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Activation of the Canonical Wnt/ β -Catenin Signalling Pathway is Rare in Canine Malignant Melanoma Tissue and Cell Lines

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Summary

Canine malignant melanoma is a highly aggressive tumour associated with a poor overall survival rate due to both local disease recurrence and its highly metastatic nature. Similar to advanced melanoma in man, canine oral melanoma is poorly responsive to conventional anti-cancer therapies. The lack of sustainable disease control warrants investigation of novel therapies, preferably targeting features specific to the tumour and different from normal cells. The Wnt signalling pathway is known to contribute to melanocytic lineage development in vertebrates and perturbation of the Wnt/ β -catenin pathway has been implicated in numerous cancer types. Alterations of the Wnt/ β -catenin pathway are suggested to occur in a subset of human melanomas, although the precise role of the Wnt/ β -catenin pathway in melanoma is yet to be defined. This study investigates the activation status of the canonical Wnt/ β -catenin pathway in canine malignant melanoma and its potential as a therapeutic target for treating this disease. The data indicate canonical Wnt/ β -catenin pathway activation is a rare event in canine oral malignant melanoma tissue and canine malignant melanoma cell lines.

Keywords

dog; malignant melanoma; Wnt/ β -catenin pathway

Introduction

Melanoma accounts for approximately 4% of all canine malignant tumours and affects the oral cavity, lip, skin, digit and eye most frequently (MacEwen *et al.*, 1986; Ramos-Vara *et al.*, 2000). Melanoma is the most common oral tumour in dogs and is an aggressive malignancy, characterized by local invasiveness and high metastatic potential (Todoroff and Brodey, 1979). Prognostic factors reported for canine oral melanomas include tumour size, location within the oral cavity (cranial versus caudal), presence of bone lysis and stage (Harvey *et al.*, 1981; MacEwen *et al.*, 1986, 1999; Proulx *et al.*, 2003). Despite advances in aggressive, multimodal therapies aimed at local and systemic disease, few affected dogs survive beyond 1 year and most succumb to recurrence of local disease, metastasis, or both

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Conflict of Interest Statement

The authors have no potential conflicts of interest to disclose.

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(Rassnick *et al.*, 2001; Freeman *et al.*, 2003; Proulx *et al.*, 2003; Boria *et al.*, 2004; Murphy *et al.*, 2005).

Canine patients are treated with aggressive surgery and/or radiation therapy, combined with systemic therapy including chemotherapy, immunotherapy, or both. Unfortunately, very few chemotherapeutic agents lead to durable remissions (Rassnick *et al.*, 2001; Alexander *et al.*, 2006). Immunotherapy recently became an appealing therapeutic approach as it utilizes a unique antigen in the hope of generating an immune response against a very specific cell type. Thus far, clinical trials evaluating tumour vaccines, such as xenogeneic human tyrosinase and allogeneic whole cell tumour vaccine expressing xenogeneic glycoprotein 100, have shown promise in the treatment of oral melanoma in dogs, although durable remissions are still lacking (Bergman *et al.*, 2003; Alexander *et al.*, 2006). It is obvious that improved therapeutic options are needed to improve disease outcome. Currently, there is a great deal of interest in developing targeted therapies that act upon altered signalling pathways in cancer cells. While targeted therapies aim to improve therapeutic efficacy, these targets must first be defined by understanding of which pathways are instrumental to melanoma development and progression. The Wnt signalling pathway may be one such target.

