Nectin-4 and p63 immunohistochemical expression in canine prostate tumourigenesis

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Short running title: Nectin-4 and p63 expression in dog prostate cancer

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Abstract

Nectin-4 is an E-cadherin-based adherens junction protein of normal epithelial cells, as well as a potent mediator of anchorage-independent cancer colony formation. It is considered a tumour-associated histological and serological marker in various human cancers. The transcription factor p63 is a basal cell marker in the normal prostate, involved in cell adhesion, as well as in the formation and survival of circulating tumour cell clusters. The aim of this study was to evaluate Nectin-4 and p63

immunohistochemical expression in 42 canine prostate tissues including 2 normal prostates, 10 benign prostatic hyperplasias (BPHs), 30 prostatic carcinomas (PCs), 1 pulmonary and 1 lymph node metastasis. From normal to neoplastic tissues, Nectin-4 showed a progressive switching from membranous (m-Nectin-4) to cytoplasmic (c-Nectin-4), regardless of the histological subtypes, except for lack of expression in solid PCs. Metastatic cells exhibited both strong membranous and cytoplasmic positivity. c-Nectin-4 expression was significantly ($p<0.0001$) increased in PCs/metastasis compared to BPHs cases and a decrease ($p<0.05$) of nuclear p63 immunostaining was also detected in the two groups. Furthermore, data showed a significant association ~~correlation~~ ($p<0.05$) between p63 and m-Nectin-4 distribution, although their colocalization was detected only in scattered cells by double immunofluorescence. Our results suggest the involvement of m-Nectin-4 in canine prostate tumourigenesis and metastatic potential, while the exact role of c-Nectin-4 expression detectable in primary PCs requires further investigations.

Keywords: Cancer, Dog, Immunohistochemistry, Nectin-4, p63, Prostate.

Introduction

Aggressive cancer cells have a tendency to self-aggregate in order to survive and proliferate in the absence of an appropriate matrix anchorage.[1] Clusters of circulating tumour cells (CTCs) have been identified in blood samples of several cancer-affected human patients, including those with prostate cancer, and therapies targeting such cell–cell contacts may represent a novel cancer therapeutic approach.[2] Nectin-4, a component of the E-cadherin-based adherens junctions in epithelial cells, encoded by the *PVRL4* (Poliovirus-Receptor-Like 4) gene, is a potent mediator of the anchorage-independent growth relying upon the formation of physical contacts between circulating cells. [1] In humans, Nectin-4 is mainly expressed in the placenta and, to a lesser extent, in tonsils, oral mucosa, trachea, oesophagus, nasopharynx, prostate, lung, and stomach.[3,4,5] Given its role as an epithelial cell receptor for canine distemper virus, Nectin-4 expression has mainly been evaluated in relation to Morbillivirus infection in dogs,[3,6,7] and its expression has been observed in the canine lung, kidney, intestine, urinary bladder,[7] brain and placenta.[3] Recently, the immunohistochemical expression of citoplasmic Nectin-4 (c-Nectin 4) has been detected in 4 out of 9 (45%) canine mammary tumour cell lines derived from three different dogs, as well as in paraffin sections

of mammary adenocarcinoma,[8] whereas lower *Nectin-4* gene expression levels were observed in canine non-tonsillar oral squamous cell carcinoma and oral melanoma compared to normal gingival controls.[9]

In humans, Nectin-4 is a well-recognised, tumour-associated histological and serological marker for several types of adenocarcinoma (lung, breast, pancreas, ovary).[10-11] Nectin-4 expression is lost or reduced in many human cancer cell lines derived from melanoma, neuroblastoma, glioma, medulloblastoma, colon cancer, prostate cancer and renal cell carcinoma, as well as in related cancer tissue samples. It has also been reported to act as a tumour suppressor, especially in ductal breast carcinoma, colorectal adenocarcinoma and renal clear cell carcinoma.[12]-[13]

Another interesting molecule that changes its expression during prostate tumourigenesis is the transcription factor p63, belonging to the p53 family. The ΔNp63 is the predominantly expressed isoform and it is normally detectable at high levels in the basal cells of the normal stratified and glandular epithelia.[14] In the skin, the ΔNp63 isoform is essential for the maintenance of the progenitor population of the basal layer, while the TAp63 isoform is required to allow their complete differentiation in association with or subsequently to ΔNp63.[15] Knockdown of p63 also causes down-regulation of cell adhesion-associated genes, resulting in cell detachment and anoikis in mammary epithelial cells and keratinocytes.16 On the other hand, over-expression of p63 up-regulate the expression of cell adhesion molecules, thus increasing cellular adhesion and enhancing CTCs formation and survival.17 The expression of both p63 isoforms is dysregulated in several human and canine tumours including prostate cancer.[18-20] In this respect, several studies have investigated p63 expression in canine prostate carcinomas (PCs),[19-23] revealing that p63+ canine PCs represent a very rare PC group showing a distinct phenotype compared to typical canine PCs.[20]

At the molecular lever, Nectin-4 and p63 are regulated by and regulate IRF6 (Interferon Regulatory Factor 6) protein in differentiated keratinocytes.[24] In particular, p63 activates IRF6 transcription, while *IRF6* gene depletion reduces Nectin-4 gene expression.24 Therefore, given the involvement of these proteins in regulating the expression of cell adhesion molecules, the evaluation of their expression and distribution in PCs may help to better understand their involvement in cell-to-cell contact during prostate cancer progression.

