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BRAF-V600E Protein Expression in Canine Malignant Cutaneous Melanoma, in Accordance with the Introduction of Biomarkers in Comparative Oncology Studies

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ABSTRACT

Background: Melanoma is the cause of death for 1.3% of all cancer patients in humans. The key role of the BRAF protein in the progression of human melanoma has been confirmed, and its prognostic significance has been revealed. Because canine cancer resembles human cancer in biological behavior and molecular abnormalities, BRAF protein may be expressed in canine melanoma, the same as human melanoma. Despite the investigation of the BRAF mutation in canine melanoma, the status of BRAF at the protein level in canine skin melanoma has not yet been examined.
Methods: Thirty-two formalin-fixed, paraffin-embedded tissue samples of canine malignant cutaneous melanoma were randomly selected. After cutting into 3-µm-thick sections, the samples were evaluated for BRAF protein expression by immunohistochemistry and using the anti-BRAF V600E (VE1) mouse monoclonal antibody
Results: The BRAF status was assessed using the Allred scoring system. Among the 32 samples examined, 21 samples were negative, and 11 cases showed high BRAF protein expression.
Conclusion: The detection of positive BRAF expression in 34.3% of canine cutaneous melanoma samples could be a step forward to improving treatment options, using dogs as an animal model in human melanoma clinical trials, and possibly identifying a new prognostic biomarker in canine melanoma.

Keywords: immunohistochemistry, BRAF, canine cutaneous melanoma, protein expression.

INTRODUCTION:

Melanoma is a potentially fatal type of human skin cancer that results from the uncontrolled growth of pigmented cells (melanocytes) in the epidermis and is one of the most lethal skin neoplasms in dogs [1, 2]. There are four types of melanoma in dogs: Oral melanoma, which is the most common malignant tumor of the oral cavity in dogs, has a prevalence rate of 62%; cutaneous melanoma, digital melanoma, and ocular melanoma have prevalence rates of 27, 10, and 1, respectively [3]. Canine cutaneous melanoma commonly manifests as small brown to black masses; further, they can indicate large, flat, and wrinkled masses [4]. Generally, melanomas arising from hair-covered skin in dogs are usually benign, but a rare type of cutaneous melanoma that could behave aggressively also occurs in dogs [3]. Surgical excision is the most effective treatment option for benign cutaneous melanoma, However, there is a lack of definitive treatments that have been suggested for canine malignant melanoma [4].

BRAF is a part of the mitogen-activated protein kinase (MAPK) pathway [5]. Since 2002, when BRAF mutations in melanoma and other types of human cancer were discovered, BRAF mutations have been reported in 6-8% of all solid human tumors [6, 7]. Mutation of the BRAF gene is a frequent event in human melanoma and is found in approximately half of all human melanoma cases [8, 9]. The prevalent mutation (higher than 90%) in human BRAF is a transversion of thymine-to-adenine in exon 15 at nucleotide 1799, which resulted in the alteration from valine to glutamic at codon 600 and is shown as BRAF V600 [10]. The BRAF V600E mutation could lead to increased or uncontrolled cell proliferation and resistance to apoptosis in melanoma cells. Although mutations of RAS and BRAF appear to be rare in canine melanoma, due to the fact that human and canine cancers have many characteristics in common, the discovery of BRAF mutations in canine tumors, including melanoma, highlights the significance of MAPK as an oncogenic signaling pathway and its involvement in canine melanoma pathogenesis [11, 12].

In spite of the studies on BRAF mutations in canine cancers, thus far there has been no report on the status of BRAF at the protein level in canine cutaneous melanoma. Considering that many types of canine cancers, including melanoma, have common characteristics with human cancers and the genes and molecular pathways involved in the development of human cancers are similar to those of canine cancers, it is hypothesized that the BRAF protein may be expressed in canine malignant cutaneous melanoma and, like BRAF in human melanoma, have a key role [13].