The Wnt signalling pathway may be split into a highly conserved ‘canonical’ pathway as well as a ‘non-canonical’ signalling pathway, which are regulated by secreted glycoproteins known as Wnts (O’Connell and Weeraratna, 2009). In the absence of canonical Wnt signalling, cytosolic β -catenin is rapidly phosphorylated by a complex of proteins, collectively termed the ‘degradation complex’, consisting of glycogen synthase kinase-3 β (GSK-3 β), adenomatous polyposis coli (APC) tumour suppressor protein and axin. The destruction complex phosphorylates serine and threonine residues in the amino-terminus of β -catenin, thereby targeting β -catenin for ubiquitination and proteosomal destruction and maintaining low baseline amounts of cytosolic β -catenin (Rubinfeld *et al.*, 1993,1996; Chien *et al.*, 2009a, b; Lucero *et al.*, 2010; White *et al.*, 2012). The activation of the canonical Wnt/ β -catenin signalling pathway involves the binding of a canonical Wnt ligand to the receptors ‘frizzled’ (FZD) and low-density lipoprotein receptor-related protein 5/6 (LRP5/6), thereby inhibiting β -catenin phosphorylation, which allows β -catenin to escape degradation and accumulate in the cytoplasm (Chien *et al.*, 2009a, b; White *et al.*, 2012). Stabilized β -catenin translocates into the nucleus, where it interacts with members of the T-cell factor/lymphoid enhancer factor (TCF/LEF) family of DNA binding proteins to promote the transcription of Wnt-responsive genes (Behrens *et al.*, 1996). Hence, the presence of cytoplasmic or nuclear β -catenin would not be expected without activation of the canonical Wnt/ β -catenin pathway. In contrast the non-canonical Wnt pathway functions independently of β -catenin and in some contexts may serve to antagonize β -catenin signalling (Nemeth *et al.*, 2007).

The activation of the canonical Wnt/ β -catenin signalling pathway is necessary for normal development and differentiation of a number of cell types, including the melanocyte (Takeda *et al.*, 2000; Dunn *et al.*, 2005). In addition, the aberrant activation of the canonical Wnt/ β -catenin signalling pathway is noted to occur in a number of human cancers including colorectal, breast and hepatocellular carcinoma (Hajra and Fearon, 2002). Given, this pathway’s known involvement with melanocyte development, together with its involvement in other cancers, the canonical Wnt/ β -catenin signalling pathway may be relevant to the development and/or progression of canine malignant melanoma. In human melanoma, the role of the Wnt/ β -catenin signalling pathway remains undefined, as demonstrated by conflicting reports using several in-vitro and in-vivo experimental models. Initial studies indicated that approximately 30% of malignant melanoma metastases displayed cytoplasmic/nuclear β -catenin, suggesting that the pathway is active in a subset of aggressive melanomas and may play an oncogenic role (Rimm *et al.*, 1999; Giles *et al.*,

2003). Further, the viability of melanoma cell lines harbouring Wnt/ β -catenin alterations is reduced by inhibition of the Wnt/ β -catenin pathway (Tarapore *et al.*, 2010). However, other reports argue that the activation of the Wnt/ β -catenin signalling pathway acts to reduce the aggressiveness of melanomas, improve survival, and may be of therapeutic benefit in some contexts (Weeraratna, 2005; Chin *et al.*, 2006; Chien *et al.*, 2009a, b; Arozarena *et al.*, 2011). It has been postulated that activation of the canonical Wnt/ β -catenin signalling pathway is necessary for development of melanoma, but for progression to more aggressive phenotypes to occur the canonical pathway must be silenced (O'Connell and Weeraratna, 2009).

A recent report indicated that β -catenin expression is increased in canine cutaneous melanoma compared with normal canine melanocytes (Han *et al.*, 2010). However, canine cutaneous melanoma often follows a more benign course of disease and does not recapitulate the aggressive nature of human cutaneous melanoma in the manner of canine oral malignant melanoma. The aim of the present study was to characterize the status of canonical Wnt signalling activity in canine malignant melanoma in order to (1) assess whether the canonical Wnt signalling pathway could serve as therapeutic target for this tumour and (2) to determine whether canine malignant melanoma could serve as a relevant model for further exploration of the Wnt signalling pathway in human melanoma.

