TITLE: 14-3-3σ Protein Expression in Canine Renal Cell Carcinomas

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14-3-3σ protein expression in canine renal cell carcinomas

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Abstract

14-3-3σ is a protein expressed in many epithelial tissues associated with essential cell functions including cell-cycle control, apoptosis and cytoskeletal integrity. There is a paucity of knowledge of the tumorigenesis of canine renal cell carcinomas (CRCC) and the histological origin of this tumor has not been established. This study analyzed the expression of 14-3-3σ, Ki67, cytokeratins and vimentin in 40 CRCC. Aberrant expression of 14-3-3σ was demonstrated in 15 (38%) cases, and was associated with a significantly shorter survival time ($p<0.002$). In contrast to CRCC, normal kidney did not express 14-3-3σ. Ki-67proliferation index did not show utility as a prognostic factor. The distal convoluted tubular epithelium in normal kidneys co-expressed cytokeratins and vimentin and thus maintenance of this co-expression pattern in CRCC suggests that most tumors arise from the distal segment of the nephron. These results suggest that 14-3-3σ is a potential negative prognostic factor and a possible therapeutic target.

Key words: 14-3-3 sigma, stratifin, human mammary epithelial marker-1, *dogs, immunohistochemistry, cytokeratin, oncology, renal cell carcinoma, kidney, urinary, vimentin*

The 14-3-3 family is a group of small, highly conserved and ubiquitously expressed acidic proteins with molecular weights between 28–33 kDa. They are found in all eukaryotic species and include seven different 14-3-3 protein isoforms in mammalian cells, each designated by a Greek letter (beta-β, epsilon-ε, gamma-γ, eta-η, theta-θ, sigma-σ, and zeta-ζ). Multiple 14-3-3 ligands are involved in various cellular processes including DNA damage signaling, cell proliferation and survival, cytoskeleton rearrangement, transcriptional control and cell cycle regulation. Specifically, the ability of 14-3-3 proteins to bind and regulate several oncogenic, as well as various tumour suppressor gene products, suggests they have a potential role in tumorigenesis.[13,17]

Unlike other family members, which are broadly expressed in the brain and play a pivotal role in neurodegenerative diseases, 14-3-3σ (also known as stratifin or human mammary epithelial marker-1) is only expressed in epithelial cells and is closely associated with tumor development. The expression of this isoform was originally identified as a response to DNA damage and *TP53* activation. *TP53* encodes the protein p53 which subsequently induces the production of 14-3-3σ in the cytoplasm. 14-3-3σ is able to bind and sequester specific cyclin-dependent kinases, CDK4, CDK2 and CDK1 that regulate the G1/S and G2/M cell cycle checkpoints preventing initiation of mitosis and allowing DNA repair.[14] Furthermore, 14-3-3σ has been shown to play an anti-apoptotic role and promote cell survival[29] and recently has been identified as a promising therapeutic target.[2,40] In veterinary medicine, 14-3-3σ has been reported as an oncoprotein in canine mammary and urinary bladder carcinomas.[34-36]

Carcinomas are the most common canine primary renal tumors comprising 4 different histologic patterns (solid [clear cell or chromophobe type], tubular, papillary and multilocular cystic)[9,19,20] that display heterogeneous biological behaviors and present an overall median survival time (MST) ranging from 359-743 days.[3,5,9] Clinical outcome

is associated with histologic subtype, mitotic index and Cox-2 expression.[5] In contrast to humans, there is a dearth of knowledge with regards to the mechanisms and molecules that contribute to the development and progression of canine renal cell carcinomas. Additionally, the histological origin of these tumors in the dog has still not been clarified and there is evidence that their immunophenotype differs from the human counterpart.[8]

In order to elucidate if 14-3-3σ plays a role in CRCC oncogenesis, as has been demonstrated for other canine tumors, this study aims to investigate the expression of the protein and its association with outcome and with various clinicopathological variables, including Ki-67labelling index. Immunohistochemical labelling will be used to compare the distribution of two major cell markers (cytokeratins and vimentin) in the normal kidney to their expression pattern in tumors to determine the cell of origin of CRCC.