Thus, the aim of this study was to evaluate the immunohistochemical expression and cellular localisation of membranous and cytoplasmic Nectin-4 and p63 in normal, hyperplastic and neoplastic canine prostate

tissues in order to investigate their possible role in the malignant transformation and invasive/metastatic properties of prostate cancer cells. In addition, since a diagnostic/prognostic marker of canine prostate cancer has not been identified so far, this preliminary study could provide the scientific basis for future investigations on Nectin-4 expression in prostate cancer tissues in association with follow up information and the possibility to detect its soluble form in the serum of canine PC patients, which may help in filling this gap.

Materials and methods

Information on age, breed and castration status of the dogs are summarized in Supplementary table n°1, n°2 and n°3.

Histological examination

In this study, 42 formalin-fixed, paraffin wax-embedded canine prostate samples were analyzed, including 2 normal prostates, 10 benign prostatic hyperplasia (BPH), and 30 primary PCs. Two PCs were associated with pulmonary (1/2) and lymph node (1/2) metastases. All cases were classified according to the human WHO classification, [25] recently adapted to canine PCs.[26] Our study did not involve human participants or live animals and an ethics approval was not required.

Immunohistochemistry for Nectin-4 and p63

Immunohistochemistry was performed using the following primary antibodies (Abs): goat polyclonal anti-human Nectin-4 (1:70, AF2659, R&D Systems, Minneapolis, MN) and mouse monoclonal anti-human p63 (1:400, clone 4A4, DAKO, Glostrup, Denmark), which identifies both p63 isoforms, i.e. ΔNp63 and TAp63. Cross-reactivity with canine tissues for these primary Abs has been previously demonstrated.[8,19,20]. After deparaffinisation and rehydration, antigen retrieval was performed submerging sections in 1M Urea (pH 8.0) in a microwave for 15 min (3 cycles, 5 min/each). To reduce non-specific binding, slides were then incubated at room temperature with 5% non-fat dried milk, 5% BSA and 5% normal horse serum (Nectin-4) or normal goat serum (p63) for 15 min each, before overnight incubation with the specific primary Ab at 4˚C. Sections were treated with 3% H_2O_2, in absolute methanol for 45 min, to inhibit

endogenous peroxidase activity and then rinsed in 0.05M Tris-buffered saline (TBS, pH 7.6) for 5 min. After incubation with secondary biotinylated horse anti-goat (Nectin-4) or horse anti-mouse (p63) (1:200; Vector Laboratories) Ab for 30 min, the reaction was visualised using the Vectastain Elite ABC System (code PK 6200, Vector Laboratories) for 30 min and 0.1% H_2O_2 in 3-3′-diaminobenzidine solution (code D5905, Sigma–Aldrich, St. Louis, Mo, USA) followed by Mayer's haematoxylin (Merck, Darmstadt, Germany) counterstaining. A negative control was performed in all instances by omitting the primary Ab and incubating tissue sections with TBS. The following positive controls were used: canine normal prostate for p63[19] and canine normal lung for Nectin-4.[7]

Double immunofluorescence

Double immunofluorescence was also performed to investigate Nectin-4-p63 and Nectin-4-Laminin co-expression in normal prostates, BPHs and PCs. For Nectin-4-p63 analysis, tissue samples were treated as described for the immunohistochemical procedure using a mixture of primary Abs, applied overnight at 4°C. The first secondary biotinylated goat anti-goat Ab (for Nectin-4) (1:200 dilution; Vector Laboratories) was applied and incubated for 30 min at room temperature and slides were then treated with Texas Red-conjugated avidin (Vector Laboratories) diluted 1:100 in a buffer consisting of 0.1 M NaHCO3 and 0.15 M NaCl, pH 8.2–8.5, for 10 min at room temperature. An avidin/biotin blocking step was performed by incubating slides for 15 min with avidin and then biotin (Avidin/Biotin Blocking Kit, Vector Laboratories) at room temperature. Another secondary biotinylated goat anti-mouse Ab (for p63) (1:200 dilution; Vector Laboratories) was applied and incubated for 30 min at room temperature and the slides were then treated with fluorescein-conjugated avidin (1:100 dilution in 0.1 M NaHCO3, 0.15 M NaCl buffer, pH 8.2–8.5; Vector Laboratories) for 10 min at room temperature. Nuclei were counterstained with 4,6-diamidino-2-phenylindole (DAPI) (Vector Laboratories). For Nectin-4-laminin analysis, dewaxed and rehydrated 3-5 μm thick tissue sections were incubated with a primary antibodies mixture (Nectin-4 pAb 1:70, R&D Systems Inc. Minneapolis US; Laminin pAb 1:100, DAKO;) overnight at 4°C, followed by incubation with biotinylated secondary anti-goat (Nectin-4) and anti-rabbit (Laminin) antibodies (Vector Laboratories UK) and avidin-conjugated fluorescein (Nectin-4) and Texas Red (Laminin) (Vector Laboratories UK). Antigen

retrieval was performed in urea buffer (pH 8.00) in a microwave for 20 minutes. Sections were mounted with Vectashield (Vector Laboratories).