Materials and Methods

Sample Selection

Archived tissue blocks of canine oral malignant melanoma from the Pathology Service at the University of Wisconsin-Madison Veterinary Medical Teaching Hospital (UWVMTH) were identified by medical records search. Canine oral malignant melanoma tissue was collected at the time of diagnostic biopsy, surgical removal or necropsy examination. Haematoxylin and eosin (HE) stained and unstained sections (5 μ m) slices were prepared from the paraffin wax-embedded tissues. Normal canine skin was collected from a dog with no evidence of melanoma presenting to the UWVMTH Oncology Service for a separate procedure following client consent.

Canine Melanoma Cell Lines

Nine canine melanoma cell lines were used for this study. The cell lines UCDK9M1, UCDK9M2, UCDK9M3m, UCDK9M4 and UCDK9M5 were provided by Dr. M. Kent of the University of California at Davis. The cell lines CML-1, CML-10C2 and CML-6M were provided by Dr. L. Wolfe of Auburn University. The cell line 17CM98 was generated by Dr. G. Hogge at the University of Wisconsin-Madison. Of the nine canine melanoma cell lines used in this study, three were generated from canine primary oral malignant melanoma (UCDK9M3, UCDK9M4 and CML-1), three were from lymph node metastases of canine oral malignant melanoma (17CM98, UCDK9M2 and UCDK9M5), one was from a metastatic skin lesion of canine oral melanoma (UCDK9M1), one was from a canine cutaneous malignant melanoma (CML-10c2) and one was from a metastatic lymph node lesion of a primary cutaneous malignant melanoma (CML-6M) (Wolfe *et al.*, 1987; Alexander *et al.*, 2006; Aina *et al.*, 2011). To the authors' knowledge, there is no relationship between any of the primary and metastatic cell lines. One human melanoma cell line (Mel888) known to harbour an activating point mutation in β -catenin was provided by Dr. M. Albertini at the University of Wisconsin-Madison and utilized as a positive control for western blotting analysis and TOP/FOPflash assays (Rubinfeld *et al.*, 1997). All cell lines were maintained in modified Eagle's medium-alpha (MEM α) supplemented with 10% fetal bovine serum (FBS) and antibiotics at 37°C in 5% CO₂.

Detection of β -Catenin by Immunohistochemistry

Immunohistochemistry (IHC) was performed on unstained sections of canine oral malignant melanoma. Slides were dewaxed in CitriSolv[®] (Fisher Scientific, Hampton, New Hampshire, USA), rehydrated through an ethanol series and rinsed in type II H₂O. Endogenous peroxidase activity was blocked with H₂O₂ 0.5% in methanol. Slides were rinsed in tap water for 5 min and heat treated in citrate buffer (0.1M) for antigen retrieval. Tissue sections were soaked in a solution of dried milk in phosphate buffered saline (PBS) (0.5g/100ml) for 5 min. To block non-specific binding, normal horse serum from the mouse IgG Vector Vectastain Elite ABC Kit (Vector Laboratories, Burlingame, California, USA) was mixed with PBS/milk solution and applied to each slide. Excess blocking solution was blotted from the slides and the mouse anti- β -catenin primary antibody (BD Transduction Laboratories, BD Biosciences, San Jose, California, USA), diluted 1 in 100 in PBS/milk solution, was applied to each slide. This antibody has been previously used to detect β -catenin expression in canine tissue (Stein *et al.*, 2011). Slides serving as negative controls were treated with the PBS/milk solution in which the primary antibody was omitted. All slides were rinsed in PBS, treated with a Vectastain Elite biotinylated anti-mouse antibody followed by Vectastain Elite ABC Reagent, rinsed in PBS and treated with 3, 3'-diaminobenzidine (DAB) substrate solution (Vector Laboratories). Slides were rinsed in PBS and counterstained with Mayer's haematoxylin, dehydrated through an ethanol series, soaked in CitriSolv and coverslipped.