Materials and Methods

Case selection

Cases included in this retrospective study were selected from a previously published series of dogs with CRRC that underwent nephrectomy (n=39).[5] One case presented with metastatic disease and was immediately euthanized and diagnosis was made at necropsy; this dog was not included in the survival analysis. A complete medical history from dogs containing signalment, clinical presentation, laboratory findings, clinical stage, histological diagnosis, use and type of adjuvant therapy and overall MST was available. Samples from five normal canine kidneys were used as controls.

Histological classification

Hematoxylin- and eosin-stained tissue sections were histologically classified according to the WHO Histological Classification of Tumors of the Urinary System of Domestic Animals by three veterinary pathologists (ASB, ALS and SLP).[19] In cases of disagreement between two or more pathologists, a consensus was reached. To further classify the solid tumors into clear cell and chromophobe-type carcinomas, additional tissue sections were stained with Hale's colloidal iron stain. In addition to subtype, other histopathological features including mitotic activity index (MAI) (mitoses per 2.37 mm^2)[21], presence of necrosis, inflammation and vascular invasion were assessed as previously described.[5]

Immunohistochemistry and evaluation

Immunohistochemical analysis was performed in forty cases. Four micrometer thick fresh-cut tissue sections from formalin-fixed, paraffin-embedded tissue blocks were labeled with mouse-monoclonal antibodies against 14-3-3σ (clone 5D7, 1:40, Santa Cruz Biotechnology, Heidelberg, Germany), Ki-67(MIB-1, 1:150, Dako, Ely, UK), cytokeratins (clone AE1/ AE3, 1:100, Dako) and vimentin (clone V9, 1:500, Dako) on a BondMax Autostainer (Leica, Milton Keynes, UK). 14-3-3σ antibody has been previously shown to identify the appropriate canine molecular weight antigens by western blot and to cross-react with canine tissues.[27,33,36] Heat-induced antigen retrieval was performed using a pH 6 buffer (Bond ER1, Leica) for 10 minutes (vimentin), 20 minutes (14-3-3σ) and 30 minutes (AE1/AE3) at 90^{o}C and a pH 9 buffer (Bond ER2, Leica) for 10 minutes at 90^{o}C for Ki-67. The Bond Polymer Refine Detection kit (Leica) was used for visualization with hematoxylin counterstain. As positive controls, normal canine urinary bladder (for 14-3-3σ), normal canine skin (for

cytokeratins and vimentin) and a hyperplastic lymph node (for Ki-67) were used in every assay. All positive controls showed: cytoplasmic immunolabeling of the urinary bladder urothelium for 14-3-3σ, nuclear immunolabeling for Ki-67, and cytoplasmic immunolabeling for cytokeratins and vimentin. When present, surrounding histologically normal renal tissue was used as an internal positive control. Negative controls were prepared by replacing the primary antibody with Leica Antibody Diluent (Leica) only. A positive reaction was indicated by the presence of distinct brown cytoplasmic or nuclear labelling in neoplastic cells.

Immunoreactivity was independently evaluated by two of the authors (ASB and SLP) and discrepancies were discussed jointly on a double-headed microscope. To evaluate correlations with clinical features, pathological variables and outcome, positive labeling for 14-3-3σ was assessed using a previously published semiquantitative scoring system.[35] The percentage (1, up to 10%; 2, 11%–50%; 3, 51%–80%; and 4, >80%) and the staining intensity (0, negative; 1, weak; 2, moderate; and 3, intense) of positive cells was recorded and a total score (TS), ranging from 0 to 12 was calculated by multiplying these two parameters for each of the studied cases. The count of Ki-67-positive cells was performed in 10 high power fields (40X) counting at least 1,000 cells for each case, using the TMARKER software.[28] Ki-67 expression was evaluated as the labeling index and was defined as the percentage of Ki-67-positive cells. For cytokeratins and vimentin, positivity was recorded when there was distinct cytoplasmic labelling of the neoplastic epithelial cells and scored semiquantitatively (0, negative; 1, weakly positive; 2, moderately positive and 3, strongly positive) as previously described.[12]

