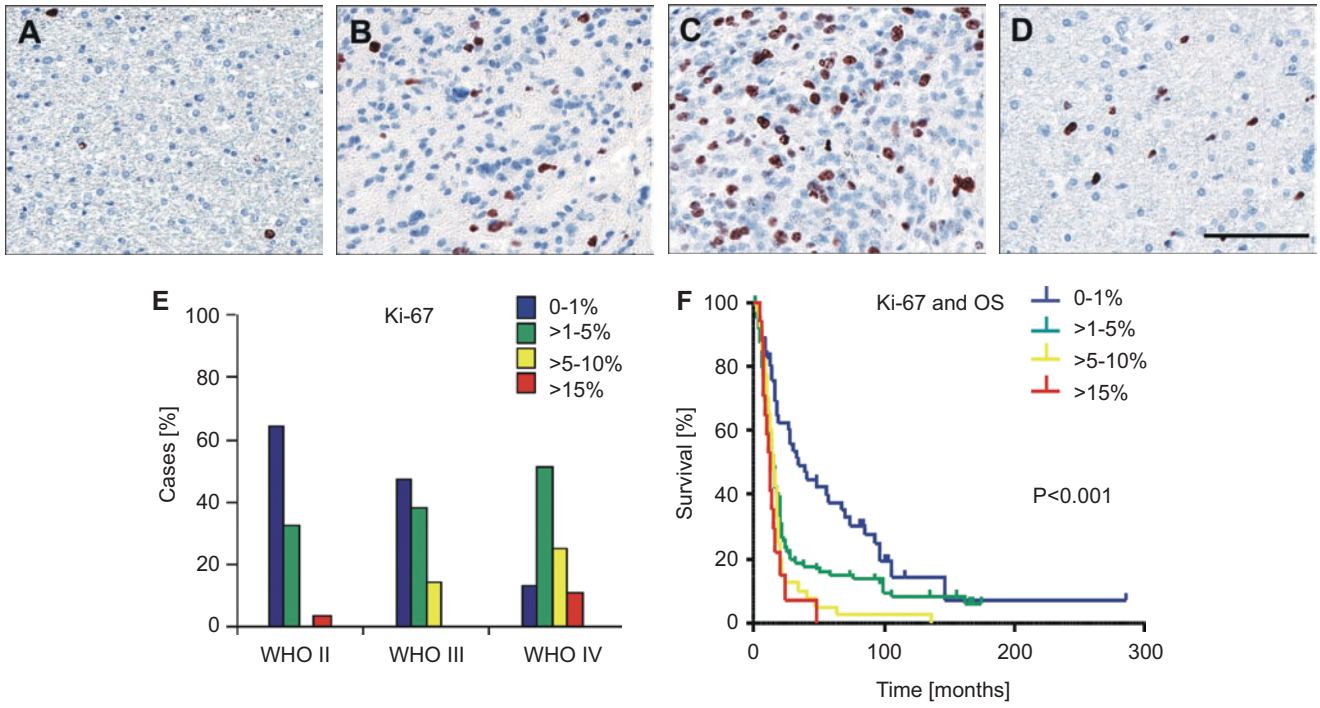

Figure 1. (A) Ki-67 labelling in astrocytic glioma WHO grade II, (B) WHO grade III and (C) glioblastoma WHO grade IV. (D) Invasion zone of a glioblastoma WHO grade IV with single positive cells. (E) WHO grade-dependent expression of Ki-67. (F) Kaplan-Meier plot illustrating the association of Ki-67 staining with survival. Scale bar = 100μm (A-D).

zones (figure 3A-D). Only a minor fraction of tumours lacked Sox-2 positivity in WHO grade II (13.3%), WHO grade III (15.3%) and WHO grade IV (18.3%), while most of the tumours showed high Sox-2 frequencies (figure 3E): In all WHO grades about half of the tumour tissues were immunoreactive in at least 10% of cells (WHO grade II: 55.5%, WHO grade III: 61.5% and WHO grade IV: 50.5%). Although Sox-2 expression was not associated with WHO grade (rho = -0.09, p = 0.19) in a direct comparison of primary tumours with corresponding recurrences that had undergone malignant transformation Sox-2 immunoreactivity was found to increase (7/16 patients) or to remain stable (7/16 patients) with tumour upgrade but rarely to decrease during tumour progression (2/16 patients). On the other hand, immunoreactivity of recurrences without malignant transformation was quite heterogeneous and either decreased (11/33 patients), remained stable (11/33 patients) or increased (11/33 patients) compared to the primary tumours of the corresponding patients. However, the difference between the distribution of patients according to Sox-2 immunoreactivity in the two previous categories was not significant (p = 0.30).

