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LABORATORY INVESTIGATION - HUMAN/ANIMAL TISSUE

Spontaneous canine gliomas: overexpression of EGFR, PDGFRα and IGFBP2 demonstrated by tissue microarray immunophenotyping

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Abstract Fifty-seven spontaneous canine gliomas were histologically classified and graded using the latest World Health Organization (WHO 2007) criteria for classification of human gliomas. A total of 19 canine astrocytomas were classified as follows: grade IV (GBM) $n = 7$; grade III $n = 5$; and grade II, $n = 7$. Thirty-eight oligodendrogliomas were classified as either grade III (anaplastic) $n = 35$ or low grade II $n = 3$. Tissue microarray (TMA) immunohistochemistry was used to evaluate tumor expression of EGFR, PDGFRa and IGFBP2, three key molecules of known pathophysiological importance in human gliomas. Findings were correlated with tumor classification and grade. Increased EGFR expression was demonstrated in 57% of GBMs, 40% of grade III and 28% of grade II astrocytomas. EGFR expression occurred in only 3% of grade III oligodendrogliomas. Increased expression of PDGFRα was demonstrated in 43% of GBMs, 20% of grade III, and 14% of grade II astrocytomas. In the oligodendroglioma series, 94% of grade III tumors overexpressed PDGFRα. IGFBP2 expression was detected in 71, 60 and 28% of GBMs, grade III and grade II astrocytomas respectively. IGFBP2 expression occurred in 48% of anaplastic and in 33% of low grade oligodendrogliomas. Expression of EGFR, PDGFRα or IGFBP2 was not detected in normal canine CNS control TMA cores. The incidence of overexpression of EGFR, PDGFRα and IGFBP2 in these canine gliomas closely parallels that in human tumors of similar type and grade. These findings support a role for the spontaneous canine glioma model in directed pathway-targeting therapeutic studies.

Keywords Canine · EGFR · Glioma · IGFBP2 · Immunohistochemistry · PDGFRα · Tissue microarray

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Introduction

Recent genetic and molecular characterization of human glial tumors has identified several key growth factors and their tyrosine kinase (TK) receptors that may prove to be exquisite therapeutic targets. Upregulation of epidermal growth factor receptor (EGFR) by gene amplification or activating mutations with protein over expression is a hallmark in up to 60% of primary human glioblastoma multiforme (GBM) [1]. Overexpression of platelet-derived growth factor (PDGF) ligands A and B and/or their TK receptors have been demonstrated in all grades of astrocytomas with highest incidence (25%) in GBMs [2, 3]. In contrast the ligands of PDGF and their corresponding receptors PDGFR-α and β almost invariably are

overexpressed in all low and high grade human oligodendrogliomas [4, 5]. PDGFR signaling quantitatively regulates tumor grade and is required to sustain high grade oligodendrogliomas in mice [6]. In human GBMs, insulin-like growth factor-binding protein 2 (IGFBP2) overexpression is a key molecular event [7, 8]. This increased expression directly correlates with both higher histological grade and decreased survival in diffuse gliomas [8–10]. Gene amplification and overexpression of IGFBP2 also enhances glioma cell mobility and tumor invasiveness [11, 12]. In a transgenic mouse brain tumor, the IGFBP2 gene exerts a key oncogenic signal in activation of the AKT pathway and with K-Ras or PDGFB, promotes tumorigenesis and the progression of both astrocytomas and oligodendrogliomas [13].

In the dog, the incidence of primary spontaneous tumors of the CNS is approximately 14.5 per 100,000 dogs, with glial cell tumors (astrocytomas, oligodendrogliomas and mixed glial tumors) accounting for about 20% of all tumors [14]. The identification of similar shared key biomarkers in these spontaneous canine gliomas would provide a valuable translational model system for human tumors and introduce the possibility for targeted therapy in dogs. The neuroimaging, gross, and histological characteristics of the canine grade IV astrocytoma (GBM) share close similarities to their human counterpart [15]. Expression of EGFR protein was demonstrated in 60% of these tumors and later confirmed in another small GBM series [16]. We have recently demonstrated in both canine astrocytomas and high grade oligodendrogliomas qualitative upregulation of mRNA of TK receptors including EGFR-1 and PDGFRα respectively [17]. Despite these encouraging similarities, there have been no further studies on identification or expression of other critical growth factors/receptors in canine gliomas.

