Survivin and Sox9: Potential Stem Cell Markers in Canine Normal, Hyperplastic, and Neoplastic Canine Prostate

Veterinary Pathology

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Journal:	*Veterinary Pathology*
Manuscript ID	VET-16-FLM-0243.R3
Manuscript Type:	Full Length Manuscript
Date Submitted by the Author:	18-Jun-2018
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Keywords:	Dog < Domestic Mammals < Species, Immunohistochemistry < Technology, prostate carcinoma, Sox9, stem cells, survivin
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Survivin and Sox9, potential stem cell markers in canine normal, hyperplastic and neoplastic canine prostate

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Abstract

Canine prostatic carcinoma is a relevant model for human prostatic carcinoma. Survivin is proposed as a cancer biomarker of malignancy in human prostatic cancer. Sox9 is a stem cell marker required for prostate development and expressed in several adult tissues. The aims of the present study were to evaluate the patterns and expression levels of two putative stem cell markers, survivin and Sox9, in canine benign prostatic hyperplasia (BPH) and prostatic carcinoma, in order to investigate their potential as **stem cell markers. Immunohistochemistry using specific antibodies was performed on 3 samples of normal prostate gland, 18 samples of canine BPH, and 16 samples of prostatic carcinoma. The basal cell layer of normal and hyperplastic prostatic lobules had nuclear Sox9 immunolabeling, and nuclear and rarely cytoplasmic survivin immunostaining, identifying them as potential stem cell markers. Significantly more frequent (≥10% of nuclei)survivin and Sox9 expression was observed in prostatic carcinoma compared with BPH. The potential co-expression of survivin with Sox9, androgen receptor (AR) and p63 was also investigated in selected BPH and prostatic carcinoma cases using immunofluorescence, and a partial co-localization was observed. Results indicate that Sox9 and survivin could be considered as markers of stemness in canine prostate cells. Given its role in proliferation, cells in the** basal cell layer with nuclear survivin expression are likely to be transit amplifying cells that maintain some stem cell proprieties.

Key words: dog; immunohistochemistry; prostate carcinoma; Sox9; stem cells; survivin.

Canine prostatic carcinoma is considered a relevant model for studying advanced, hormone-refractory prostatic carcinoma in men.[1,18] Besides man, the dog is the only species to spontaneously develop prostate cancer and these cancers have certain features in common: age of onset, frequency of invasion, sites of metastases, and histological features.[18]

It has been proposed that cancer contains a minor population of cells that can self-renew while simultaneously giving rise to tumour cells: cancer stem cells (CSCs).[33] Human[15] and canine[25] prostatic tumours appear to contain a subpopulation of cells with stem cell features. CSCs are highly tumorigenenic, have a high metastatic potential, and show a relatively high resistance to traditional cancer therapies.[24] These findings have prompted the emergence of a new field of study in cancer treatment involving the targeting of CSCs; accordingly this requires the identification of new cancer stem cell markers.

Survivin represents a well-known cancer therapy-resistance factor that is overexpressed in several tumour types.[2] A role for survivin has been proposed in tumour initiation and progression, as well as in the maintenance of cancer stem cells.[30] It has been proposed that survivin be used as a biomarker for malignancy in early screening for human prostatic cancer.[31] The overexpression of survivin has been implicated in the development of prostatic carcinoma, leading investigators to evaluate the efficacy of survivin inhibitors as a possible new therapeutic option.[35] High expression of survivin has been observed in putative cancer stem cells isolated from prostatic adenocarcinoma in a murine model.[20,21] However, no data have been published regarding the expression and significance of survivin in canine prostatic tumours.

Sox9 is a stem cell marker expressed in several adult tissues and is required for human prostate development.[14,32] It contributes to the development of human prostatic carcinoma[7] and is, therefore, considered a

potential prognostic marker in human prostatic carcinoma patients.[37] However, although Sox9 is expressed in a large proportion of prostate cancers, its relevance to prognosis varies depending on the molecular environment: It has recently been demonstrated that loss of Sox9 expression was associated with prostatic carcinoma recurrence in ERG-positive and PTEN-deleted prostate cancers.[6]

Based on the hypotheses that survivin and Sox9 could represent markers of cancer stem cells and malignancy in canine prostatic tumours, the present study aimed to characterize the immunohistochemical (IHC) patterns and levels of expression of survivin and Sox9 in canine benign prostatic hyperplasia (BPH) and prostatic carcinoma.

