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

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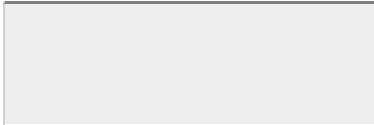
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# Veterinary Pathology

## Survivin and Sox9, potential stem cell markers in canine normal, hyperplastic and neoplastic canine prostate

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Keywords:	Dog < Domestic Mammals < Species, Immunohistochemistry < Technology, prostate carcinoma, Sox9, stem cells, survivin
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 ( $\geq 10\%$ 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

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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 properties.

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Manuscripts

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3 **1 Survivin and Sox9, potential stem cell markers in canine normal,**  
4 **2 hyperplastic and neoplastic canine prostate**

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23

24 **Abstract**

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

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

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3 51 Canine prostatic carcinoma is considered a relevant model for studying  
4 52 advanced, hormone-refractory prostatic carcinoma in men.<sup>1,18</sup> Besides man, the  
5 53 dog is the only species to spontaneously develop prostate cancer and these  
6 54 cancers have certain features in common: age of onset, frequency of  
7 55 invasion, sites of metastases, and histological features.<sup>18</sup>

11 56 It has been proposed that cancer contains a minor population of cells that  
12 57 can self-renew while simultaneously giving rise to tumour cells: cancer  
13 58 stem cells (CSCs).<sup>33</sup> Human<sup>15</sup> and canine<sup>25</sup> prostatic tumours appear to contain  
14 59 a subpopulation of cells with stem cell features. CSCs are highly  
15 60 tumorigenic, have a high metastatic potential, and show a relatively high  
16 61 resistance to traditional cancer therapies.<sup>24</sup> These findings have prompted  
17 62 the emergence of a new field of study in cancer treatment involving the  
18 63 targeting of CSCs; accordingly this requires the identification of new  
19 64 cancer stem cell markers.

23 65 Survivin represents a well-known cancer therapy-resistance factor that is  
24 66 overexpressed in several tumour types.<sup>2</sup> A role for survivin has been  
25 67 proposed in tumour initiation and progression, as well as in the  
26 68 maintenance of cancer stem cells.<sup>30</sup> It has been proposed that survivin be  
27 69 used as a biomarker for malignancy in early screening for human prostatic  
28 70 cancer.<sup>31</sup> The overexpression of survivin has been implicated in the  
29 71 development of prostatic carcinoma, leading investigators to evaluate the  
30 72 efficacy of survivin inhibitors as a possible new therapeutic option.<sup>35</sup> High  
31 73 expression of survivin has been observed in putative cancer stem cells  
32 74 isolated from prostatic adenocarcinoma in a murine model.<sup>20,21</sup> However, no  
33 75 data have been published regarding the expression and significance of  
34 76 survivin in canine prostatic tumours.

35 77 Sox9 is a stem cell marker expressed in several adult tissues and is  
36 78 required for human prostate development.<sup>14,32</sup> It contributes to the  
37 79 development of human prostatic carcinoma<sup>7</sup> and is, therefore, considered a

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3 80 potential prognostic marker in human prostatic carcinoma patients.<sup>37</sup>  
4  
5 81 However, although Sox9 is expressed in a large proportion of prostate  
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7 82 cancers, its relevance to prognosis varies depending on the molecular  
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9 83 environment: It has recently been demonstrated that loss of Sox9 expression  
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11 84 was associated with prostatic carcinoma recurrence in ERG-positive and  
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13 85 PTEN-deleted prostate cancers.<sup>6</sup>

14 86 Based on the hypotheses that survivin and Sox9 could represent markers of  
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16 87 cancer stem cells and malignancy in canine prostatic tumours, the present  
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18 88 study aimed to characterize the immunohistochemical (IHC) patterns and  
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20 89 levels of expression of survivin and Sox9 in canine benign prostatic  
21  
22 90 hyperplasia (BPH) and prostatic carcinoma.

