c-Kit immunoexpression patterns differ in melanotic and amelanotic canine oral melanomas

Análise qualitativa quanto à diferença da imunoexpressão do gene c-Kit em melanomas melânicos e amelânicos da cavidade oral em cães

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Abstract

Melanomas are the most common oral malignancy in dogs. Cell proliferation and connexin expression has been shown to differ in canine melanotic and amelanotic oral melanomas. This study aimed to analyze the c-Kit protein expression in melanotic and amelanotic melanomas from canine buccal cavity. A total of 34 canine buccal melanomas (19 melanotic and 15 amelanotic).were collected. The amelanotic melanomas presented faster evolution and higher incidence of metastasis than melanotic tumors. A significantly higher number of c-Kit positive cells were observed in amelanotic neoplasms. In addition, the intensity of c-Kit immunolabeling was predominantly stronger in amelanotic melanomas. These results confirm a potential role for c-Kit in canine oral melanomas with clear differences in expression patterns between the two histological types of tumor, melanotic and amelanotic. This study highlights the importance of a detailed study of c-Kit mutations in canine oral melanomas to better understand the molecular mechanisms implicated in the development of this disease.

Keywords: Melanotic melanoma. Amelanotic melanoma. c-Kit. Immunohistochemistry. Dogs.

Resumo

Melanomas são as mais frequentes neoplasias malignas da cavidade bucal de cães. Sabe-se que a proliferação de células e expressão de conexina diferem em melanomas melanóticos e amelanóticos da cavidade bucal de cães. Este estudo analisou a expressão da proteína c-Kit em melanomas melanóticos e amelanóticos da cavidade bucal canina. Um total de 34 melanomas bucais caninos (19 melanóticos e 15 amelanóticos) foram coletados. Os melanomas amelanóticos apresentaram evolução mais rápida e maior incidência de metástase. Foi constatado um número significativamente maior de células positivas para c-Kit em neoplasias amelanóticas. Além disso, a intensidade de imunomarcação de c-Kit foi predominantemente mais forte em melanomas amelanóticos. Estes resultados confirmam um papel potencial para c-Kit em melanomas orais caninos, com diferenças claras em padrões de expressão entre os dois tipos histológicos de tumor, melanóticos e amelanóticos. Este trabalho destaca a importância de um estudo detalhado das mutações c-Kit em melanomas orais caninos para ser possível a melhor compreensão dos mecanismos moleculares envolvidos no desenvolvimento da doença.

Palavras-chave: Melanoma melanótico. Melanoma amelanótico. C-kit. Imuno-histoquimica. Cães.

Introduction

Melanomas account for 7% of all malignant canine tumors (SMITH; GOLDSCHMIDT; MCMANUS, 2002) and are the most common oral malignancy in dogs (BERGMAN, 2007). They are characterized by local invasion and high rate of distant metastases (PROULX et al., 2003). Because of the prevalence and aggressive nature of oral cavity tumors in dogs, studying their molecular characteristics is mandatory. The primary factors that determine the biological behavior of an oral melanoma in dogs are site, size, stage and histologic parameters. (SPANGLER; KASS, 2006). Based only on histological features,

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the melanotic malignant neoplasm is classified by the Veterinary WHO bulletin in three subtypes of cell morphologic aspects: epithelioid or polygonal, spindle or fibromatous and mixed - epithelioid and spindle (GOLDSCHMIDT et al., 1998). Another classification is based on the ability of melanoma cells to produce or not melanin, classifying melanomas into melanotic and amelanotic types, respectively. However, the combined lack of pigmentation and the indistinct cellular morphology of many oral malignant melanomas often make definitive diagnosis difficult when based on microscopic examination alone (SMEDLEY et al., 2011a). Although the histological appearance has not been used as prognostic value (SMEDLEY et al., 2011b), the lack of melanin appears to play an important role in prognosis due to the signals of higher aggressiveness seen in amelanotic melanomas. (TAJIMA; URA-ISHIKO; HAYASHI, 1996; CHOI; KUSEWITT, 2003). Canine melanotic and amelanotic buccal melanomas have recently been shown to differ regarding cell proliferation and connexin expression (TEIXEIRA et al., 2014), and amelanotic buccal melanomas were considered more aggressive than their melanotic counterparts.

The c-Kit is a tyrosine kinase receptor whose aberrant activation is implicated in a variety of tumors. Its ligand, the glycoprotein stem cell factor (SCF), recruits and activates a high number of intracellular signal transducers implicated in tumor progression, such as phosphoinositide-3 kinase (PI3K), AKT, Src, mitogen-activated protein kinase (MAPK), janus kinase (JAK), transcription factors (e.g. STAT) and phospholipase (PL) Cγ. (SMALLEY; SONDAK; WEBER, 2009).

The role of c-Kit signaling in melanoma is controversial. Although c-Kit activity is critical to melanocyte development and migration, its expression tends to be reduced in most melanomas, mainly in the invasive and metastatic types (NATALI et al., 1992). Transfection of c-Kit into c-Kit-negative highly metastatic human melanoma cells significantly inhibited tumor growth and metastasis in nude mice (HUANG et al., 1998). Exposure of c-Kit positive melanoma cells to SCF in vitro and in vivo triggered apoptosis of these cells but not of normal melanocytes. Loss of c-Kit receptor may thus allow melanoma cells to escape SCF/c-Kit-mediated apoptosis (HUANG et al., 1998). In addition, Newmann et al. (2012) have shown that Kit expression anywhere within the resected mucosal melanomas correlated with significantly longer survival.