Material and Methods:

Thirty-two formalin-fixed paraffin-embedded blocks previously diagnosed as canine cutaneous malignant melanoma were reviewed again by a pathologist and confirmed. Clinical data, such as their age, gender, and anatomical location of tumors, were obtained from the files of the animals. For immunohistochemistry (IHC) staining, 3-μm-thick sections were cut. The slides were kept overnight at room temperature to dry. The sections were deparaffinized with xylene and hydrated with a descending series of ethanol concentrations. Antigen retrieval was performed by the heat method, and the activity of endogenous peroxidases was blocked using hydrogen peroxide (H2O2). After incubation with the primary antibody (anti-BRAF V600E (VE1) at a dilution of 1:250, the slides were incubated with the secondary antibody. For immunohistochemical detection, diaminobenzidine (DAB) staining was performed. Hematoxylin was used for background staining. After the sections were washed with water, an ascending series of ethanol concentrations was used for dehydration. After being cleared with xylene, the slides were mounted. Phosphate-buffered saline (PBS) was added in the negative control section instead of the primary antibody. According to the manufacturer's advice, canine colorectal cancer samples were used as a positive control, which showed positive expression of BRAF after the primary antibody was added, which was a sign of the antibody's correct function.

BRAF expression was assessed by the Allred scoring system. The Allred score (A-Score) is calculated based on

Table 1. Clinicopathological data of 32 canine cutaneous malignant melanoma

Variable	No.	Frequency (%)
Age (years)		
<6	11	34.3
6-10	19	59.3
>10	2	6.2
Tumor size (cm)		
<1	2	6.2
1-3	21	65.6
>3	9	28.1
Laterality		
Head & neck	5	15.6
Trunk	7	21.8
Hand & foot	20	62.5
Histologic invasion status		
In situ	10	31.2
Invasion	22	68.7
Allred score		
0-1	14	43.7
2-3	1	3.1
4-6	11	34.3
7-8	6	18.7
BRAF status		
Negative (Allred score 0–5)	21	65.6
Positive (Allred score 6-8)	11	34.3

both the proportion score (PS) and the intensity score (IS). The proportion score was the percentage ratio of positive BRAF-stained tumor cells to the total number of cells, classified as PS0 (0%), PS1 (>0–1%), PS2 (≥1–10%), PS3 (>10–33%), PS4 (>33–66%), and PS5 (>66–100%). The intensity score measured staining intensity by visual assessment and was scored as 0 (negative), 1 (weak), 2 (moderate), or 3 (strong). The total score (TS) was calculated as the sum of the PS and IS and ranged from 0 to 8. TS ≤ 5 was considered negative, and TS ≥ 6 was considered to have positive staining for BRAF V600E [14].

Results:

There were 18 males and 14 females among the 32 malignant cutaneous melanoma specimens. The age of the dogs ranged between 3 and 12 years old (median, 6). BRAF expression was assessed by IHC and evaluated by the Allred scoring system. Negative BRAF expression (Allred score 0–5) was identified in 21/32 (65.6%) specimens, and 11/32 (34.3%) cases showed positive cytoplasmic BRAF protein expression (Allred score 6-8) (Figure -1).