Quantification of immunolabelling and statistical analysis

M-Nectin-4 (membranous) and c-Nectin-4 (cytoplasmic) were semiquantitatively assessed for each sample in 10 randomly selected high-power fields (400x) as follows: absent (0% positive cells), low (>0–<10%), moderate (≥10– <50%), high (≥50%-<75%) and very high (≥75%). Labelling intensity for Nectin-4 was also graded as weak (+), moderate (++) or strong (+++). The number of p63-positive nuclei, for each sample, was calculated in 10 randomly selected high-power (400X) fields counting at least 1000 normal, hyperplastic or neoplastic epithelial cells, and expressed as a percentage. Positivity was evaluated in a double blinded study (MM and LDS) for each case. Differences among BPHs and PCs/Metastasis tissues regarding Nectin-4 immunoreactivity and nuclear expression scores of p63 were assessed by the Fisher's exact test. For Nectin-4, samples were divided into two categories based on the percentage of positive cells: <10% positive cells (absent and low immunoreactivity) versus >10% positive cells (moderate, high and very high immunoreactivity). For p63, the following groups were considered: <10% positive nuclei (absent and low number of positive nuclei) versus >10% positive nuclei (moderate and high number of positive nuclei)[17] Differences in the percentage of p63 expression according to m-nectin-4 and c-nectin-4 distribution were investigated with a Kruskal-Wallis ~~Correlation between m-nectin-4 and c-nectin-4 distribution and percentage of p63 expression was carried out by Kruskal-Wallis~~ test followed by Dunn's Multiple Comparison test. All statistical analyses were performed using GraphPad statistical software, with $p<0.05$ considered to be significant.

Results

Immunohistochemical expression of Nectin-4 and p63 in normal prostates and BPHs.

All normal (2/2) and hyperplastic (10/10) tissues showed a moderate (+/++) m-Nectin-4 expression, localised at the basolateral surface of the cells. Moreover, BPHs were frequently characterised by low (+) c-Nectin-4 staining, mainly localised at the basal side (Figure 1A,B).

In normal prostates and BPHs, nuclear p63 expression was confined to the basal cells, forming a discontinuous layer along the basement membrane (Figure 2A,B). The percentage of positive nuclei in the two normal prostates was 35.6% and 37% respectively, while it ranged from 0% to 14.50% in BPHs samples, with an average of 6.70% (Table 1).

Immunohistochemical expression of Nectin-4 and p63 in different histological subtypes of PCs.

Cribriform PCs (6/30)

A variable, low to high c-Nectin expression was observed in cribriform PCs, whereas the membranous positive staining was generally high or very high (Figure 1C). Interestingly, a decreased expression of c- and m-Nectin-4 was detected in areas characterised by cribriform lesions in which neoplastic cells infiltrate the surrounding stroma (Figure 1D). The percentage of p63-positive cells ranged from 0.60 % to 4.80%, with an average of 1.75% (Table 2).

Solid undifferentiated PCs (4/30)

Neoplastic cells lacked both Nectin-4 and p63 expression in solid undifferentiated PCs (Figure 1C), with the exception of a single case characterised by scattered signet-ring cells and large areas of necrosis (Table 2).

Small acinar/ductal PCs (6/30)

A heterogeneous staining pattern was observed in small acinar/ductal samples showing moderate to high (++/+++) m-Nectin-4, with a single case exhibiting low membranous expression. Cytoplasmic labelling varied from <10% to ≥10–<50%, with a generally moderate intensity of staining (Figure 1E). P63-positive nuclei of neoplastic cells ranged from 4.30% to 7.30%, with an average of 5.88% (Table 2).

Mixed histological subtypes of PCs (14/30)

In mixed PCs, m-Nectin-4 expression of the different histotypes observed within each tumour reflected the pattern of expression already described for the individual tumours (Table 3). The staining was maintained in acinar and cribriform lesions, while lacking in solid areas. c-Nectin-4 was generally present in more than 75% cells, except for the solid type in which the signal was absent. Two cases (n. 2 and n. 12 in Table 3) contained multifocal sarcomatoid changes that showed a diffuse, low m-Nectin-4 and moderate c-Nectin-4 expression. In all mixed PCs, nuclear p63 immunolabeling was absent or detectable in a low number of cells (nuclear score <10%) (Figure 2C-D), with only two cases -characterised by a papillary pattern- showing a high nuclear score (12.30% - 19.60%) (supplementary Fig. 1).

Metastases and emboli

Metastatic lesions showed high (+++) m-Nectin-4 and moderate (++) c-Nectin-4 staining (Figure 1F). In the lymph node, clusters of metastatic cells with a strong membranous staining were observed within the medullary cords. Emboli in peritumoral lymphatic vessels observed in two PCs showed a strong c-Nectin-4 expression. Metastatic cells in the lung were p63-negative, while rare positive p63-positive nuclei (0.6%) were observed within the lymph node (Table 4).

Double immunofluorescence

Evaluation of m-Nectin-4-p63 co-expression revealed an alternate p63 distribution characterised by variable intensity of m-Nectin-4 (+/+++) in all cases, although colocalization of both proteins was only observed in scattered cells (Figure 3 and supplementary Fig. 2 and Fig. 3).

Nectin-4/laminin double staining showed an evident expression of m- and c-Nectin-4 in the basal cells of the cribriform structures, surrounded by a well-formed basal membrane characterized by an intense laminin staining, while the expression decreased in structures with a thin or absent basal membrane and in cells infiltrating the surrounding stroma.

Statistical analysis

C-Nectin-4 and p63 immunostaining showed a significant decrease ($p<0.0001$ and $p<0.05$ respectively) in PCs/metastasis when compared to BPH cases. Furthermore, the percentage of p63+ nuclei was significantly higher in samples with very-high m-Nectin-4 distribution than in all other samples($p<0.05$) (Supplementary Fig. 4). On the other hand, a tendency towards an association between c-Nectin-4 immunostaining and decreased percentage of p63+ nuclei was only observed (Table 2).