Univariate and multivariate analysis did not identify any association between survival and expression of Sox-2, (figure 3F). Interestingly however, augmented Sox-2 expression correlated with increased Ki-67 levels (rho = 0.21, p = 0.001) as in the case of nestin.

Discussion

The tumour stem cell hypothesis in gliomas has been challenged lately by the discovery of tumorigenic cell populations lacking the stem cell phenotype previously used to characterize SLGCs (Beier et al. 2007, Wang et al. 2008). Therefore it is of fundamental importance to better characterize the tumour-driving populations and to understand how these cells influence the clinical behaviour of a given tumour. To address these questions, we established a tissue-microarray and studied the expression of the two neural stem cell-associated markers nestin and Sox-2 in a set of 283 primary astrocytic gliomas and 52 additional recurrences of WHO grade II-IV, which is the largest cohort of cases reported so far in this regard (Almqvist et al. 2002, Dahlstrand et al. 1992, Ma et al. 2008, Strojnik et al. 2007). In agreement with other studies we showed that nestin, and Sox-2 are abundantly expressed in the majority of tumour biopsies and that the amount of nestin-positive tumour cells increases in a tumour grade-dependent manner. In addition to previous studies we show that nestin expression in tumour cells was significantly correlated with tumour proliferation and seemed to be involved in tumour recurrence and tumour progression. Finally, nestin expression turned out to be associated with worse clinical outcome independently of patient age, tumour grade and extent of resection.