To expand on these findings, this study was designed to detect immunocytochemically, using a canine high-throughput glioma tissue microarray (TMA) construct, any changes in expression of EGFR, PDGFRα, and IGFBP2 in 57 canine gliomas. These results were then correlated with the glioma histologic type and grade.

Materials and methods

Canine brain tumors

Archival tissues were selected from a continuous clinical series of 57 dogs, that had been admitted to the Veterinary Medical Teaching Hospital (VMTH) of the School of Veterinary Medicine, University of California Davis for neurological evaluation and which subsequently had a complete necropsy examination. For inclusion in the study, each dog had a brain tumor diagnosed histologically as either an oligodendroglioma or an astrocytoma. Archival tissues were obtained from either surgical biopsies or at necropsy. Tumors were classified and graded according to the latest criteria of the human WHO classification of tumors of the CNS [18]. Paraffin-embedded blocks processed from formalin-fixed tissue and selected for sampling for the canine glioma TMA included 19 astrocytomas (7 grade IV (GBM), 5 grade III (AA), 7 grade II (A) astrocytomas) and 38 oligodendrogliomas (35 grade III (AO) and 3 grade II (O) tumors) (see Fig. 1).

Tissue microarray construction

Representative areas of tumor tissue for core sampling were delineated by two human neuropathologists (A.W.B., G.N.F.) on hematoxylin-eosin (HE) stained sections, from the original paraffin blocks for the tissue microarray assembly. Then using an Automated Tissue Arrayer (Beecher Instruments, Inc., Sun Prairie, WI), duplicate 0.6 mm diameter cores were extracted from the corresponding areas of donor paraffin embedded blocks and inserted into the TMA recipient block in predetermined sites according to the tumor type (Fig. 1). Appropriate human and canine CNS duplicate core samples from normal gray and white matter were included as negative control tissues. In addition duplicate cores of appropriate human gliomas, obtained from surgical resection or necropsy, in the archives of the Department of Pathology, MD Anderson Cancer Center, Houston were included as positive control tissue for each antibody.

Immunohistochemistry

Tissue sections 5 μm thick were cut from the TMA recipient paraffin block for initial microscopic screening of HE-stained sections for suitability of each core, and then

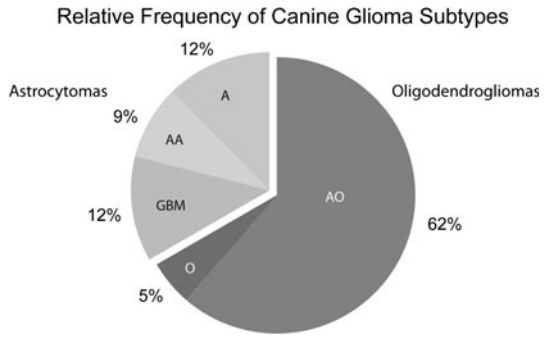

Fig. 1 Diagram of the relative frequency (%) of the canine oligodendroglioma and astrocytoma subtypes included in this study. *A* astrocytoma Grade II, *AA* anaplastic astrocytoma Grade III, *GBM* glioblastoma multiforme Grade IV, *O* oligodendroglioma Grade II, *AO* anaplastic oligodendroglioma Grade III

for subsequent immunohistochemical staining and evaluation (Fig. 2). Both construction of the canine glioma TMA and immunohistochemical staining for EGFR, PDGFRα, and IGFBP2 were done at the Tissue Microarray and Automated Image Acquisition Laboratory, The University of Texas M.D. Anderson Cancer Center, Houston, TX as described previously [19]. After core removal, sections of one randomly selected tumor of each type were immunostained with each of the antibodies to confirm either the positive or negative immunoreactivity demonstrated on the TMA sections.