23  
24 91 A further objective was to study the co-localization of survivin with Sox9,  
25  
26 92 p63 and AR in a subset of canine prostatic hyperplastic and neoplastic  
27  
28 93 lesions. p63 has been identified as a prostate basal cell marker required  
29  
30 94 for normal prostatic development<sup>12,28</sup> and seems to be required for  
31  
32 95 maintenance of progenitor/stem cells.<sup>28</sup> It has also been shown that p63 is  
33  
34 96 discontinuously expressed in the basal cells of both normal canine  
35  
36 97 prostatic acini and hyperplastic lobules but downregulated in canine  
37  
38 98 prostatic carcinoma.<sup>29</sup>

39 99 The androgen/AR axis controls the growth and development of prostate tissue  
40  
41 100 as well as prostatic carcinoma progression, and AR differentially  
42  
43 101 influences the characteristics of normal stem cells and prostate cancer  
44  
45 102 stem cells.<sup>9</sup> Recently it was shown that AR is expressed in canine BPH and,  
46  
47 103 to a lesser extent, in Prostatic carcinoma.<sup>9,29</sup> In the prostate, survivin  
48  
49 104 expression seems to be regulated by androgen. Survivin  
50  
51 105 expression/overexpression in human prostatic carcinoma has been proposed as  
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53 106 one of the molecular mechanisms of progression to androgen independence.  
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55 107 The overexpression of survivin appeared to be sufficient to induce  
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57 108 androgen-independent growth of androgen-dependent cells.<sup>36</sup>



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5 110 **Material and methods**6  
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8 111 **Histological examination**

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10 112 The present study was carried out using 37 formalin-fixed, paraffin wax-  
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12 113 embedded samples of canine prostate tissues from different dogs: 16 BPH, 16  
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14 114 primary prostatic carcinoma, and one necropsy sample of normal canine  
15  
16 115 prostate gland retrospectively collected from the University archives, with  
17  
18 116 unknown fixation time. In addition, fresh material from four additional  
19  
20 117 dogs, including two normal prostate glands and two BPH cases, was formalin  
21  
22 118 fixed for a maximum of 24 h. These additional samples were used  
23  
24 119 specifically to investigate the pattern of Sox9 expression with IHC and  
25  
26 120 the possible effects of formalin fixation time on the immunostaining.

27 121 Histological diagnosis was performed using haematoxylin and eosin (HE)-  
28  
29 122 stained slides, according to WHO guidelines.<sup>17</sup> Prostatic carcinoma samples  
30  
31 123 were further classified based on the histological subtypes<sup>27</sup> and the  
32  
33 124 Gleason-like grading system.<sup>26</sup>

34  
35 125 **Immunohistochemistry**

36  
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38 126 Immunohistochemistry (IHC) was performed using specific primary antibodies  
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40 127 (Table S1), according to a previously described technique.<sup>3</sup> Briefly,  
41  
42 128 deparaffinized and rehydrated sections were incubated with 3% H<sub>2</sub>O<sub>2</sub> in  
43  
44 129 absolute methanol for 45min to inhibit endogenous peroxidase activity and  
45  
46 130 then rinsed in 0.05M Tris-buffered saline (TBS, pH 7.6) for 5min. Antigen  
47  
48 131 retrieval was performed by heat treatment in Tris-EDTA buffer, pH9.0 in a  
49  
50 132 microwave oven for 5min (four cycles). After the last treatment, sections  
51  
52 133 were left for 20min in the buffer for cooling. To reduce non-specific  
53  
54 134 binding, slides were then incubated with normal goat serum (code MR\*HRP-  
55  
56 135 650, Biospa, Milan, Italy) for 10min at room temperature before overnight  
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58 136 incubation with the primary Ab in a humidified chamber at 4°C. After

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3 137 rinsing with TBS, immune complexes were treated at room temperature for  
4 138 30min with secondary biotinylated goat anti-mouse or anti-rabbit antibodies  
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6 139 (1:200 dilution; Vector Laboratories Inc., Burlingame, California, USA) and  
7  
8 140 subsequently visualized using an avidin-biotin complex (ABC) method  
9  
10 141 (Vectastain ABC Kit, Vector Laboratories) for 30min. Peroxidase activity  
11  
12 142 was detected by a 5min application of 0.1% H<sub>2</sub>O<sub>2</sub> in 3-3'-diaminobenzidine  
13 143 solution (code D5905, Sigma-Aldrich, St. Louis, Mo, USA) followed by  
14 144 counterstaining with Mayer's haematoxylin (Merck, Darmstadt, Germany) for  
15 145 1min before rinsing, dehydrating and mounting. A negative control was  
16  
17 146 performed in all instances by omitting the primary Ab and incubating tissue  
18  
19 147 sections with TBS and/or replacing it by an Ab of irrelevant specificity  
20  
21 148 (rabbit anti-human von Willebrand factor polyclonal Ab, from DAKO,  
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23 149 Glostrup, Denmark). Sections of canine tissues known to display expression  
24  
25 150 of the investigated molecules, as indicated in Table S1, were used as  
26  
27 151 positive controls.