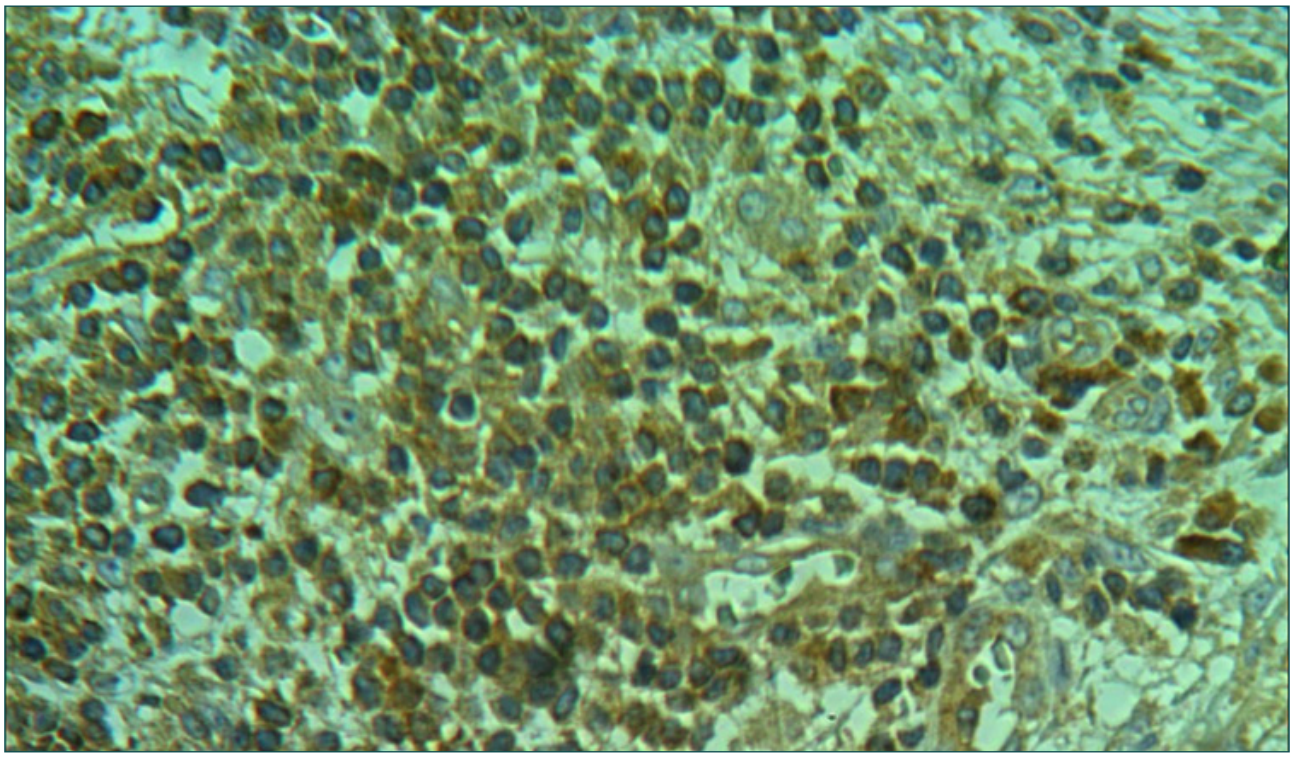

Figure 1. High BRAF protein expression in canine cutaneous malignant melanoma (400x).

Discussion:

Even with significant advances in treatment options, malignant melanoma remains a lethal skin cancer in humans and dogs with a poor prognosis [2, 15]. Oncogenic BRAF mutations occur in ~ 60% of melanoma patients, and the V600E mutation accounts for roughly 90% of these mutations [13]. Regardless of some limitations due to drug resistance, BRAF protein inhibitors such as Vemurafenib have led to advances in treating melanoma patients with BRAF V600E mutations [16, 17]. In addition to its importance in therapeutic aspects, in a study performed by Lars A. Akslen et al. in 2016, after examining the relationship between BRAF protein expression and clinicopathological variables such as tumor thickness, mitotic count, and ulceration, BRAF protein was found as a prognostic marker associated with poor survival in human melanoma [18]. Since dogs are the appropriate animal model for studying melanoma, and BRAF is so essential in human melanoma, investigating BRAF status at the protein level in canine cutaneous melanoma may be useful in some ways for both humans and dogs [19].

To our knowledge, this is the first study reporting the expression of BRAF protein in canine malignant cutaneous melanoma. The investigation conducted by Mochizuki H et al. in 2015 indicated that after sequencing the BRAF gene in 54 canine melanoma cases (47 oral, 6 cutaneous, and 1 ocular), a transversion of T to A at nucleotide 1349 was detected, resulting in the amino acid switching from valine to glutamic acid at codon 450 (shown as V450E). The BRAF V450E mutation was identified in only 6% of cases (one cutaneous and two mucosal melanoma) and considered to correspond to the human BRAF V600E mutation [6]. In the study mentioned, it was noted that the BRAF mutation is associated with skin exposure to ultraviolet, and the reason for the low frequency of BRAF mutations in dogs is that the skin of dogs is covered with hair, and the hair coat protects them from UV exposure [20, 21]. Although it was thought that even if the BRAF protein is expressed in canine cutaneous melanoma, it would express in a small number of samples, in our study the BRAF V600E-mutated protein was identified in 18/32 (56.2%) cases, which is a notable percentage. Since direct sequencing of tumor DNA is the gold standard method for detecting BRAF mutations, further studies for a detailed examination of BRAF mutations in canine malignant cutaneous melanoma are recommended [22].

