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Presence of neural progenitors in spontaneous canine gliomas: A histopathological and immunohistochemical study of 20 cases

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ABSTRACT

Gliomas are the most common primary brain tumours in humans and are associated with a poor prognosis. An accurate animal model of human glioma tumorigenesis is needed to test new treatment strategies. Dogs represent a promising model because they develop spontaneous diffusely-infiltrating gliomas. This study investigated whether spontaneous canine gliomas contain cancer stem cells previously identified in all grades of human gliomas.

Twenty spontaneous cases of canine gliomas were graded according to the human WHO classification. The expression of different markers of lineage differentiation was evaluated with immunohistochemistry as follows: nestin and CD133 for neural stem cells, doublecortin for neuronal progenitor cells, Olig2 for glial progenitor cells, glial fibrillary acidic protein, vimentin and S-100 for mature glial cells, and NeuN and βIII-tubulin for mature neurons. Gliomas were characterised as follows: five grade II (oligodendrogliomas); nine grade III (seven anaplastic oligodendrogliomas, one anaplastic astrocytoma, one anaplastic oligoastrocytoma); six grade IV (glioblastomas).

Immunohistochemical evaluation revealed that (1) nestin and CD133 were expressed in all grades of gliomas with a higher proportion of positive cells in high-grade gliomas; (2) the expression of S-100 protein and Olig2 did not differ substantially between astrocytic and oligodendroglial tumours, and (3) all gliomas were negative for mature neuron markers. The results demonstrated the presence of undifferentiated neural progenitors in all grades of spontaneous canine gliomas, confirming the relevance of this animal model for further studies on cancer stem cells.

Introduction

Gliomas are the most common primary brain tumours in adult people, representing 81% of all malignant brain tumours (Ostrom et al., 2014). Diffusely infiltrating gliomas are classified as low-grade (grade II) and high-grade (grades III and IV) according to their degree of malignancy (Louis et al., 2007). They are associated with a poor prognosis largely because of their widespread invasiveness and resistance to multimodal treatments.

Recent studies have indicated that all grades of human gliomas contain putative cancer stem cells (CSCs), a small subpopulation of cells thought to be responsible for initiating and maintaining cancer growth through their ability to self-renew (Reya et al., 2001; Singh et al., 2004; Beier et al., 2007; Rebetz et al., 2008). In agreement with the cancer stem cell hypothesis, gliomas are hierarchically organised: self-sustaining CSCs at the apex have the potential to differentiate into astrocytic, oligodendroglial, and neuronal lineages, and give rise to malignant progenitors, lineage-restricted precursors, and differentiated cells (Singh et al., 2004). It is also suspected that CSCs are

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responsible for resistance to chemo- and/or radiotherapy leading to local relapse after treatment (Bao et al., 2006; Eyler and Rich, 2008). Immunohistochemically, CSCs show expression of markers such as nestin and prominin-1 (CD133) (Singh et al., 2003; Galli et al., 2004; Dell'Albani, 2008).

Efforts are currently being made to decipher the oncogenic mechanisms of these tumours in order to identify new therapeutic targets and improve response to treatment. Although rodent glioma models have been used in preclinical glioma research for over 30 years (Das et al., 2008), their reliability has increasingly been questioned (Sughrue et al., 2009). In contrast, the dog presents an attractive model because of its close evolutionary relationship with humans, its greater brain size compared to rodent models, and the spontaneous nature of gliomas, which are the second most frequent intracranial tumours in this species, with a prevalence of 32% (Chen et al., 2013).

The CSC hypothesis has been investigated in a wide variety of canine tumours (Wilson et al., 2008; Cocola et al., 2009; Fujii et al., 2009; Penzo et al., 2009; Cogliati et al., 2010; Ferletta et al., 2011; Michishita et al., 2011; Nemoto et al., 2011; Blacking et al., 2012; He et al., 2014). However, only one case of canine glioblastoma containing CSCs has been published (Stoica et al., 2009).

Evaluation of lineage commitment of tumour cells in human infiltrating gliomas has demonstrated that low-grade glioma cells are reminiscent of glial-progenitors, while high-grade glioma cells maintain glial progenitor-like features and additionally exhibit enhanced expression of neural precursors (Rebetz et al., 2008). So far, the lineage commitment and differentiation blockage of tumour cells in spontaneous canine gliomas have not been investigated or compared with those aspects of human infiltrating gliomas.

The aim of the present study was to investigate the expression of markers of glial and neuronal lineage differentiation hierarchy in 20 spontaneous canine infiltrating gliomas and to determine whether canine tumours show a lineage commitment similar to their human counterparts.

Material and methods

Case selection

Twenty canine gliomas were retrospectively selected from the databases (2008–2012) of the Veterinary Neuropathology group of the Universitat Autònoma de Barcelona (UAB, Spain) and the Laboratoire d'Anatomie Pathologique Vétérinaire du Sud-Ouest (LAPVSO, France). All of the samples were obtained during necropsy performed immediately following a presumptive diagnosis of glioma. The diagnosis was made by a Board-certified neuroradiologist (SA, CF, LC, KG) based on clinical criteria, magnetic resonance imaging features, and cerebrospinal fluid analysis. All owners gave their written consent for necropsy and histopathological analysis.

