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Histological and immunohistochemical investigation of canine prostate carcinoma with identification of common intraductal carcinoma component

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Abstract

A limited number of species, including men and dogs, spontaneously develop prostate cancer (PC). The histological and molecular relevance of canine PC as a model for the disease in men remains controversial. To address this challenge, this study aimed to assess the histomorphology and expression of basal cell, urothelial and neuroendocrine markers [p63, high molecular weight cytokeratin (HMWCK), Uroplakin 3 (UPIII), neuron-specific enolase (NSE)] in canine PC ($n = 41$). Based on histomorphology, 10/41 (24%), 21/41 (51%) and 9/41 (22%) were classified as adenocarcinoma (AC), urothelial carcinoma (UC), and mixed carcinoma, respectively. Tumour inflammation was common, frequently severe [20/41 (49%)], and associated with neutering ($p < .02$) and urothelial differentiation ($p < .02$). Most (36/40, 90%) cancers contained only rare cells with basal cell marker expression or were negative. The expression of UPIII was absent or weak in the majority (33/38, 87%) of tumours, with moderate to strong staining in the remaining cases. NSE expression in PC was rare and limited to 2/14 (14%) cases. Tumour extension into benign ducts and glands was a common finding with presence in 17/39 (44%) of carcinomas with and without urothelial differentiation. In conclusion, we confirm that canine PC is characterized by absent or weak expression of basal cell and urothelial markers. Although rare, NSE expression, potentially indicating neuroendocrine differentiation, is reported for the first time in canine PCa. Intraductal carcinoma of the prostate with concurrent invasive PCa (IDCP-inv) is a frequent, not previously described, finding in dogs with PC.

KEYWORDS
canine, histopathology, immunohistochemistry, p63, prostate carcinoma, uroplakin

1 | INTRODUCTION

In men, prostate cancer (PC) is common occurring in one of every seven men during their lifetime.[1] This is in contrast to animals, which rarely develop this malignancy spontaneously. In fact, only one animal species, the dog, is known to develop PC on a regular basis.[2–5] The dog has therefore been nominated as an animal model for human PC.[3,6–8] Canine PC is characterized by high tumour grade and aggressive

biological behaviour with presence of metastasis in up to 40% of dogs at the time of diagnosis.[2,9] Prostate carcinoma (PCa) is the most common type of prostatic tumour,[10] arising from glandular, ductal or urethral epithelial cells. In men, urothelial cancers are distinguished from PC. However, in canine PCa the cell of origin frequently remains unknown.[2,3,11,12] Despite certain limitations, such as commonly highly aggressive disease with short survival times and androgen insensitivity, pet dogs with spontaneous PCa are a promising and still underexploited animal model.[6] Spontaneous disease occurrence, similarities in benign and neoplastic prostate anatomy and histology, shared environment between men and dogs, utility for therapeutic studies and the available canine genome sequence are some of the main advantages, which the canine model can provide. Further investigations are needed in order to better evaluate the histological and molecular relevance of canine PC as a model for the disease in men.

Prostate basal cell markers, including p63 and high molecular weight cytokeratin (HMWCK), are routinely used for the diagnosis of human PCa when assessing basal cell layer continuity.[13–17] This however cannot be applied to dogs since the canine prostate basal cell layer is discontinuous even in the benign gland.[18,19] In most PCa of men and dogs, the expression of basal cell markers is either absent or restricted to a low number of neoplastic cells,[19–21] with the exception of one rare subtype of p63 positive PCa.[19,21] P63 and HMWCK are also used as urothelial markers, and may, ideally together with additional prostate glandular (PSA, PSAP, NKX3.1) and urothelial (GATA3, CK7, CK20) markers, help to distinguish poorly differentiated human glandular prostatic from urothelial carcinoma (UC).[14,22–25] To our best knowledge, the suitability of p63 and HMWCK for discriminating between prostate glandular and urothelial carcinoma has however not yet been examined in dogs.

UPIII, a highly specific but less sensitive marker for urothelial differentiation, may be used in cases of human or canine PCa where UC is a differential diagnosis.[26–29] UPIII expression was demonstrated to be common in canine PCa, leading to a suggested conclusion that canine PCa may have its origin in the prostatic ducts based on the IHC expression pattern of eight different markers.[11] To date, only a limited number of studies report the expression of UPIII in canine prostate tissue.[11,21]

Neuroendocrine (NE) differentiation, that is, cells with NE morphology and expression of NE markers, is an uncommon but relevant feature of PCa due to its known association with tumour progression, poor prognosis and an androgen receptor negative state.[30–32] PCa with NE differentiation is molecularly distinct from pure NE prostate tumours. PCa cells are thought to become NE-like cells through transdifferentiation even though the exact mechanism are only now being deciphered.[32,33] In benign canine prostates, the number of cells with NE phenotype was observed to increase after castration, confirming the association with androgen-independence in dogs.[34] To date, studies reporting NE differentiation in canine PCa are lacking.

Intraductal carcinoma of the prostate (IDCP) refers to the presence of prostate carcinoma cells within expanded, pre-existing non-neoplastic prostatic ducts and acini.[35,36] IDCP is has been classified as a separate entity by the WHO 2016, comprising two distinct diseases: (i) IDCP associated with invasive carcinoma (IDCP-inv) and (ii) pure IDCP. IDCP-inv corresponds primarily to a growth pattern, whereas the latter is considered a rare precursor lesion of PCa.[37,38] IDCP-inv is not uncommon in men with invasive PCa and has been shown to be associated with high tumour grade, advanced stage and poor disease outcome.[39,40] Due to its clinical relevance, pathologists are advised to record the presence of this lesion. In canine PCa, the presence of IDCP has not been reported and is described for the first time in the present study.

The aim of this study was to assess the histomorphology and immunohistochemical expression of basal cell, urothelial and neuroendocrine markers in canine PCa, in comparison with non-neoplastic prostate tissue.