The BRAF V600E protein expression level in 370 melanocytic lesions (232 primary melanoma, 138 metastatic melanoma, 25 naevocytic naevi, and 24 dysplastic naevi) was investigated by G. Safaee Ardekani et al. in 2013. BRAF protein expression was significantly higher in primary melanoma compared to dysplastic naevi, and BRAF expression was even higher in metastatic melanoma compared to primary tumors. High BRAF protein expression was linked to tumor ulceration, increased tumor thickness, and higher American Joint Committee on Cancer (AJCC) stages. This study's findings demonstrated that BRAF protein plays an essential role in melanoma progression, and a prognostic value for BRAF protein was considered [9].

Despite the indisputable significance of murine models in cancer research, they have revealed a couple of limits: genetically engineered mouse models are costly and lack the same genetic background and mutagenic load as human cancers; patient-derived xenograft mouse models have compromised immune systems, so they do not accurately replicate the behavior of naturally occurring cancers in humans [23, 24]. It is also acknowledged that mice were not as efficient as expected in some aspects of drug research. Mice's bone marrow is generally less sensitive to many cytotoxic agents induced by chemotherapy than human bone marrow and can tolerate higher drug concentrations than human patients. Therefore, mice are unsuitable for use in the assessment of the adverse effects of novel chemotherapies or combinational approaches with chemotherapeutic agents [25]. Another limitation of conventional laboratory models, such as mice, is that sometimes, even when the histology of the tumors is the same, response to treatments and tumor development observed in mouse models are not predictive of what occurs in humans [23]. Furthermore, these models do not adequately represent

some essential features that define human cancer, including genomic instability, growth over long periods of time, the function of the immune system, and the noteworthy heterogeneity in cells of the tumor, the tumor microenvironment, and the stroma [26]. In light of the drawbacks mentioned above and the point that successful translation from rodent models to clinical cancer trials has a rate of less than 8%, researchers are becoming more interested in spontaneously occurring cancers in pet animals, particularly dogs [24, 27]. The processes of tumor initiation and progression are impacted by the same factors in dogs and humans, including nutrition, sex, age, and environment [25]. Canine cancers show the same pattern of cancer development and have clinical and histological appearance, biological behavior, tumor genetics, molecular pathways and targets, and responses to traditional regimens, such as chemotherapy, radiation therapy, and surgery, similar to those occurring in humans [24]. Additional features that contribute to the advantages of using the dog as an animal model are a more rapid progression of the disease and a shorter overall lifespan, characteristics that are difficult to replicate in other animal models [26, 27].

Compared to mice, the genome sequence of dogs is more similar to the human genome, and all 19,000 dog-identified genes are orthologous, or at least akin to human genes. As a result of that, there is more sequence similarity between the human and canine proteins than between the human and mouse for many cancer-associated proteins [23, 25]. At the protein level, antibodies utilized in humans for IHC also frequently work in dogs, while mice often need an antibody specific for the mouse protein, further highlighting the similarity between human and dog genes [28 -30]. Considering the superiority of the dog as an animal model over traditional laboratory models in some key aspects of the fields of cancer research and drug development, it appears that dog tumors represent the main features of human cancer better than any other model, and conducting more research on dogs with a focus on comparative oncology and translational research can benefit both species and bridge the gap between in vitro and in vivo studies [23, 25].

Conclusion:

IIn this study, we investigated the BRAF status at the protein level in malignant cutaneous melanoma in dogs. The IHC results revealed that 21/32 (65.6%) of tumor tissues showed negative protein expression and 11/32 (34.3%) were positive for BRAF-V600E. Identification of BRAF V600E-mutated Protein in more than half of the canine malignant cutaneous melanoma samples could lead to the development of targeted therapy options in canine melanoma, using dogs as an animal model for studying human melanoma. Of course, after examining the relationship between BRAF protein expression and clinicopathologic features in future studies, it may be possible to consider a prognostic value for BRAF in canine melanoma as well as in humans.

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