A further objective was to study the co-localization of survivin with Sox9, p63 and AR in a subset of canine prostatic hyperplastic and neoplastic lesions. p63 has been identified as a prostate basal cell marker required for normal prostatic development[12,28] and seems to be required for maintainance of progenitor/stem cells.[28] It has also been shown that p63 is discontinuously expressed in the basal cells of both normal canine prostatic acini and hyperplastic lobules but dowregulated in canine prostatic carcinoma.[29]

The androgen/AR axis controls the growth and development of prostate tissue as well as prostatic carcinoma progression, and AR differentially influences the characteristics of normal stem cells and prostate cancer stem cells.[9] Recently it was shown that AR is expressed in canine BPH and, to a lesser extent, in Prostatic carcinoma.[9,29] In the prostate, survivin expression seems to be regulated by androgen. Survivin expression/overexpression in human prostatic carcinoma has been proposed as one of the molecular mechanisms of progression to androgen independence. The overexpression of survivin appeared to be sufficient to induce androgen-independent growth of androgen-dependent cells.[36]

Material and methods

Histological examination

The present study was carried out using 37 formalin-fixed, paraffin wax-embedded samples of canine prostate tissues from different dogs: 16 BPH, 16 primary prostatic carcinoma, and one necropsy sample of normal canine prostate gland retrospectively collected from the University archives, with unknown fixation time. In addition, fresh material from four additional dogs, including two normal prostate glands and two BPH cases, was formalin fixed for a maximum of 24 h. These additional samples were used specifically to investigate the pattern of Sox9 expression with IHC and the possible effects of formalin fixation time on the immunostaining.

Histological diagnosis was performed using haematoxylin and eosin (HE)-stained slides, according to WHO guidelines.[17] Prostatic carcinoma samples were further classified based on the histological subtypes[27] and the Gleason-like grading system.[26]

Immunohistochemistry

Immunohistochemistry (IHC) was performed using specific primary antibodies (Table S1), according to a previously described technique.[3] Briefly, deparaffinized and rehydrated sections were incubated with 3% H2O2 in absolute methanol for 45min to inhibit endogenous peroxidase activity and then rinsed in 0.05M Tris-buffered saline (TBS, pH 7.6) for 5min. Antigen retrieval was performed by heat treatment in Tris-EDTA buffer, pH9.0 in a microwave oven for 5min (four cycles). After the last treatment, sections were left for 20min in the buffer for cooling. To reduce non-specific binding, slides were then incubated with normal goat serum (code MR*HRP-650, Biospa, Milan, Italy) for 10min at room temperature before overnight incubation with the primary Ab in a humidified chamber at 4°C. After

rinsing with TBS, immune complexes were treated at room temperature for 30min with secondary biotinylated goat anti-mouse or anti-rabbit antibodies (1:200 dilution; Vector Laboratories Inc., Burlingame, California, USA) and subsequently visualized using an avidin-biotin complex (ABC) method (Vectastain ABC Kit, Vector Laboratories) for 30min. Peroxidase activity was detected by a 5min application of 0.1% H2O2 in 3-3′-diaminobenzidine solution (code D5905, Sigma-Aldrich, St. Louis, Mo, USA) followed by counterstaining with Mayer's haematoxylin (Merck, Darmstadt, Germany) for 1min before rinsing, dehydrating and mounting. A negative control was performed in all instances by omitting the primary Ab and incubating tissue sections with TBS and/or replacing it by an Ab of irrelevant specificity (rabbit anti-human von Willebrand factor polyclonal Ab, from DAKO, Glostrup, Denmark). Sections of canine tissues known to display expression of the investigated molecules, as indicated in Table S1, were used as positive controls.

Double immunofluorescence

Double immunofluorescence was used to qualitatively investigate survivin-Sox9, survivin-p63 and survivin-AR nuclear co-expression in 16 selected cases (eight BPH and eight prostatic carcinoma) based on our previous published study[26], using specific antibodies (Table S1). Tissue samples were treated as described for the immunohistochemical procedure. A sequential protocol was used for double staining. Primary antibodies were applied overnight at 4°C. The first secondary Ab, biotinylated goat anti-rabbit (for survivin) (1:200 dilution; Vector Laboratories, Burlingame, CA, USA) was applied and incubated for 30min at room temperature, and slides were then incubated with fluorescein-conjugated avidin (1:100 dilution in 0.1M NaHCO3, 0.15M NaCl buffer, pH 8.2–8.5; Vector Laboratories) for 10min at room temperature. An avidin/biotin blocking step was performed by incubating slides for 15min with avidin and then biotin (Avidin/Biotin