Histology and morphological diagnosis

Representative tissue samples were fixed in 10% formalin, processed into 5 µm paraffin-embedded sections, and stained with haematoxylin and eosin (HE) for microscopic evaluation. Gliomas were evaluated by six experts (FF, AD, CD, MD, DF and MP) in a blind study, according to the criteria defined by the World Health Organisation (WHO) for human tumours of the central nervous system (Louis et al., 2007). This classification has been updated more recently compared with the WHO animal grading system (Koestner et al., 1999), and recent veterinary publications have used the human WHO scheme to characterise canine gliomas (Higgins et al., 2010; Young et al., 2011; York et al., 2012; Bentley et al., 2013).

Morphological diagnosis was a two-step process: (1) identification of tumour phenotype and (2) grading. Diagnosis of oligodendroglial tumours relied on the recognition of neoplastic cells with well-defined membranes, cytoplasm clearing, and round and hyperchromatic nuclei, typically organised in a 'honeycomb' pattern. Astrocytic tumours were identified by elongated neoplastic cells with scant eosinophilic cytoplasm, organised in a loosely structured matrix. In oligoastrocytoma, two neoplastic cell populations with astrocytic and oligodendroglial phenotypes, respectively, were intermingled.

High-grade gliomas were distinguishable from low-grade gliomas by an increased degree of cytonuclear atypia (all high-grade gliomas) and an increased frequency of necrosis and/or endoluminal proliferation of endothelial cells leading to glomeruloid-like vessels (all high-grade gliomas except anaplastic astrocytoma). The mitotic index (i.e. number of mitoses per 10 high-power fields) was calculated for each sample but no threshold value was used in the grading scheme. Typical pseudopalisading of neoplastic cells around necrotic foci was a pathognomic feature of glioblastoma. Additional features observed were the growth pattern (relationship with the surrounding tissue), mucinous secretion, and inflammation.

Immunohistochemistry

The immunohistochemical (IHC) markers we used were characteristic of glial and neuronal lineage differentiation hierarchy: nestin and CD133 as stem cell markers; Olig2 protein and doublecortin (DCx) as glial and neuronal progenitor cell markers, respectively; glial fibrillary acidic protein (GFAP) and vimentin (VIM) as mature astrocyte markers; S-100 protein as a mature oligodendroglial and astrocytic marker; and NeuN protein (NeuN) and βIII-tubulin as mature neuron markers. The Iba1 microglial marker was used for the evaluation of the inflammatory-associated response. The nuclear antigen Ki-67 was used as a marker of cellular proliferative activity.

Sections 5-µm thick were mounted on capillary glass slides, deparaffinised, and rinsed with water. The primary antibodies used are summarised in Table 1. When antigen retrieval was necessary, sections were heated for 20 min in a water-bath or

Table 1
Immunohistochemical markers used for the study of canine gliomas: main features.

	Antibody name	Company	Dilution	Pretreatment
Nestin	Rabbit anti-nestin polyclonal antibody	Abcam ab5968	1:500	Citrate buffer 10 mM pH 6.0, 20 min water bath + 30 min RT
CD133	Rabbit anti-CD133 polyclonal antibody	Abcam 19898	1:200	Citrate buffer 10 mM pH 6.0, 20 min water bath + 30 min RT
DCx	Rabbit anti-doublecortin polyclonal antibody	Abcam ab18723	1:1000	Citrate buffer 10 mM pH 6.0, 20 min water bath + 30 min RT
ßIII	Mouse anti-ßIII tubulin monoclonal antibody	Chemicon MAB1637	1:200	Citrate buffer 10 mM pH 6.0, 20 min water bath + 30 min RT
NeuN	Mouse anti-neuronal nuclei monoclonal antibody	Chemicon MAB377	1:500	Citrate buffer 10 mM pH 6.0, 20 min water bath + 30 min RT
Olig2	Rabbit anti-Olig2 polyclonal antibody	Chemicon AB9610	1:100	Citrate buffer 10 mM pH 6.0, 20 min water bath + 30 min RT
GFAP	Rabbit anti-glial fibrillary acidic protein polyclonal antibody	Dako Z0334	1:500	Citrate buffer 10 mM pH 6.0, 20 min water bath + 30 min RT
S100	Rabbit anti-S100 polyclonal antibody	Dako Z0311	1:1000	Citrate buffer 10 mM pH 6.0, 20 min water bath + 30 min RT
VIM	Mouse anti-vimentin clone V9 monoclonal antibody	Dako M0725	1:200	Without pretreatment
Ki-67	Mouse anti-Ki-67 antigen monoclonal antibody	Dako M7240	1:100	Citrate buffer 10 mM pH 6.0, 4 min PC + 30 min RT
Iba1	Goat anti-Iba1 polyclonal antibody	Abcam ab5076	1:600	Citrate buffer 10 mM pH 6.0, 20 min water bath + 30 min RT

RT, room temperature; PC, pressure cooker.