Detection of β -catenin in Malignant Melanoma Cell Lines by Western Blotting

Lysate from the Mel888 cell line served as a positive control as this human melanoma cell line has a known activating mutation in the serine 37 residue of β -catenin causing its stabilization. Briefly, cells were lysed using a mammalian protein extraction reagent (MPER; Pierce, Rockford, Illinois, USA) and protein lysates collected. Proteins from cell lysates were separated on a 7.5% sodium dodecyl sulphate polyacrylamide gel at 150V for 1.5 h. Proteins were transferred onto a nitrocellulose membrane at 100V for 1 h and blocked with tris-buffered saline (TBS) containing 5% non-fat dry milk and 1% bovine serum albumin for 1 h. The membranes were incubated overnight with mouse anti- β -catenin antibody (as used for IHC) diluted 1 in 2,000 in blocking solution. Primary antibody was removed by washing in TBS/0.05% Tween-20 (TBST) three times for 5 min. Membranes were exposed to a horseradish peroxidase-conjugated anti-mouse IgG secondary antibody (for 1 h at room temperature), washed in TBST three times for 5 min and treated with a chemiluminescence substrate (Pierce). Blots were visualized after exposure to film and analyzed using a Gel Logic 100 Imaging System (Kodak, Rochester, New York, USA). The public domain Java image process program, ImageJ, was used for quantification of blots and comparison of relative protein amounts between cell lines.

In-Vitro Assessment of Canonical Wnt Signalling Activity in Canine Malignant Melanoma

Luciferase Reporter Assay for Wnt/ β -Catenin Activity—Cell lines were distributed in a six-well plate at a density that would achieve 40–50% confluence within 24 h. Cells were then transfected transiently using Lipofectamine LTX and PLUS reagents (Invitrogen, Carlsbad, California, USA), according to the manufacturer's protocol, to introduce either 2.5 μ g TOPflash or 2.5 μ g FOPflash reporter plasmid, together with 0.5 μ g of TK-*Renilla* luminescent reporter plasmid (TCF Reporter Plasmid Kit; Millipore, Temecula, California, USA). The TOPflash luciferase reporter plasmid contains TCF4 binding sites upstream of the luciferase gene, resulting in luciferase activity in the presence of active Wnt/ β -catenin signalling, while the FOPflash reporter plasmid contains mutated TCF4 binding sites. The TK-*Renilla* plasmid serves as a control for transfection efficiency. Twenty-four hours after transfection, cells were harvested and luciferase and *Renilla* luminescence were measured

using the Dual-Luciferase Reporter Assay System (Promega, Madison, Wisconsin, USA) on a luminometer (BioTek Synergy HT Multimode Microplate Reader and Gen5 software; BioTek Instruments, Winooski, Vermont, USA). The relative luciferase units for each transfection were adjusted by *Renilla* activity in the same sample and each corrected TOPflash luciferase value was normalized to the corresponding corrected FOPflash value. Three independent transfections were performed, with each sample assayed in triplicate.

Relative Expression of β -Catenin Target Genes in Canine Malignant Melanoma

Canonical Wnt signalling pathway activity in canine malignant melanoma cell lines was determined by assessing downstream target gene expression by quantitative polymerase chain reaction (qPCR). Total RNA was isolated from cell lines using Trizol (Invitrogen) and purified by PureLink RNA Mini Kit (Ambion, Life Technologies, Carlsbad, California, USA) according to the manufacturer's instructions. cDNA was synthesized from 250ng of total RNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Life Technologies, Carlsbad, California, USA) according to the manufacturer's protocol. qPCR was performed using TaqMan Gene Expression Master Mix with TaqMan Gene Expression Assays (Applied Biosystems) according to the manufacturer's protocol on a Bio-Rad iCycler machine with a Bio-Rad iQ5 Multicolor Real-Time PCR Detection System. Assays include: canine Axin2 (Cf02631333_m1), canine Mitf-M (Cf02690540_m1) and canine Lef (Cf02686727_m1) (Applied Biosystems). Ct values were normalized to 18S expression (4352930E). Normal canine skin was used to determine relative expression of mRNA targets in canine melanoma cell lines using the $\delta\delta$ Ct method (Yuan *et al.*, 2008). Gene expression of samples was measured in triplicate.