2 | MATERIALS AND METHODS

2.1 | Samples

The protocol and procedures employed in this study were ethically reviewed and approved by the Ethics committee at the University of Nottingham School of Veterinary Medicine and Science (permission number 1669 160 208). Formalin-fixed and paraffin-embedded benign and malignant prostate tissue from 104 male dogs [$n = 41$ carcinoma, $n = 17$ normal glands, $n = 15$ benign prostate hyperplasia (BPH), $n = 16$ glandular ($n = 14$ post-castration, $n = 2$ due to starvation) atrophy, $n = 14$ neonatal or premature gland] was included. The following case information was available: age at the time of prostate tissue sampling/disease diagnosis, neutering status and dog breed. No follow-up data was available. Within the PCa cases, the following breeds were represented: $n = 12$ Labrador retriever, $n = 5$ cross breeds, $n = 24$ various. All dogs with assessed benign gland were intact except for 12 out of 14 cases with diffuse glandular atrophy. Cases with cancer were either neutered ($n = 24$), entire ($n = 7$) or of unknown neutering status ($n = 10$). The mean age (in years except for neonatal/premature glands) of dogs belonging to the different groups were as follows: neoplasia: 9.63 (*SD* 1.63); normal: 3.41 (*SD* 2.82); hyperplasia: 8.07 (*SD* 2.56); atrophy: 8.53 (*SD* 4.17); neonatal/premature: 71 days (*SD* 67.13).

2.2 | Histology

Haematoxylin and eosin (HE)-stained tissue sections were histologically assessed by a board-certified veterinary pathologist (SdB), with the support of two certified veterinary pathologists (LGR and FG), and an experienced human uropathologist (MAR). As recently proposed by Palmieri et al.,[41] all tumours were classified based on their histomorphology as: (1) prostatic urothelial carcinoma (UC); (2) prostatic adenocarcinoma (AC); and (3) prostatic carcinoma with mixed urothelial and glandular phenotypes. Based on their growth pattern, UC were further subclassified as (i) solid, (ii) papillary or (iii) cribriform and AC as (i) simple tubular, (ii) intra-alveolar (papillary, cribriform) or (iii) solid. For each tumour, the mitotic activity was measured by counting the number of mitotic figures per 10 (or less in small tissue

fragments) consecutive high-power fields (HPF) in the area of the highest mitotic activity. The presence of inflammation was assessed in all cancer cases and semi-quantified as follows (according to percentage of affected tissue): 0 = absent, 1 = mild (<5%), 2 = moderate (5–50%), 3 = severe (>50%).

2.3 | Immunohistochemistry

Immunohistochemistry (IHC) of full tissue sections was performed for p63 ($n = 40$ PCa, n = 60 non-neoplastic [$n = 17$ normal, $n = 14$ neonatal/premature, $n = 13$ BPH, $n = 16$ atrophy] prostates), UPIII ($n = 39$ PCa, $n = 2$ normal urinary bladder, $n = 4$ non-neoplastic prostate tissue adjacent to carcinoma), as well as for few selected cases for the following markers before staining tissue microarrays (TMA): CK5/6 ($n = 2$ PCa, $n = 1$ normal, $n = 2$ neonatal/premature prostates), CK14 ($n = 2$ PCa, $n = 1$ normal, $n = 2$ neonatal/premature prostates), and NSE ($n = 2$ PCa, $n = 1$ normal, $n = 2$ neonatal/premature prostates). In addition, TMA [containing one to six 0.6 mm diameter tissue cores per case of $n = 45$ PCa and $n = 68$ ($n = 4$ benign cancer-adjacent tissue, $n = 13$ neonatal/premature, $n = 9$ BPH, $n = 22$ normal, $n = 11$ prostatitis, $n = 9$ atrophy) non-neoplastic prostates corresponding to the same cases of which full tissue sections were assessed as indicated above] were stained for CK5/6, CK14 and NSE. Information about the used primary antibodies is given in Table 1. The antibodies (with the same clones) used in this study were previously reported for the use in the canine species.[11,29,42–48] For UPIII, the antibody supplier confirms cross-reactivity for canine tissue. Two to three μm paraffin-embedded sections were mounted on positively charged slides (Colour Frosted Plus, Biosystems, Muttenz, Switzerland), dried for 35 min at 60°C and subsequently dewaxed, pre-treated and stained on Bond-III immunostainers (Leica Biosystems, Melbourne, Australia). After dewaxing (Bond Dewax solution; Leica Biosystems), slides were subject to a heat induced epitope retrieval step, using a Tris-EDTA (Bond Epitope Retrieval 2; pH 9 for CK14, CK5/6, NSE, p63) and citrate based buffer (Bond Epitope Retrieval 1, pH 6 for UPIII) for 20 to 40 min at 95° to 100°C (Table 1). To reduce non-specific binding of primary antibodies a protein block solution was applied for 10 min at room temperature, as for all following steps. Then the slides were incubated with the primary antibody for 15 min. All further steps were performed using reagents of the Bond Polymer Refine Detection Kit (Leica Biosystems, DS9800) as follows: Endogenous peroxidase was blocked for 5 (CK5/6, CK14, NSE) to 10 (p63, UPIII) min, then a rabbit-anti-mouse secondary antibody was applied (8 min), followed by a peroxidase-labelled polymer (8 min). Both these reagents were supplemented with 2% dog serum to block non-specific binding (LabForce, Nunningen, Switzerland). Finally, slides were developed in 3,3′-diaminobenzidine / H2O2 (10 min), counterstained with haematoxylin, and mounted. In negative controls the primary antibody was replaced with wash buffer. Known positive controls were stained in parallel with each series.

The following semi-quantitative scoring system was used for p63, CK5/6, CK14 and NSE: 0 = negative; 1 = rare (<5%) stained cells; 2 = moderate number (5–70%) of stained cells; 3 = large number (>70%) of stained cells. For UPIII, staining intensity and distribution were scored separately, using the following semi-quantitative system including staining intensity and distribution. UPIII staining intensity was scored as 0 = negative; 1 = weak; 2 = moderate; 3 = strong. UPIII staining distribution (according to percentage of stained neoplastic cells): 1 = multifocal, <10%; 2 = multifocal, 10%–80%; 3 = multifocal to diffuse, >80%.

