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ORIGINAL ARTICLE

Effective detection of *BRAF*^{V595E} mutation in canine urothelial and prostate carcinomas using immunohistochemistry

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Abstract

Canine urothelial carcinoma (UC) and prostate carcinoma (PC) frequently exhibit the *BRAF*^{V595E} mutation, akin to the *BRAF*^{V600E} mutation common in various human cancers. Since the initial discovery of the *BRAF* mutation in canine cancers in 2015, PCR has been the standard method for its detection in both liquid and tissue biopsies. Considering the similarity between the canine *BRAF*^{V595E} and human *BRAF*^{V600E} mutations, we hypothesized that immunohistochemistry (IHC) using a BRAF^{V600E}-specific antibody could effectively identify the canine mutant BRAF^{V595E} protein. We tested 122 canine UC (bladder $n = 108$, urethra $n = 14$), 21 PC, and benign tissue using IHC and performed digital droplet PCR (ddPCR) on all 122 UC and on 14 IHC positive PC cases. The results from ddPCR and IHC were concordant in 99% (135/136) of the tumours. Using IHC, *BRAF*^{V595E} was detected in 72/122 (59%) UC and 14/21 (65%) PC. Staining of all benign bladder and prostate tissues was negative. If present, mutant BRAF staining was homogenous, with rare intratumour heterogeneity in three (4%) cases of UC. Additionally, the *BRAF*^{V595E} mutation was more prevalent in tumours with urothelial morphology, and less common in glandular PC or UC with divergent differentiation. This study establishes that BRAF^{V600}-specific IHC is a reliable and accurate method for detecting the mutant *BRAF*^{V595E} protein in canine UC and PC. Moreover, the use of IHC, especially with tissue microarrays, provides a cost-efficient test for large-scale screening of canine cancers for the presence of *BRAF* mutations. This advancement paves the way for further research to define the prognostic and predictive role of this tumour marker in dogs and use IHC to stratify dogs for the treatment with BRAF inhibitors.

KEYWORDS
BRAF, canine, immunohistochemistry, prostate carcinoma, urothelial carcinoma

1 | INTRODUCTION

Urothelial carcinoma (UC) of the bladder and urethra is the most common lower urinary tract tumour in dogs.[1–3] Due to the propensity of distant metastases and the anatomic location, the long term prognosis is poor and cure is rare; however, many dogs with UC that receive treatment can have improved quality for relatively long periods of time.[1,2]

There is a significant predisposition for Scottish Terriers, West Highland White Terriers, Shelties and others breeds for tumour development, and other risk factors include female sex and being spayed or neutered.[3–6] The high breed predisposition indicates an underlying genetic basis for the disease.[3,6–9] In multiple studies, the activating *BRAF*^{V595E} mutation, the canine homologue of human *BRAF*^{V600E}, was identified as somatic driver mutation to be frequently present in 65%–87% of UC of dogs.[1,5,6,8,10,11] BRAF is a serine–threonine protein kinase and immediate downstream effector of RAS. It activates the MAP kinase extracellular signal regulated kinase (MEK), which then phosphorylates extracellular signal-regulated kinases 1 and 2 (ERK1 and ERK2), and thereby orchestrates cell growth, differentiation, proliferation, senescence and apoptosis.[12]

In humans, the activating *BRAF*^{V600E} mutation is particularly prevalent in melanoma, where it is present in nearly half of the cases.[13] It significantly increases protein kinase activity, resulting in constitutive BRAF-MEK-ERK signalling that drives tumour growth.[14] The high frequency of the *BRAF*^{V600E} mutations in melanoma and other human cancers, including colorectal and thyroid carcinoma, implies that this oncogene may be an attractive therapeutic target. Indeed, in recent years, various inhibitors that specifically target *BRAF*^{V600E} mutations (e.g., vemurafenib, dabrafenib and encorafenib) have successfully entered the clinic.[14] Hence, in addition to being a diagnostic and prognostic marker, the *BRAF* mutation has also predictive relevance.[15,16]

Interestingly, canine UC and human muscle-invasive bladder cancer share many similarities at the cellular and molecular level, with similar propensity and site of metastases, as well as response to therapy.[1,3,4,17,18] The dog is therefore considered a highly relevant animal model for studies of human UC.[3,11] However, in contrast to dogs, *BRAF* mutations are only sporadically described in human muscle-invasive UC.[11,17,19]

In canine cancers, the *BRAF*^{V595E} mutation is not only prevalent in UC of the bladder and urethra but also in prostatic carcinomas (PC),[6,20] while it remains absent in other investigated canine cancer types.[6] Canine PC shares the high metastatic potential and invasive nature with UC.[21] It may also originate from various epithelial tissues in the prostate, mirroring human prostate cancer.[22] The exact cellular origin of canine prostate cancer often remains elusive due to small biopsy samples and the complex nature of these tumours characterized by varied differentiation, significant inflammation and necrosis.[23] This complexity raises questions about the exact role of the *BRAF*^{V595E} mutation in canine prostate tumours: is it a characteristic of UC in the prostatic urethra, true prostate adenocarcinoma or both?[8,20]

For the detection of *BRAF* mutations in human cancer multiple methods exist, including Sanger sequencing, pyrosequencing, next-generation sequencing, immunohistochemistry (IHC) and PCR.[24–26] Test sensitivity, specificity, cost, turnaround time and requirements for equipment and experienced staff vary between the different methods. Most commonly, initial detection of cases with *BRAF*^{V600E} or its corresponding mutant protein is performed by IHC, in combination with a molecular test.