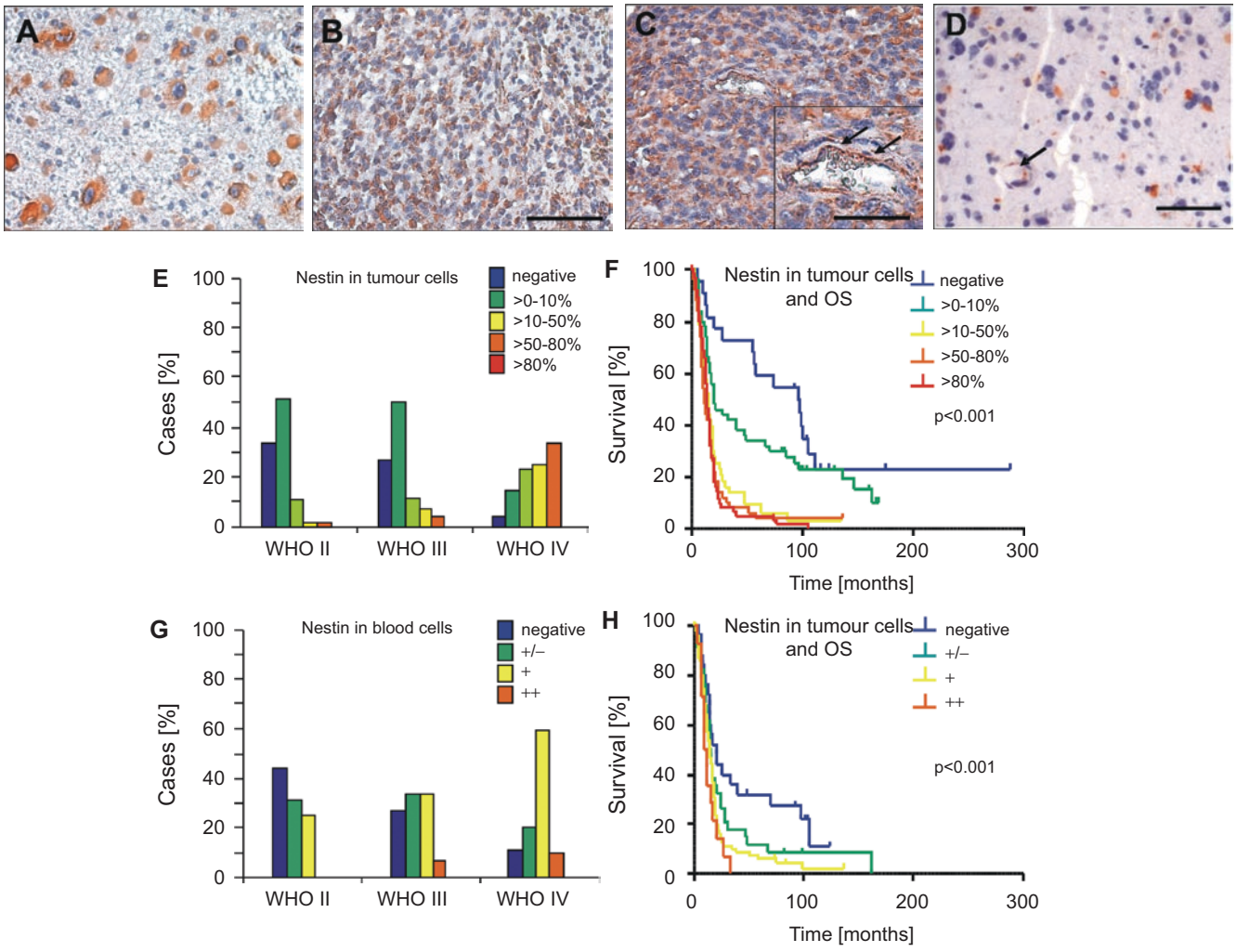

Figure 2. (A) Nestin immunoreactivity in astrocytic glioma WHO grade II, (B) WHO grade III and (C) glioblastoma WHO grade IV. (D) Invasion zone of an astrocytic glioma WHO grade III with single positive cells. Arrows in small inserts indicate immunoreactivity in endothelial cells. (E) WHO grade-dependent expression of nestin expression in tumour cells. (F) Kaplan-Meier plot illustrating the association of nestin expression in tumour cells and survival. (G) WHO grade-dependent expression of nestin on endothelial cells. (H) Kaplan-Meier plot illustrating the association of nestin expression on endothelial cells with survival. Scale bar = 50μm (A-D).

Although nestin is mainly found during mammalian CNS development it may be re-expressed in the adult organism under certain pathological conditions such as neoplastic transformation (Veselska et al. 2006). Other reports have analyzed nestin expression in brain tumours (Almqvist et al. 2002, Dahlstrand et al. 1992, Ma et al. 2008, Strojnik et al. 2007): Even though numbers of tumour specimens analyzed in these reports varied from 20 to 87 patients, all studies describe 1) abundant nestin expression in primary CNS tumours compared with normal brain tissue and 2) increased nestin expression in higher tumour grades. Strojnik and co-workers further identified nestin as a strong prognostic marker for decreased overall survival in a study comprising 87 CNS tumours (Strojnik et al. 2007). This fits well to our results obtained by univariate survival analysis and could be confirmed in a multivariate setting where we excluded known prognostic confounders such as age at time of diagnosis, extent of tumour resection and WHO grade. Still, with regard to the wide distribution of nestin described in previous reports and in our study, it must be argued that the undifferentiated (or less differentiated) tumour compartment is broader than initially expected. Thus nestin seems to be suitable to detect different types of glioma cells with a reduced differentiation. For instance, AC133/CD133, only marks a small subpopulation of undifferentiated tumour cells located within the nestin-positive tumour population (Singh et al. 2003, Zeppernick et al. 2008). Altogether, these results could help to identify new and more selective markers using nestin as a first, rough means to indentify the tumour compartment with a more immature phenotype. Interestingly, nestin immunoreactivity could also be located within tumour infiltration zones, and might