All PCa samples with available basal cell marker staining (40/41) were assessed for the presence of intraductal or intraglandular carcinoma spread (IDC) based on histomorphology and p63 staining. As reported by Guo and Epstein[39] IDC spread was defined as the presence of malignant epithelial cells filling large acini and prostatic ducts, with preservation of basal cells and (i) solid or dense cribriform pattern or (ii) loose cribriform or micropapillary patterns with marked nuclear atypia or comedonecrosis. IDC was not further classified as either UC with intraductal spread, or IDC of the prostate (IDCP) with or without invasive carcinoma. If present, the IDC were assessed for the following features: growth pattern (solid, dense cribriform, loose cribriform or micropapillary), necrosis (present or absent), extent of IDC as a percentage of the total tumour area assessed (1: <10%, 2: 10%–49%, 3: 50%–90%, 4:>90%), and UPIII staining (positive or negative).

2.4 | Statistical analysis

Statistics were performed using SPSS v.26.0 (IBM Corp., Armonk, New York, USA). The Chi-square test was used to test for associations

TABLE 1 Primary antibodies used for immunohistochemistry

Antibody	Clone	Source[a]	Code	Dilution	Pre-treatment[b]
Cytokeratin 14 (CK14)	LL002	Leica Biosystems	NCL-L-LL002	1:150	H2(40)95
Cytokeratin 5/6 (CK5/6)	D5/16B4	Dako (Agilent)	M 7237	1:100	H2(30)100
Neuron Specific Enolase (NSE)	MRQ-55	Cell Marque	CMC30622000	1:100	H2(20)95
P63	DAK-p63	Agilent Technologies	M731701-2	1:100	H2(30)99
UroplakinIII (UPIII)	AU-1	Cell Marque	345 M-15	1:100	H1(30)99

[a]Leica Biosystems (Novocastra), Newcastle-upon-Tyne, UK; Dako, Glostrup, Denmark; Cell Marque, Rocklin, CA, USA; Agilent Technologies, Cheshire, UK.
[b]Pre-treatment with Epitope Retrieval Buffer Type 1 (citrate based, pH 6) or 2 (Tris-EDTA based, pH 9) (Leica Biosystems) for (x) minutes at 95°C, 99°C or 100°C on Bond-III immunostainers (Leica Biosystems, Melbourne, Australia).

between the different clinical and histological parameters. $p < .05$ was considered as significant.

3 | RESULTS

The examined cancerous prostate tissue was collected from dogs of variable age, neutering status and breed (Table 2). Most cases were seen in Labrador retrievers (12/41, 29%) and cross breeds (5/41, 12%), presumably representing two of the most common breeds in the UK (https://www.thekennelclub.org.uk/, accessed 6 May 2020). Based on histomorphology, 10/41 (24%), 21/41 (51%) and 9/41 (22%) were classified as AC, UC, and mixed carcinoma, respectively (Table 2). One case of UC presented with extensive squamous differentiation. Two of the tumours classified as mixed carcinoma were assigned to this group due to a lack of convincing either glandular or urothelial features. Instead, they were characterized by a diffusely anaplastic or squamous morphology. One case could not be classified as any of the three tumour groups due to poor tissue quality. AC were

FIGURE 1 Canine prostate carcinoma with predominantly cribriform growth, classified as mixed glandular and urothelial carcinoma. Haematoxylin and eosin (HE) stain. Bar indicates 200 μm. Inset. Same case depicting an area with a different, more anaplastic (solid) growth pattern. HE stain. Bar indicates 200 μm

FIGURE 2 Poorly differentiated, suspected primary glandular, canine prostate carcinoma. Neoplastic cells appear anaplastic and a highly discohesive growth is evident. HE stain. Bar indicates 100 μm. Inset: Same case at higher magnification. HE stain. Bar indicates 50 μm

TABLE 2 Clinical information of affected dogs and tumour inflammation in $n = 41$ canine

Prostate carcinoma subtype	Incidence	Mean age (years) (SD, range)	Neutering[a]			Breed(top three)	Tumour inflammation[b]				
			1	0	Unknown		0	1	2	3	Combined 2 and 3
Adeno-carcinoma	10/41 (24%)	9.11 (1.97, 5–11)	2/10 (20%)	5/10 (50%)	3/10 (30%)	2/10 (20%) Labrador 2/10 (20%) Border collie 2/10 (20%) Rottweiler	0/10	5/10 (50%)	2/10 (20%)	3/10 (30%)	5/10 (50%)
Urothelial carcinoma	21/41 (51%)	9.80 (1.58, 6–12)	15/21 (71%)[e]	0/21	6/21 (29%)	6/21 (29%) Labrador 3/21 (14%) Cross breed 2/21 (10%) Yorkshire terrier	1/21 (5%)	2/21 (10%)	5/21 (24%)	13/21 (62%)	18/21 (86%)[d]
Mixed carcinoma	9/41 (22%)	10.75 (1.71, 9–13)	5/9 (56%)	0/9	4/9 (44%)	4/9 (44%) Labrador 1/9 (11%) Various	0/9	1/9 (11%)	1/9 (11%)	3/9 (33%)	4/9 (44%)
Total	$N = 41$[c]	9.63 (1.63, 5–13)	24/41 (59%)	7/41 (17%)	10/41 (24%)	12/41 (29%) Labrador 5/41 (12%) Cross breed 2/41 (5%) Various	2/41 (5%)	11/41 (27%)	8/41 (19%)	20/41 (49%)	28/41 (68%)

Note: prostate carcinoma cases, indicated for each tumour subtype.

[a]0: Entire; 1: Neutered.

[b]0: absent; 1: mild; 2: moderate; 3: severe.

[c]In one case, the carcinoma subtype could not be determined.

[d]$p < .05$ (Chi-square test).

[e]$p < .01$ (Chi-square test).