In contrast, in dogs, PCR, either quantitative (qPCR) or digital (dPCR), is currently the only well-established method to detect the *BRAF*^{V595E} mutation.[20,27] Both PCR techniques are highly specific, however, dPCR outperforms qPCR due to its higher sensitivity.[28] PCR can be performed using liquid or tissue biopsies, including urine, cytologic smears or formalin-fixed paraffin-embedded (FFPE) material.[29] However, as with any diagnostic tool, there are certain limitations. The specific PCR for detecting *BRAF*^{V595E} is not available in all diagnostic institutions and alternative test methods for canine samples are currently lacking. For screening a larger series of cases by PCR, sample pooling is possible, but does not allow to separate test results for the individual cases. Furthermore, spatial analysis of the mutation, including the evaluation of intratumour heterogeneity or the correlation of mutation with defined histopathologic features, is not possible using PCR. IHC provides a cost-efficient and widely established alternative test method to address these limitations. Indeed, IHC is one of the most frequently used methods for the evaluation of *BRAF*^{V600E} mutation status in human medicine, and is especially valuable because it provides spatial information and can detect intratumoural heterogeneity.[30]

Studies reporting the performance of IHC for mutated BRAF protein detection are currently lacking in the veterinary literature. Due to the homologous mutation of canine and human BRAF, we hypothesized that IHC for anti-human BRAF^{V600E} protein would reliably detect BRAF^{V595E} protein in canine tissue. In order to investigate this, we evaluated 143 cases of canine UC and PC with different mutation status by IHC and compared these results with the corresponding digital droplet PCR (ddPCR) test results.

2 | MATERIALS AND METHODS

2.1 | Samples

FFPE canine tissue of 108 bladder UC, 14 urethra UC, 21 PC (prostatic urethral UC and prostatic adenocarcinomas) and 60 benign prostate were included (normal mature $n = 11$; normal pre-puberty $n = 15$; prostatitis $n = 11$; hyperplasia $n = 11$; castration induced atrophy $n = 12$). In 30 and one cases of UC and PC, respectively, benign tissue was also available adjacent to tumour tissue, which included benign urothelium or non-neoplastic prostate gland. For all cases, FFPE tissue blocks and tissue microarrays (TMAs) were available. TMAs were created with the TMA Grand Master (3DHistech, Budapest, Hungary) with multiple (1–10) cores for each tumour and core diameters of 0.6 mm. The following case information was provided: age at the time of biopsy sampling, sex, neutering status and dog breed. For UC, the most common breeds were crossbreed

($n = 25$), Labrador Retriever ($n = 12$), Cocker Spaniel ($n = 8$), West Highland White Terrier ($n = 7$), Jack Russell Terrier ($n = 6$), Cavalier King Charles Spaniel ($n = 6$), and Scottish Terrier ($n = 5$) (complete breed list provided as supporting information). Out of 122 UC cases, 83 (68%) dogs were female (63 neutered, 2 intact, 18 of unknown neutering status), 39 (32%) were male (22 neutered, 3 intact, 14 of unknown neutering status). The mean age at diagnosis was 10 years (range 4–14 years). For prostate tumours, the following breeds were represented: Labrador Retriever ($n = 8$), Jack Russell Terrier ($n = 2$), Yorkshire Terrier ($n = 2$), Cross breed ($n = 2$), Fox Terrier ($n = 1$), Staffordshire Bullterrier ($n = 1$), Welsh Corgi ($n = 1$), Rottweiler ($n = 1$), Border Collie ($n = 1$) and Belgian Shepherd ($n = 1$). Out of 21 PC cases, 16 (80%) dogs were neutered, 3 dogs were intact and one of unknown neutering status. The mean age at diagnosis was 9.5 years (range 6–13 years).

2.2 | Histology

Haematoxylin and eosin (HE) stained tissue sections were prepared from all TMAs and from all corresponding FFPE blocks. Slides were then scanned using the NanoZoomer S360MD Slide scanner system (Hamamatsu Photonics, Shizuoka, Japan) for digital evaluation using the NDP.view2 viewer (HAMAMATSU Photonics K.K., Hamamatsu City, Japan). Bladder and urethral tumours were classified as either low- or high-grade UC based on the most recent canine grading system of Meuten.[31] Furthermore, these tumours were categorised based on their histomorphology as: (1) conventional urothelial, (2) urothelial with divergent (glandular, squamous or both) differentiation or (3) other (anaplastic or other differentiation). Prostate tumours were categorised based on the most recent classification system by Palmieri et al.[32] as (1) glandular (adenocarcinoma), (2) urothelial or (3) mixed (any combination of urothelial, glandular or other).

2.3 | Immunohistochemistry

IHC was initially performed on TMAs and, in a second step, on 38 ($n = 36$ UC, $n = 2$ PC) corresponding FFPE blocks which showed positive staining on the TMA and which consisted of a large, typically transmural, tumour tissue sample. Tissue blocks were cut using a microtome and mounted on a microscope slide. Briefly, the FFPE tissue was pretreated with ULTRA cell conditioning solution 1 (Tris EDTA) for 72 min at 99°C and was then incubated with the mouse-anti-human primary BRAF V600E mutation specific (VE1) antibody from Roche Diagnostics (material number: 08033706001, catalogue number: 760–5095) for 60 min at 36°C. For detection, the OptiView DAB IHC Detection Kit (Ventana Medical Systems, Roche, Basel, Switzerland) was used according to the manufacturer's instructions. Slides were then scanned, and digitally reviewed (same scanner and software as indicated above). The immunostaining was localized to the cytoplasm and was categorised as either absent or present. Intratumour staining was classified as either homogeneous (distributed regularly and evenly throughout the tumour tissue) or heterogeneous (regional staining differences with focal or multifocal negative areas). Non-neoplastic tissues as indicated above served as (internal) negative control. Tissue from $BRAF^{V600E}$ mutated human colon carcinoma was used as a positive control. For the validation of IHC positive canine cases, ddPCR was performed individually for each case.