Discussion

Dogs with naturally occurring prostate cancer are relevant models for the disease in humans and pre-clinical studies of new diagnostic and therapeutic approaches in dogs may provide benefit for both species with prostate cancer.[27] Murine, human and canine Nectin-4 protein shares high homology,[3] and the critical domains for binding measles virus are completely conserved in the last two species.[8]

The present study focused on the significance of Nectin-4 expression in normal, hyperplastic and neoplastic canine prostatic tissues, suggesting that changes in Nectin-4 expression and function may be involved in prostate tumourigenesis and malignant progression. The first novel finding of this study was the detection of m-Nectin-4 expression in normal canine prostate tissue. In addition, Nectin-4 immunostaining switched from membranous in normal and BPHs samples to predominantly cytoplasmic in most of PCs, then disappearing in solid undifferentiated tumours. In this respect, the weak intensity of c-Nectin-4 expression detected in neoplastic cribriform areas characterized by stromal invasion and thin or discontinuous basal membrane, as well as its strong expression observed in metastases, is of particular interest. In fact, this finding could be in agreement with the loss of adhesion molecules facilitating the migratory phase of tumour cells and their re-expression during the typical clustering mode in metastasis of prostate tumours, although a higher number of normal and neoplastic cases are necessary to confirm this hypothesis.

To date, there are only two reports on the expression of Nectin-4 in several normal and neoplastic tissue in dogs, although there are some discrepancies in the expression patterns.[7,28] Cytoplasmic Nectin-4 has been found to be expressed in neoplastic canine mammary tissues,[8] as well as in four canine mammary tumour

cell lines, where it is likely correlated with malignancy. The latter study was carried out by using the same polyclonal antibody applied in the present investigation. In several human cancers,[29] Nectin immunostaining has been described as predominantly cytoplasmic or both membranous and cytoplasmic,[30] although the significance of this different distribution is still debated. M-Nectin-4 expression was significantly associated with a lower metastasis-free survival rate in breast cancer patients with luminal-A (oestrogen receptor-positive [ER+], and/or progesterone receptor-positive [PR+], human epidermal growth factor receptor 2 [HER-]) tumours, whereas high c-Nectin-4 expression was significantly associated with higher rates of disease-free survival and local relapse-free survival. On the other hand, the absence or a severe reduction of the cytoplasmic form in luminal-A tumours with undetectable cell membrane Nectin-4 has been associated with a higher risk of relapse.[31]

Two different studies [31,32] used the same commercial goat polyclonal antibody that we have applied in our samples, in both cases detecting Nectin-4 expression in the cytoplasm of breast cancer cells. However, M-Rabet et al. (2017)[33] recently tested this antibody on Nectin-4 mRNA negative triple-negative breast cancers detecting both cytoplasmic and nuclear staining by IHC. Despite recognising Nectin-4 by Western Blotting, this polyclonal antibody resulted in a high background signal on Nectin-4 mRNA negative MDA-MB-231 cells, when compared to the human anti-Nectin 4 monoclonal antibody N41. We did not detect any nuclear positivities in our cases and, even if, according to the findings of M-Rabet et al. (2017),[33] the observed cytoplasmic expression may be argued as a background, this will not explain the different degree and intensity of immunoreactivity observed in each histotype examined, as well as the complete absence of cytoplasmic expression in solid tumours. These findings also suggest the importance of further investigating the prognostic significance of membranous and cytoplasmic Nectin-4 in canine prostate cancer.

Loss of junctional molecule expression (i.e. E-Cadherin) in less differentiated invasive carcinomas is a well-known phenomenon, which occurs through multiple mechanisms, e.g. complete or partial gene deletion, promoter inactivation by methylation, chromatin rearrangement[34] and, as far as Nectin-4 is concerned, enzimatic cleavage of the membranous protein. In particular, the extracellular domain of Nectin-4 can be proteolytically cleaved to release a soluble fragment (sN4). [35] The soluble form is generated by the activity of TACE-ADAM-17 (Tumor Necrosis Factor-α-Converting Enzyme-

Metallopeptidase-Domain-17), that cuts the Nectin-4 protein ectodomain. [32] To date, sN4 has been detected in the serum of cancer patients, suggesting its potential use to diagnose or predict cancer evolution, as described in ovarian,[11,37] lung,[38,39] and breast cancers. [38] Since over-expression of TACE-ADAM-17 has been found in human prostatic tumour cell lines and tissue samples,[40] and Nectin-4 expression has been detected in prostate cancer tissues, the serum levels of its soluble form may be of potential interest to be investigated in future studies, both in humans and dogs with prostate cancer. On the other hand, over-expression of m-Nectin-4 may induce lamellipodia formation through activation of small GTPase Rac1 (Ras-related C3 botulinum toxin substrate 1), enhancing migration on Matrigel of fibroblast-like cells, determining the formation of cell clusters, and favouring neoplastic invasion during metastasis. [10] In our study, it is important to underline that both lymph node and pulmonary metastases showed tumour cells clusters with a strong m-Nectin-4 expression. Besides promoting anchorage-independent growth, Nectin-4 can also favour adhesion of cancer cells in different tissues: in vitro studies have demonstrated that blocking Nectin-4 with specific antibodies induces loss of neoplastic cells ability to adhere to pulmonary endothelial cells.[1] It is likely that Nectin-4 can modulate a spectrum of still incompletely defined biological activities, depending on its level and intracellular localisation, both in normal and neoplastic tissues. [31] Nectin-4 was also found to be mainly expressed in breast cancer cell lines with a luminal-like phenotype, as well as to be absent or weakly expressed in cells with a basal-like phenotype.[41]