#### 30 152 **Double immunofluorescence**

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33 153 Double immunofluorescence was used to qualitatively investigate survivin-  
34 154 Sox9, survivin-p63 and survivin-AR nuclear co-expression in 16 selected  
35 155 cases (eight BPH and eight prostatic carcinoma) based on our previous  
36  
37 156 published study<sup>26</sup>, using specific antibodies (Table S1). Tissue samples were  
38  
39 157 treated as described for the immunohistochemical procedure. A sequential  
40  
41 158 protocol was used for double staining. Primary antibodies were applied  
42  
43 159 overnight at 4°C. The first secondary Ab, biotinylated goat anti-rabbit  
44  
45 160 (for survivin) (1:200 dilution; Vector Laboratories, Burlingame, CA, USA)  
46  
47 161 was applied and incubated for 30min at room temperature, and slides were  
48  
49 162 then incubated with fluorescein-conjugated avidin (1:100 dilution in 0.1M  
50 163 NaHCO<sub>3</sub>, 0.15M NaCl buffer, pH 8.2-8.5; Vector Laboratories) for 10min at  
51  
52 164 room temperature. An avidin/biotin blocking step was performed by  
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54 165 incubating slides for 15min with avidin and then biotin (Avidin/Biotin  
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3 166 Blocking Kit, code SP-2001, Vector Laboratories) at room temperature. A  
4 167 further protein blocking step was performed by incubating slides with  
5 168 normal goat serum (Vector Laboratories) for 15min at room temperature,  
6 169 before the overnight application of the second primary Ab. The second  
7 170 secondary Ab, biotinylated goat anti-rabbit (for Sox9, AR) or anti-mouse  
8 171 (for p63) (1:200 dilution; Vector Laboratories) was applied and incubated  
9 172 for 30min at room temperature, and slides were then incubated with Texas  
10 173 Red-conjugated avidin (Vector Laboratories) diluted 1:100 in a buffer  
11 174 composed of 0.1M NaHCO<sub>3</sub> and 0.15M NaCl, pH 8.2-8.5, for 10min at room  
12 175 temperature. Nuclei were counterstained with 4',6-diamidino-2-phenylindole  
13 176 (DAPI) (Vector Laboratories).

#### 23 177 **Quantification of immunolabelling and statistical analysis**

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25  
26 178 Nuclear and cytoplasmic survivin and nuclear Sox9 immunolabelling were  
27 179 assessed semiquantitatively by two pathologists (LB and MR) in ten randomly  
28 180 selected HPF (40X) and graded as percentage of all neoplastic cells as  
29 181 follow: 0, absent; 1 (low), >0 and <10% of cells; 2 (moderate), 10 to <25%  
30 182 of cells; 3 (high), 25 to <50% of cells; 4 (very high), >50% of cells.

31 183 Labelling intensity of cytoplasmic survivin was graded as no (0), weak (-  
32 184 /+), moderate (+) or strong (++) labelling.

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35 185 Fisher's exact test was used to compare semiquantitatively assessed  
36 186 immunoreactivity in BPH and prostatic carcinoma. For this purpose, both  
37 187 survivin cytoplasmic score and survivin and Sox9 nuclear score were  
38 188 evaluated. The cases were grouped according to the nuclear score as  
39 189 follows: <10% positive nuclei (absent and very low number of positive  
40 190 nuclei) versus ≥10% positive nuclei (low/moderate and high number of  
41 191 positive nuclei). This choice depended on the distribution of nuclear score  
42 192 values for each protein and on several statistical analyses performed.  
43 193 Analyses were performed using SPSS statistical software, with P <0.05  
44 194 considered to be significant.

195

**196 Results****197 Histological examination**

198 Histological features are reported in Table S2 (Please, refer to the online  
199 version of the manuscript for supplemental material). In half of the  
200 prostatic carcinoma cases (8/16), a single histological pattern was seen  
201 (four papillary, three solid/undifferentiated and one small acinar/ductal);  
202 a mixed pattern was observed in the other 8 cases. Necrosis was observed in  
203 12/16 (75%) cases and 2/16 (12.5%) were characterized by abundant fibrous  
204 tissue (formerly termed "scirrhous prostatic carcinoma").