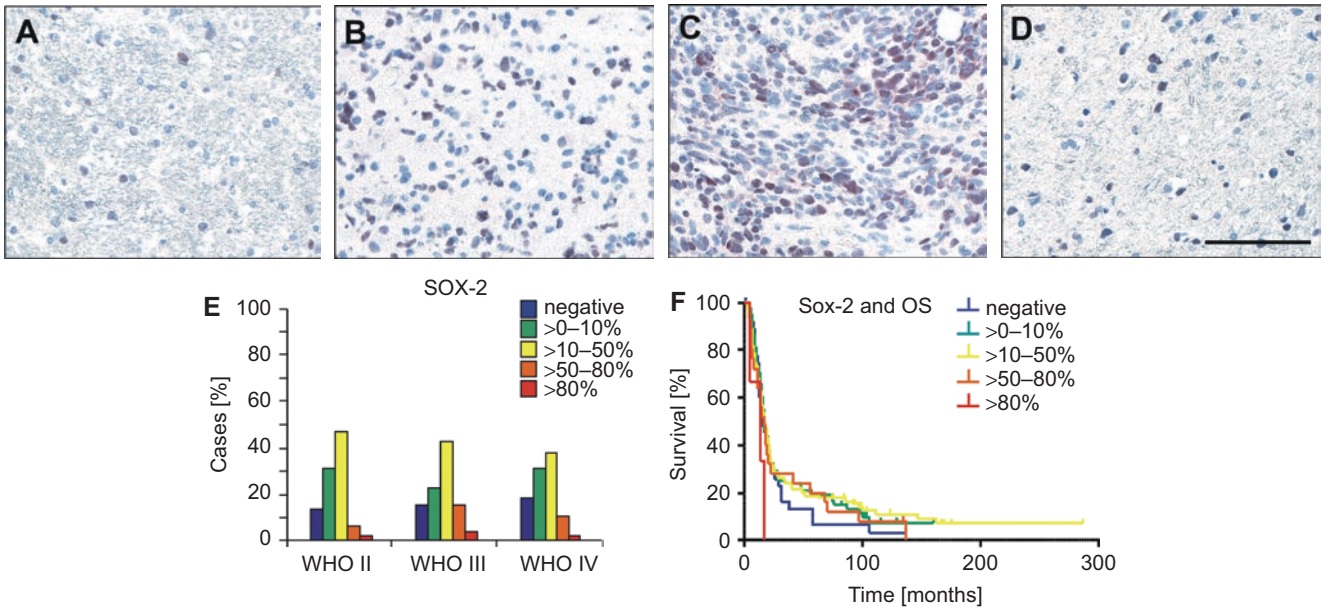

Figure 3. (A) Sox-2 labelling in astrocytic glioma WHO grade II, (B) WHO grade III and (C) glioblastoma WHO grade IV. (D) Invasion zone of a glioblastoma WHO grade IV with single positive cells. (E) WHO grade-dependent expression of Sox-2. (F) Kaplan-Meier plot illustrating the association of Sox-2 expression with survival. Scale bar = 100μm (A-D).

characterize stem-like tumour cells with particular migratory activity. However, presence of invading normal neural stem cells, which are known to be attracted to tumour sites and express nestin as well, can not be excluded (Walzlein et al. 2008). In addition, nestin could also be detected in newly formed capillaries (Almqvist et al. 2002, Dahlstrand et al. 1992, Ma et al. 2008, Strojnik et al. 2007, Walzlein et al. 2008, Colin C et al. 2007). In our study the amount of nestin-expressing endothelial cells increased with tumour malignancy and was well correlated with tumour grade. However, we were able to show that the relative amount of nestin$^+$/CD31$^+$ endothelial cells remained constant despite the tumour grade-dependent increase in the absolute number of CD31$^+$ cells. It is thus likely that increased numbers of nestin-positive blood vessels mirror an increased level of angiogenesis in high-grade tumours. Altogether, our data establish a strong link between nestin-positive tumour cells and tumour growth.

Sox-2 is a transcription factor universally marking neural progenitor and stem cells throughout the vertebrate CNS, capable of inhibiting neuronal differentiation and maintaining progenitor characteristics in these cells (Graham et al. 2003). In a study involving 72 astrocytomas, Sox-2 expression in tumours was found to be higher than in normal brain tissue and to increase with tumour grade (Ma et al. 2008), whereas our own results involving 335 gliomas tissues derived from 283 patients neither revealed tumour grade-dependency nor a significant correlation with patient prognosis. However, the abundant expression of Sox-2, which is already present in low-grade tumours as well as its correlation with tumour proliferation, suggests that Sox-2 might be involved in early stages of tumour formation. In line with this assumption individual tumours often showed increased Sox-2 expression after malignant transformation. It is thus tempting to speculate that Sox-2 is not only involved in early stages of gliomagenesis but also participates in tumour progression.

Conclusion

In summary, we have described abundant expression of two stem cell-associated markers in a large cohort of astrocytic tumours. We showed a correlation of Sox-2 and nestin immunoreactivity with tumour proliferation and reported a tumour grade-dependent occurrence of nestin with a significant influence on patient prognosis. Altogether, these results suggest that immature cell populations influence different aspects of tumour growth in glioma and significantly impact on patient prognosis.

Declaration of interest

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