Blocking Kit, code SP-2001, Vector Laboratories) at room temperature. A further protein blocking step was performed by incubating slides with normal goat serum (Vector Laboratories) for 15min at room temperature, before the overnight application of the second primary Ab. The second secondary Ab, biotinylated goat anti-rabbit (for Sox9, AR) or anti-mouse (for p63) (1:200 dilution; Vector Laboratories) was applied and incubated for 30min at room temperature, and slides were then incubated with Texas Red-conjugated avidin (Vector Laboratories) diluted 1:100 in a buffer composed of 0.1M $NaHCO_3$ and 0.15M NaCl, pH 8.2–8.5, for 10min at room temperature. Nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) (Vector Laboratories).

Quantification of immunolabelling and statistical analysis

Nuclear and cytoplasmic survivin and nuclear Sox9 immunolabelling were assessed semiquantitatively by two pathologists (LB and MR) in ten randomly selected HPF (40X) and graded as percentage of all neoplastic cells as follow: 0, absent; 1 (low), >0 and <10% of cells; 2 (moderate), 10 to <25% of cells; 3 (high), 25 to <50% of cells; 4 (very high), >50% of cells. Labelling intensity of cytoplasmic survivin was graded as no (0), weak (-/+), moderate (+) or strong (++) labelling.

Fisher's exact test was used to compare semiquantitatively assessed immunoreactivity in BPH and prostatic carcinoma. For this purpose, both survivin cytoplasmic score and survivin and Sox9 nuclear score were evaluated. The cases were grouped according to the nuclear score as follows: <10% positive nuclei (absent and very low number of positive nuclei) versus ≥10% positive nuclei (low/moderate and high number of positive nuclei). This choice depended on the distribution of nuclear score values for each protein and on several statistical analyses performed. Analyses were performed using SPSS statistical software, with $P < 0.05$ considered to be significant.

Results

Histological examination

Histological features are reported in Table S2 (Please, refer to the online version of the manuscript for supplemental material). In half of the prostatic carcinoma cases (8/16), a single histological pattern was seen (four papillary, three solid/undifferentiated and one small acinar/ductal); a mixed pattern was observed in the other 8 cases. Necrosis was observed in 12/16 (75%) cases and 2/16 (12.5%) were characterized by abundant fibrous tissue (formerly termed "scirrhous prostatic carcinoma").

Immunohistochemistry

The IHC results are summarized in Table 1 and S2.

Survivin expression in normal gland, BPH and prostatic carcinoma

Survivin-positive nuclei were present among the basal/reserve cell layer of normal (Fig.1) and hyperplastic prostatic lobules [0-10% of cells in the 11/16 (68,75%) cases with positive labeling] (Fig.2). Even if a single case of normal gland was analyzed, normal prostatic tissue surrounding the BPH lesions was also evaluated, showing the same pattern of expression of the molecule. Most of the BPH cases (12/16, 75%) showed patchy cytoplasmic immunostaining, with a low to moderate (0-25% of neoplastic cells) expression in most of the cases (10/12, 83,3%). In contrast, prostatic carcinomas had more diffuse cytoplasmic expression as well as nuclear expression of survivin. Cytoplasmic staining was observed in all the prostatic carcinoma samples, with half of the cases (8/16) showing low to moderate expression (0-25% of neoplastic cells) and the other half high expression (>25% of neoplastic cells). Positive nuclei were present in all except one case of prostatic carcinoma, with most (9/16, 56%) showing

moderate to high expression (10 to >50% of positive nuclei)(Fig.3), present mainly among the neoplastic cells with basal cell morphology in the areas with tubular-papillary pattern .

Sox9 expression in normal gland, BPH and prostatic carcinoma

Sox9-positive nuclei were observed in scattered cells among the basal/reserve cell layer of 2 of 3 normal prostatic glands (Fig.4) and 5 of 15 hyperplastic lobules (Fig.5). Of these positive cases, two normal glands and 2 BPH cases were freshly sampled, while the other 3 BPH cases were from the archive and the fixation time was not known, suggesting that prolonged fixation may interfere with Sox9 immunostaining.[34] Among prostatic carcinomas, 9/16 cases were positive and most (7/9) showed a moderate to high expression (>25% of neoplastic cells with nuclear immunolabelling)(Fig.6).