Statistical analysis

Expression of 14-3-3σ, Ki-67, cytokeratins and vimentin were compared with histological subtype, MAI, metastasis at diagnosis, necrosis and inflammation. A chi-squared and a Fisher's exact test were used for studying categorical variables. Non-parametric analysis was performed with the Kruskal-Wallis 1-way ANOVA test. Overall median survival time was defined as time from nephrectomy for CRCC treatment until death. Dogs that were lost to follow-up or still alive at the end of the study period or died were censored.

Survival analysis using the Kaplan-Meier product limit method was conducted to estimate disease-related MST, for the whole population of CRCC dogs. To compare estimated disease-related MST between categories the logrank test (univariate analysis) was employed. Multivariate analysis using Cox-regression analysis was performed to demonstrate combined predictor variables found significant in univariate analysis. All statistical analyses were carried out using SPSS software (version for Windows 22.0 Armonk, IBM Corp., NY, USA). In all comparisons, $P < 0.05$ was accepted as defining statistical significance.

Results

Clinical findings

For the 39 dogs with clinical follow-up, the median age at diagnosis was 8 years (range, 2–12 years). There was a male to female ratio of 1.05:1, including 20 males (9 intact) and 19 females (5 intact). Breed distribution amongst dogs was as follows: 12 cross breeds, 7 Labrador Retrievers, 5 Boxers, 3 Golden Retrievers, 2 German Shepherds, 2 Dalmatians and one each of Cocker Spaniel, Rottweiler, Weimaraner, Japanese Akita, Galgo Español, Bullmastiff, English Bulldog and Collie (Supplemental Table S1).

The overall median survival time (MST) for dogs diagnosed with RCC was 540 days (95% confidence interval [CI], 407–673). Of the 39 dogs, 29 were dead (74%) at the end of the data collection period, 8 (21%) were alive and 2 (5%) were lost to follow-up. Death due to neoplastic disease occurred in 18/39 dogs (46%). Death was documented as unrelated to RCC in 5 (13%) and of unknown cause in 6 dogs (15%).

The most commonly reported presenting signs were weight loss in 15 dogs (38%), hematuria in 12 dogs (31%), inappetence or anorexia in 11 dogs (28%) and lethargy in 8 dogs (20%). A palpable abdominal mass was evident on physical examination in 17 dogs (43%). In 3 cases (8%), the renal tumor was an incidental finding during a health screening or staging prior to surgery. Other less common presenting complaints included, vomiting, abdominal pain and diarrhea. For the 18 cases in which tumor size was provided, the average size was 10.5 cm in largest diameter with a range of 5 to 16 cm.

Histological findings

Forty RCC cases were available for assessment. Following histological examination these were categorized as tubular (n=21, 53%), papillary (n=9, 22%) or solid (n=10, 25%). Within the group of tumors exhibiting a tubular pattern, 12 out of 21 (57%) cases were well-differentiated (composed of easily recognizable tubules formed by cells with mild to moderate atypia) and 9 out of 21 (43%) poorly-differentiated (composed of closely-packed sheets of cells with rare tubules formed by epithelial cells with marked atypia). Using colloidal iron stain the solid group was subclassified into chromophobe RCC (n=9, 90%) that were positive with colloidal iron stain, and clear cell RCC (n=1,

10%) that exhibited large cytoplasmic vacuoles and eccentric nuclei and were negative with colloidal iron stain.