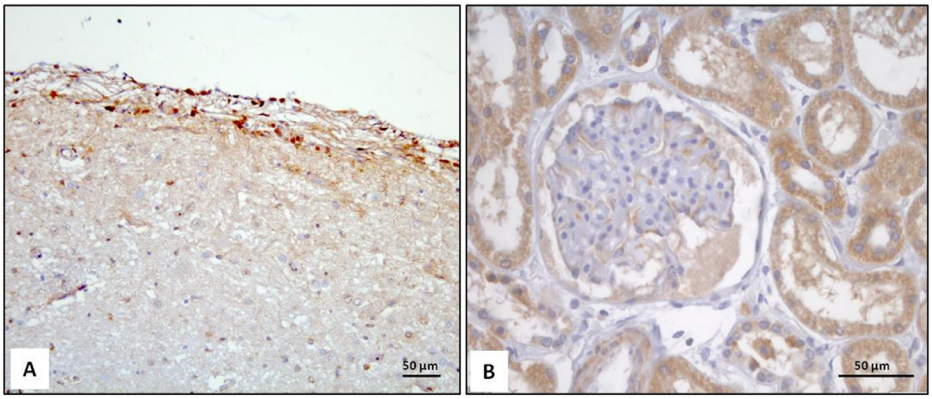

Fig. 1. Immunohistochemical evaluation of markers of neural stem cells in control tissues. (A) Presence of nestin^{+} cells located in the subventricular zone of an adult dog. (B) Expression of CD133 protein by the epithelial tubular cells of the kidney of adult dog.

4 min in a pressure cooker with 10 mM citrate buffer pH 6.0, cooled for 30 min at room temperature, and rinsed in phosphate-buffer saline (PBS) (Table 1). Previously, sections were treated 35 min with 3% peroxide to block endogenous peroxidase activity. Non-specific binding was blocked by normal goat or rabbit serum 30% diluted with PBS for 1 h. Samples were incubated overnight with primary antibodies at 4 °C (nestin, Olig2 and VIM) or 40 min at room temperature (all other antibodies tested). Sections were rinsed with PBS and incubated for 40 min with a labelled polymer according to the manufacturer's instructions (mouse kit K4007 or rabbit kit K011, Dakocytomation). For Iba1, the incubation was performed using a secondary goat anti-rabbit antibody and then with a standard ABC Peroxidase staining kit (Thermo Scientific, kit number 32020) diluted with PBS, for 1 h at room temperature. Staining was completed by a 10 min incubation with 3,3′-diaminobenzidine (DAB) and counterstaining in haematoxylin for 3 s. The positive control used for GFAP, Olig2, S-100, VIM, DCx, NeuN, and βIII-tubulin was normal canine brain tissue including grey and white matter. The positive control for nestin was brain tissue of the same canine brains, including the subventricular zone in order to verify cell specificity of this marker (Fig. 1A). Positive control for CD133 was adult canine healthy kidney tissue, because it is expressed by tubular epithelial cells (Fig. 1B). In all experiments, negative controls were obtained by omitting the primary antibody.

Evaluation of immunohistochemical data

A semiquantitative, four-class, proportion score was defined as the percentage of positive tumour cells labelled by the different markers: 0, <5% positive cells; 1, 5–30% positive cells; 2, 30–60% positive cells; 3, 60–90% positive cells; 4, >90% positive cells. Ten high-power fields were evaluated in each case. The proliferation index was estimated by counting Ki-67 stained and unstained cells in 10 high-power fields (approximatively 2000 cells) and then expressed as a percentage. Only nuclear staining was considered positive, and areas with the most positivity were chosen for evaluation.

Results

Clinical features

Breed, sex, age, and clinical signs of all dogs included in the study are summarised in Table 2. Eighty percent of the cases were Boxers

Table 2
Clinical data of dogs included in the study. M, male; F, female. Age expressed in years.

Case	Breed	Sex	Age	Neurological signs	Tumour localisation
1	Boxer	F	8	Head pressing, gait abnormality, right circling, depressed mental status, absent left proprioceptive reactions	Right prosencephalon
2	Boxer	F	9	Clusters of generalised tonic–clonic seizures, depressed mental status, right circling	Right prosencephalon
3	French bulldog	M	4	Generalised tonic–clonic seizures, autonomous signs, depressed mental status, vestibular signs, absent right proprioceptive reactions	Left prosencephalon
4	Boxer	M	10.5	Right blindness, absent menace response (right eye), decreased right proprioceptive reactions, mild cerebellar ataxia	Left prosencephalon
5	French bulldog	M	8	Seizures, behavioural changes, myosis	Right prosencephalon
6	Boxer	F	8	Seizures	Left prosencephalon
7	Bulldog	M	6	Left proprioceptive deficits, seizures	Right prosencephalon
8	French bulldog	F	5	Behavioural changes, depressed mental status, left circling, absent right proprioceptive reactions	Left prosencephalon
9	Boxer	F	13	Generalised tonic–clonic seizures, right circling, decreased right proprioceptive reactions, right facial hemispasm and hyperesthesia	Right prosencephalon/VII cranial nerve nucleus
10	Boxer	F	7	Weight loss, head tilted to the right side, left walking from 10 days	Left mesencephalon
11	Boxer	M	10	Status epilepticus, generalised tonic–clonic seizures.	Left prosencephalon
12	Boxer	F	12	Ataxia, circling, recumbency, head tilted to the left side	Left prosencephalon/mesencephalon
13	French Bulldog	M	13	Depressed mental status, tremors, epileptiform seizures	Right prosencephalon
14	French bulldog	F	4	Depressed mental status, right hemiparesis, absent menace response (right eye), absent right proprioceptive reactions	Left prosencephalon
15	French bulldog	M	4	Behavioural changes, generalised tonic–clonic seizures	Left prosencephalon
16	Boxer	F	10	Spinal ataxia of forelimbs, left circling, absent menace response (right eye), bilateral blindness, right proprioceptive deficit	Left prosencephalon
17	Jack Russell	M	3	Seizures	Left prosencephalon
18	Golden retriever	M	7	Right circling, behavioural changes	Right prosencephalon
19	Cross breed	F	11	Balance disorder, vestibular ataxia, left proprioceptive deficit, nystagmus	Right rhombencephalon
20	Scottish terrier	F	10	Behavioural changes, seizures	Right prosencephalon

Table 3
Histopathological diagnoses and features in glial tumours.