Double immunofluorescence

From a qualitative evaluation of the immunofluorescence-stained slides, only a partial co-localization of survivin and p63 (survivin+/Sox9+) was observed. In BPH, survivin-/p63+ nuclei were more numerous (5-10%), survivin+/p63- nuclei were scattered and rare, and survivin+/p63+ nuclei were few (<5% of cells were double-positive) and observed among basal cells (Fig.7). In contrast, in prostatic carcinoma, survivin+/p63- nuclei were more numerous (10-25%),and survivin+/p63+ nuclei were scattered and few (<5% of cells were double-positive). p63 cytoplasmic immunostaining was also evident (Fig.8).

In BPH cases, a higher number of survivin-/AR+ cells (>50%) was observed compared to survivin+/AR+ cells (5-10%), while no survivin+/AR- cells were observed (Fig.9). Nuclear immunolabelling in prostatic carcinoma was characterized by numerous survivin+/AR+ cells (25-50%) and survivin+/AR-

cells were also evident (10-25%)(Fig.10). Several neoplastic cells showed an intense aberrant AR cytoplasmic expression as well as cytoplasmic immunolabelling for survivin.

In BPH cases, survivin+/Sox9- cells were more numerous (between 10 and 25%) than survivin+/Sox9+ cells (<5% of double positive cells), while no survivin-/Sox9+ cells were observed (Fig.11). In the prostatic carcinoma cases, a higher number of survivin+/Sox9+ (double-immunostained) cells was observed (Fig.12), with intense nuclear and faint cytoplasmic immunolabelling, and a few (<5%) survivin+/Sox9- and survivin-/Sox9+ cells.

Statistical Analysis

Both nuclear survivin and Sox9 expression appeared to be higher (≥10% of neoplastic cells with positively labeled nuclei) in prostatic carcinoma compared with BPH cases ($p<0.01$ for both). No significant differences were observed in cytoplasmic survivin expression comparing BPH with prostatic carcinoma cases.

Discussion

This study is the first to characterize the IHC expression of survivin and Sox9 in normal, hyperplastic and neoplastic canine prostate. The main aims were to verify if Sox9 and survivin are expressed by canine prostatic epithelial cells and if they could represent prostatic stem cell markers.

The nuclear immunostaining observed in the basal cell layer of normal prostate gland is consistent with Sox9 as a stem cell marker in canine prostate, similar to what has been reported in the human literature.[7] In contrast to our predictions, the first round of testing with Sox9 immunohistochemistry, using cases selected from the University's archives, showed no positive cells in the normal glands, and only a few positive

samples among the BPH cases. A second round of testing, using freshly sampled prostatic tissue fixed in formalin for 24 hours, revealed positive staining in all four cases, and it was more intense and evident than that observed in samples with longer fixation time. This suggests that prolonged formalin fixation time decreased Sox9 antigen detection, indicating that this antigen is more vulnerable to degradation than the other investigated molecules (such as survivin, p63, AR).[34]

The role of survivin in stem cells[16] might indicate that the positive cells found in the basal cell layer of normal glands are stem cells. However, since survivin also has a role in proliferation, it is also possible that these cells represent partially differentiated, proliferating, transit amplifying cells that maintain some stem cell properties.

The prostate basal cell marker p63 had already been shown to be involved in epithelial development by regulating stem cell/transit amplifying cells, their differentiation, and cell death.[8,22] Results obtained from the immunofluorescence study showed that in BPH survivin and p63 are expressed in different cells, with only partial, minimal co-localization. Our hypothesis is that they mark transit amplifying cells in these lesions, an undifferentiated population of cells in transition between stem cells and differentiated cells, with intermediate features. This would support the previously reported theory that at least two biologically distinct populations of basal cells exist in the canine prostate gland[19] and that these cells can be involved in the development/maintenance of BPH.

Other recent studies characterizing the expression of stem cell markers have shown that co-expression of CD44 and CD133 detects stem cells in canine prostatic cancer cell lines.[23,24] Further studies should be done in order to investigate whether survivin and Sox9 are co-expressed with these markers.