14-3-3σ expression

14-3-3σ expression was not detected in histologically normal canine kidney (glomeruli, proximal convoluted tubules, distal convoluted tubules, loop of Henle and collecting ducts) surrounding carcinomatous areas (n = 12) or in two normal canine kidney samples used as controls (Fig.1). In contrast, compressed and reactive convoluted tubules, located at the periphery of renal carcinomas, occasionally displayed moderate to intense cytoplasmic immunoreaction (Fig. 1 inset), but these positive areas were not considered in the generation of a TS. As there was no protein labelling in normal kidney, cases with a TS ≥ 1 were considered positive and consequently to neo-express 14-3-3σ. Within the tumors, positive protein expression occurred in 15/40 RCC (38%), (12 tubular, 2 papillary and 1 solid), with TS ranging from 1 to 12 (median 5.2), and 26/40 cases were negative (62%). The positive to negative ratio between the three histological tumour subtypes (tubular, papillary and solid) were 12:9, 2:7 and 1:9. Seven cases had a TS < 3, considered as low 14-3-3σ-expression (Fig. 2) and 8 cases had a TS ≥ 4, considered as high 14-3-3σ-expression (Figs. 3 and 4). Of this latter group 6 cases were tubular, 2 cases were papillary and one case was solid (clear cell type). Two tubular and one papillary carcinoma showed the highest TS (12). The predominant staining pattern was cytoplasmic, but in 13 of 15 positive cases (86%) the nucleus also exhibited positive labelling. There were no cases with an exclusive nuclear staining pattern. In all positive cases the immunoreaction varied from those with heterogeneous staining, characterized by groups of cells without expression or mildly positive, or cases with multifocal strongly positive cells, to cases with diffuse and

intense staining. Within the positive cases, mitotic figures including those with bizarre morphology exhibited moderate to strong immunopositivity to 14-3-3σ (Fig. 3 inset). Positive cases that contained areas with clusters and solitary bizarre neoplastic cells at the front of invasion into the adjacent renal parenchyma and vascular invasion within lymphatics showed intense expression of the protein (Fig. 4 inset). Furthermore, one of the positive cases had tissue from a lung metastasis available that was positively labelled. Stromal tissue, fibroblasts and areas of necrosis were not immunolabeled for 14-3-3σ.

Ki-67 expression

Ki-67 was detected only in cell nuclei without cytoplasmic immunolabeling in any of the samples. Normal canine kidney did not express Ki-67 and histologically normal kidney adjacent to carcinomas sporadically showed positive cells in the tubular lining epithelium. Ki-67 proliferative index (PI) ranged from 0.0% to 56.0% (mean 15.7%) of cells. The mean Ki-67 values for each tumor type, tubular, papillary and solid were 20.2%, 14.5% and 3.0% respectively. In 16 of 40 (40%) cases there was no immunolabeling for Ki-67. There was no correlation between Ki-67 expression and mitotic activity index ($P > 0.05$).

Cytokeratins and vimentin expression

In normal canine kidney and in the non-neoplastic renal tissue of the cases with carcinoma, the cytokeratin cocktail (which immunoreacts with keratins 1 and 2, 3, 4, 5, 6, 7, 8, 10, 13, 14, 15 16 and 19) labelled the epithelial cells forming the distal convoluted tubule and the collecting tubule and duct, while the proximal convoluted tubules and the glomeruli were negative (Fig. 5). Variable proportions of neoplastic

cells were immunolabeled in 33/40 (82.5%) cases whereas 7 (17.5%) cases were negative. Within the positive cases, 15 (45.5%) were moderately to strongly positive and 18 (54.5%) were weakly positive in randomly distributed cell clusters. The staining pattern was mainly cytoplasmic and diffuse to finely granular, with frequent membranous highlighting (Fig. 6).

In normal renal parenchyma, vimentin was expressed in glomerular endothelial cells, interlobular arteries and veins, peritubular capillaries, vasa recta and the interstitium as well as in epithelial cells lining distal convoluted tubules (Fig. 7). In CRCC, vimentin was expressed in neoplastic epithelial cells in 34 cases (85%) while 6 cases (15%) did not express this marker. Of the positive cases, 26 (76.5%) were moderately to strongly positive (Fig. 8) and 8 (23.5%) were weakly positive.

Of the 40 cases, 27 (67.5%) co-expressed cytokeratins and vimentin to some extent, 7 cases (17.5%) were cytokeratins negative and vimentin positive, 6 cases (15%) were cytokeratins positive and vimentin negative.