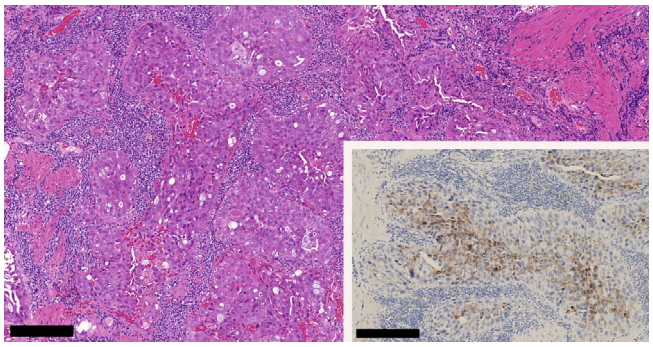

FIGURE 3 Urothelial carcinoma in the canine prostate gland. Neoplastic nests are embedded in a severely inflamed stroma. HE stain. Bar indicates 200 μm. Inset: UPIII immunohistochemistry of the same case with moderate apical membranous staining. Bar indicates 200 μm

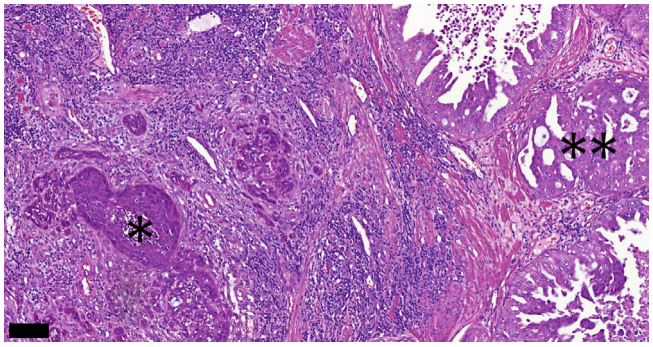

FIGURE 4 Canine prostatic carcinoma with mixed urothelial and glandular morphology. Two different growth patterns are evident, consisting of more basaloid appearing, suspected squamous (*), and cribriform (**) areas. Note the markedly inflamed tumour stroma. HE stain. Bar indicates 100 μm

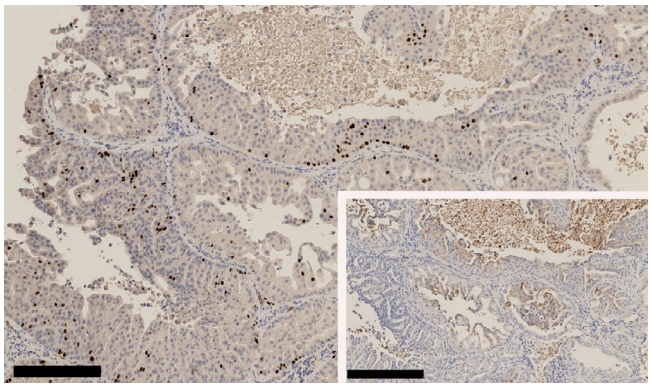

FIGURE 5 P63 immunohistochemistry of an urothelial carcinoma with cribriform growth pattern in the canine prostate gland. A low number of neoplastic cells show strong nuclear staining. Bar indicates 200 μm. Inset: UPIII immunohistochemistry of the same case and region with weak apical membranous staining. Bar indicates 200 μm

further classified as simple tubular (5/10; 50%), intra-alveolar (3/10; 30%) and solid (2/10; 20%), whereas all UC were cribriform (Figures 1-3) (Table 2). No neuroendocrine morphology, neither focal nor diffuse, was observed in any of the assessed tumours. The mitotic activity varied markedly between the individual carcinomas, with 0–74, 0–43 and 0–49 mitotic figures per 10 HPF for UC, AC and mixed carcinomas, respectively. The mean number of mitotic figures per HPF was similar in all three carcinoma subtypes, with 1.33 (*SD* 1.75), 1.44 (*SD* 1.48) and 1.90 (*SD* 1.99) mitoses for UC, AC and mixed carcinomas, respectively. No significant differences in the mitotic count was observed between UC and non-UC tumours.

All but two (39/41, 95%) of PCa were accompanied by a certain degree of inflammatory reaction, which was severe in 20/39 (51%), moderate in 8/39 (21%), and mild in 11/39 (28%) of cases (Table 2). The inflammation typically comprised a multifocal to coalescing lymphoplasmacytic infiltrate (Figure 4). The presence of moderate to severe inflammation was more common in neutered (19/24, 79%) compared with entire dogs (2/7, 29%) ($p < .02$). Due to the small sample size and Cramer-V value of 0.453, the effect size however was considered only moderate. The same association with the presence of moderate to severe inflammation was true for dogs with UC (18/21, 86%) compared with non-UC (10/20, 50%) ($p < .02$). When comparing dogs with UC versus non-UC, neutering was significantly more common in the UC group (15/15, 100% versus 9/16, 56%) ($p < .005$). No significant age differences were observed between UC and glandular PCa or between entire and neutered dogs, respectively.

IHC staining for p63 and HMWCK (i.e., CK5/6 and CK14) in benign prostate glandular tissue was limited to basal cells. In normal and hyperplastic glands, the basal cell layer was discontinuous, whereas neonatal/premature glands presented with a continuous layer of basal cells. In atrophic glands, a mixture of continuous and, to a lesser extent, discontinuous basal cell layers were observed. The urothelium lining prostate ducts and the urethra showed consistent p63 and CK5/6 staining of basal and intermediate cells. However, a multifocally discontinuous layer of stained basal cells was seen in distal non-urothelial portions of the duct. In contrast to p63 and CK 5/6, CK14 was less sensitive with only rare staining of basal and urothelial cells. In premature glands, CK5/6 stained basal cells were restricted to the duct-acinar transition area and outermost peripheral acini and CK14 staining was negative altogether.

P63 staining was performed in 40/41 PCa cases and was present in 24/40 (60%). It was characterized by a consistently strong nuclear signal in a varying number of neoplastic cells. Twenty out of 40 (50%) PCa had rare (<5%), individual p63 positive cells (Figure 5), 16/40 (40%) lacked p63 expression, and 4/40 (10%) demonstrated p63 positivity in >70% of neoplastic cells. Moderate p63 staining (5%–70% positive cells) was not observed. The four cases with high p63 expression consisted of two well-differentiated carcinomas, histomorphologically interpreted as urothelial, and two (one classified as urothelial and one as mixed) carcinomas with squamous differentiation. In the two cases with urothelial morphology, p63 staining was restricted to basal and intermediate epithelial cells. In a large proportion of PCa (17/39, 44%), neoplastic cells were observed