In human prostatic tissue, survivin expression is regulated by androgen stimulation.[36] In the canine prostatic tissue studied here, the presence of survivin+/AR- cells in prostatic carcinoma, together with the increased expression of survivin in prostatic carcinoma compared to BPH, would suggest a role for survivin in the progression to androgen independence in the dog as well. The survivin+/AR- cells could represent a subpopulation of androgen-independent tumour cells. These may be important in the early stages of development of prostate cancer and, if present in human prostatic carcinoma, may be the cause of the failure of androgen ablation therapy that occurs in most human advanced cases.[10] Our findings would suggest the presence, in canine prostatic carcinoma, of a subpopulation of neoplastic cells with low levels of AR and high levels of survivin and some features of stem cells (prostatic stem cells or transit amplifying cells). This supports the possibility that this tumour derives from basal/stem cell-like epithelial cells expressing low levels of AR.[11,18]

The origin of CSCs within solid tumours has not yet been clarified and would appear to vary based on tumour type: tumour cells could originate from normal tissue stem cells or from partially differentiated transit amplifying cells that undergo oncogenic transformation to produce CSCs.[13] Further studies should be done to better characterize this subpopulation and its features of stemness, and to investigate the possible regulation of the AR-activated pathways on survivin expression in canine prostatic tissue and prostatic neoplasms.

Acknowledgements

We thank the Veterinary Pathology Diagnostic Centre of the Utrecht University for providing tissue blocks of canine prostatic lesions, as well as Marina Baffoni and Saskia Essen-van Dorresteijn for their technical support.

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Figure Legends

Figures 1-3. Immunohistochemistry for survivin. Figure 1. Normal prostate gland, dog 1. Scattered positive nuclei among the basal cell layer (arrows) and weak, patchy cytoplasmic immunostaining are present. Figure 2. Benign prostatic hyperplasia (BPH), dog 7. Weak cytoplasmic immunostaining and scattered positive nuclei (arrows), some of which are in the basal cell layer (arrowhead) of hyperplastic prostatic lobules. Figure 3. Prostatic carcinoma, dog 35. Numerous positive nuclei and weak, patchy cytoplasmic immunostaining among the neoplastic cells of a solid prostatic carcinoma, with intensely positive mitotic figures (arrow). Figures 4-6. Immunohistochemistry for Sox9. Figure 4. Normal prostate gland, dog 2. Positive nuclei are evident in the basal cell layer (arrows). Figure 5. BPH, dog 15. Several positive nuclei are shown, mainly present in the basal

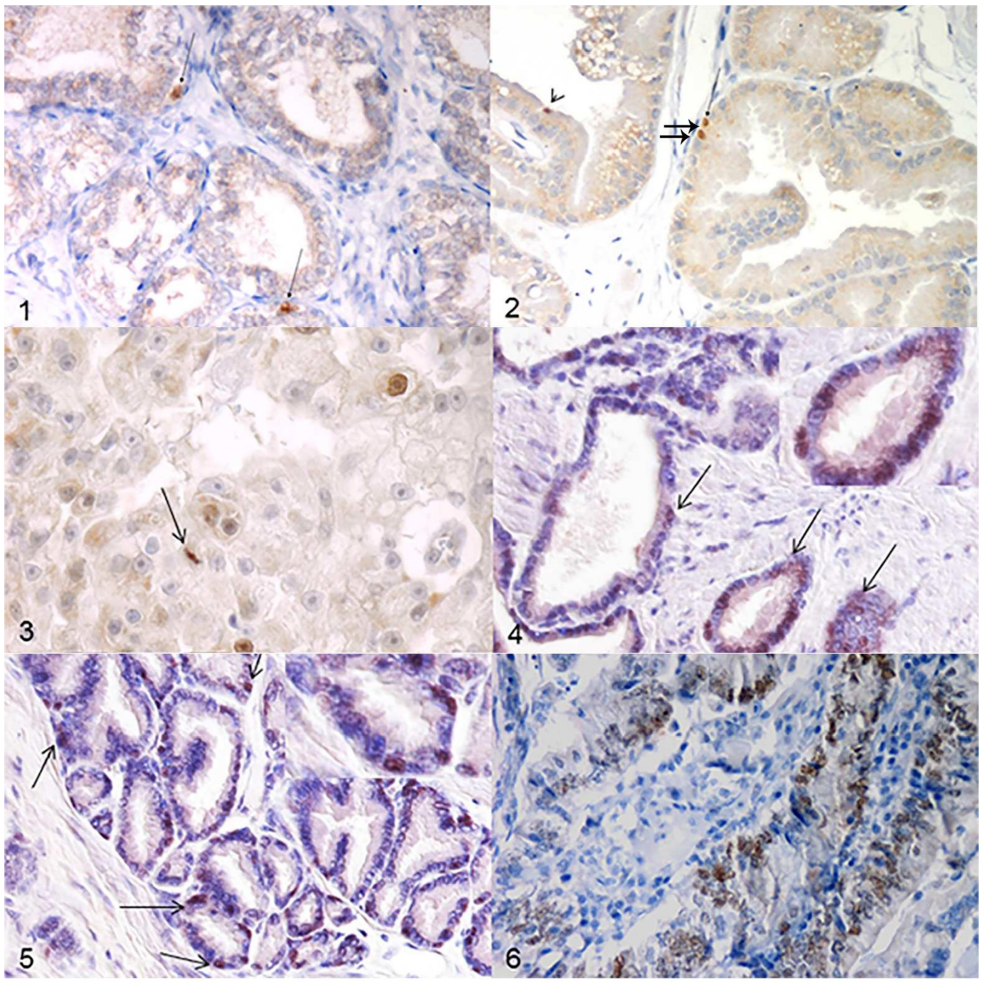
cell layer(arrows). Figure 6. Prostatic carcinoma, dog 22. Numerous positive nuclei were observed among the neoplastic cells of a papillary prostatic carcinoma.