**205 Immunohistochemistry**

206 The IHC results are summarized in Table 1 and S2.

**207 Survivin expression in normal gland, BPH and prostatic carcinoma**

208 Survivin-positive nuclei were present among the basal/reserve cell layer of  
209 normal (Fig.1) and hyperplastic prostatic lobules [0-10% of cells in the  
210 11/16 (68,75%) cases with positive labeling] (Fig.2). Even if a single case  
211 of normal gland was analyzed, normal prostatic tissue surrounding the BPH  
212 lesions was also evaluated, showing the same pattern of expression of the  
213 molecule. Most of the BPH cases (12/16, 75%) showed patchy cytoplasmic  
214 immunostaining, with a low to moderate (0-25% of neoplastic cells)  
215 expression in most of the cases (10/12, 83,3%). In contrast, prostatic  
216 carcinomas had more diffuse cytoplasmic expression as well as nuclear  
217 expression of survivin. Cytoplasmic staining was observed in all the  
218 prostatic carcinoma samples, with half of the cases (8/16) showing low to  
219 moderate expression (0-25% of neoplastic cells) and the other half high  
220 expression (>25% of neoplastic cells). Positive nuclei were present in all  
221 except one case of prostatic carcinoma, with most (9/16, 56%) showing

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3 222 moderate to high expression (10 to >50% of positive nuclei) (Fig.3), present  
4 223 mainly among the neoplastic cells with basal cell morphology in the areas  
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6 224 with tubular-papillary pattern .  
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9 225 **Sox9 expression in normal gland, BPH and prostatic carcinoma**

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11 226 Sox9-positive nuclei were observed in scattered cells among the  
12  
13 227 basal/reserve cell layer of 2 of 3 normal prostatic glands (Fig.4) and 5 of  
14  
15 228 15 hyperplastic lobules (Fig.5). Of these positive cases, two normal glands  
16  
17 229 and 2 BPH cases were freshly sampled, while the other 3 BPH cases were from  
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19 230 the archive and the fixation time was not known, suggesting that prolonged  
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21 231 fixation may interfere with Sox9 immunostaining.<sup>34</sup> Among prostatic  
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23 232 carcinomas, 9/16 cases were positive and most (7/9) showed a moderate to  
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25 233 high expression (>25% of neoplastic cells with nuclear  
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27 234 immunolabelling) (Fig.6).  
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31 236 **Double immunofluorescence**

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33 237 From a qualitative evaluation of the immunofluorescence-stained slides,  
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35 238 only a partial co-localization of survivin and p63 (survivin+/Sox9+) was  
36  
37 239 observed. In BPH, survivin-/p63+ nuclei were more numerous (5-10%),  
38  
39 240 survivin+/p63- nuclei were scattered and rare, and survivin+/p63+ nuclei  
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41 241 were few (<5% of cells were double-positive) and observed among basal cells  
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43 242 (Fig.7). In contrast, in prostatic carcinoma, survivin+/p63- nuclei were  
44  
45 243 more numerous (10-25%), and survivin+/p63+ nuclei were scattered and few  
46  
47 244 (<5% of cells were double-positive). p63 cytoplasmic immunostaining was  
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245 also evident (Fig.8).

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50 246 In BPH cases, a higher number of survivin-/AR+ cells (>50%) was observed  
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52 247 compared to survivin+/AR+ cells (5-10%), while no survivin+/AR- cells were  
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54 248 observed (Fig.9). Nuclear immunolabelling in prostatic carcinoma was  
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56 249 characterized by numerous survivin+/AR+ cells (25-50%) and survivin+/AR-

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3 250 cells were also evident (10-25%) (Fig.10). Several neoplastic cells showed  
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5 251 an intense aberrant AR cytoplasmic expression as well as cytoplasmic  
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7 252 immunolabelling for survivin.

8  
9 253 In BPH cases, survivin+/Sox9- cells were more numerous (between 10 and 25%)  
10  
11 254 than survivin+/Sox9+ cells (<5% of double positive cells), while no  
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13 255 survivin-/Sox9+ cells were observed (Fig.11). In the prostatic carcinoma  
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15 256 cases, a higher number of survivin+/Sox9+ (double-immunostained) cells was  
16  
17 257 observed (Fig.12), with intense nuclear and faint cytoplasmic  
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19 258 immunolabelling, and a few (<5%) survivin+/Sox9- and survivin-/Sox9+ cells.