Immunohistochemical staining for EGFR, PDGFRα and IGFBP2 was done using a streptavidin–biotin unlabeled immunoperoxidase technique (ABC-Elite, Vector Laboratories, Burlingame, CA) with diaminobenzidine (DAB) as the chromogen for EGFR and PDGFα and aminoethyl carbazole (AEC) for the IGFBP2 antibody. All immunohistochemistry was done using an autostainer (Autostainer Plus, Dako Corp., NY). Antibodies applied were a mouse monoclonal antibody to EGFR Clone 31G7 (Zymed Laboratories Inc., South San Francisco, CA) diluted 1:50 following pretreatment of sections with protease Type 24 at room temperature for 2 min; a rabbit polyclonal antibody to PDGFRα (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) diluted 1:50 with pretreatment of sections in a citrate buffer pH 6.0, for 45 min at 93°C; and a polyclonal goat antibody to IGFPB2 (C-18) diluted at 1:1000 at 4°C overnight (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). All secondary antibody incubation was done at room temperature for 60 min. Meyer's hematoxylin was used as a nuclear counterstain. For both the human and canine negative control tissues an antibody of the same source and isotype but of irrelevant specificity was substituted at the same dilution for each of the primary antibodies.

Fig. 2 Canine glioma TMA. **a** HE stained tissue micro-array showing block grouping for GBM, A, AA, O and AO tumor subtypes. **b** PDGFRα immunohistochemically stained TMA

Scoring of the immunoreactivity data

Evaluation with semi-quantitative scoring of positive or negative immunoreactivity of cells within the cores for both EGFR, PDGFRα, IGFPB2 was done as follows: with less than 5% of immunoreactive cells, tumors were graded as negative; from 6 to 25% immunoreactive cells as +; 26–50% as ++; and 51–100% immunoreactive cells as +++. Only strong intracytoplasmic immunoreactivity for EGFR, PDGFRα and IGFPB2 was scored as positive and then graded. The number of positive immunoreactive tumors was calculated as a percentage of the total number of those tumors classified within each histological type and grade (Fig. 3).

Results

Immunohistochemical staining

Astrocytomas

Positive cytoplasmic immunoreactivity for EGFR was demonstrated in 4/7 (57%) of the grade IV GBM's, 2/5 (40%) of grade III astrocytomas and 2/7 (28%) of grade II astrocytomas (Table 1). Positive immunoreactivity for PDGFRα occurred in 3/7 (43%) of grade IV, 1/5 (20%) of grade III and in 1/7 (15%) of grade II astrocytomas (Table 2). The highest number of tumors with positive IFGBP2 immunoreactivity was 5/7 (71%) of GBMs, 3/5 (60%) of grade III and the lowest in 2/5 (28%) of low grade astrocytomas (Figs. 4 and 5).

Oligodendrogliomas

Positive cytoplasmic immunoreactivity for EGFR was detected in 1/35 (3%) of grade III oligodendrogliomas and 0/3 of grade II oligodendrogliomas (Table 1). Positive immunoreactivity for PDGFRα occurred in 32/35 (94%) of grade III oligodendrogliomas and in 0/3 grade II