Figures 7-8: Double immunofluorescence for survivin and p63; nuclei counterstained with 4',6-diamidino-2-phenylindole (DAPI). Figure 7: BPH, dog 10. Only a partial co-localization of survivin and p63 was observed, showing a higher number of survivin-/p63+ nuclei in basal cells and few survivin+/p63+ cells. Figure 8.: prostatic carcinoma, dog 19. More frequent survivin+/p63- and only few survivin+/p63+ nuclei were observed with infrequent p63 cytoplasmic immunostaining. Figures 9-10: Double immunofluorescence for survivin and AR; nuclei counterstained with DAPI. Figure 9:: BPH, dog 6. Higher number of nuclear survivin-/AR+ cells compared to survivin+/AR+ cells was observed in BPH cases, without any nuclear survivin+/AR- cells. Most of the cells show cytoplasmic survivin expression. Figure 10: prostatic carcinoma, dog 22. Rare cells had nuclear labelling for both survivin and AR (arrow), but survivin+/AR- nuclear labelling was also evident. Figures 11-12: Double immunofluorescence for survivin and Sox9; nuclei counterstained with DAPI. Figure 11: BPH, dog 18. Most of the cells have survivin+/Sox9- cytoplasmic labeling (green arrow). Figure 12: prostatic carcinoma, dog 32. Numerous neoplastic cells in the prostatic carcinoma cases evaluated showed survivin+/Sox9+ double immunostaining (arrows), with intense nuclear and faint cytoplasmic survivin immunolabelling.