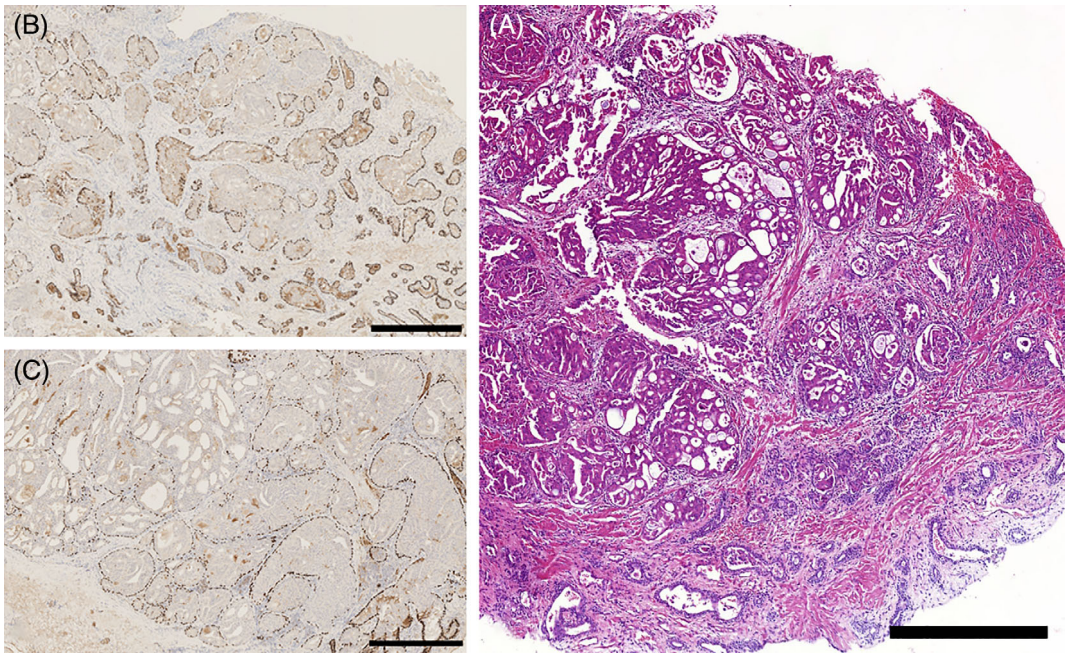

FIGURE 6 Intraductal and intraglandular tumour spread in the canine prostate corresponding to a case of intraductal carcinoma of the prostate with concurrent invasive carcinoma with mixed urothelial and glandular morphology (A,B) and a case of urothelial carcinoma with cribriform growth pattern (C). Intraductal and intraglandular tumour extension is evident with remaining p63 positive benign basal cells of pre-existing ducts and glands (B,C). A: HE stain. Bar indicates 500 μm. (B,C): P63 immunohistochemistry. Bar indicates 500 μm

TABLE 3 Incidence and histological characterization of intraductal or intraglandular carcinoma (IDC) spread in canine urothelial and non-urothelial prostate carcinoma

Tumour type	Number of cases with IDC	Extent of IDC[a]				Growth pattern of IDC		Presence of necrosis in IDC
		1	2	3	4	Solid	Dense cribriform	
PCa (AC or mixed carcinoma)	4/18 (22%) • 2/9 (22%)[b] AC • 2/9 (22%) mixed	1/4 (25%)	1/4 (25%)	2/4 (50%)	0/4	2/4 (50%)	2/4 (50%)	2/4 (50%)
UC	13/21 (62%)[c]	10/13 (77%)	1/13 (8%)	1/13 (8%)	1/13 (8%)	9/13 (69%)	4/13 (31%)	0/13
Total	17/39 (44%)	11/17 (65%)	2/17 (12%)	3/17 (17%)	1/17 (6%)	11/17 (65%)	6/17 (35%)	2/17 (12%)

Abbreviations: AC, adenocarcinoma; IDC, intraductal carcinoma spread; PCa, prostate carcinoma; UC, urothelial carcinoma; UPIII, UroplakinIII.
[a]1: <10%; 2: 10%–49%; 3: 50%–90%; 4: >90% of assessed total tumour tissue.
[b]In one case, the presence of IDC could not be assessed due to missing basal cell (p63) staining.
[c]$p < .05$ (Chi-square test).

intraluminally in variably expanded non-neoplastic ducts and glands, with preserved p63 stained basal cell layers, compatible with intraductal or intraglandular carcinoma (IDC) spread (Figure 6) (Table 3). Despite discontinuity of the remaining basal cell layer, as seen in benign canine prostate glands, this layer was interpreted to be preserved due to the absence of neoplastic proliferations breaking through basal cell lining. The extent of IDC within the examined tumour area was less than 10% in most cases (11/17; 65%), whilst in 2/17 (12%), 3/17 (18%) and 1/17 (6%) of cases, IDC took up 10%–49%, 50%–90% and > 90% of the tumour section, respectively. IDC growth was predominantly solid (11/17, 65%), with a dense cribriform pattern in the remaining cases (6/17, 35%). Necrosis within IDC was an uncommon feature (2/17, 12%). The majority (13/17; 76%) of tumours with IDC corresponded to UC. In these cases, IDC remained UPIII negative in 6/13 (46%), with weak, moderate or strong UPIII staining in 3/13 (23%), 2/13 (15%) and 2/13 (15%), respectively. IDC spread was associated with the presence of moderate to severe tumour inflammation (15/17, 88% versus 2/17, 12%) ($p < .03$).

CK5/6 and CK14 expression was limited to 4/18 (22%) PCa. One of these cases was characterized by diffuse squamous differentiation, with strong expression of CK5/6, CK14 and p63. The three other cases were multifocally CK14 positive in regions with squamous differentiation with a lack of CK5/6 and minimal to absent p63 and UPIII expression. Based on histomorphology, the CK14 positive areas were found to correspond to regions with squamous differentiation.