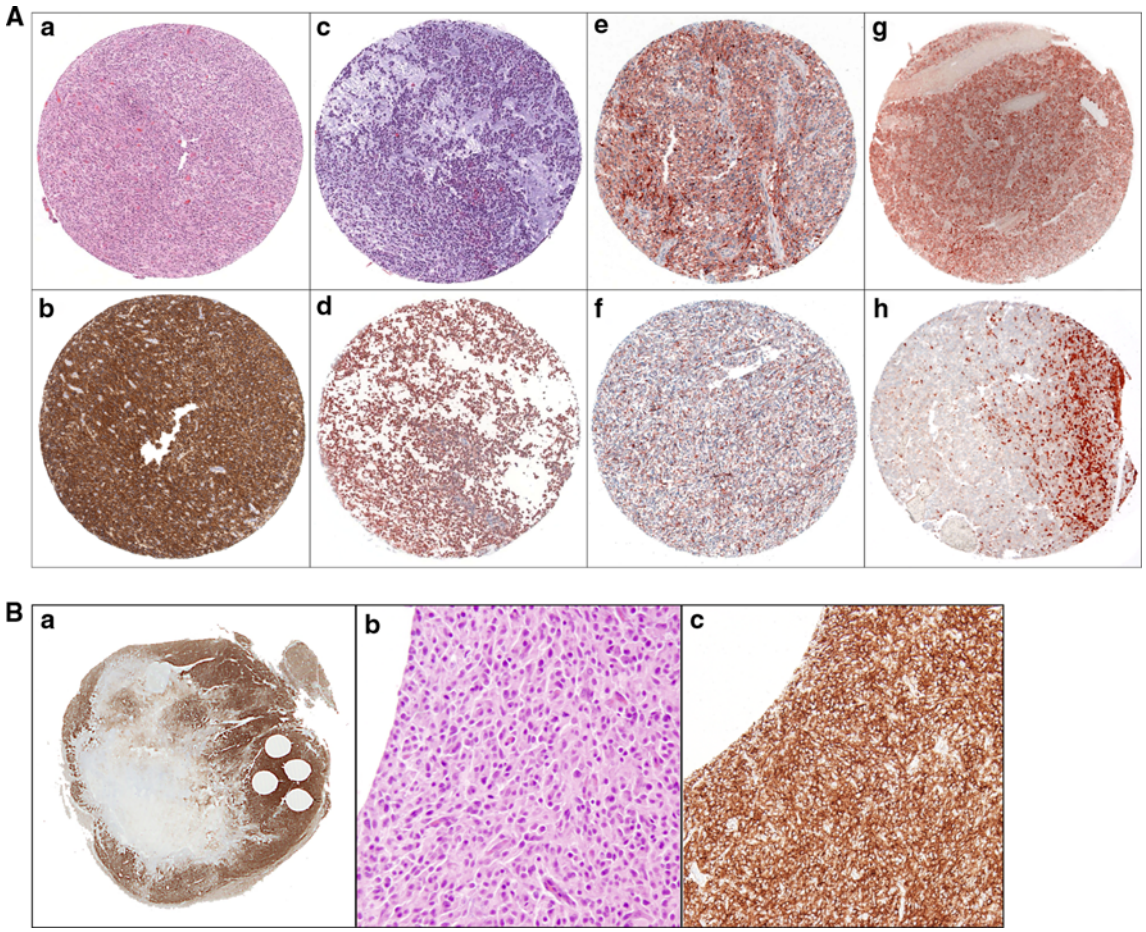

Fig. 3 **A** Representative HE and immunostaining of TMA human and canine glioma cores. (*a*) HE stained GBM with (*b*) EGFR positive immunoreactivity; (*c*) Grade III oligodendroglioma HE; (*d*) same tumor immunoreactive for PDGFα; (*e*) GBM positive for PDGFα; (*f*) Grade II astrocytoma immunoreactive for PDGFα; (*g*) Human GBM as EGFR positive control core; (*h*) canine oligodendroglioma immunoreactive for IGFBP2. **B** Positive immunoreactivity to EGFR demonstrated on a tissue section of a canine GBM after the TMA core removal. (*a*) Note the site of the four punch core biopsies on tissue section. (*b*) HE staining of the GBM adjacent to the core. (*c*) Note the positive EGFR immunoreactivity similar to the intensity and degree seen in the corresponding core section in **A**(*b*)

Table 1 Results of immunostaining for EGFR expression in 19 astrocytomas and 38 oligodendrogliomas

Tumor type	Number cases	EGFR−	EGFR+	EGFR++	EGFR+++	% EGFR positive
A	7	5	0	2	0	28
AA	5	3	0	2	0	40
GBM	7	3	0	3	1	57
O	3	3	0	0	0	0
AO	35	34	0	0	1	3

A astrocytoma Grade II, *AA* anaplastic astrocytoma Grade III, *GBM* glioblastoma multiforme Grade IV, *O* oligodendroglioma Grade II, *AO* anaplastic oligodendroglioma Grade III

oligodendrogliomas (Table 2). One of three (33%) of the low grade oligodendrogliomas were IGFBP2 immunoreactive compared with 17 of the 35 (48%) high grade oligodendrogliomas (Table 3, Fig. 4). No immunoreactivity was detected in normal gray or white matter of control canine or human brain tissue except for variable endothelial staining with IGFBP2 antibody (Fig. 6).