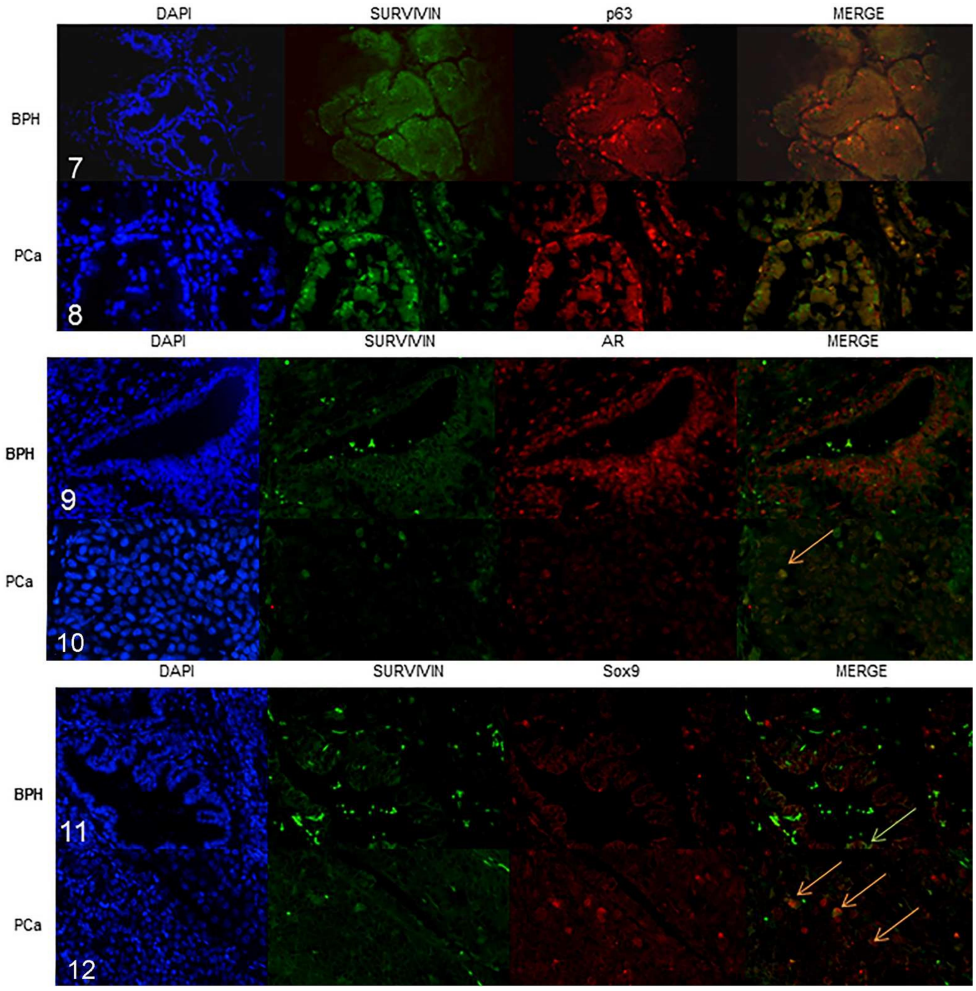
Table 1. Expression of survivin and Sox9 in normal prostate, benign prostatic hyperplasia, and prostatic carcinoma of dogs. The data show immunohistochemistry grades.

Case N°	Nucelar survivin	Cytoplasmic survivin	Sox9
Normal gland			
1	1	0	0
2*	-	-	1
3*	-	-	1
BPH			
4	1	1, +	0
5	0	1, ++	0
6	1	1, -/+	0
7	1	1, +	0
8	0	1, ++	0
9	1	1, -/+	0
10	1	2, ++	0
11	1	2, ++	0
12	0	1, -/+ to +	1
13	1	4, ++	0
14	1	0	3
15	1	3,++	0
16	0	0	0
17	1	0	0
18	1	1,+	1
19	0	0	0
20*	-	-	1
21*	-	-	1
PCa			
22	1	1, -/+	0
23	2	4, ++	4
24	1	4, +/++	4
25	1	2, ++	0
26	1	1, -/+	-
27	2	1, +/++	4
28	4	4, ++	4
29	1	4,++	-
30	4	4, +/++	4
31	1	1, -/+ with small clusters ++	1
32	2	3, ++	0
33	2	Cribiform: 3, +, Rest: 4, +++	2
34	2	3, +	2
35	3	1, +/++	1
36	0	1, -/+	0
37	3	1, -/+	0

Grades for immunolabelling, as percentage of all neoplastic cells: 0, absent; 1, >0 and <10% of cells; 2, 10 to <25% of cells; 3, 25 to <50% of cells; 4, >50% of cells. BHP: benign prostatic hyperplasia. PCa: prostatic carcinoma. *: maximum 24 h of formalin fixation time.



180x180mm (300 x 300 DPI)



180x181mm (300 x 300 DPI)

Supplemental Table S1 - Methods used for immunohistochemistry.

MOLECULE	ANTIBODY TYPE	SOURCE	WORKING DILUTION	POSITIVE CONTROL
Sox9	Rabbit PAb	Santa Cruz Biotecnology	1:700	Canine normal skin[5]
Survivin	Rabbit PAb	NOVUS Biologicals	0.7 µg/ml	Canine sabaceous carcinoma[4]
p63	Mouse MAb	DAKO	1:400	Canine prostatic hyperplasia[26]
AR	Rabbit PAb	Santa Cruz Biotecnology	1:500	Canine prostatic hyperplasia[26]

PAb: polyclonal antibody; MAb: monoclonal antibody.

Supplementary table S2. The table sows tumour type, prostatic carcinoma histo-patterns, Gleason/like score, presence of necrosis and immunohistochemistry grades.