2.4 | Digital droplet PCR

In a first step, ddPCR was performed on all UC of the bladder and urethra, independent of their IHC staining result. Based on the high concordance of IHC and ddPCR in the UC cases, only IHC positive prostate tumours were tested with ddPCR. For DNA extraction, 3×10 µm sections were prepared from the FFPE full tissue blocks. The extraction was performed using the QIAamp® DNA FFPE Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Isolated DNA was examined for the presence of the BRAF mutation c.1784T > A by ddPCR using a mutation-specific TaqMan® assay as described by Mochizuki et al.[20] Analysis was performed using the DropletReader (Bio-Rad, Feldkirchen, Germany) and the QuantaSoft™ Software (Bio-Rad, Feldkirchen, Germany). For bladder and urethra UC with negative IHC result, ddPCR was performed in pools of two. If the result from the pool was positive, the ddPCR was repeated on the individual tissue blocks.

2.5 | Statistical analysis

Statistical analysis was performed with NCSS 2023 (23.0.2) software. Pearson's Chi-Square test was performed to test for association of *BRAF* mutation with tumour classification, neutering, sex and tissue type; two-sample *t*-test was performed to test for differences in age of dogs with *BRAF*-mutated versus non-mutated tumours. $p < .05$ was considered statistically significant.

3 | RESULTS

3.1 | Histology

All bladder and urethra tumours were defined as high-grade carcinoma, except for one case of low-grade bladder UC in an 8-years-old male intact Labrador Retriever. The majority of these tumours corresponded to a conventional UC (90/122; 74%). Half of the remaining cases presented either as UC with divergent, squamous or glandular, differentiation (16/122; 13%), or as non-urothelial or poorly differentiated tumours (16/122; 13%). Inflammation of the tumour stroma was common (108/122; 89%), characterized by a multifocal predominantly lymphoplasmacytic infiltrate of variable severity, with occasional formation of lymphoid follicles. In transmural samples, invasion into the muscular layer was observed in 20/40 (50%) cases. The majority of prostate tumours were categorised as mixed carcinoma

given their heterogeneous glandular, urothelial or poorly differentiated histomorphology (10/21; 48%). Remaining cases were approximately equally either UC (6/21; 29%) or prostatic adenocarcinoma with predominance of neoplastic acini, glands or ducts (5/21; 24%). As for the bladder and urethra tumours, stromal inflammation was frequent and consisted of a mixed population neutrophils and mononuclear leukocytes (18/21; 90%).

3.2 | BRAF V600E IHC

Following the identical antigen retrieval and staining protocol as recommended for human tissue, canine tissue was immunolabelled with a mutation-specific mouse monoclonal antibody that was raised against a synthetic peptide representing the $BRAF^{V600E}$ mutated amino acid sequence from amino acids 596–606. The sequence of this peptide (GLATEKSRWSG) is identical to the canine $BRAF^{V595E}$ protein.[9] The IHC staining allowed direct visualization of the mutant protein in the tumour tissue, at single-cell resolution. In contrast to the wild-type protein which is located intranuclear, mutant BRAF is expressed in the cytoplasm, which was confirmed by the performed IHC staining in all cases. Out of 122 cases of bladder and urethra UC, 70 (57%) were positive for $BRAF^{V600E}$ IHC (Table 1). The majority of conventional UC stained positive (62/90; 69%), whereas the opposite was true for UC with divergent (7/16; 44%) or non-urothelial differentiation (1/16; 6%). Positive immunolabelling was characterized as homogeneous cytoplasmic, variably intense, staining of tumour cells with sharp demarcation to surrounding non-neoplastic tissue, which remained unstained (Figure 1). The transition of malignant to benign tissue was available in 28 out of 70 IHC positive cases and the highly specific staining of tumour cells was confirmed in all cases. Intratumour staining was homogenous in all but three tumours, where immunolabelling was more heterogeneous, with multifocal negative areas or variations in staining intensity. In two of these cases, staining variation corresponded to different histomorphological differentiation, with positive staining for urothelial morphology and negative staining in regions with divergent, non-urothelial differentiation. The third tumour with heterogeneous immunostaining had diffuse urothelial morphology and staining variation could not be

Bladder and urethra carcinoma		ddPCR		
Histological subtype	**IHC**	**Positive**	**Negative**	**Total**
Conventional urothelial	Positive	62	0	62
	Negative	1	27	28
Urothelial with divergent differentiation	Positive	7	0	7
	Negative	0	9	9
Non-urothelial or poorly differentiated	Positive	1	0	1
	Negative	0	15	15
Total		71	51	122

Abbreviations: ddPCR, digital droplet PCR; IHC, immunohistochemistry.

TABLE 1 $BRAF^{V595E}$ mutation status of canine bladder and urethra tumours tested by ddPCR and IHC. All cases with positive IHC were confirmed by ddPCR with consistent results. One small sample of bladder UC stained negative with IHC, but was found to be BRAF mutated by ddPCR.