Immunocytochemistry for β -Catenin Expression in Malignant Melanoma

Cells were collected using trypsin-EDTA, washed in PBS and suspended in PBS. Cells (5×10^4) were adhered to charged slides (Fisher Scientific, Pittsburgh, Pennsylvania, USA) by cytocentrifugation (Shandon Cytospin 2) at 500 rpm for 5 min. Slides were fixed in methanol/acetone, placed in slide boxes wrapped in foil and stored at -20°C until needed for immunocytochemistry (ICC). Prior to performing ICC, slides were allowed to warm to room temperature. Cells were washed in TBST for 5 min, the periphery of the slides were marked with PAP pen (Scientific Device Laboratory, Des Plaines, Illinois, USA) and endogenous peroxidase activity was quenched by incubating in H_2O_2 0.3% in methanol for 10 min. To block non-specific binding, normal horse serum from the mouse IgG Vector Vectastain Elite ABC Kit was mixed with PBS/milk solution and applied to each slide. Excess blocking solution was blotted from the slides and the mouse anti- β -catenin primary antibody (as used for IHC; diluted 1 in 100 in PBS/milk) was applied to each slide. Slides serving as negative controls were treated with the PBS/milk solution in which the primary antibody was omitted. All slides were rinsed in PBS, treated with a Vectastain Elite Biotinylated Anti-Mouse Antibody followed by Vectastain Elite ABC Reagent, rinsed in PBS and treated with DAB substrate solution. Slides were rinsed in PBS, counterstained with Mayer's haematoxylin and coverslipped.

Results

Determination of β -Catenin Expression in Clinical tumour Samples

Twelve archived canine oral melanomas were used to determine the expression pattern of β -catenin by IHC. Of the 12 samples, nine expressed β -catenin and three did not label for β -catenin expression. Of the positively labelled tumors, four had solely cytoplasmic expression, four had both cytoplasmic and membranous labelling and one had both cytoplasmic and nuclear labelling (Fig. 1).

Evaluation of Canonical Wnt Signalling Activity in Canine Malignant Melanoma Cell Lines

To evaluate the expression of β -catenin protein, a hallmark of canonical Wnt signalling activity, western blotting was performed with nine canine malignant melanoma cell lines. β -catenin was detected in all cell lines evaluated, although the amount of β -catenin detected in all of the canine melanoma cell lines was less than that detected in the Mel888 cell line, which is known to harbour mutated β -catenin resulting in its stabilization (Fig. 2).

To evaluate canonical Wnt signalling pathway activity, the combination of a luciferase reporter assay and qPCR of downstream targets were performed on all canine melanoma cell lines. For the TOP/FOPflash assays, a ratio >1 indicates that there is canonical Wnt signalling activity above a background level. As expected, the Mel888 cell line displayed a very high TOP/FOPflash ratio, indicating an active canonical Wnt pathway consistent with the stabilizing point mutation within β -catenin. Surprisingly, none of the canine melanoma cell lines displayed TOPflash activity significantly above background FOPflash activity (Fig. 3). Of all the canine melanoma cell lines, the UCDK9M5 cell line had the highest average ratio at 1.9, while the 17CM98 cell line had the lowest at 0.8. These findings suggest the canonical Wnt signalling pathway is not active in canine malignant melanoma cell lines.

To validate the results of the TOP/FOPflash assays, the expression of target genes downstream of β -catenin was assessed using qPCR on all canine melanoma cell lines. The mRNA expression levels in canine melanoma cell lines were compared with those in normal canine skin. The target genes assessed were Axin2, Lef and Mitf (Takeda *et al.*, 2000; Hovanes *et al.*, 2001; Yan *et al.*, 2001; Jho *et al.*, 2002; Barolo, 2006). The relative expression of both Axin2 and LEF were repressed in most canine melanoma cell lines compared with normal canine skin. LEF expression was not detected in the UCDK9M2, UCDK9M3 or UCDK9M4 cell lines. The low relative expression levels of Axin2 and LEF would suggest that the canonical signalling pathway is not active in canine malignant melanoma cell lines. Conversely, the relative expression of MITF was increased in all canine melanoma cell lines compared with normal skin.