### 20 259 **Statistical Analysis**

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23 260 Both nuclear survivin and Sox9 expression appeared to be higher ( $\geq 10\%$  of  
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25 261 neoplastic cells with positively labeled nuclei) in prostatic carcinoma  
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27 262 compared with BPH cases ( $p < 0.01$  for both). No significant differences were  
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29 263 observed in cytoplasmic survivin expression comparing BPH with prostatic  
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31 264 carcinoma cases.

### 32 265 33 34 35 266 **Discussion**

36  
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38 267 This study is the first to characterize the IHC expression of survivin and  
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40 268 Sox9 in normal, hyperplastic and neoplastic canine prostate. The main aims  
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42 269 were to verify if Sox9 and survivin are expressed by canine prostatic  
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44 270 epithelial cells and if they could represent prostatic stem cell markers.

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46 271 The nuclear immunostaining observed in the basal cell layer of normal  
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48 272 prostate gland is consistent with Sox9 as a stem cell marker in canine  
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50 273 prostate, similar to what has been reported in the human literature.<sup>7</sup> In  
51  
52 274 contrast to our predictions, the first round of testing with Sox9  
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54 275 immunohistochemistry, using cases selected from the University's archives,  
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56 276 showed no positive cells in the normal glands, and only a few positive

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3 277 samples among the BPH cases. A second round of testing, using freshly  
4 278 sampled prostatic tissue fixed in formalin for 24 hours, revealed positive  
5 279 staining in all four cases, and it was more intense and evident than that  
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7 280 observed in samples with longer fixation time. This suggests that prolonged  
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9 281 formalin fixation time decreased Sox9 antigen detection, indicating that  
10 282 this antigen is more vulnerable to degradation than the other investigated  
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12 283 molecules (such as survivin, p63, AR).<sup>34</sup>  
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16 284 The role of survivin in stem cells<sup>16</sup> might indicate that the positive cells  
17  
18 285 found in the basal cell layer of normal glands are stem cells. However,  
19  
20 286 since survivin also has a role in proliferation, it is also possible that  
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22 287 these cells represent partially differentiated, proliferating, transit  
23  
24 288 amplifying cells that maintain some stem cell properties.  
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26 289 The prostate basal cell marker p63 had already been shown to be involved in  
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28 290 epithelial development by regulating stem cell/transit amplifying cells,  
29  
30 291 their differentiation, and cell death.<sup>8,22</sup> Results obtained from the  
31  
32 292 immunofluorescence study showed that in BPH survivin and p63 are expressed  
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34 293 in different cells, with only partial, minimal co-localization. Our  
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36 294 hypothesis is that they mark transit amplifying cells in these lesions, an  
37  
38 295 undifferentiated population of cells in transition between stem cells and  
39  
40 296 differentiated cells, with intermediate features. This would support the  
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42 297 previously reported theory that at least two biologically distinct  
43  
44 298 populations of basal cells exist in the canine prostate gland<sup>19</sup> and that  
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46 299 these cells can be involved in the development/maintenance of BPH.

47  
48 300 Other recent studies characterizing the expression of stem cell markers  
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50 301 have shown that co-expression of CD44 and CD133 detects stem cells in  
51  
52 302 canine prostatic cancer cell lines.<sup>23,24</sup> Further studies should be done in  
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54 303 order to investigate whether survivin and Sox9 are co-expressed with these  
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56 304 markers.  
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3 305 In human prostatic tissue, survivin expression is regulated by androgen  
4 306 stimulation.<sup>36</sup> In the canine prostatic tissue studied here, the presence of  
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6 307 survivin+/AR- cells in prostatic carcinoma, together with the increased  
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8 308 expression of survivin in prostatic carcinoma compared to BPH, would  
9  
10 309 suggest a role for survivin in the progression to androgen independence in  
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12 310 the dog as well. The survivin+/AR- cells could represent a subpopulation of  
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14 311 androgen-independent tumour cells. These may be important in the early  
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16 312 stages of development of prostate cancer and, if present in human prostatic  
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18 313 carcinoma, may be the cause of the failure of androgen ablation therapy  
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20 314 that occurs in most human advanced cases.<sup>10</sup> Our findings would suggest the  
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22 315 presence, in canine prostatic carcinoma, of a subpopulation of neoplastic  
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24 316 cells with low levels of AR and high levels of survivin and some features  
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26 317 of stem cells (prostatic stem cells or transit amplifying cells). This  
27  
28 318 supports the possibility that this tumour derives from basal/stem cell-like  
29  
30 319 epithelial cells expressing low levels of AR.<sup>11,18</sup>