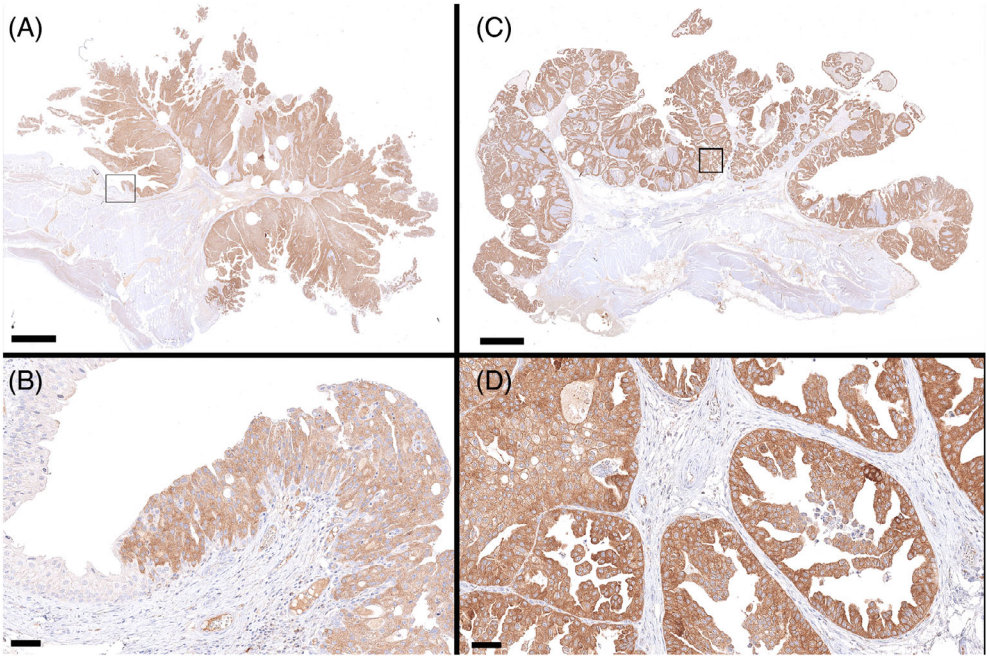

FIGURE 1 Two cases of $BRAF^{V595E}$ mutated canine bladder urothelial carcinoma with diffuse cytoplasmic staining, $BRAF^{V600E}$ immunohistochemistry. (A) Overview of the first case, size bar indicates 2.5 mm. (B) Higher magnification of (A), showing the transition of tumour to benign tissue demonstrating highly specific staining of tumour cells with negative staining of adjacent benign urothelium. Size bar indicates 50 µm. (C) Overview of the second case, size bar indicates 2.5 mm. (D) Higher magnification of (C), size bar indicates 50 µm.

explained by variation in tumour morphology. The single case with low-grade UC had negative IHC staining.

Out of 21 PC, 14 (65%) were positive by IHC, with homogenous intratumour staining of glandular, urothelial and mixed carcinomas (Table 2). All tested benign prostate tissues ($n = 60$) stained negative (Figure 2). Occasional non-specific IHC staining was seen in low numbers of stromal leukocytes, predominantly plasma cells, characterized by weak cytoplasmic immunolabelling.

TABLE 2 *BRAF*^{V595E} mutation status of benign and malignant canine prostate tissue tested by ddPCR and IHC. All cases with positive IHC were confirmed by ddPCR with consistent results.

		ddPCR			
Prostate lesion	**IHC**	**Positive**	**Negative**	**NP**	**Total**
Adenocarcinoma	Positive	1	0		1
	Negative			4	4
Urothelial carcinoma	Positive	5	0		5
	Negative			1	1
Mixed carcinoma	Positive	8	0		8
	Negative			2	2
Non-neoplastic	Positive				0
	Negative			65	65
Total		14	0	72	86

Abbreviations: ddPCR, digital droplet PCR; IHC, immunohistochemistry; NP, not performed.

3.3 | digital droplet PCR

All 122 bladder and urethra UC were tested by ddPCR, which confirmed a high specificity (100%) and sensitivity (99%) of the BRAF IHC (Table 1). All 70 cases with positive IHC staining were confirmed to be BRAF mutated by ddPCR. Out of 52 UC with negative IHC, 51 cases were confirmed to be negative by ddPCR. The one remaining IHC negative tumour, which was tested BRAF positive by ddPCR consisted of a very small tissue sample of a conventional bladder UC. All 14 PC with positive IHC were confirmed to bear the *BRAF*^{V595E} mutation by ddPCR.

3.4 | Correlation BRAF mutation with tumour type, sex, neutering and breed

Bladder and urethra tumours with conventional urothelial differentiation were more likely to be *BRAF*-mutated compared to UC with