Finally, given the apparent discrepancy between the results of western blotting, which indicated β -catenin expression by all melanoma cell lines, and the assays measuring canonical pathway activity, which indicated little or no canonical pathway activation, the subcellular location of β -catenin in the cell lines was investigated. ICC was performed in order to determine the localization of β -catenin protein expression in canine malignant melanoma cell lines. The positive control Mel888 cell line displayed intense labelling of the nucleus, cytoplasm and membrane (Fig. 5). All canine malignant melanoma cell lines displayed intense membranous labelling. However, in contrast to the Mel888 cell line, there was no evidence of nuclear β -catenin labelling in any of the canine melanoma cell lines (Fig. 5). These findings are consistent with β -catenin being present within melanoma cells, but not actively participating in canonical Wnt signalling in canine malignant melanoma.

Discussion

Canine malignant melanoma is a highly aggressive disease in need of improved therapies. Targeted therapies are likely to play a role in the treatment of this disease in the future. Ideally, targeted therapies are based on a thorough understanding of altered molecules or pathways known to contribute to the development or progression of the cancer in question. We were interested in determining the status of the canonical Wnt signalling pathway in canine malignant melanoma, especially in the oral form and its metastatic derivatives. Our aims were twofold; firstly to determine whether the canonical Wnt signalling pathway is a potential therapeutic target and secondly to determine if canine malignant melanoma is a

relevant comparative model for the study of Wnt signalling abnormalities in human melanoma.

Using ICC, nuclear β -catenin was present in < 10% of the tumour tissue samples assessed. The percentage of canine oral malignant melanomas labelling for the presence of nuclear β -catenin in this study is similar to studies of human melanoma, in which 6–30% are positive for expression of nuclear β -catenin (Bachmann *et al.*, 2005; Larue and Delmas, 2006). In studies of human melanoma, the presence of nuclear β -catenin has been associated with a less invasive phenotype and improved patient survival (Bachmann *et al.*, 2005; Chien *et al.*, 2009a, b). Unfortunately, the small sample size and multitude of treatments received by the dogs in the present study precluded the ability to determine if a similar relationship existed for canine oral malignant melanoma. When evaluating canine cutaneous melanocytic tumors, Han *et al.* (2010) found nuclear β -catenin to be infrequent as well. In that study only two cutaneous melanomas were classified as malignant and both of these samples had < 1% of their nuclei labelled. In contrast, the majority of cases were considered benign melanocytomas and there was no nuclear labelling for β -catenin (Han *et al.*, 2010).

Interestingly, approximately 30% of the canine oral malignant melanoma samples evaluated herein expressed cytoplasmic β -catenin. Han *et al.* found > 90% of the cells in cutaneous melanocytomas and cutaneous malignant melanomas to express cytoplasmic β -catenin (Han *et al.*, 2010). Historically, the presence of cytoplasmic β -catenin has been taken to mean the canonical Wnt signalling pathway is active, otherwise β -catenin should be in a membrane complex with E-cadherin or absent due to its rapid ubiquitin-mediated proteosomal degradation. Recently, it was reported that different amino acid residues within β -catenin may be phosphorylated, resulting in its cytoplasmic localization, but failure to translocate into the nucleus to upregulate the target genes of the canonical Wnt signalling pathway (Kuphal and Bosserhoff, 2011). While the significance of this stable, yet transcriptionally-inactive form of β -catenin remains under study, the IHC results suggest that investigations of canine oral malignant melanoma may contribute to better understanding the function of this form of β -catenin. Work is currently underway to determine the phosphorylation status of β -catenin in these canine samples to further validate this model.