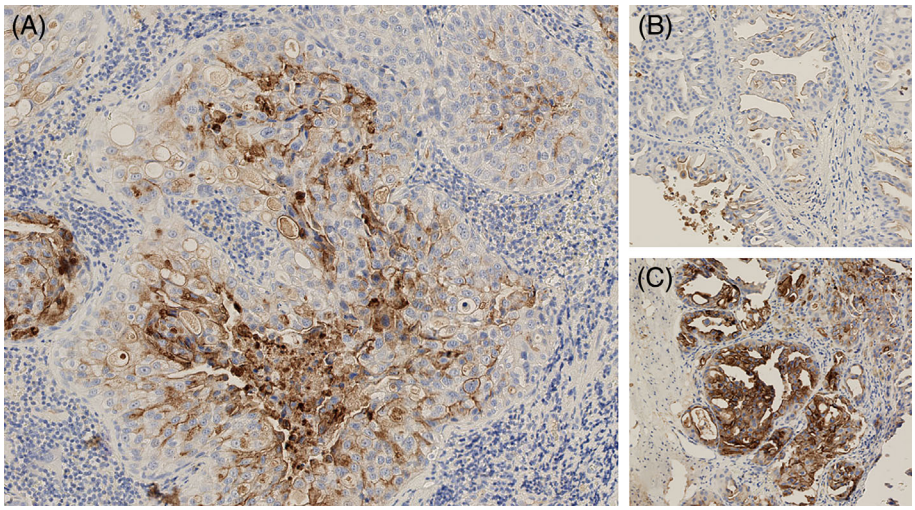

FIGURE 7 UroplakinIII immunohistochemistry of three different histomorphologically classified as urothelial carcinomas in the canine prostate, characterized by a variably intense, multifocal, apical membranous staining of neoplastic cells. Moderate (A) (same case as shown in Figure 3), weak (B) (same case as shown in Figure 5) and strong (C) staining

TABLE 4 Combined p63, HMWCK and UroplakinIII immunohistochemical staining of 37 canine prostate carcinomas

	No of cases showing indicated UroplakinIII staining result				
P63/HMWCK	**Negative**	**Weak**	**Moderate**	**Strong**	**Total**
Double negative					
$P63^-/HMWCK^-$	12[a]	3	0	0	15
Rare p63 positivity[b]					
$P63^{+/-}/HMWCK^-$	6	7	4	1	18
Double positive[c]					
$P63^+/HMWCK^+$	4[d]	0	0	0	4
Total	22	10	4	1	37

[a]One case with multifocal CK14 expression ($P63^-/CK5/6^-/CK14^+$).
[b]<5% p63 positive cells.
[c]>70% p63 positive cells.
[d]One case with negative CK14 and not performed CK5/6 staining ($P63^+/CK14^-$).

Expression of UPIII was assessed in 38/41 PCa. It was characterized by a variably intense membranous staining, which was most pronounced along luminal surfaces. UPIII staining was negative in 22/38 (58%) of cases and showed a weak (11/38, 29%), moderate (4/38, 11%), or strong (1/38, 3%) staining intensity (Figure 7). The distribution and percentage of stained cells was rare (<10% of cells) in 12/16 (75%) of cases, multifocal (10–80% of cells) in 2/16 (13%), and multifocal to diffuse (>80% of cells) in 2/16 (13%). When comparing the histomorphological diagnosis and UPIII staining, 14/21 (67%) UC stained positive with weak, moderate and strong staining intensities in 9/14 (64%), 4/14 (29%) and 1/14 (7%) of cases, respectively. UC with weak staining intensities presented with <10% of UPIII positive cells, whereas the one case with strong staining showed >80% of UPIII stained cells. The number of UPIII positive cells in UC with moderate staining intensities varied markedly, ranging from <10% ($n = 1$), 10%–80% ($n = 2$) to >80% ($n = 1$) of stained cells. All but one case of AC remained UPIII negative. The single AC case with strong UPIII staining was characterized by severe autolytic and freezing tissue artefacts and additional strong UPIII positivity in non-urothelial epithelial cells in the lungs and kidney. UPIII staining in mixed (glandular and urothelial) carcinomas was limited to 2/9 (22%) cases, which showed weak staining of <10% of cells in areas with urothelial morphology. In non-neoplastic prostate tissue, duct as well as urethra demonstrated apical membranous UPIII staining. The co-expression of basal cell markers and UPIII could be assessed in 37/41 cases. Results are provided in Table 4. Double negative PCa were most common (12/37, 32%), followed by PCa with weak UPIII and rare p63 staining (7/37, 19%). The four PCa cases with strong p63 expression did not express UPIII.

Immunohistochemical expression of NSE was observed in two PCa. The first case, with available IHC staining of the full tumour tissue cross section, presented with multifocal cytoplasmic staining of approximately 5%–10% of tumour cells with a variable, weak to strong staining intensity (Figure 8). This case corresponded to a primary acinar adenocarcinoma with multifocal mucoid and cystic morphology. The second case, where IHC staining was restricted to one tissue core within the TMA, was characterized by weak to moderate cytoplasmic NSE expression in >90% of neoplastic cells. This case corresponded to a UC with strong UPIII staining.

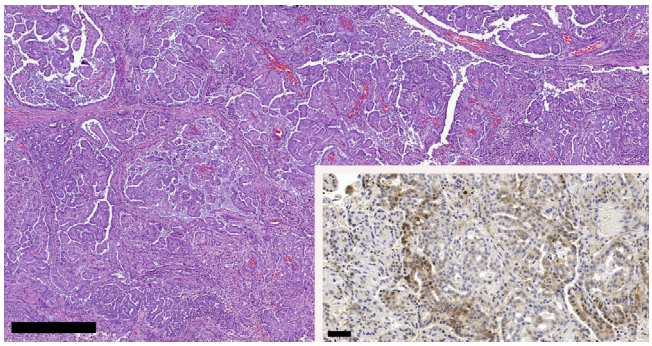

FIGURE 8 Primary glandular canine prostate carcinoma with evident papillary growth. HE stain. Bar indicates 500 μm. Inset: Same case with IHC staining of neuron-specific enolase. Multifocal weak to moderate cytoplasmic staining of tumour cells is present. Immunohistochemistry. Bar indicates 50 μm

4 | DISCUSSION

Even though less common than in men, PC occurs spontaneously and commonly in dogs.[2–5] The cell of origin remains unknown in many cases of canine PC, primarily due to the indistinguishable histomorphology of advanced disease at the time of diagnosis. Whilst the prognostic significance of the different histologic canine tumour subtypes remains largely unknown, there is a tendency for a higher metastatic rate in cancers with a mixed morphology.[2] In addition, knowing the histogenesis of canine PC is crucial when considering using PC in the dog as a spontaneous animal model for PC in men as has been suggested in several occasions.[3,6–8] When comparing UC versus primary glandular or mixed glandular and urothelial PCa, the present study shows a higher ratio of neutered dogs in UC compared to non-urothelial tumours. Similar findings have been described in a study with 76 dogs, which reported a higher risk of prostate adenocarcinoma in entire dogs compared with carcinomas with a mixed (including urothelial) morphology.[2]

Canine PCa is further classified as (i) prostatic UC; (ii) prostatic AC; and (iii) prostatic carcinoma with mixed urothelial and glandular phenotypes, based on the histomorphological appearance.[3,49,50] Poorly differentiated PCa are not uncommon in dogs, making the histomorphological distinction between AC and UC difficult. In the present study, 9 out of 41 (22%) PCa were histomorphologically classified as mixed glandular and urothelial carcinomas. Two of these tumours were assigned to this group due to the absence of convincing either glandular or urothelial features. Instead, they appeared diffusely anaplastic or had a diffuse squamous morphology, which did not allow distinction between UC and AC.