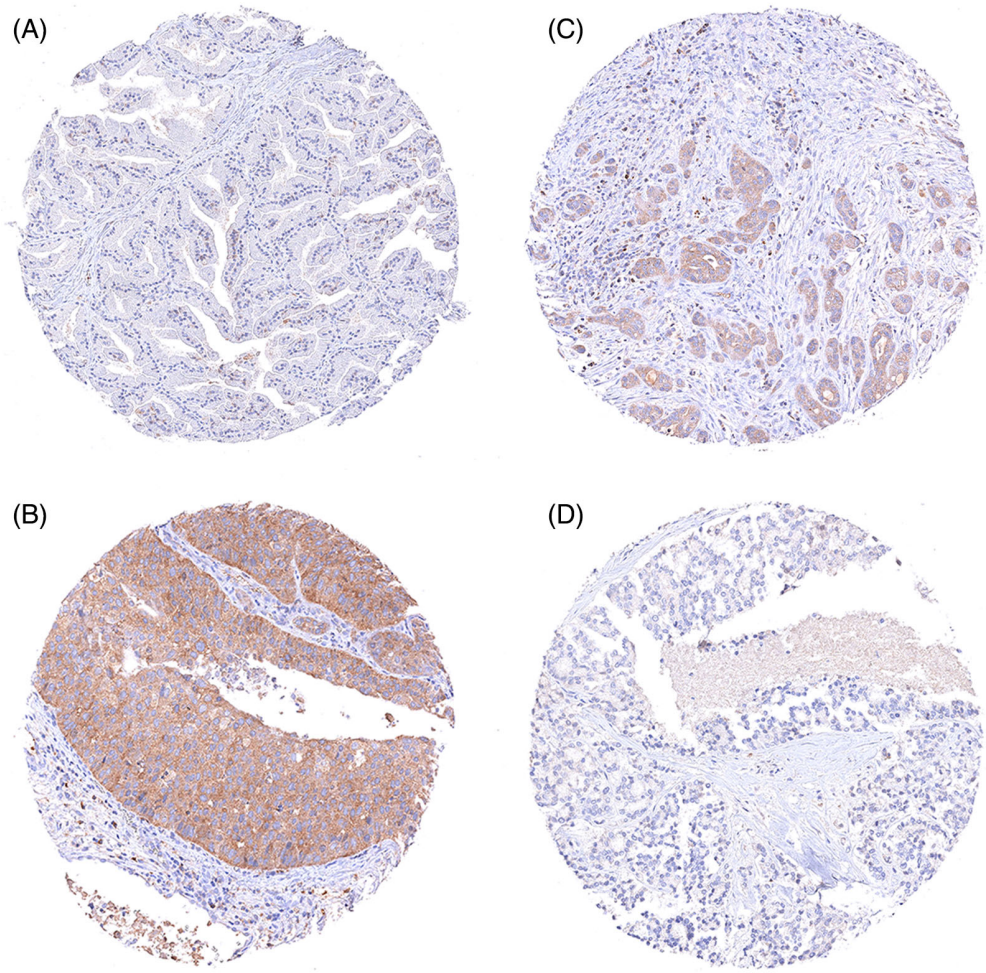

FIGURE 2 Canine tissue microarray cores of benign prostate (A) and prostate carcinoma (PC) (B–D), *BRAF*^{V600E} immunohistochemistry. (A) Normal prostate with negative staining, (B) BRAF mutated PC with diffuse cytoplasmic staining, (C) Highly invasive BRAF mutated PC with positive staining, and (D) BRAF non-mutated PC with negative staining.

divergent or tumours with non-urothelial histomorphology ($p < .0001$). No correlation was found between sex, castration status, tissue type (bladder vs. urethra), age, breed and this specific mutation.

BRAF mutated PC was only observed in castrated dogs ($n = 14$), whereas prostate tumours of entire dogs lacked this specific mutation ($n = 4$). Two of the remaining three cases of non-mutated PC were found in neutered dogs of the same breed (Yorkshire terrier). Despite limitation due to low case numbers, *BRAF* mutation was confirmed to be associated with castration ($p < .01$).

Following the same trend as bladder and urethra tumours, prostate carcinomas (PC) with urothelial differentiation (UC or mixed) were more likely to be *BRAF* mutated ($p < .05$).

4 | DISCUSSION

In our study, we established that an anti-human $BRAF^{V600E}$ IHC is a reliable and accurate method for detecting the mutant $BRAF^{V595E}$ protein in canine UC and PC. IHC provides a cost-effective and familiar approach for pathologists, regularly used in human cancer diagnostics to identify the $BRAF^{V600E}$ mutation.[26,33] For dogs, however, mutation-specific BRAF IHC has not yet been set up and canine studies investigating mutant BRAF protein expression in tissue are currently not available in the literature. Given the genetic similarity of the *BRAF* mutations in dogs and humans, we hypothesized that the mutated protein is detected in canine UC and PC using a human anti-$BRAF^{V600E}$ IHC protocol. This hypothesis was confirmed by our parallel testing of 143 canine cases with ddPCR, demonstrating a 100% specificity and a high sensitivity (99%) of the $BRAF^{V600E}$ IHC for detecting the $BRAF^{V595E}$ mutant protein in canine urothelial and prostate tumours. The one case of UC with a negative IHC and a positive ddPCR test result consisted of a very small tumour sample, which might explain the lack of staining.

In human cancers, the presence of $BRAF^{V600E}$ has been reported to correlate with specific histomorphological tumour subtypes. For instance, in thyroid cancer, it is strongly linked with either the papillary or anaplastic morphology,[34] and in melanomas, it is associated with pigmentation, scatter of intraepidermal melanocytes and solar elastosis.[35] Our recent study using artificial intelligence to analyse canine urinary bladder tumours revealed that *BRAF* mutations in these cases frequently corresponded with papillary growth, smooth and pushing rather than invasive tumour fronts.[7] The present investigations support a parallel between the morphological implications of BRAF mutations in both human and canine cancers. In contrast, Yamasaki and colleagues' recent investigation into the cytomorphological differences in UC cell lines with and without the $BRAF^{V595E}$ mutation found no significant variances.[36] This suggests that in vivo morphologies may be influenced by the tumour microenvironment or obscured in vitro by cell culture conditions.

In the present study, 72/122 (59%) UC and 13/21 (65%) PC were $BRAF^{V595E}$ mutated. This is in accordance with the prevalence of the mutation reported in the literature using PCR,[5–7,9,20] despite our limitation of a small sample size of PC cases. In the dog, $BRAF^{V595E}$ is considered a diagnostic marker for UC and PC.[5,20] However, it is important to keep in mind that the absence of $BRAF^{V59E5}$ does not exclude UC and PC since tumours lacking this specific mutation will not be detected with this method. Therefore, in cases where a tumour of the urinary tract or prostate is suspected, but $BRAF^{V595E}$ is absent, other diagnostic tools, for example, cytology, histology or imaging techniques, need to be performed to assess whether a neoplastic process is present or not.