As far as p63 is concerned, this molecule confirmed its expression pattern in normal and hyperplastic canine prostate, which is typically confined to basal cells, forming a discontinuous layer along the basement membrane,[19] a finding in agreement with the discontinuity of the basal cell layer of canine prostate gland, differently from humans.[42] On the other hand, nuclear p63 immunolabelling was frequently absent or detectable in a low percentage of neoplastic cells in PC cases, confirming that p63+ canine PCs represent a very rare PC subtype.[20] Our study also investigated for the first time the possible relationship between p63 and Nectin-4 expression in canine prostate tissues. In this respect, even though Nectin-4-p63 coexpression by double immunofluorescence was rarely detectable, a significant association ~~correlation~~ between very high m-nectin-4 distribution and high levels of p63+ nuclei was revealed, thus suggesting a possible, IRF6-dependent or -independent, link between Nectin-4 and p63 in the canine prostate.

Conclusions

In conclusion, this is the first study describing Nectin-4 expression in canine prostate tissue and correlating its expression with the basal cell marker p63. The significance of Nectin-4 cytoplasmic expression remains to be clarified, and the relation ~~correlation~~ between p63 nuclear localization and m-nectin-4 distribution requires further investigations. Our results also pave the way for future studies directed to investigate the presence of Nectin-4 soluble form in the serum of canine PC patients, with the aim to compensate the lack of a veterinary diagnostic marker (biomarker) for canine prostate cancer.

Conflict of Interest statement

All the authors have no conflicts of interest to declare.

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Figure legends

Figure 1 Prostate gland; dog. Immunohistochemistry for Nectin-4. (A) BPH showing m-Nectin-4 expression only in the basolateral side. (B) BPH showing m-Nectin-4 and c-Nectin-4 expression localised in the latero/basal side. (C) Mixed PC showing high$^{(+++)}$ m-Nectin-4 and low$^{(+)}$ c-Nectin-4 expression in the cribriform lesion (arrow) and absent expression in the solid undifferentiated part (arrowhead). (D) Cribriform PC showing high$^{(+++)}$ m-Nectin-4 and low/moderate $^{(+/++)}$ c-Nectin-4 expression in cancer cells. The expression decreases where the cells infiltrate the surrounding stroma (arrow). (E) Small acinar/ductal PC showing low$^{(+)}$ to moderate$^{(++)}$ m-Nectin-4 in cancer cells. (F) Clusters of cancer cells expressing high$^{(+++)}$ m-Nectin-4 in a lung metastasis.

Figure 2 Prostate gland; dog. Immunohistochemistry for p63. (A-B) Nuclear p63 immunostaining discontinuously detectable in the basal cell layer of both normal acini (A) and hyperplastic lobules (B). (C-D) Variable p63 expression in canine PCs: low nuclear p63 expression in solid PC (C) and in isolated cells of cribriform carcinoma (D).

Figure 3 Prostate gland; dog. Nectin-4-p63 double-immunofluorescence. From left to right: red Nectin-4, green nuclear p63 and merge. (A-C) A BPH case showing m-Nectin-4 positive cells with strong labelling (arrows), which are negative for nuclear p63 (arrowheads). (D-F) A p63-positive PC case showing diffuse c-Nectin-4 and multifocal m-Nectin-4 expressing cells, most of which are negative for p63.

Supplementary Figure 1. Prostate gland; dog. Immunohistochemistry for p63. Canine PC characterized by a papillary pattern showing a high p63 nuclear score.

Supplementary Figure 2. Prostate gland; dog. Histological image of the canine BPH case illustrated in Figure 3 (A-C).

Supplementary Figure 3. Prostate gland; dog. Histological image of the canine PC case illustrated in Figure 3 (D-F).

Supplementary Figure 4. M-Nectin/p63 correlation. Bar graph represents mean+SEM of the percentage of p63 positive nuclei for each group of prostate tissues grouped on the basis of m-nectin-4 distribution. Statistically significant differences between groups are shown by asterisks (*$p<0.05$).

	Diagnosis	m-Nectin-4		c-Nectin-4	p63
		Distribution	Intensity	Distribution	
1	Normal	Very high	+	Absent	37%
2	Normal	Very high	+	Absent	35.6%

	Diagnosis	m-Nectin-4		c-Nectin-4		p63
		Distribution	Intensity	Distribution	Intensity	
1	Benigne prostatic hyperplasia, cystic	Very high	+/++	Low	+	0%
2	Benigne prostatic hyperplasia, fibrosis	Very high	+/++	Low	+/++	0%
3	Benigne prostatic hyperplasia	Very high	+/++	Low	+	0%
4	Benigne prostatic hyperplasia	Very high	+/++	Low	+/++	7.5%
5	Benigne prostatic hyperplasia	Very high	+/++	Low	+	5.34%
6	Benigne prostatic hyperplasia	Very high	+/++	Low	+/++	0%
7	Benigne prostatic hyperplasia, squamous metaplasia	Very high	+/++	Low	+	9.34%
8	Benigne prostatic hyperplasia	Very high	+/++	Low	+/++	13.27%
9	Benigne prostatic hyperplasia	Very high	+/++	Low	+	11.12%
10	Benigne prostatic hyperplasia	Very high	+/++	Low	+/++	14.5%

Table 1. Summary of semiquantitatively assessed scores of immunohistochemical labelling in canine normal prostate gland and BPH.