The role of IHC as diagnostic tool remains controversial, especially since some canine PCa may express both urothelial and glandular prostate markers, such as PSA, PSMA, UPIII, CK5, CK7, CK14 and CK18.[2,3,11,51] Uroplakin, a transmembrane protein expressed by apical urothelial (umbrella) cells, is known to be a specific marker for terminal urothelial differentiation in both men and dogs,[27,29] however its sensitivity is often only in the range of 40%–60%.[27,41,52–54] In a study of 90 canine PCa, 47 cases expressed UPIII,[15] with similar results in a previous work by Lai et al.[11] Based on the staining with UPIII and other markers, Lai et al.[11] proposed that canine PCa may derive from prostatic ducts rather than from acini. Similarly, to the previously reported findings, a significant proportion (17/41, 41%) of all PCa expressed UPIII in the present study. The majority (14/17, 82%) of UPIII positive tumours corresponded to UC, confirming the specific staining of this marker, as reported previously.[28,54] One third of UC and most of the mixed carcinomas were UPIII negative, which was an expected finding due to the previously reported moderate sensitivity of this marker.[27,52–55] All but one case of AC remained UPIII negative. The UPIII positive staining in one AC was considered artefactual due to the presence of severe post-mortem tissue changes in this case. We confirm that UPIII is highly specific for urothelial differentiation and that convincing membranous UPIII staining is restricted to the urethra and proximal ducts in the benign canine prostate.

P63, which belongs to the p53 family,[56] is one of basal cell, as well as urothelial cell, markers when diagnosing PCa in men. In this setting, its two main purposes are: (i) as a basal cell marker, to help to distinguish neoplastic from non-neoplastic prostate tissue by assessing the continuity of the glands' basal cell layer,[15–17] and (ii) as a marker for urothelial differentiation, to aid in the differentiation between poorly differentiated adenocarcinoma and high-grade UC.[22–24,57,58] In dogs, the first aim of p63 immunodetection is not directly applicable since the basal cell layer in the healthy canine prostate gland is discontinuous,[18,19] which has been confirmed in the present study. In men, p63 immunodetection is known to be highly specific and moderately sensitive for UC, when compared to primary glandular PCa.[22,23] To date, the specificity and sensitivity of p63 for urothelial differentiation in dogs with PCa has not been reported. In the few available canine studies which evaluated p63 in PCa, the tumours were characterized by rare or absent expression of p63,[19–21] with the exception of a very rare subtype of glandular PCa, which aberrantly expresses p63.[21] In the canine cases of the present study, p63 expression was limited to rare (<5%) cells in most (90%) PCa, which is in agreement with previous studies.[20] The remaining four cases, consisting of two PCa with squamous differentiation and two well differentiated, suspected UC, were characterized by strong expression of p63. The two cases with squamous morphology most likely represent UC with squamous differentiation, which is common in canine and human UC.[1,59,60] The differential diagnosis of primary prostate squamous cell carcinoma cannot be ruled out, but is considered less likely due its rare occurrence.[59] While squamous cell carcinomas are well known to express p63 in both men and dogs,[3,61,62] little is known about the level of p63 expression in canine urothelial tumours. In a study including 25 dogs with bladder UC, p63 has been shown to be rarely detected in neoplastic cells, in contrast to a strong expression in urothelial cells of normal and inflamed bladder tissue.[63] As far as the authors are aware, canine studies comparing p63 expression between urothelial and primary glandular PCa are lacking.

When combining the basal cell markers (p63, HMWCK) and UPIII staining, most PCa were either triple negative or weakly positive in the present study. In triple negative PCa, UC cannot be excluded with

certainty since these markers have limited sensitivities and because poorly differentiated human and canine urothelial or non-urothelial, PCa are known to have only rare or absent expression of these markers.[19,20,64] Considering the known high specificity of UPIII, it can be assumed that UPIII positive tumours or tumour areas correspond to urothelial differentiation. Interestingly, the two cases comprising relatively well differentiated neoplastic urothelium with strong p63 expression were UPIII negative. Based on histomorphology, these two cases were suggestive of UC even though a ductal or, less likely, glandular origin could not be ruled out with certainty. The six UPIII negative cases with rare p63 and absent HMWCK expression are difficult to interpret and cannot be further classified as either urothelial or primary glandular PCa. Finally, all cases with moderate or strong UPIII expression, which convincingly represent UC, were only p63 positive in a low number of cells and were negative for HMWCK. In the examined canine prostate UC, higher expression of UPIII does not, therefore, appear to correspond to higher p63 expression. In fact, a previous study reported that only p63 negative tumour cells expressed UPIII.[64] It was proposed that loss of p63 is required for terminal urothelial differentiation, in both normal as well as neoplastic urothelium. To the authors' best knowledge, studies evaluating the combined staining pattern of UPIII and p63 in canine PCa are lacking.

CK5/6 and CK14 expression was absent in all but four canine PCa. One UPIII negative PCa with diffuse squamous differentiation was strongly positive for CK5/6 and CK14, as well as p63. This is an expected finding since all three are squamous specific markers for human and canine tissue.[65–67] Three further cases were multifocally CK14 positive with lack of CK5/6 and absent or minimal p63 and UPIII expression. In these cases, CK14 positive tumour areas were interpreted to correspond to regions with squamous differentiation. None of the UPIII positive PCa showed any expression of CK5/6. This may seem surprising as both markers are known to reliably stain a significant proportion of UC.[25,67] However, false CK5/6 negative cases are considered likely due to the following reasons: i) UPIII expression was only weak and restricted to less than 10% of the tumour tissue section in the majority of cases and ii) CK5/6 staining was performed on TMAs, representing only a small fraction of the entire tumour. In contrast to p63, multifocal staining of low numbers of individual neoplastic cells was not observed for HMWCK.