31  
32 320 The origin of CSCs within solid tumours has not yet been clarified and  
33  
34 321 would appear to vary based on tumour type: tumour cells could originate  
35  
36 322 from normal tissue stem cells or from partially differentiated transit  
37  
38 323 amplifying cells that undergo oncogenic transformation to produce CSCs.<sup>13</sup>  
39  
40 324 Further studies should be done to better characterize this subpopulation  
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42 325 and its features of stemness, and to investigate the possible regulation of  
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44 326 the AR-activated pathways on survivin expression in canine prostatic tissue  
45  
46 327 and prostatic neoplasms.

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#### 49 329 **Acknowledgements**

50 330 We thank the Veterinary Pathology Diagnostic Centre of the Utrecht  
51  
52 331 University for providing tissue blocks of canine prostatic lesions, as well  
53  
54 332 as Marina Baffoni and Saskia Essen-van Dorresteijn for their technical  
55  
56 333 support.

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33 452 **Figure Legends**

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36 453 Figures 1-3. Immunohistochemistry for survivin. Figure 1. Normal prostate  
37  
38 454 gland, dog 1. Scattered positive nuclei among the basal cell layer (arrows)  
39  
40 455 and weak, patchy cytoplasmic immunostaining are present. Figure 2. Benign  
41  
42 456 prostatic hyperplasia (BPH), dog 7. Weak cytoplasmic immunostaining and  
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44 457 scattered positive nuclei (arrows), some of which are in the basal cell  
45  
46 458 layer (arrowhead) of hyperplastic prostatic lobules. Figure 3. Prostatic  
47  
48 459 carcinoma, dog 35. Numerous positive nuclei and weak, patchy cytoplasmic  
49  
50 460 immunostaining among the neoplastic cells of a solid prostatic carcinoma,  
51  
52 461 with intensely positive mitotic figures (arrow). Figures 4-6.  
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54 462 Immunohistochemistry for Sox9. Figure 4. Normal prostate gland, dog 2.  
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56 463 Positive nuclei are evident in the basal cell layer (arrows). Figure 5.  
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58 464 BPH, dog 15. Several positive nuclei are shown, mainly present in the basal

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3 465 cell layer(arrows). Figure 6. Prostatic carcinoma, dog 22. Numerous  
4 466 positive nuclei were observed among the neoplastic cells of a papillary  
5 467 prostatic carcinoma.

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9 468 Figures 7-8: Double immunofluorescence for survivin and p63; nuclei  
10 469 counterstained with 4',6-diamidino-2-phenylindole (DAPI). Figure 7: BPH,  
11 470 dog 10. Only a partial co-localization of survivin and p63 was observed,  
12 471 showing a higher number of survivin-/p63+ nuclei in basal cells and few  
13 472 survivin+/p63+ cells. Figure 8.: prostatic carcinoma, dog 19. More frequent  
14 473 survivin+/p63- and only few survivin+/p63+ nuclei were observed with  
15 474 infrequent p63 cytoplasmic immunostaining. Figures 9-10: Double  
16 475 immunofluorescence for survivin and AR; nuclei counterstained with DAPI.  
17 476 Figure 9:: BPH, dog 6. Higher number of nuclear survivin-/AR+ cells  
18 477 compared to survivin+/AR+ cells was observed in BPH cases, without any  
19 478 nuclear survivin+/AR- cells. Most of the cells show cytoplasmic survivin  
20 479 expression. Figure 10: prostatic carcinoma, dog 22. Rare cells had nuclear  
21 480 labelling for both survivin and AR (arrow), but survivin+/AR- nuclear  
22 481 labelling was also evident. Figures 11-12: Double immunofluorescence for  
23 482 survivin and Sox9; nuclei counterstained with DAPI. Figure 11: BPH, dog 18.  
24 483 Most of the cells have survivin+/Sox9- cytoplasmic labeling (green arrow).  
25 484 Figure 12: prostatic carcinoma, dog 32. Numerous neoplastic cells in the  
26 485 prostatic carcinoma cases evaluated showed survivin+/Sox9+ double  
27 486 immunostaining (arrows), with intense nuclear and faint cytoplasmic  
28 487 survivin immunolabelling.