In-vitro assays were used to address the question of whether the canonical Wnt pathway is active in canine malignant melanoma. While the western blotting results indicate that β -catenin protein is expressed in canine malignant melanoma cell lines, it is present in much lower amounts relative to a human melanoma cell line known to harbour an activating β -catenin mutation. However, the western blots provided no true indication as to whether β -catenin is active in the canonical Wnt signalling pathway. The findings of minimal TOP/FOPflash activity in any of the canine melanoma cell lines suggest that the canonical Wnt signalling pathway is not active in these lines. Similarly, the low relative expression of Axin2 and LEF mRNA support the argument that the canonical Wnt signalling pathway is not active in canine malignant melanoma. The increased MITF expression relative to normal skin may be more indicative of the lack of pigmentation in that sample of skin, as opposed to an active canonical Wnt signalling pathway.

Because β -catenin was present in all cell lines evaluated by western blotting, but canonical Wnt signalling activity was lacking based on low TOP/FOPflash ratios and target gene expressions, ICC was used to localize β -catenin. All cell lines were found to have strong membranous labelling for β -catenin; however, no nuclear β -catenin was detected in any of the cell lines. These findings indicate that the β -catenin detected by western blotting is from the membranous portion of the cell and is not actively participating in the canonical Wnt signalling pathway, consistent with the TOP/FOPflash assays and qPCR results.

Similar to the highly invasive melanoma in man, these findings indicate that canine oral malignant melanoma does not have an active canonical Wnt signalling pathway. While β -catenin was detected in all cell lines, it was most likely from the membranous portion of cells. These results, in conjunction with those of Han *et al.* (2010), suggest that the targeted suppression of active β -catenin is not likely to be a relevant therapeutic target in canine malignant melanoma. Conversely, it is possible that the activation of canonical Wnt/ β -catenin may hold therapeutic potential for canine malignant melanoma. While additional studies are necessary to validate this in the dog, studies utilizing human melanoma cell lines and tissue have found the activation of this pathway to be associated with less aggressive behaviour (Chien *et al.*, 2009a, b; Arozarena *et al.*, 2011). Finally, the current findings suggest that the highly aggressive canine malignant melanoma is comparable to the more invasive forms of human melanoma, which lack canonical Wnt signalling activity, and may serve as a valuable model for the human disease in order to address questions regarding the canonical Wnt signalling pathway in melanoma. However, additional work is necessary to determine the relevance of the non-canonical Wnt pathway in canine malignant melanoma, which seems to be important in the progression of human melanoma (O'Connell and Weeraratna, 2009).

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Fig. 1. β -catenin in canine oral malignant melanoma. Representative images of canine oral malignant melanoma labelled for presence of β -catenin displaying (A) predominantly cytoplasmic labelling, (B) membranous and cytoplasmic labelling, (C) nuclear labelling, and (D) negative control. IHC.

Fig. 2.

Western blot analysis for presence of β -catenin in canine melanoma cell lines. Mel888, a human melanoma cell line containing a stabilizing point mutation in β -catenin, was used as a positive control. Quantification of blots performed using ImageJ and numerical values represent the quantity of β -catenin in each cell line relative to Mel888.

Fig. 3. Luciferase reporter assay to assess Wnt/ β -catenin pathway activity in melanoma cell lines. TOPflash and FOPflash values are corrected to respective *Renilla* luminescence as a transfection control. Mel888, a human melanoma cell line containing a stabilizing point mutation in β -catenin, was used as a positive control.

Fig. 4. Relative expression of β -catenin target genes in canine malignant melanoma cell lines compared with normal canine skin. Relative expression of (A) Axin2 mRNA, (B) LEF mRNA, and (C) MITF mRNA.

Fig. 5.

β -catenin in melanoma cell lines. (A) Mel888 with primary antibody omitted (negative control). (B) Mel888 with positive cytoplasmic/nuclear labelling. (C) CML-1 with primary antibody omitted (negative control). (D) CML-1 with membranous labelling. Results of CML-1 IHC are representative of all canine melanoma cell lines tested. ICC.