Case number	Diagnosis	GR	ATYP	MI	MSEC	NEC	VASC	INF	RNT
1	Oligodendroglioma	HC	Low	6	Yes	No	CB	No	Compressive
2	Oligodendroglioma	HC	Low	4	Yes	No	CB/G	Yes	Infiltrative
3	Oligodendroglioma	HC	Low	10	Yes	No	CB/G	No	Infiltrative
4	Oligodendroglioma	HC	Low	7	Yes	Yes	CB/G	No	Infiltrative
5	Oligodendroglioma	HC	Low	8	No	No	G	Yes	Infiltrative
6	Anaplastic oligodendroglioma	S	Moderate	9	Yes	Yes	G	No	Compressive + infiltrative areas
7	Anaplastic oligodendroglioma	S	Moderate	16	Yes	No	G	No	Infiltrative
8	Anaplastic oligodendroglioma	S	Moderate	7	Yes	No	G/CB	No	Compressive
9	Anaplastic oligodendroglioma	S	Moderate	2	No	No	CB/H	Yes	Infiltrative
10	Anaplastic oligodendroglioma	S	Moderate	12	No	Yes	CB	No	Compressive + infiltrative areas
11	Anaplastic oligodendroglioma	S	High	15	No	No	CB/H	No	Infiltrative
12	Anaplastic oligodendroglioma	S	Moderate	25	No	No	CB	No	Infiltrative
13	Anaplastic astrocytoma	S	High	10	No	No	CB/H	No	Infiltrative
14	Anaplastic oligoastrocytoma	S/HC	High	10	No	No	CB/H	Yes	Infiltrative
15	Glioblastoma	S	High	12	Yes	Yes	G	No	Infiltrative
16	Glioblastoma	S	High	15	No	No	CB	No	Compressive
17	Glioblastoma	S	High	2	No	Yes	G	No	Compressive
18	Glioblastoma	S	High	2	No	Yes	G	No	Compressive + infiltrative areas
19	Glioblastoma	S	High	17	No	Yes	H	Yes	Compressive + infiltrative areas
20	Glioblastoma	S	High	28	Yes	Yes	H	No	Infiltrative

GR, growth; ATYP, cytonuclear atypia; MI, mitotic index; MSEC, mucinous secretion; NEC, necrosis; VASC, vascular features; INF, inflammation; RNT, relation with surrounding nervous tissue; HC, honeycomb; S, solid growth; CB, capillary branching; H, haemorrhages; G, glomeruloid vessels.

and French bulldogs and most tumours were located in the prosencephalon (18/20, 90%). The mean age of affected dogs was 8 years (range 3–13 years). Both sexes were equally affected (9 males and 11 females).

Histological characterisation of canine gliomas

Tumours were microscopically characterised as follows: five grade-II gliomas (all oligodendrogliomas); nine grade-III gliomas (seven anaplastic oligodendrogliomas, one anaplastic astrocytoma, and one anaplastic oligoastrocytoma), and six grade-IV astrocytomas (all glioblastomas). The histological features of each tumour are summarised in Table 3 and representative images are displayed in Fig. 2.

Immunohistochemical features of canine gliomas

Expected patterns of immunostaining for each marker of lineage differentiation hierarchy were observed in positive controls. In canine healthy brain tissue, nestin$^+$ cells were found in the subventricular zone adjacent to the lateral ventricles (Fig. 1A), a neurogenic niche that has been previously described in dogs (Lim et al., 2012; Walton et al., 2013).

Proportion scores for markers of neuronal and glial lineage differentiation hierarchy are summarised in Table 4. Grade II-gliomas demonstrated low proportion scores for the stem cell markers nestin and CD133 (median 1 for both markers), but higher positivity was observed in the majority of high-grade gliomas, especially in glioblastomas for which the median proportion score for nestin was 3 (range 1–4) (Figs. 3A, B). With the markers of neuronal progenitor cells, expression of DCx was lacking in more than half of the cases (11/20). Samples with DCx expression were represented by low-grade (4/5) and high-grade (5/15) gliomas. Irrespective of the grade or morphological type of tumour, DCx expression was generally low, with 7/9 DCx$^+$ samples exhibiting a proportion score of 1. In contrast, expression of Olig2 was detected at high levels in almost all cases (18/20). The pattern of immunoreactivity for Olig2 appeared to be the same regardless of the morphological diagnosis or grade of malignancy. Indeed, the median proportion score was equal to 3 for oligodendroglial (range: 2–4) and astrocytic (range:

Table 4
Immunohistochemical features (proportion score) for the different glial tumours.