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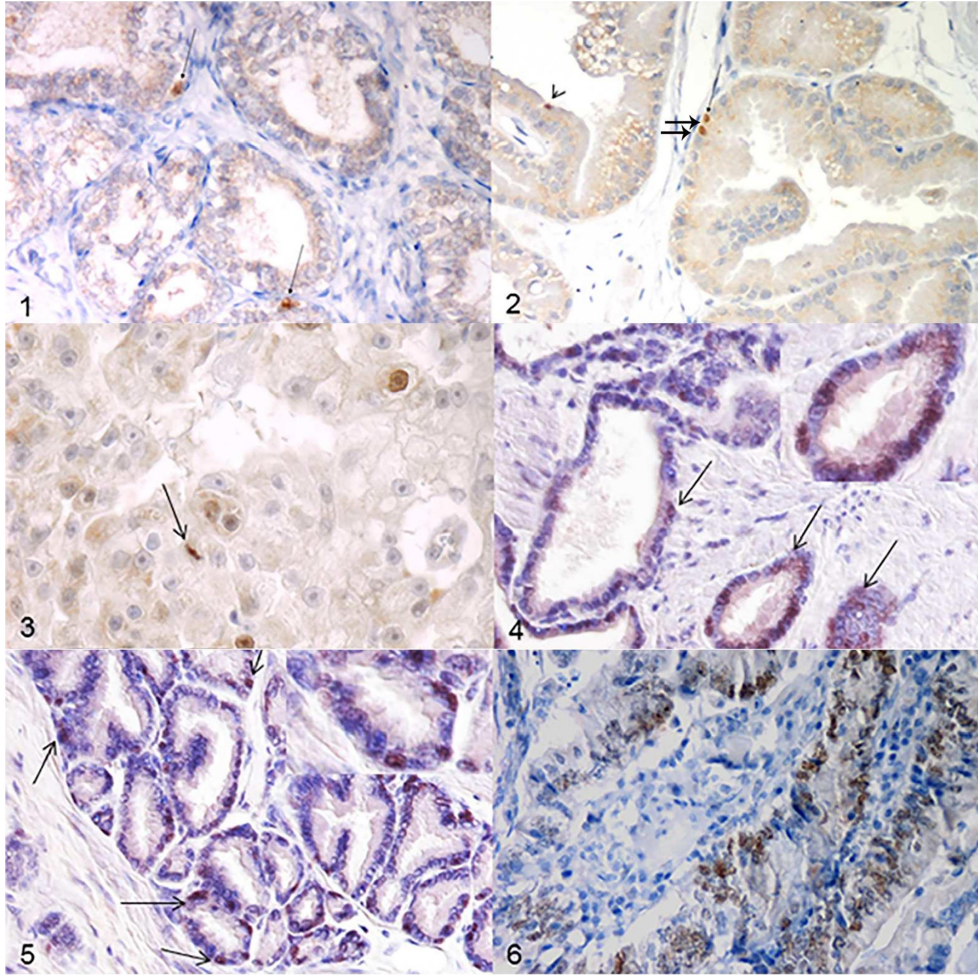
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Table 1. Expression of survivin and Sox9 in normal prostate, benign prostatic hyperplasia, and prostatic carcinoma of dogs. The data show immunohistochemistry grades.

Case N°	Nuclear survivin	Cytoplasmic survivin	Sox9
<b>Normal gland</b>			
1	1	0	0
2*	-	-	1
3*	-	-	1
<b>BPH</b>			
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
<b>PCa</b>			
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