A number of studies have investigated whether $BRAF^{V595E}$ is also prognostic or predictive for UC.[5,37,38] Thus far, none of these studies was able to demonstrate a correlation between *BRAF* mutation status, overall survival, and disease-free interval. This is in contrast to human papillary thyroid carcinomas, where $BRAF^{V600E}$ has been shown to be significantly associated with increased cancer-related mortality.[15] A large retrospective study on human melanomas also found that the $BRAF^{V600E}$ mutation is associated with increased mortality.[39]

The therapeutic implications of BRAF mutations are noteworthy. BRAF inhibitors (BRAFi), including vemurafenib and dabrafenib, have shown significant clinical benefits in human patients with $BRAF^{V600E}$ mutant cancer and have been approved by the FDA.[40,41] However, the efficacy of BRAFi varies across different cancer types and therapy resistance remains a major challenge. Recently, combination therapies with MEK and/or EGFR inhibitors have gained attention due to their improved performance compared to BRAFi monotherapy. To evaluate the predictive relevance of $BRAF^{V595E}$ in dogs, BRAFi have been tested in canine patients with naturally occurring *BRAF*-mutated UC and a few canine UC cell lines with or without *BRAF* mutation. Rossman et al. performed a clinical trial with vemurafenib in 34 pet dogs with BRAF mutant UC.[42] Treatment led to partial remission in 9 out of 24 dogs, with a median progression-free interval of 181 days. The responses to this BRAF inhibitor in dogs did mimic those reported in men, including good initial response, followed by drug resistance. Maeda et al. tested the effect of dabrafenib on two different canine UC cell lines with mutant or wild-type BRAF.[38] This drug was shown to be effective in BRAF mutant but not wild-type tumour cells. However, both cell lines were relatively resistant to this BRAF inhibitor. Jung et al. compared the anti-tumour effect of sorafenib and vemurafenib in three canine and one human BRAF mutated TCC cell lines. In contrast to human tumours and the one investigated human cell line in their study, canine cell lines were more sensitive to sorafenib than vemurafenib.[10] Sorafenib is an important drug for liver cancer therapy in men, primarily through the induction of apoptosis and ferroptosis and it is not a specific BRAF inhibitor, in contrast to vemurafenib.[43] As Jung and colleagues suggest, the different sensitivity of BRAF mutant cancer to sorafenib and vemurafenib in men and dogs may indicate different cancer genetics in men and dogs, innate resistance to vemurafenib in canines, or different drug binding affinity to the BRAF protein in humans and dogs. Variable effects of vemurafenib have already been observed in previous canine UC cell line studies. Decker et al. investigated three BRAF mutants and one wild-type canine UC cell line.[9] Two of three mutant cell lines displayed a clear reduction in pMEK after exposure to vemurafenib, whereas levels of pMEK remained high in the remaining mutant cell line. The wild-type

cell line, which was the same as used by Maeda et al., was characterized by low pMEK levels and no response to vemurafenib. Cronise et al. found BRAF mutant canine UC cell lines to be insensitive to vemurafenib, however MAPK inhibitors were effective in mutant and BRAF wild-type cell lines.[44] Furthermore, ErbB inhibitors were identified to have a synergistic effect with MAPK inhibitors, promoting combination therapies for canine UC.

Noteworthy, efficacy of, and resistance to BRAF/MAPK pathway targeted therapies may potentially be influenced by intratumour *BRAF* mutation heterogeneity. Tumour heterogeneity for mutant BRAF is described to occur in 3% to 15% of melanomas.[45] Whether mutant *BRAF* heterogeneity influences patient outcomes or the response to BRAF/MEK inhibitors in human melanoma remains controversial in the literature.[46,47] While this heterogeneity's impact on patient outcomes in human melanoma is debated, it appears to be a rare occurrence in canine cancers, based on our findings.

The $BRAF^{V600E}$ mutation has been confirmed to be linked to malignant transformation and is known to be one of the earliest events in tumorigenesis.[48] Of note, this mutation is not purely restricted to malignant lesions, but it is also found in benign tissue changes such as melanocytic nevi, endosalpingiosis and Langerhans cell histiocytosis.[49] It is therefore key, as for any molecular test, that the *BRAF* mutation status is interpreted together with histopathology and clinical information. Thus far, canine $BRAF^{V595E}$ is considered cancer-specific and has not yet been detected in benign tissue.[29,50] This is also consistent with our results from the prostate, where all non-neoplastic prostate samples were negative for $BRAF^{V595E}$ on IHC. However, as mentioned before, a limitation of this study is that non-neoplastic lesions of the lower urinary tract, for example, cystitis was not specifically examined.

The detection of *BRAF* mutations, including the canine-specific $BRAF^{V595E}$, represents one important method for cancer diagnosis, in addition to traditional diagnostic approaches like histology, cytology and imaging. With the establishment of a $BRAF^{V595E}$-specific IHC for canine tissue, we now have a cost-efficient and readily available test for detecting these mutations. While BRAF-targeting therapies in dogs are still evolving, the utility of knowing the BRAF mutation status in patients with confirmed cancer may be useful to stratify patients for such targeted therapy.

In conclusion, IHC represents a reliable, highly sensitive and specific method to detect mutant $BRAF^{V595E}$ in canine urothelial and prostate tumours. With the availability of TMAs and IHC, cost-efficient testing for large-scale screening of canine cancers for the presence of *BRAF* mutations have become feasible and enable further research to define the prognostic and predictive role of this tumour marker in dogs.