Case	Morphologic diagnosis	Nestin	CD133	DCx	βIII tubulin	NeuN	Olig2	GFAP	S100	VIM
1	Oligodendroglioma	1	0	1	0	0	3	1	1	1
2	Oligodendroglioma	1	1	1	0	0	3	1	3	2
3	Oligodendroglioma	1	1	3	0	0	3	1	1	1
4	Oligodendroglioma	1	0	1	0	0	3	1	1	2
5	Oligodendroglioma	1	1	0	0	0	4	0	4	0
6	Anaplastic oligodendroglioma	1	0	0	0	0	3	0	4	1
7	Anaplastic oligodendroglioma	2	0	0	0	0	4	0	0	0
8	Anaplastic oligodendroglioma	0	1	1	0	0	2	1	4	1
9	Anaplastic oligodendroglioma	1	1	0	0	0	3	1	1	2
10	Anaplastic oligodendroglioma	3	1	0	0	0	3	0	0	0
11	Anaplastic oligodendroglioma	1	1	1	0	0	4	1	4	1
12	Anaplastic oligodendroglioma	3	3	0	0	0	3	0	2	3
13	Anaplastic astrocytoma	3	3	0	0	0	0	3	3	0
14	Anaplastic oligoastrocytoma	1	3	1	0	0	2	3	1	2
15	Glioblastoma	4	1	2	0	0	4	2	1	2
16	Glioblastoma	1	0	1	0	0	4	4	1	4
17	Glioblastoma	3	3	0	0	0	3	3	2	3
18	Glioblastoma	1	1	0	0	0	3	3	3	2
19	Glioblastoma	3	3	0	0	0	0	0	2	1

Correspondence between the proportion score and the percentage of positively stained tumour cells: 0, <5%; 1, 5–30%; 2, 30–60%; 3, 60–90%; 4, >90%.