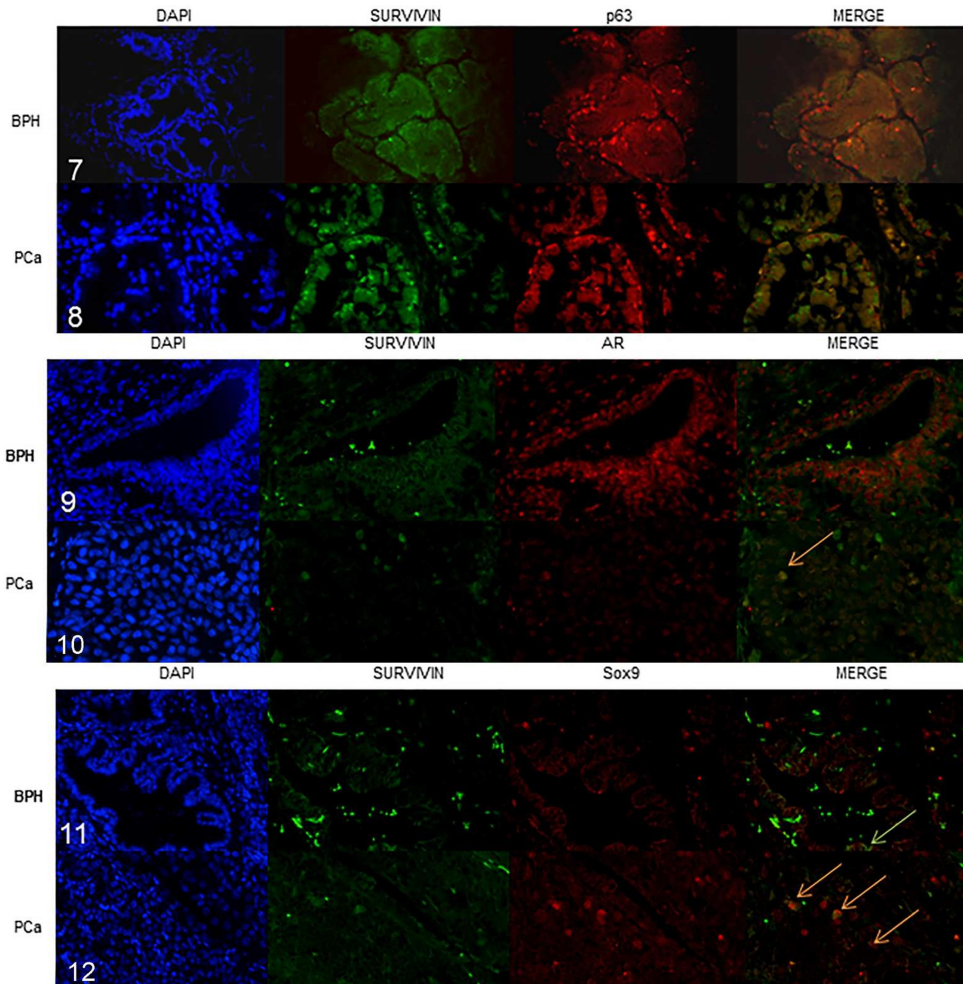
Grades for immunolabelling, as percentage of all neoplastic cells: 0, absent; 1, >0 and <10% of cells; 2, 10 to <25% of cells; 3, 25 to <50% of cells; 4, >50% of cells. BPH: benign prostatic hyperplasia. PCa: prostatic carcinoma. \*: maximum 24 h of formalin fixation time.

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180x180mm (300 x 300 DPI)





180x181mm (300 x 300 DPI)

**Supplemental Table S1 - Methods used for immunohistochemistry.**

<b>MOLECULE</b>	<b>ANTIBODY TYPE</b>	<b>SOURCE</b>	<b>WORKING DILUTION</b>	<b>POSITIVE CONTROL</b>
Sox9	Rabbit PAb	Santa Cruz Biotechnology	1:700	Canine normal skin <sup>5</sup>
Survivin	Rabbit PAb	NOVUS Biologicals	0.7 µg/ml	Canine sebaceous carcinoma <sup>4</sup>
p63	Mouse MAb	DAKO	1:400	Canine prostatic hyperplasia <sup>26</sup>
AR	Rabbit PAb	Santa Cruz Biotechnology	1:500	Canine prostatic hyperplasia <sup>26</sup>

PAb: polyclonal antibody; MAb: monoclonal antibody.

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2 **Supplementary table S2.** The table shows tumour type, prostatic carcinoma histo-  
3 patterns, Gleason/like score, presence of necrosis and immunohistochemistry  
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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, cribriform, 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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60**Supplemental Table S1 - Methods used for immunohistochemistry.**

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

PAb: polyclonal antibody; MAb: monoclonal antibody.

**Supplementary Table S2.** The table shows 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, cribriform, papillary	9	Yes	2	3, ++	0
33	PCa	Cribriform, signet-ring, papillary	10	Yes	2	Cribriform: 3, +, Rest: 4, +++	2
34	PCa	Cribriform, 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. BPH: benign prostatic hyperplasia. BHP: benign prostatic hyperplasia. PCa: prostatic carcinoma. \*: maximum 24 h of formalin fixation time.