Statistical analysis

In univariate analysis, there was a statistically significant association between high 14-3-3σ-expression (TS > 4) and a shorter survival time (P = 0.002; (Fig. 9). Furthermore, shorter survival time was significantly associated with a high (% = 3-4) percentage of positive cells (P = 0.001), and high stain intensity (stain intensity = 2-3; P = 0.000). A higher 14-3-3σ TS was positively correlated with the poorly-differentiated tubular subtype (P = 0.03) and with a higher (> 30) mitotic activity index (P = 0.003). Furthermore, in multivariate analysis, 14-3-3σ-expression remained an independent predictor of prognosis (P = 0.013). 14-3-3σ expression was not correlated with metastasis at the time of diagnosis, necrosis or type of inflammation, nor with Ki-67,

vimentin or cytokeratins expression ($P > 0.05$). No association was observed between Ki-67 index and MST.

Discussion

In the present study, the expression and prognostic significance of various markers of cell proliferation, tumor progression and cell characterization were investigated in a large case series of canine RCC. To the author's knowledge this is the first analysis of 14-3-3σ in canine RCC, and the first evidence that this protein may be a significant prognostic factor in canine neoplasms.

14-3-3σ expression has become the focus of much research in human medicine. The levels of mRNA, protein expression and methylation status have been studied in a variety of human cancers.[7,22,32,39] As 14-3-3σ can be induced in a p53-dependent manner, preventing mitotic catastrophe, and because its expression is silenced in various cancers, such as breast and head and neck carcinoma, it has been regarded as a tumor suppressor.[6,18] Subsequent studies have suggested that its role in tumorigenesis is tumor-dependent, as an increasing number of investigations have found over-expression and neo-expression of the protein being associated with malignancy.[16,23,32,39] Similarly, the relevance of this protein as a tissue differentiation marker and as an oncoprotein has been reported in canine mammary and urinary bladder carcinomas.[34-36] In the current study, immunohistochemical analysis revealed the absence of 14-3-3σ immunolabelling in normal kidney and is in accordance with previous studies using western blot and immunohistochemistry in both humans and dogs.[22,24,33,36] Interestingly, 14-3-3σ was neo-expressed in 37.5% CRCC which is more than double the 16.4% described in human RCC.[22] Additionally, positive cases that had vascular invasion showed positive labeling in embolic neoplastic cells. While in

humans the role of 14-3-3σ remains to be confirmed, we have demonstrated that its neo-expression in a subset of CRCC is significantly associated with a shorter survival time and is an independent prognostic factor. These results are compatible with previous studies in human and canine mammary basal carcinomas, characterized by a poor prognosis, that also over-express 14-3-3σ.[16,36] It has now been established that 14-3-3σ can promote lung, liver and pancreatic carcinomas.[31,32,38,39] Similar to our findings, these tumors arise from tissues that do not constitutively express the protein. These findings could support the hypothesis that a mutant form of 14-3-3σ, which retains anti-apoptotic and proliferative abilities, favors invasion and metastasis, but lacks tumor suppressive activity of the wild type protein, may be produced during tumorigenesis. Further studies, analyzing ligands of 14-3-3σ and possible mutations are required to clarify its precise role in canine RCC.

The histological origin of canine RCC has not been established. In a previous study[12] including tubulopapillary, papillary, papillary-cystic, sarcomatoid and solid variants, all cases were positive for uromodulin, also known as Tamm-Horsfall glycoprotein, which is exclusively synthesized in the kidney.[10] It was suggested that, because bovine renal cell tumors were also positive for uromodulin[15], the origin of canine RCC was most likely the distal convoluted tubule. However, the bovine study also reported that in the normal bovine kidney, uromodulin was present in epithelial cells of both the proximal and the distal convoluted tubule and, consequently, it was not possible to establish the histogenesis of RCC with this antibody. In the current study we attempted to provide insights into the origin of CRCC by immunohistochemistry for broad-spectrum keratin (AE1/AE3) and vimentin in both normal and canine RCC. To the best of our knowledge the expression pattern of vimentin and cytokeratins in normal kidney in comparison