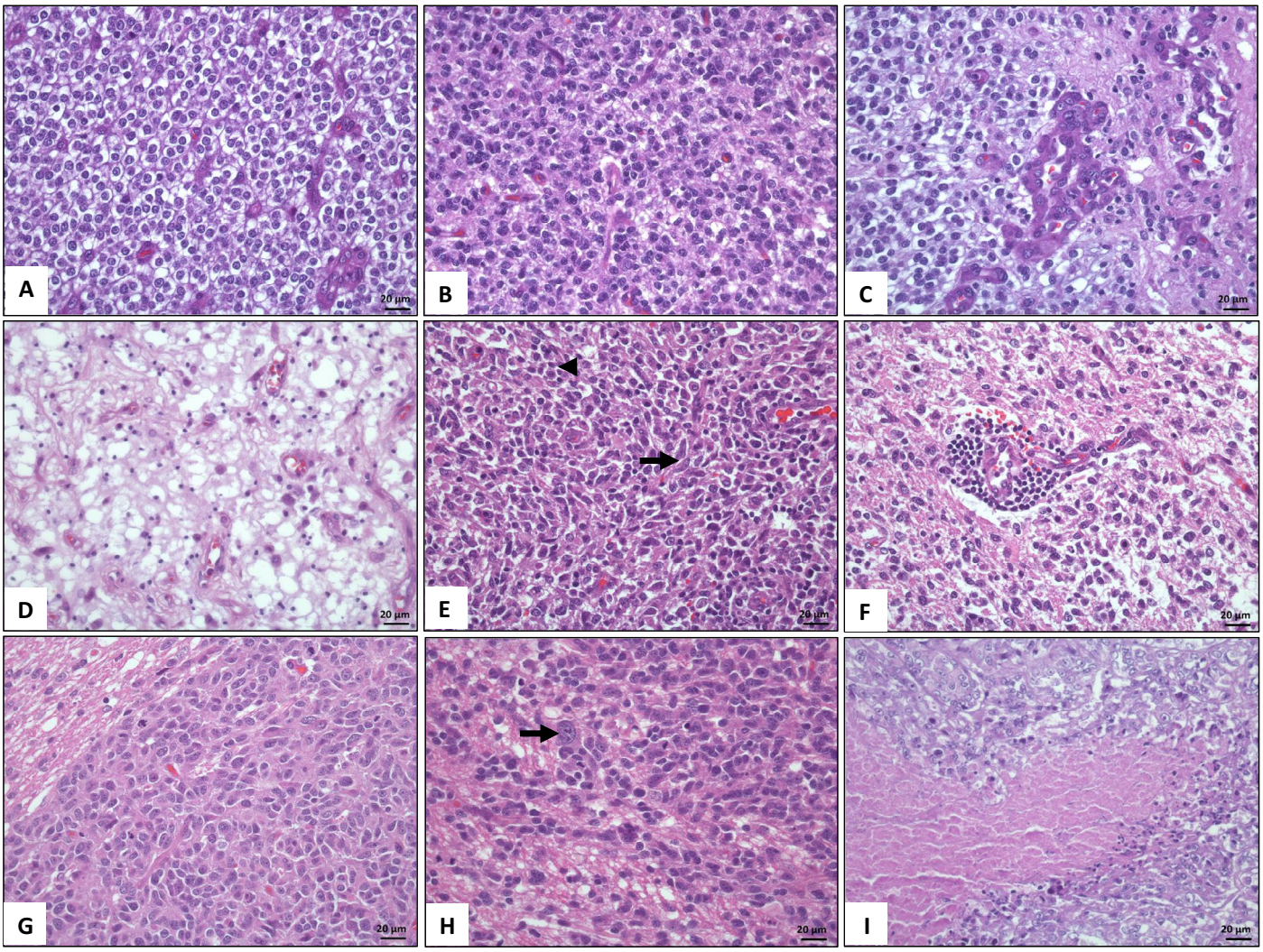

Fig. 2. Histopathological features of canine gliomas. (A, B) Honeycomb and solid growth patterns displayed by oligodendrogliomas and anaplastic oligodendrogliomas, respectively. (C) Glomeruloid-like vessels found in the margin of anaplastic oligodendrogliomas. (D) Anaplastic astrocytoma showing a diffuse infiltration of pleomorphic cells and numerous blood vessels. (E) Anaplastic oligoastrocytoma showing two cell populations: one represented by round cells with poorly defined eosinophilic cytoplasm (arrowhead, oligodendrocytes) and a second by elongated cells with an ill-defined eosinophilic cytoplasm (arrow, astrocytes). (F) Perivascular non-proliferative cuffs in anaplastic oligoastrocytoma that evidence an inflammatory associated response. (G, H) Compressive and infiltrative growth patterns displayed by glioblastomas. Note the giant nucleus frequently observed in this grade of tumour. (I) Typical pseudopalisading of neoplastic cells around a necrotic focus observed in most glioblastomas.

0–4) tumours, and also considering low-grade (range: 3–4) and high-grade (range: 0–4) gliomas (Table 4).

Distinctly different patterns of immunoreactivity for GFAP and VIM were evident between oligodendroglial and astrocytic tumours. Oligodendroglial tumours, irrespective of the grade of malignancy, had a low proportion score (range 0–1; median 1 for GFAP and range 0–3; median 1 for VIM; Fig. 4B), while astrocytic tumours showed a high proportion score with strong immunostaining (range 0–4; median 3 for GFAP and range 0–4; median 2 for VIM). Regarding S-100 protein expression, similar proportion scores were found for oligodendroglial (range 0–4; median 1.5) and astrocytic tumours (range 1–3; median 2). The inflammatory response was evaluated using the microglial marker Iba1, demonstrating a high level of expression corresponding to macrophages in perivascular cuffs, especially in the anaplastic oligoastrocytoma (Fig. 2F).

Nine of the tumour samples were found to be negative to Ki-67 immunostaining. In these tumours, cells assumed to be activated (endothelial cells in glomeruloid-like vessels) were not labelled, so the tumours were not considered when calculating the proliferation index. In tumour samples for which Ki-67 immunostaining was successful (cases 1–5, 8, 9, 14–16), mean proliferation index increased from low-grade to high-grade gliomas (6.5% in grade II gliomas, 8.5% in grade III gliomas and 9.5% in grade IV gliomas; Fig. 3G). These results were in agreement with the increase in mean mitotic index from low-grade to high-grade gliomas (Table 3).

Discussion

In this study, we investigated for the first time the IHC expression of characteristic markers of glial and neuronal lineage differentiation in 20 canine spontaneous gliomas. The study achieved two objectives: (1) gaining greater insight into the morphological and IHC characteristics of infiltrating canine gliomas (including lineage commitment and differentiation blockage of tumour cells), and (2) providing data regarding the potential of canine tumours for use as a relevant animal model for human glioma studies.

In order to investigate the similarities of phenotypic features of spontaneous canine and human gliomas, the human WHO classification was used to classify the canine gliomas. The tumours included in the study consisted of gliomas of differing phenotype