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CONFLICT OF INTEREST STATEMENT

The authors Alexandra Kehl and Heike Aupperle-Lellbach declare no conflict of interest; however, they are employed at LABOKLIN GmbH & Co, which is offering genetic diagnostic tests for routine diagnostics in veterinary medicine. All other 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.

REFERENCES

1. Knapp DW, Dhawan D, Ramos-Vara JA, et al. Naturally-occurring invasive urothelial carcinoma in dogs, a unique model to drive advances in managing muscle invasive bladder cancer in humans. *Front Oncol*. 2020;9:1493.
2. Fulkerson CM, Knapp DW. Management of transitional cell carcinoma of the urinary bladder in dogs: a review. *Vet J*. 2015;205:217-225.
3. de Brot S, Robinson BD, Scase T, et al. The dog as an animal model for bladder and urethral urothelial carcinoma: comparative epidemiology and histology. *Oncol Lett*. 2018;16:1641-1649.
4. de Brot S, Grau-Roma L, Stirling-Stainsby C, et al. A Fibromyxoid stromal response is associated with muscle invasion in canine urothelial carcinoma. *J Comp Pathol*. 2019;169:35-46.
5. Grassinger JM, Merz S, Aupperle-Lellbach H, Erhard H, Klopfleisch R. Correlation of BRAF variant V595E, breed, histological grade and cyclooxygenase-2 expression in canine transitional cell carcinomas. *Vet Sci*. 2019;6:31.
6. Mochizuki H, Breen M. Comparative aspects of BRAF mutations in canine cancers. *Vet. Sci*. 2015;2:231-245.
7. Küchler L, Posthaus C, Jäger K, et al. Artificial intelligence to predict the BRAF V595E mutation in canine urinary bladder urothelial carcinomas. *Animals*. 2023;13:1-16.
8. Mochizuki H, Kennedy K, Shapiro SG, Breen MB. BRAF mutations in canine cancers. *PLoS One*. 2015;10:1-9.
9. Ostrander E, Decker B, Parker HG, et al. Homologous mutation to human BRAF V600E is common in naturally occurring canine bladder cancer—evidence for a relevant model system and urine-based diagnostic test. *Mol Cancer Res*. 2012;17:1310-1314.
10. Jung H, Bae K, Lee JY, et al. Establishment of canine transitional cell carcinoma cell lines harboring BRAF V595E mutation as a therapeutic target. *Int J Mol Sci*. 2021;22:9151.
11. Wong K, Abascal F, Ludwig L, et al. Cross-species oncogenomics offers insight into human muscle-invasive bladder cancer. *Genome Biol*. 2023;24:1-29.
12. Dhillon AS, Hagan S, Rath O, Kolch W. MAP kinase signalling pathways in cancer. *Oncogene*. 2007;26:3279-3290.
13. Davies H, Bignell GR, Cox C, et al. Mutations of the BRAF gene in human cancer. *Nature*. 2002;417:949-954.
14. Poulikakos PI, Sullivan RJ, Yaeger R. Molecular pathways and mechanisms of BRAF in cancer therapy. *Clin Cancer Res*. 2022;28:4618-4628.
15. Xing M, Alzahrani AS, Carson KA, et al. Association between BRAF V600E mutation and recurrence of papillary thyroid cancer. *J Clin Oncol*. 2015;33:42-50.
16. Araque KA, Gubbi S, Klubo-Gwiezdzinska J. Updates on the management of thyroid cancer. *Horm Metab Res*. 2020;52:562-577.