with renal carcinomas has not been studied to date. This comparison is essential in order to validate the use of a given antibody as a differentiation marker as this allows pathologists to identify if its expression is modified (the antigen may be lost, under- or over-expressed or even neo-expressed) in a neoplasm.[27] Interestingly, here we show that cytokeratins and vimentin are co-expressed in the distal convoluted tubule and, for cytokeratins, this expression extends to the collecting tubule and duct. The co-expression was preserved in 67.5% of the tumors. Vimentin, a marker of mesenchymal cells, is expressed in a large proportion of canine RCC and it has been proposed that this could represent the acquisition of an epithelial to mesenchymal phenotype.[12] However, given we demonstrated the co-expression of cytokeratins and vimentin in the normal distal convoluted tubule epithelium, this supports the hypothesis of this cell type as the possible origin of canine RCC. This would be in agreement with the above study that used uromodulin expression to support the same hypothesis.[12] In humans, clear cell RCC account for 70% to 80% of renal carcinomas and papillary carcinoma is second in frequency accounting for 10% to 15% of renal carcinomas. These tumors share a similar immunoprofile, and most of them express cytokeratins, vimentin and CD10 (a marker of proximal convoluted tubules) and are negative for CD117 (positive in chromophobe carcinomas). Based on this combination of markers, it is known that clear cell RCC arise from proximal convoluted tubules.[30,37] In addition, papillary carcinomas are thought to arise from distal convoluted tubules.[1] Taken together, these data suggest that many canine RCC arise from a different segment of the nephron than that of human RCC.

Ki-67 protein is expressed in all active phases of the cell cycle (G1, S, G2, and M phases) and is a well-documented marker of cell proliferation in different malignancies.

Although there was no significant correlation between Ki-67 expression and clinicopathological features, the Ki-67 index tended to be higher in 14-3-3σ-positive cases. In 40% of cases there was no immunolabeling for Ki-67, nonetheless these tissues were reactive for cytokeratins and vimentin. One explanation for this lack of labeling may reside in different sensitivity to fixation and it is known that fixation times can affect some antigens more than others. There is a single case report of a metastatic canine renal cell carcinoma that labelled with Ki-67 but these authors did not specify the source of the antibody or give a labeling index.[4] In human RCC there are conflicting results regarding Ki-67. In one study it served as an independent predictor of oncological outcomes in patients with localized clear-cell renal cell carcinoma[11] while in another there was no correlation between grade, stage and outcome.[26] Overall it seems that Ki-67 is not yet recognized as a reliable predictor of prognosis for RCC.[25]

In summary, this study shows for the first time that 14-3-3σ protein is neo-expressed in a significant number of canine RCC and its presence is positively associated with a shorter survival time. Furthermore, it seems that in contrast to humans, most canine RCC may arise from distal segments of the nephron.