Nectin 4 expression was classified as membranous (localized at cell-cell boundaries) or cytoplasmic (uniformly distributed throughout the cytoplasm). Sample were grouped into five categories based on the number of positive cells for each type of Nectin 4 expression.

Immunolabelling distribution: absent (0% labelled cells), low (>0– <10% labelled cells), moderate (≥10– <50% labelled cells), high (≥50%-<75% labelled cells) and very high (≥75% labelled cells).

Immunolabelling intensity: weak labelling (+), moderate labelling (++), strong labelling (+++).

	Diagnosis: carcinoma at single histotype (histological pattern) - small acinar /ductal	m-Nectin-4		c-Nectin-4		p63
		Distribution	Intensity	Distribution	Intensity	
1	Small /acinar ductal PC	Moderate	++	Moderate	++	4.3%
2	Small acinar/ ductal PC	Moderate	++	Moderate	++	7.3%
3	Small acinar /ductal PC	Moderate	++	Moderate	++	5.6%
4	Small acinar/ductal PC	Absent	-	Low	+	6.7%
5	Small acinar/ductal PC	High	+++	High	+++	6.2%
6	Small/ ductal PC	High	++	Low	+	5.2%
	Diagnosis: carcinoma at single histotype (histological pattern) – solid	**m-Nectin-4**		**c-Nectin-4**		**p63**
		Distribution	**Intensity**	**Distribution**	**Intensity**	
1	Solid PC with scattered signet- ring cells, large areas of necrosis.	Absent	-	Low	+	1.6%
2	Solid	Absent	-	Low	+	0%
3	Solid with neoplastic cell from polygonal to spindle, multifocal necrosis, multinucleated giant cells, focal bone metaplasia	Absent	-	Absent	-	0%
4	Solid with multifocal large signet-ring cells	Absent	-	Low	+	0%
	Diagnosis: carcinoma at single histotype (histological pattern) – cribriform	**m-Nectin-4**		**c-Nectin-4**		**p63**
		Distribution	**Intensity**	**Distribution**	**Intensity**	
1	Cribriform PC with comedonecrosis and mineralisation; multifocal signet-ring cells	Absent	-	High	+	4.8%
2	Cribriform PC with comedonecrosis with occasionalsignet ring cells	High	+++	High	++	0.8%
3	Cribriform + infiltrative aspects	High	++	Very High	+++	1.5%
4	Cribriform	Low	+	Very High	+	1.6%
5	Cribriform	Low	++	Very High	+	1.2%
6	Cribriform PC with comedonecrosis.	High	++	Moderate	++	0.6%

Table 2. Summary of semiquantitatively assessed scores of immunohistochemical labelling in canine PCs.

Nectin 4 expression was classified as membranous (localised at cell-cell boundaries) or cytoplasmic (uniformly distributed throughout the cytoplasm). Samples were grouped into five categories based on the number of positive cells for each type of Nectin 4 expression.

Immunolabelling distribution: absent (0% labelled cells), low (>0– <10% labelled cells), moderate (≥10– <50% labelled cells), high (≥50%-<75% labelled cells) and very high (≥75% labelled cells).

Immunolabelling intensity: weak labelling (+), moderate labelling (++), strong labelling (+++).

	Diagnosis: carcinomas at mixed histotypes (histological patterns)	m-Nectin-4		c-Nectin-4		p63
		Distribution	Intensity	Distribution	Intensity	
1	- Cribriform - Small acinar/ductal - Solid Large areas of necrosis and haemorrhages	Moderate Absent Absent	++ - -	Low Absent Absent	+ - -	0% 0% 0%
2	- Cribriform with central necrosis - Papillary - Multifocal sarcomatoid trasformation Occasional signet ring cells	High Absent Low	+++ - +	High High moderate	++ ++ ++	0% 3,4% 0,6%
3	- Cribriform - Solid	Moderate Absent	+++ -	Moderate Absent	+ -	1,5% 0%
4	- Papillary - Cribriform with occasional comedonecrosis - Small acinar/ductal Bone metaplasia	High High Moderate	++/+++ +++ ++	High High Moderate	++/++ ++/++ ++	0% 0% 4,3%
5	- Multifocal small/acinar + mucinous carcinoma Perineureal invasion and mucinous fibroplasia	Low	++	High	+	5,3%
6	- Cribriform - Papillary	High Moderate	+/++ +	High High	++ +	3,3% 3,3%
7	- Cribriform with comedonecrosis - Small acinar/ductal	Absent Moderate	- +	Very High Very high	+++ +/++	2,4% 4%
8	- Cribriform without comedonecrosis - Solid - Small acinar Collagenous micronodules	Moderate Absent Moderate	+/+++ - +	Very high Low Very High	++/+++ + +/++	2,7% 0% 4,8%
9	- Cribriform - Solid Signet ring cells	Moderate Absent	++ -	Very high High	+++ +	2,6% 0%
10	- Solid - Cribriform	Absent Moderate	- +++	Low High	+ ++/+++	0% 3%
11	- Solid - Small acinar	Absent Absent	- -	Low High	+ ++	0% 4,8%
12	- Small acinar/ductal - Solid - Sarcomatoid transformation Signet ring cell	Moderate Absent Low	+++ - +	Moderate Low Moderate	+ + ++	0% 0% 0%
13	- Small acinar/ductal - Papillary	Low High	+++ ++	Moderate Low	+++ ++	0% 12,3%
14	- Cribriform - Papillary - Solid Perineural invasion	High High Absent	+++ +/+++ -	Moderate Moderate Absent	++ ++ -	1,7% 19,6% 0%

Table 3. Summary of semiquantitatively assessed scores of immunohistochemical labelling in canine prostate gland PCa.