17. Iyer G, al-Ahmadie H, Schultz N, et al. Prevalence and co-occurrence of actionable genomic alterations in high-grade bladder cancer. *J Clin Oncol*. 2013;31:3133-3140.
18. Boulalas I, Zaravinos A, Delakas D, Spandidos DA. Mutational analysis of the BRAF gene in transitional cell carcinoma of the bladder. *Int J Biol Markers*. 2009;24:17-21.
19. Longo T, McGinley KF, Freedman JA, et al. Targeted exome sequencing of the cancer genome in patients with very high-risk bladder cancer. *Eur Urol*. 2016;70:714-717.
20. Mochizuki H, Shapiro SG, Breen M. Detection of BRAF mutation in urine DNA as a molecular diagnostic for canine urothelial and prostatic carcinoma. *PLoS One*. 2015;10:1-12.
21. Axiak SM, Oncology D, Bigio A. Canine Prostatic Carcinoma. *Compend Contin Educ Vet*. 2012;34:E1-E5.
22. LeRoy BE, Nadella MVP, Toribio RE, Leav I, Rosol TJ. Canine Prostate Carcinomas Express Markers of Urothelial and Prostatic Differentiation. *Vet Pathol*. 2004;140:131-140.
23. de Brot S, Lothion-Roy J, Grau-Roma L, et al. Histological and immunohistochemical investigation of canine prostate carcinoma with identification of common intraductal carcinoma component. *Vet Comp Oncol*. 2022;20:38-49.
24. Mendez LD, Wolsefer NS, Asa SL, Wasman J, Yoest JM, Stojanov IJ. The diagnostic utility of BRAF VE1 mutation-specific immunohistochemistry in ameloblastoma. *Mod Pathol*. 2022;35:1570-1577.
25. Cheng LY, Haydu LE, Song P, et al. High sensitivity sanger sequencing detection of BRAF mutations in metastatic melanoma FFPE tissue specimens. *Sci Rep*. 2021;11:9043.
26. Singarayer R, Mete O, Perrier L, et al. A systematic review and meta-analysis of the diagnostic performance of BRAF V600E immunohistochemistry in thyroid histopathology. *Endocr Pathol*. 2019;30:201-218.
27. Rasteiro AM, Sá E Lemos E, Oliveira PA, Gil da Costa RM. Molecular markers in urinary bladder cancer: applications for diagnosis, prognosis and therapy. *Vet Sci*. 2022;9:107.
28. Gentilini F, Palgrave CJ, Neta M, et al. Validation of a Liquid Biopsy Protocol for Canine BRAFV595E Variant Detection in Dog Urine and its Evaluation as a Diagnostic Test Complementary to Cytology. *Front Vet Sci*. 2022;9:1-11.
29. Aupperle-Lellbach H, Grassinger J, Hohloch C, Kehl A, Pantke P. Diagnostische Aussagekraft der BRAF-Mutation V595E in Urinproben, Ausstrichen und Bioptaten beim kaninen Übergangszellkarzinom. *Tierärztliche Prax Ausgabe K Kleintiere/Heimtiere*. 2018;46:289-295.
30. Ito T, Tanaka Y, Murata M, Kaku-Ito Y, Furue K, Furue M. BRAF Heterogeneity in Melanoma. *Curr Treat Options Oncol*. 2021;22:20.
31. Meuten DJ, Meuten TLK. *Tumors of the Urinary System in Tumors in Domestic Animals*. 5th ed. John Wiley & Sons Inc; 2017.
32. Palmieri C, Foster RA, Grieco V, et al. ScienceDirect histopathological terminology standards for the reporting of prostatic epithelial lesions in dogs. *J Comp Pathol*. 2019;171:30-37.
33. Schirosi L, Strippoli S, Gaudio F, et al. Is immunohistochemistry of BRAF V600E useful as a screening tool and during progression disease of melanoma patients ? *BMC Cancer*. 2016;16:905.
34. Dolezal JM, Trzcinska A, Liao CY, et al. Deep learning prediction of BRAF-RAS gene expression signature identifies noninvasive follicular thyroid neoplasms with papillary-like nuclear features. *Mod Pathol*. 2021;34:862-874.
35. Broekaert SMC, Roy R, Okamoto I, et al. Genetic and morphologic features for melanoma classification. *Pigment Cell Melanoma Res*. 2010;23:763-770.
36. Yamasaki H, Uematsu Y, Okano K, et al. Establishment and characterization of urothelial carcinoma cell lines with and without BRAF mutation (V595E) in dogs. *In Vitro Cell Dev Biol Anim*. 2022;15:898-911.
37. Sakai K, Maeda S, Saeki K, et al. ErbB2 Copy Number Aberration in Canine Urothelial Carcinoma Detected by a Digital Polymerase Chain Reaction Assay. *Vet Pathol*. 2020;57:56-65.
38. Maeda S, Yoshitake R, Chambers JK, et al. BRAF mutation associates CCL17 expression and regulatory T cell recruitment in urothelial carcinoma of dogs. *Vet Pathol*. 2021;58:971-980.
39. Ny L, Hernberg M, Nyakas M, et al. BRAF mutational status as a prognostic marker for survival in malignant melanoma : a systematic review and meta-analysis. *Acta Oncol*. 2020;59:833-844.
40. Atkins MB, Lee SJ, Chmielowski B, et al. Combination Dabrafenib and Trametinib versus combination Nivolumab and Ipilimumab for patients with advanced BRAF -mutant melanoma: the DREAMseq trial - ECOG-ACRIN EA6134. *J Clin Oncol*. 2023;41:186-197.
41. Bouffet E, Hansford JR, Garrè ML, et al. Dabrafenib plus Trametinib in pediatric glioma with BRAF V600 mutations. *N Engl J Med*. 2023;389:1108-1120.
42. Rossman P, Zabka TS, Ruple A, et al. Phase I/II trial of vemurafenib in dogs with naturally occurring, BRAF-mutated urothelial carcinoma. *Mol Cancer Ther*. 2021;20:2177-2188.
43. Li Q, Chen K, Zhang T, et al. Understanding sorafenib-induced ferroptosis and resistance mechanisms: implications for cancer therapy. *Eur J Pharmacol*. 2023;955:175913.
44. Cronise KE, Hernandez BG, Gustafson DL, Duval DL. Identifying the ErbB / MAPK signaling Cascade as a therapeutic target in canine bladder cancer. *Mol Pharmacol*. 2019;96:36-46.
45. Yancovitz M, Litterman A, Yoon J, et al. Intra- and inter-tumor heterogeneity of BRAF V600Emutations in primary and metastatic melanoma. *PLoS One*. 2012;7:1-8.
46. Wilmott JS, Menzies AM, Haydu LE, et al. BRAF V600E protein expression and outcome from BRAF inhibitor treatment in BRAF V600E metastatic melanoma. *Br J Cancer*. 2013;108:924-931.
47. Manfredi L, Meyer N, Tournier E, et al. Highly concordant results between immunohistochemistry and molecular testing of mutated V600E BRAF in primary and metastatic melanoma. *Acta Derm Venereol*. 2016;96:630-634.
48. Nikiforova MN, Kimura ET, Gandhi M, et al. BRAF mutations in thyroid tumors are restricted to papillary carcinomas and anaplastic or poorly differentiated carcinomas arising from papillary carcinomas. *J Clin Endocrinol Metab*. 2003;88:5399-5404.
49. Sholl LM. A narrative review of BRAF alterations in human tumors: diagnostic and predictive implications. *Precis Cancer Med*. 2020;3:26.
50. Grassinger J, Gmbh L, Kg C. Nachweis der BRAF-mutation bei kaninen Prostataerkrankungen detection of BRAF mutation in canine prostatic diseases Einleitung. *Tierarztl Prax Ausg K Kleintiere Heimtiere*. 2019;47:313-320.

SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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