Discussion

The overall patterns and incidence of positive immunoreactivity in the canine glioma TMA to EGFR, PDGFRα and IGFBP2, within each of the canine glioma histologic subtypes and grades, was strikingly similar to those in comparable human gliomas. Identification of such shared

Table 2 Results of immunostaining for PDGFRα in 19 astrocytomas and 38 oligodendrogliomas

Tumor type	Number cases	PDGFRα−	PDGFRα+	PDGFRα++	PDGFRα+++	% PDGFRα positive
A	7	6	1	0	0	15
AA	5	4	1	0	0	20
GBM	7	4	2	1	0	43
O	3	3	0	0	0	0
AO	35	3	7	12	13	94

A astrocytoma Grade II, *AA* anaplastic astrocytoma Grade III, *GBM* glioblastoma multiforme Grade IV, *O* oligodendroglioma Grade II, *AO* anaplastic oligodendroglioma Grade III

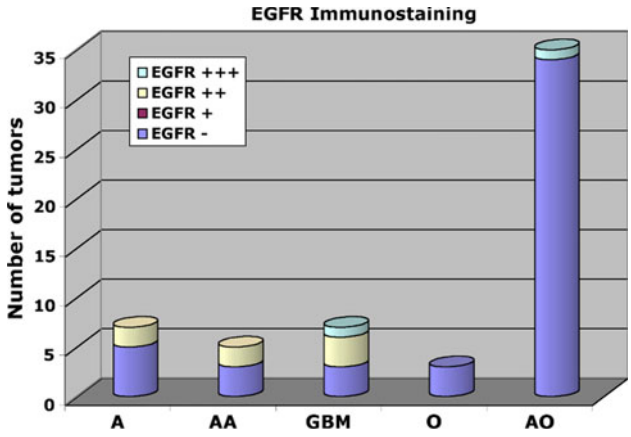

Fig. 4 Histogram of expression of EGFR immunoreactivity in canine gliomas. *A* astrocytoma Grade II, *AA* anaplastic astrocytoma Grade III, *GBM* glioblastoma multiforme Grade IV, *O* oligodendroglioma Grade II, *AO* anaplastic oligodendroglioma Grade III

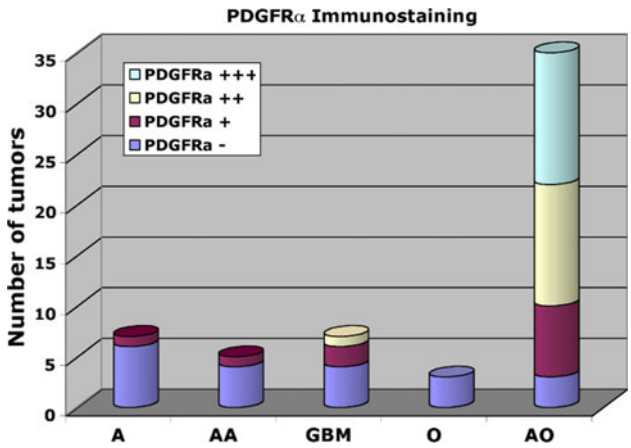

Fig. 5 Histogram of expression of PDGFα immunoreactivity in canine gliomas. *A* astrocytoma Grade II, *AA* anaplastic astrocytoma Grade III, *GBM* glioblastoma multiforme Grade IV, *O* oligodendroglioma Grade II, *AO* anaplastic oligodendroglioma Grade III

peptide growth factors implies that common signaling pathways might play critical roles in these and maybe other primary spontaneous brain tumors in the dog. These results also confirm that in canine tissue the TMA can be used as a uniform rapid immuno-profiling and screening tool for identification of these and other molecular targets [19]. However, these results do not necessarily confirm or imply a direct relationship between receptor overexpression and corresponding gene amplification. Overexpression was defined for this study simply as positive immunoreactivity of antigen not occurring in normal tissue control cores.

Firstly, in the canine astrocytomas, the overexpression of EGFR protein found in 57% of the GBMs parallels that occurring in up to 60% of human primary GBMs [1, 20]. The decreasing positive EGFR immunoreactivity in the lower grade (III and II) astrocytomas of 40 and 28% respectively also matches that trend reported in comparable human astrocytomas [2, 21]. Importantly this overall increased expression parallels the upregulation of mRNA EGFR-1 we have reported in these canine astrocytomas suggesting similar activation of this TK receptor-mediated pathway to those in human astrocytomas [17]. In human oligodendrogliomas antigenic EGFR expression increases from low to high grade tumors with 90% of all tumors exhibiting some focal reactivity [22, 23]. However this finding contrasts with only 3% positivity in all of the canine oligodendrogliomas. Remarkably this is the only discordant difference between the human and canine gliomas in similarities of growth factor expression.