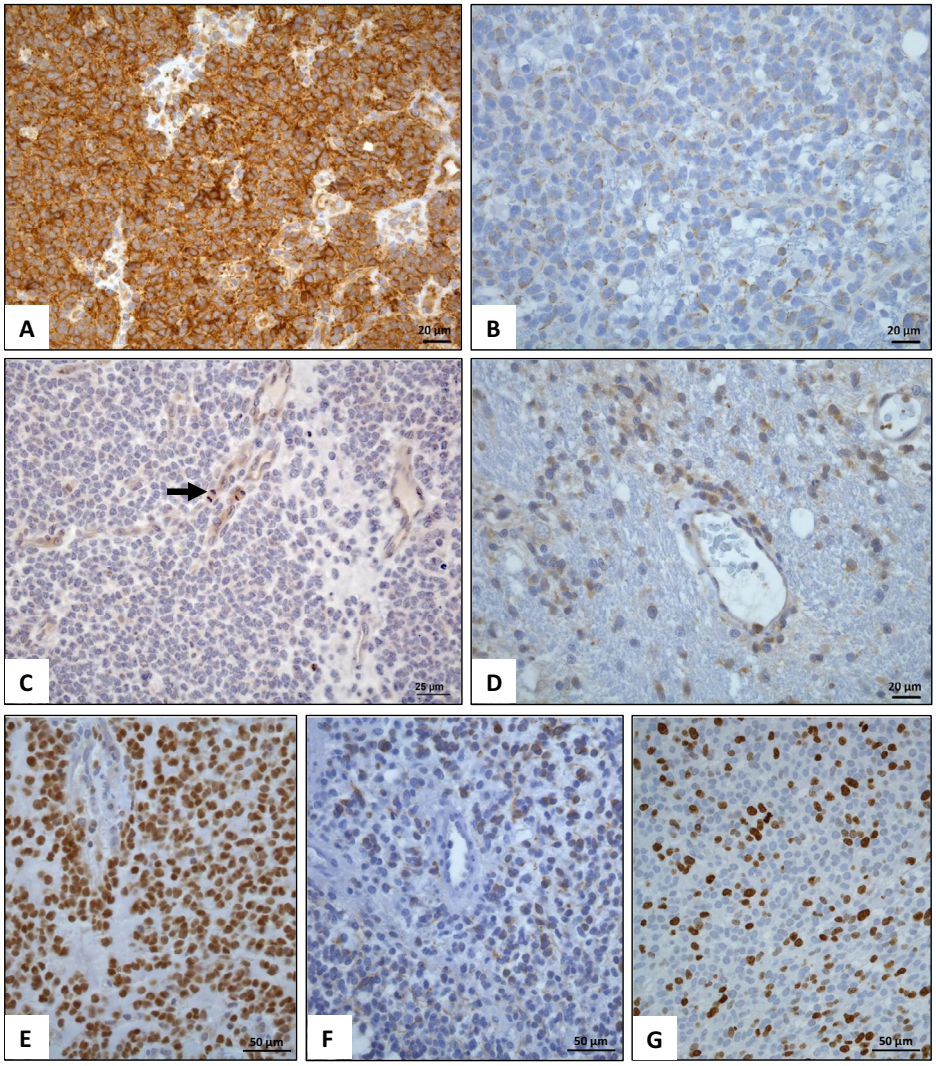

Fig. 3. Immunohistochemical features of glioblastomas. (A) A high proportion score and strong, diffuse cytoplasmic nestin immunolabelling were observed for this marker in case 15. (B) A lower proportion score and a weaker intensity with a fibrillary pattern could also be observed, as in case 16. (C, D) CD133 immunoreactivity observed in both neoplastic and endothelial cells, but with a weaker intensity in the latter. Note the evident presence of $CD133^+$ cells in case 15 (arrow). (E) Representative example of neoplastic cells exhibiting obvious Olig2 nuclear expression. (F) Cytoplasmic expression of DCx by tumour cells in case 16. (G) Ki-67 immunohistochemistry revealed a high proliferation index in glioblastomas.

and grade of malignancy: grade II and III oligodendrogliomas, grade III and IV astrocytomas, and a grade III oligoastrocytoma. The analysis of the expression of multiple cell markers characteristic of glial and neuronal genesis hierarchy in these various samples allowed us to assess the lineage commitment of canine glioma cells (Singh et al., 2004; Dell'Albani, 2008).

In order to evaluate whether CSCs were present in the different tumour samples, the glycoprotein CD133 and the intermediate filament proteins nestin were used as markers of 'stemness'. Nestin and CD133 immunolabelling were compared, and if both were expressed, this was taken to indicate the presence of undifferentiated progenitors. $Nestin^+CD133^+$ progenitors typify a less differentiated subpopulation compared to $nestin^+CD133^-$ cells (Zeppernick et al., 2008). Combining these two markers also allowed exclusion of $CD133^+$ cells that are not CSCs (epithelial cells or activated endothelial cells for example) and non-specific CD133 labelling (secondary to tissue disturbances due to the pre-treatment step of the IHC process). A better approach would have been to perform double labelling, either by associating nestin with CD133 to ensure that positive cells were actual undifferentiated cells, or associating one of these stem cell markers with EGFRvIII in order to confirm the neoplastic nature of the progenitors. Unfortunately, the small amount of available tissue for each glioma sample prevented us from carrying out such an analysis.

Expression of both nestin and CD133 was found in the different types and grades of canine gliomas investigated, while the proportion of samples exhibiting putative CSCs was equivalent for grade II gliomas and high-grade gliomas. This observation is in line with studies of human gliomas showing that the proportion of $CD133^+$ cells in the tumour increases with the degree of malignancy and is correlated with the prognosis of patients (Beier et al., 2007; Rebetz et al., 2008; Zeppernick et al., 2008; Zhang et al., 2008). Unfortunately, in our study this trend was not found to be statistically significant, perhaps due to the heterogeneous representation of glioma grades (5 low-grade vs. 15 high-grade) and the limited number of cases. Further investigations using a greater number of cases will be necessary to confirm or reject the present findings.

The lack of immunoreactivity to βIII-tubulin and NeuN indicates that canine glioma cells do not show neuronal differentiation. This finding is in contrast with IHC features of high-grade human gliomas in which the expression of βIII-tubulin along with other markers of neurons such as neuron specific enolase (NSE) has been