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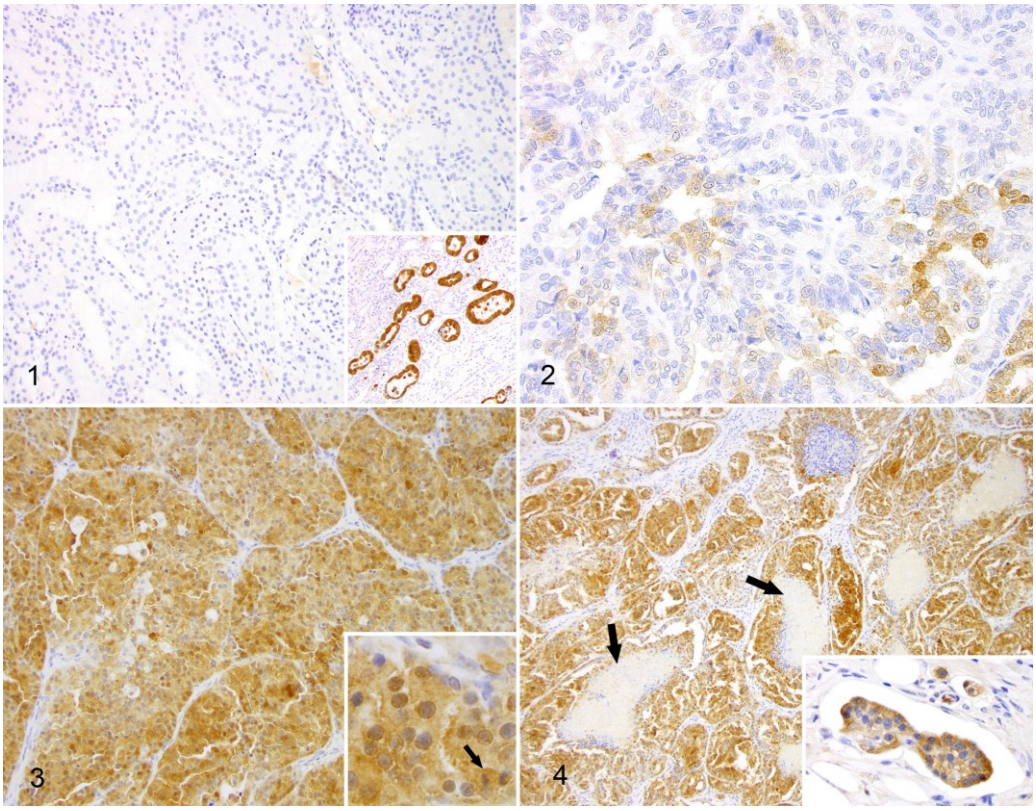
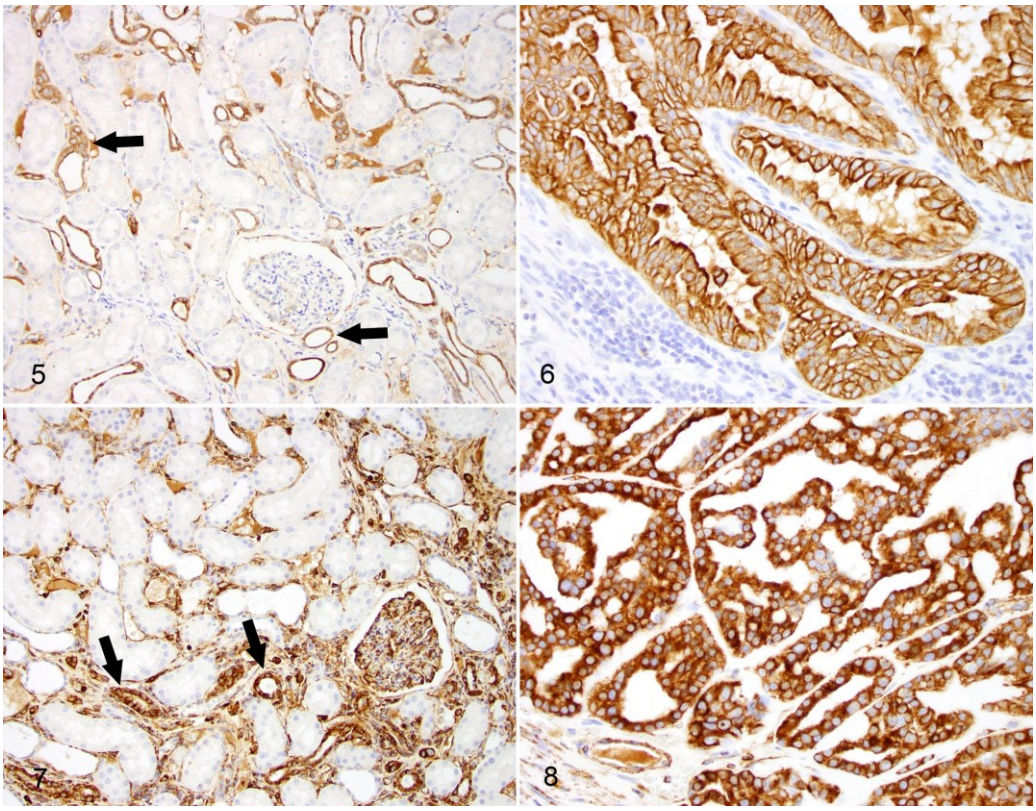
Figure legends