Case N°	Histotype	Histo-pattern	Gleason/like score	Necrosis	Nuclear survivin	Cytoplasmic survivin	Sox9
1	Normal gland				1	0	0
2	Normal gland*				-	-	1
3	Normal gland*				-	-	1
4	BPH				1	1, +	0
5	BPH				0	1, ++	0
6	BPH				1	1, -/+	0
7	BPH				1	1, +	0
8	BPH				0	1, ++	0
9	BPH				1	1, -/+	0
10	BPH				1	2, ++	0
11	BPH				1	2, ++	0
12	BPH	Cystic with prostatitis and PIN areas			0	1, -/+ to +	1
13	BPH	With prostatitis			1	4, ++	0
14	BPH	With prostatitis			1	0	3
15	BPH				1	3,++	0
16	BPH				0	0	0
17	BPH	With prostatitis			1	0	0
18	BPH	With prostatitis			1	1,+	1
19	BPH	With prostatitis			0	0	0
20	BPH*				-	-	1
21	BPH*				-	-	1
22	PCa	Papillary	8	No	1	1, -/+	0
23	PCa	Small acinar/ductal, solid, signet ring	10	Yes	2	4, ++	4
24	PCa	Papillary, solid	9	Yes	1	4, +/++	4

25	PCa	Papillary	8	Yes	1	2, ++	0
26	PCa	Small acinar/ductal, solid	9	No	1	1, -/+	-
27	PCa	Papillary, small acinar/ ductal, solid with abundant fibrous stroma	9	Yes	2	1, +/++	4
28	PCa	Solid	10	Yes	4	4, ++	4
29	PCa	Cribiform, solid	10	Yes	1	4,++	-
30	PCa	Solid with squamous metaplasia and abundant fibrous stroma	8	Yes	4	4, +/++	4
31	PCa	Papillary with cystic structures	9	No	1	1, -/+ with small clusters ++	1
32	PCa	Solid, cribiform, papillary	9	Yes	2	3, ++	0
33	PCa	Cribiform, signet-ring, papillary	10	Yes	2	Cribiform: 3, +, Rest: 4, +++	2
34	PCa	Cribiform, papillary	10	Yes	2	3, +	2
35	PCa	Solid	10	Yes	3	1, +/++	1
36	PCa	Papillary	8	No	0	1, -/+	0
37	PCa	Small acinar /ductal	7	Yes	3	1, -/+	0

Grades for immunolabelling, as percentage of all neoplastic cells: 0, absent; 1, >0 and <10% of cells; 2, 10 to <25% of cells; 3, 25 to <50% of cells; 4, >50% of cells. BHP: benign prostatic hyperplasia. BHP: benign prostatic hyperplasia. PCa: prostatic carcinoma. *: maximum 24 h of formalin fixation time.

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Supplementary Table S2. The table shows tumour type, prostatic carcinoma histo-patterns, Gleason/like score, presence of necrosis and immunohistochemistry grades.

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3	Normal gland*				-	-	1
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5	BPH				0	1, ++	0
6	BPH				1	1, -/+	0
7	BPH				1	1, +	0
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16	BPH				0	0	0
17	BPH	With prostatitis			1	0	0
18	BPH	With prostatitis			1	1,+	1
19	BPH	With prostatitis			0	0	0
20	BPH*				-	-	1
21	BPH*				-	-	1
22	PCa	Papillary	8	No	1	1, -/+	0
23	PCa	Small acinar/ductal, solid, signet ring	10	Yes	2	4, ++	4
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25	PCa	Papillary	8	Yes	1	2, ++	0
26	PCa	Small acinar/ductal, solid	9	No	1	1, -/+	-
27	PCa	Papillary, small acinar/ ductal, solid with abundant fibrous stroma	9	Yes	2	1, +/++	4
28	PCa	Solid	10	Yes	4	4, ++	4
29	PCa	Cribiform, solid	10	Yes	1	4,++	-
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32	PCa	Solid, cribiform, papillary	9	Yes	2	3, ++	0
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35	PCa	Solid	10	Yes	3	1, +/++	1
36	PCa	Papillary	8	No	0	1, -/+	0
37	PCa	Small acinar /ductal	7	Yes	3	1, -/+	0

Grades for immunolabelling, as percentage of all neoplastic cells: 0, absent; 1, >0 and <10% of cells; 2, 10 to <25% of cells; 3, 25 to <50% of cells; 4, >50% of cells. BHP: benign prostatic hyperplasia. BHP: benign prostatic hyperplasia. PCa: prostatic carcinoma. *: maximum 24 h of formalin fixation time.