Nectin 4 expression was classified as membranous (localized at cell-cell boundaries) or cytoplasmic (uniformly distributed throughout the cytoplasm). Sample were grouped into five categories based on the number of positive cells for each type of Nectin 4 expression.

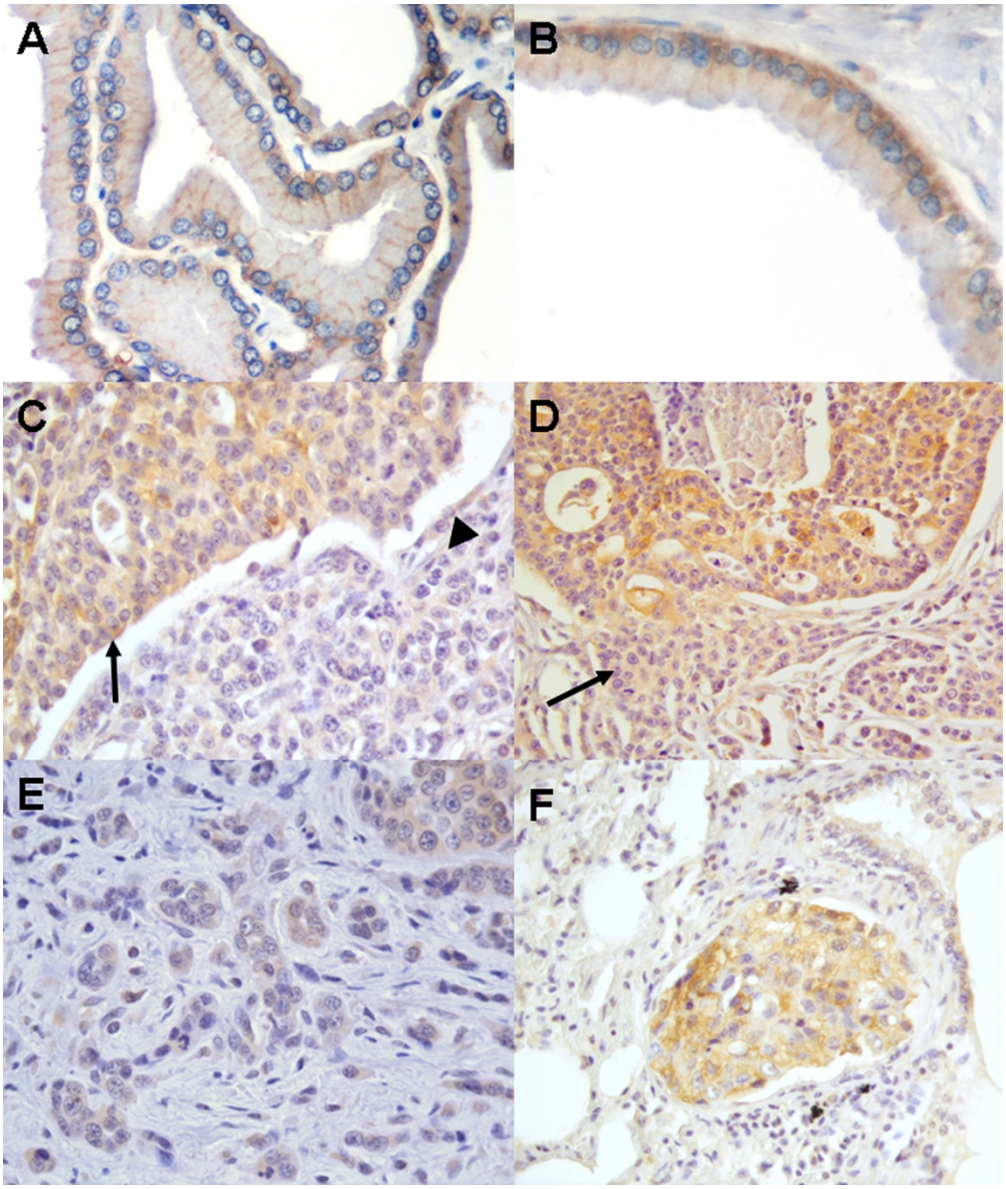
Immunolabelling distribution: absent (0% labelled cells), low (>0– <10% labelled cells), moderate (≥10– <50% labelled cells), high (≥50%-<75% labelled cells) and very high (≥75% labelled cells).

Immunolabelling intensity: weak labelling (+), moderate labelling (++), strong labelling (+++).

	Diagnosis	m-Nectin-4		c-Nectin-4		p63
		Distribution	Intensity	Distribution	Intensity	
11*	Lung metastasis	Very high	+++	Low	+/++	0%
10*	Lymphatic vessels emboli	Very high	++	Very High	+++	0%
14*	Lymphatic vessels emboli	Very High	+++	Very High	++	0%
13*	Lymph node metastasis	Very high	+++	Low	++	0.9%

Table 4. Summary of semiquantitatively assessed scores of immunohistochemical labelling in canine PCa metastasis.

* = Cases refer to the respective cases of mixed tumors.