In benign, that is, normal as well as hyperplastic, canine prostates examined in the present study, basal cell staining confirmed the previously reported discontinuous basal cell layer in dogs.[18–20] In contrast to p63 and CK5/6, IHC for CK14 revealed only rare staining of basal cells. Previous studies, which used a CK14 antibody with the same clone (LL002) report similar observations, with either absent or rare CK14 expression in basal cells of benign dog glandular prostate tissue.[11,42,45] This is in contrast to the benign human gland where CK14 positive basal cells are prominent.[68] Given the lack of CK14-positive cells and comparably low number of CK5-positive basal cells, the canine prostate is considered to have a more differentiated phenotype compared to the same gland in men.[42]

In the still developing post-natal canine prostate, the basal cell layer is known to be continuous until shortly after puberty,[18] which was confirmed in the present study. Interestingly, the currently examined canine premature glands presented with a diffuse p63 staining of basal cells whereas the CK5/6 basal cell staining was restricted to the duct-acinar transition area and outermost peripheral acini. This specific CK5/6 staining pattern was interpreted to correspond to a distinctive basal cell differentiation and proliferation program, which was previously shown to spread radially from the urethra to the peripheral acini.[18] In human prostates, a similar well-defined pattern of radial differentiation of the epithelial cords is reported.[49,69,70]

In men, there is evidence that inflammation can initiate the neoplastic process.[71] The presence of stromal inflammatory cells in benign as well as malignant human prostate glands is a common finding.[71] This was confirmed in the examined canine PCa which presented with a frequent and often severe stromal inflammatory reaction. Interestingly, the inflammation was more severe in UC compared to AC or mixed (glandular and urothelial) PCa. Similar findings have been published in men, with stromal inflammation more common in UC compared to glandular PCa.[1]

A large proportion (44%) of the studied canine PCa showed intraductal or intraglandular carcinoma (IDC) spread. The extent of IDC within the examined tumour area was less than 10% in most (65%) cases. The predominant IDC growth pattern was solid in the majority of cases, and necrosis within IDC was uncommon. Based on the histomorphology, the majority of the examined PCa with IDC were suspected to represent UC, corresponding to UC with intraductal spread, as reported by Wobker et al.[36] IDC was, however, also observed as a concurrent feature of invasive PCa in some of the primary glandular non-urothelial carcinomas studied in the present canine cohort. In men, this phenomenon is referred to as intraductal carcinoma of the prostate (IDCP) with invasive carcinoma (IDCP-inv).[1,38,39,72] IDCP (with or without concomitant invasive carcinoma) in men is defined as malignant epithelial cells filling large acini and prostatic ducts, with preservation of basal cells and (i) solid or dense cribriform pattern or (ii) loose cribriform or micropapillary pattern with either marked nuclear atypia (nuclear size 6 × normal or lager) or non-focal comedonecrosis.[39] Additional but not required diagnostic minor criteria have been described, including number of affected glands, irregular glandular branching and mitotic activity among others.[72] In the present study, high-grade prostatic intraepithelial neoplasia (HGPIN) was considered as a differential diagnosis for IDC-P as well as for intraductal spread of UC. Due to the predominantly solid growth pattern, which is absent in HGPIN,[39] and the frequently highly pleomorphic neoplastic cells, HGPIN was however considered unlikely. Whilst IDCP-inv is a yet undescribed entity in dogs, or any other animal species that develops spontaneous PC, the histomorphology of this entity in men closely resembles the same feature in the herein examined canine tissues. Considering the typically aggressive behaviour and advanced disease stage at diagnosis of PC in dogs, the presence of IDCP-inv and its association with poor prognosis and advanced tumour stage is not an unexpected finding. The present study also confirms for dogs the known challenge in men to histomorphologically distinguish IDCP from UC with intraductal spread.[36,39]

Neuroendocrine differentiation was rare in the studied canine PCa and only suspected based on the IHC expression of NSE, without convincing evidence of characteristic NE histomorphology. The NSE staining requires cautious interpretation for the following reasons: i) NSE should not be used as a sole marker for NE differentiation due to its limited specificity,[73] and ii) false negative cases are possible since NSE staining was only performed on TMA, representing only a minor portion (one 0.6 mm diameter core) of the total tumour volume. NSE expression was limited to one case each of primary glandular and of urothelial carcinoma. NE differentiation is known to occur in as many as 10% of PCa but is rarely described in UC of men.[74,75] This feature becomes more extensive following androgen deprivation therapy and with cancer progression.[76] To date, NE differentiation has however not yet been described in canine prostate tumours. In order to confirm NE differentiation in the studied cases, further investigation with more specific NE (i.e., chromogranin, synaptophysin) markers is needed.

In conclusion, the examined canine PCa were characterized by poor differentiation, which made the histomorphological distinction between urothelial and primary glandular carcinomas difficult. Most canine PCa showed absent or weak expression of basal cell and urothelial markers. Tumour inflammation was common, typically multifocal to coalescing, lymphoplasmacytic and frequently severe. Extensive inflammation was significantly more common in neutered compared with sexually intact dogs and more frequent in PCa with urothelial differentiation. Although rare, NSE expression, potentially indicating neuroendocrine differentiation, is reported for the first time in canine PCa. Intraductal or intraglandular tumour spread of both primary glandular as well as urothelial PCa was a common finding. In non-urothelial PCa, intraductal spread corresponds to an intraductal carcinoma of the prostate with concurrent invasive PCa (IDCP-inv), which is described for the first time in dogs with PC. In summary, canine PC is characterized by frequent intraductal and intraglandular tumour spread, stromal inflammation and weak to absent expression of basal and urothelial cell markers.

ETHICAL STATEMENT

The study was approved by the University of Nottingham Animal Welfare Ethical Review Body.

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CONFLICTS OF INTEREST

The authors do not have any conflicts of interest to disclose.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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