Secondly, the almost uniform overexpression of PDGFRα in the canine anaplastic oligodendrogliomas closely matches that in most human oligodendrogliomas. The expression of PDGFRα is highest in human GBMs and decreases in parallel with the lower grades [24]. Likewise there was a similar decreasing trend of overexpression of PDGFRα in all grades of canine astrocytomas, highest (43%) in GBM's and lowest (15%) in Grade II astrocytomas. This consistently high expression implies that both autocrine and paracrine stimulation might also be pivotal events in canine oligodendrogliomas as suggested for both human tumors and experimental mouse models [4, 25]. Our immunohistochemical findings also parallel the elevated mRNA levels of PDGFRα we have detected predominantly in high grade canine

Table 3 Results of immunostaining for IGFBP2 in 19 astrocytomas and 38 oligodendrogliomas

Tumor type	Number cases	IGFBP2−	IGFBP2+	IGFBP2++	IGFBP2+++	% IGFBP2 positive
A	7	5	2	0	0	28
AA	5	2	3	0	0	60
GBM	7	2	3	2	0	71
O	3	2	1	0	0	33
AO	35	18	1	8	8	48

A astrocytoma Grade II, *AA* anaplastic astrocytoma Grade III, *GBM* glioblastoma multiforme Grade IV, *O* oligodendroglioma Grade II, *AO* anaplastic oligodendroglioma Grade III

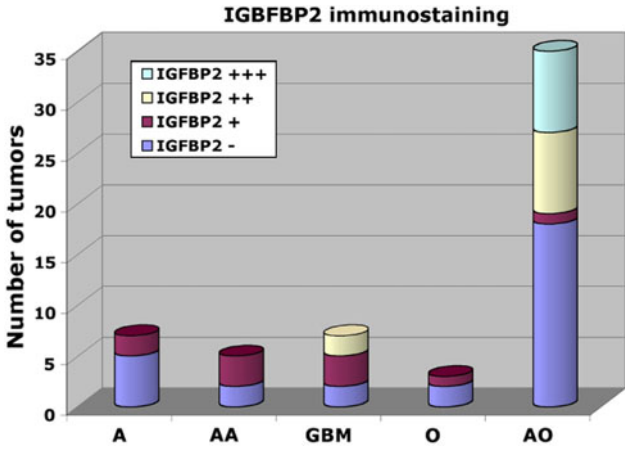

Fig. 6 Histogram of expression of IGFBP2 immunoreactivity in canine gliomas. *A* astrocytoma Grade II, *AA* anaplastic astrocytoma Grade III, *GBM* glioblastoma multiforme Grade IV, *O* oligodendroglioma Grade II, *AO* anaplastic oligodendroglioma Grade III

oligodendrogliomas, again implying a functional signaling pathway analogous to that in man [17].

Thirdly, as with human gliomas, IGFBP2 expression in the canine astrocytomas increased with the higher tumor grade with about 70% of GBMs immunoreactive compared with 28% of lower grade II tumors [7, 9, 10]. Increased expression of IGFBP2 in human higher grade astrocytomas is predictive of poorer prognosis [10]. In human GBMs, there is a significant correlation between overexpression of IGFBP2 and elevated MMP-2 expression and in turn increased invasiveness and malignancy [11]. IGFBP2 plays a key role in the activation of the Akt pathway and acts synergistically with K-Ras or PDGFB in the development and progression of both astrocytomas and oligodendrogliomas in mice [11, 13, 25]. Parallel upregulation of IGFBP2 expression in these canine astrocytomas implies that it has a comparable role in tumorigenesis and progression. In human oligodendrogliomas IGFBP2 expression increases with higher grade of tumor with 15 and 35% positive in grades II and III respectively, again surprisingly comparable with a relative 33 and 48% incidence respectively in the canine oligodendrogliomas [10].

In summary immunocytochemical demonstration of overexpression of canine EGFR, PDGFRα and IGFBP2 reveals an exciting potential for molecular targeting of these TK receptors in therapeutic studies of canine gliomas. The antibodies used were all raised against relevant human proteins suggesting close homology with their canine counterparts and indicating that application of current therapeutic targeting of these molecules in human tumors may prove useful for validation in these spontaneous canine gliomas. Based on this TMA-derived data, the canine spontaneous CNS gliomas represents a valuable translational model system for screening the effectiveness of experimental therapeutic targeting strategies that are directed at TK receptors in comparable human tumors.

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