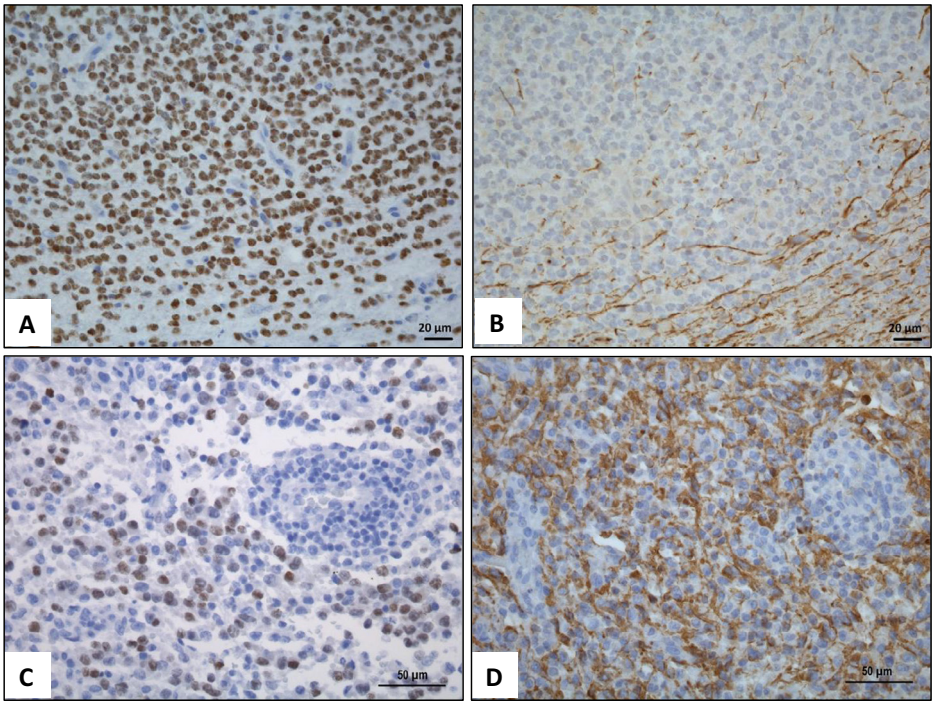

Fig. 4. Immunohistochemical features of oligodendroglial tumours and anaplastic oligodendroglioma. (A) Olig2 positivity in most of the nuclei of oligodendrogliomas. (B) Lack of expression of GFAP by neoplastic oligodendrocytes but positivity of entrapped reactive astrocytes. (C, D) Anaplastic oligoastrocytoma showed both strong Olig2 and GFAP positivity, in the nuclei of neoplastic oligodendrocytes and the cytoplasm of neoplastic astrocytes, respectively.

reported (Katsetos et al., 2007; Rebetz et al., 2008). Further evaluation of canine glioma cells, either with IHC or Western-blotting, and with different neuronal markers including NSE, will be necessary to confirm this observation.

Two markers were used to identify lineage-restricted progenitors: the transcription factor Olig2 expressed by oligodendrocyte precursor cells and the microtubule-associated protein DCx detected in neuronal precursor cells. Regardless of glioma type or grade, expression of DCx was weak or absent while the proportion score for Olig2 was high. As with human gliomas, our results suggest that both astrocytic and oligodendroglial tumours are endowed with oligodendrocyte lineage differentiation potential (Ligon et al., 2004; Rebetz et al., 2008; Rhee et al., 2009).

As expected, astrocytic tumours showed high median proportion scores for GFAP and VIM whereas median proportion scores for these markers were low in oligodendroglial tumours. The co-expression of GFAP and VIM detected in oligodendroglial tumours can be attributed to entrapped reactive astrocytes in the tumour, while GFAP expression without VIM expression suggests the presence of immature neoplastic oligodendroglial cells. As described in human gliomas, both the astrocytic and oligodendroglial tumours in our study showed similar immunoreactivity to the S-100 protein, which demonstrates the limited usefulness of this marker in the classification of spontaneous infiltrating canine gliomas.

The proliferation index tended to increase with the grade of glioma malignancy. Although this finding needs to be confirmed in a larger cohort of canine tumour samples, the higher proliferative potential of neoplastic cells in high-grade gliomas of our study, confirmed by the Ki-67 marker, is in agreement with previously published reports in humans (Hu et al., 2013).

Taken together, the lack of expression of neuronal markers and the scarcity of expression of DCx, combined with the diffuse expression of Olig2 and the presence of nestin and CD133 positive cells, suggest that tumour-initiating cells demonstrate a glial progenitor-like phenotype in canine gliomas. This finding is partially in agreement with the description of lineage commitment of glioma cells in human patients. Glial progenitor-like phenotype has been described for all human gliomas but high-grade gliomas show additional expression of markers for neuronal lineage differentiation (Rebetz et al., 2008).

Owing to the heterogeneous representation of the various glioma types and the limited number of cases in our study, a statistical analysis was not appropriate. Despite the absence of statistically significant results, this proof of concept study shows the feasibility of detecting progenitor cell markers with IHC in spontaneous canine tumour samples and the presence of these cells in all grades of diffuse gliomas. The results are a further step in demonstrating the value of spontaneous models in comparative oncology. New therapeutic strategies specifically targeting CSCs are being developed (Cho et al., 2013) and the dog could serve as a translational model for such treatments. Additional studies with a larger cohort will be necessary to allow rigorous statistical analysis of pre-planned outcome measures. We plan to extend our investigation using a greater number of canine glioma samples, currently included in a multicentre study, to evaluate IHC as well as biological characteristics of primary culture cells (capacity for neurosphere formation, multi-lineage differentiation, and tumour initiation in immunocompromised xenografted rodents).

Conclusions

To the best of our knowledge, this is the first study to evaluate lineage commitment of tumour cells in spontaneous canine gliomas. Our results suggest that, as in human infiltrating gliomas, canine glioma cells are reminiscent of glial-progenitor cells with enhanced nestin and CD133 expression in high-grade compared to low-grade gliomas. Similarities of histological and immunohistochemical features between human and canine gliomas lend support to use of the dog as a relevant animal model for preclinical evaluation of new treatment strategies.

Conflict of interest statement

Royal Canin provided financial support for this study but played no role in the study design, in the collection, analysis and interpretation of data, or in the manuscript writing or submission for publication. None of the authors has any other financial or personal relationships that could inappropriately influence or bias the content of the paper.

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