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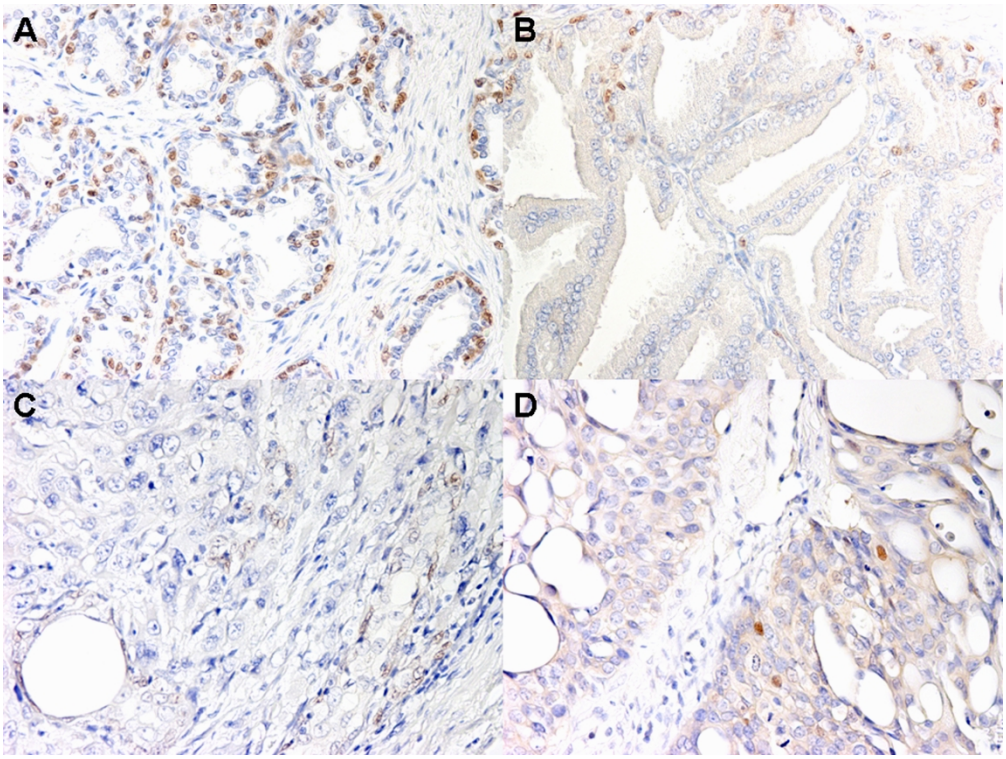

Figure 2 Prostate gland; dog. Immunohistochemistry for p63. (A-B) Nuclear p63 immunostaining discontinuously detectable in the basal cell layer of both normal acini (A) and hyperplastic lobules (B). (C-D) Variable p63 expression in canine PCs: low nuclear p63 expression in solid PC (C) and in isolated cells of cribriform carcinoma (D).

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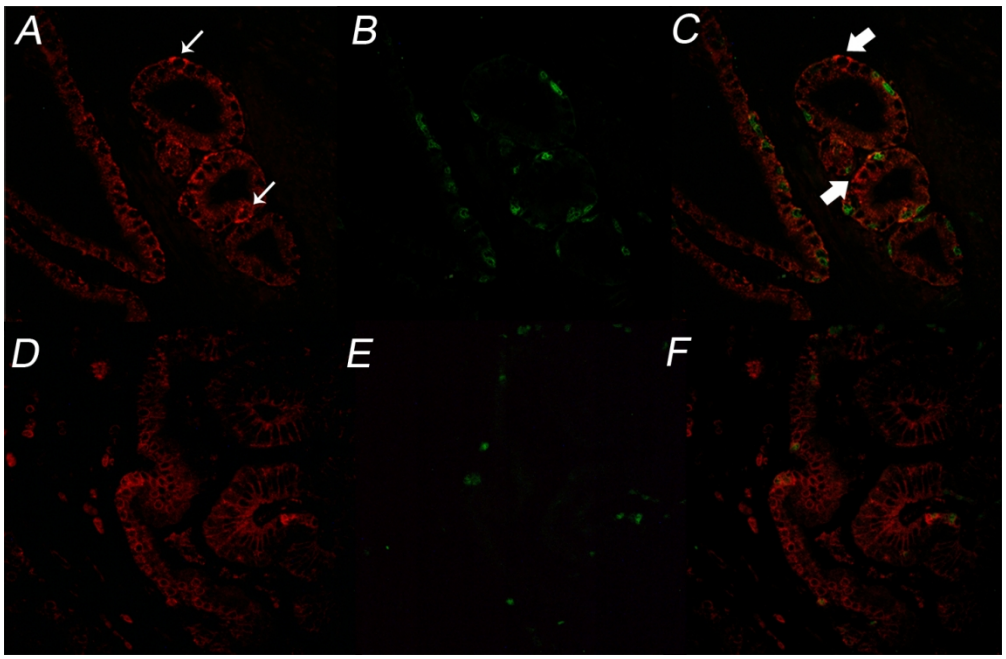

Figure 3 Prostate gland; dog. Nectin-4-p63 double-immunofluorescence. From left to right: red Nectin-4, green nuclear p63 and merge. (A-C) A BPH case showing m-Nectin-4 positive cells with strong labelling (arrows), which are negative for nuclear p63 (arrowheads). (D-F) A p63-positive PC case showing diffuse c-Nectin-4 and multifocal m-Nectin-4 expressing cells, most of which are negative for p63.