Figures 1-4. Kidney, dog. Immunohistochemistry for 14-3-3σ protein**. Figure 1.** Normal renal parenchyma does not express 14-3-3σ protein. Inset: Occasional non-neoplastic (reactive) renal tubules, immediately adjacent to a tumor, show intense immunoreaction. **Figure 2.** A well-differentiated tubular canine renal cell carcinoma (CRCC) with multifocal weak immunoreaction (total score, TS = 3). **Figure 3.** A poorly-differentiated tubular CRCC with intense expression (TS = 12). Inset: Neoplastic cells have both cytoplasmic and nuclear labeling. Note the immunolabeled mitotic figure (arrow). **Figure 4.** A well-differentiated tubular CRCC infiltrating the stroma with labeling of neoplastic cells but non-labelling in areas of necrosis (arrows). (TS = 12). Inset: Immunolabeling of neoplastic cells within lymphatic vessels.



Figures 5 and 6. Kidney, dog. Immunohistochemistry for cytokeratins. **Figure 5.** In normal renal parenchyma, cytokeratins are expressed in distal convoluted tubules (arrows). Proximal convoluted tubules are negative. **Figure 6.** A well-differentiated tubular renal cell carcinoma with intense cytoplasmic labelling. **Figures 7 and 8.** Kidney, dog. Immunohistochemistry for vimentin. **Figure 7.** In normal renal parenchyma vimentin is expressed in the interstitium, glomerular capillaries and epithelial cells of distal convoluted tubules (arrows). Proximal convoluted tubules are negative. **Figure 8.** A well-differentiated tubular renal cell carcinoma with intense cytoplasmic labelling.

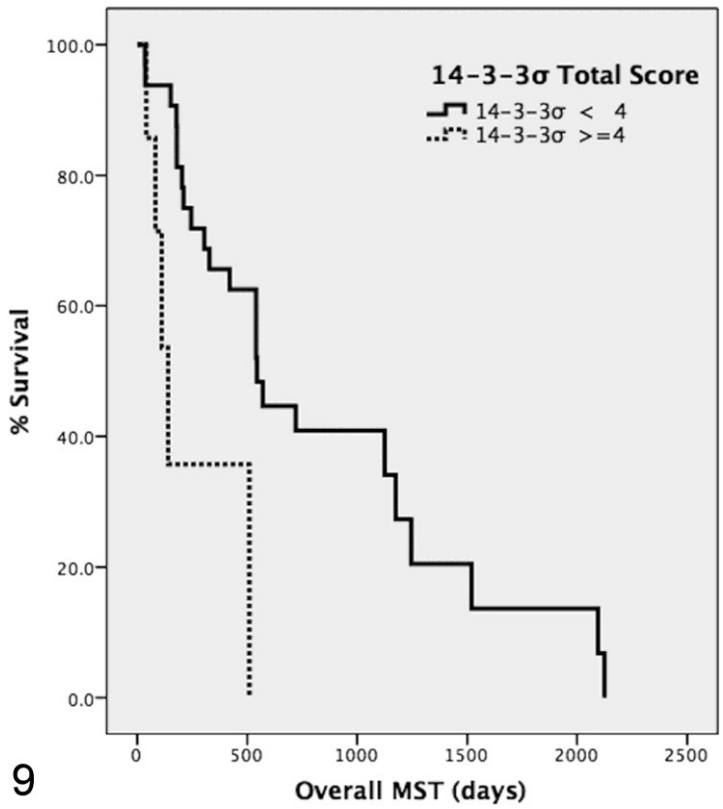


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Figure 9. Kaplan-Meier survival curve for overall median survival time according to 14-3-3σ total score (TS). A TS greater than 4 (solid line) is significantly associated with a shorter survival time (P = 0.002). Median survival time (MST).