The aim of the present study was to analyze the c-Kit protein expression in melanotic and amelanotic melanomas from oral cavity of dogs as a first step to define the role of the tyrosine kinase receptor in this disease.

Material and Methods

Animals

Canine buccal melanoma samples were obtained from the Laboratory of Comparative Dentistry, Service of Surgery, Veterinary Hospital of the School of Veterinary Medicine and Animal Science, University of São Paulo, Brazil. Age, gender, melanoma location in buccal cavity and clinical characteristics, such as metastasis signal in lymph nodes or lungs, were recorded. All animals underwent radiological evaluation and chest X-ray diagnosis in order to verify the presence of lung metastasis. All dogs were then submitted to surgery in order to remove the tumor. The term tumor evolution refers to the time period reported by the owners to the clinician that they noticed the tumor mass shown in tables 1 and 2. This study has been approved by the Committee on Ethics for the Use of Animals of the School of Veterinary Medicine and Animal Science of the University of São Paulo, Brazil. The survival time of the animals after the surgical procedure has been properly analyzed by the Kaplan Meyer curve.

Tumor samples and histopathological analysis

Tumor samples were harvested after the surgical procedures with prior permission of the animal

Table 1 – Characterization of patients with melanotic melanoma. Clinical and tumor evolution – School of Veterinary Medicine and Animal Science of the University of São Paulo – 2011

Case	Age/years	Gender	Location	Tumor Evolution	Metastasis
1	12	M	Gingiva	Progression in 3 months	No
2	11	M	Gingiva	Progression in 5 months	No
3	14	F	Gingiva	Progression in 4 months	Lung
4	8	M	Gingiva	Progression in 5 months	No
5	8	M	Gingiva	Progression in 6 months	No
6	9	F	Gingiva	Progression in 3 months	No
7	11	F	Gingiva	Progression in 1 month	No
8	12	M	Gingiva	Progression in 3 months	No
9	10	M	Gingiva	Progression in 4 months	No
10	8	F	Gingiva	Progression in 14 days	No
11	12	M	Gingiva	Progression in 4 months	No
12	8	M	Gingiva	Progression in 2 months	No
13	9	F	Gingiva	Progression in 3 months	No
14	10	F	Gingiva	Progression in 3 months	No
15	11	F	Gingiva	Progression in 5 months	No
16	11	M	Lips	Progression in 6 months	No
17	9	M	Lips	Progression in 2 months	No
18	7	M	Palate	Progression in 3 months	Nodes/Lung
19	10	M	Palate	Progression in 5 months	Node

Table 2 – Expression of c-Kit by immunohistochemistry (IHC) in melanotic and amelanotic melanomas – School of Veterinary Medicine and Animal Science of the University of São Paulo – 2011

Tumor type	Score of C-Kit Expression	% Score C-Kit Expression	% C-Kit Membrane Location	% C-Kit Cytoplasm Location
Melanotic melanoma	(-)	36.9%	-------	--------
	(+)	36.9	75%	50%
	(++)	15.8%	25%	25%
	(+++)	10.5%	-------	25%
Amelanotic melanoma	(-)	33.4%	------------	----------
	(+)	6.7%	12.5%	---------
	(++)	20%	25%	50%
	(+++)	39.9%	62.5%	50%

owners. A total of 34 melanomas from the oral cavity of dogs were collected, consisting of 19 melanotic and 15 amelanotic specimens. Representative slices of the tumors were properly fixed in neutral buffered formalin (10%). The 34 samples were routinely processed for embedding in paraffin wax. The 5 µm thick sections were stained with hematoxylin and eosin and were examined by two pathologists using a Nikon Eclipse E-800 microscope (Nikon, Tokyo, Japan). Digital photomicrographs were captured by Image ProPlus software (Media Cybernetics, Silver Spring, MD, USA). All melanomas were classified according to veterinary WHO classification of tumors (GOLDSCHMIDT et al., 1998).

Immunohistochemistry

Antibodies and respective dilutions used in this study are presented in table 3. Immunohistochemistry for HMB45 and Melan-A was used in order to prove that amelanotic tumors were indeed melanomas. Both types of melanomas were submitted to immunohistochemistry for c-Kit. In order to perform immunohistochemistry in melanotic melanomas, removal of melanin was necessary. Therefore, slides

were submitted to antigen retrieval by boiling them in citrate buffer in a microwave oven after deparaffinization and rehydration, as described previously, with modifications (HSU; RAINE; FANGER, 1981). After antigen retrieval, slides were immersed in $KMnO_4$ solution for 30 min, washed in phosphate buffered saline (PBS) for 5 min and immersed in a solution containing oxalic acid for 1 min. The LSAB kit (Dako, California) was used and stained by diaminobenzidine (DAB) with modifications (SULAIMON; KITCHELL; EHRHART, 2002). Negative controls were performed by omitting the primary antibody in a tissue slide during immunohistochemical reaction.

Semi-quantitative c-Kit analyses

The immunohistochemical labeling was evaluated semi-quantitatively and quantitatively. The intensity of the immunolabeling is represented by the number of positively labeled melanoma cells and was scored as follows: (-) no positive cells, (+) less than 30% of positive cells; (++) from 30% to 60% of positive cells and (+++) more than 60% of positive cells. The positive cells were then quantified in an image analysis system. For each sample, 1,000 cells were counted in 40X magnification field, with positive and negative cells recorded. The procedure was performed manually in the Image Pro Plus System (version 4.5 of Windows, 2002 edition). The percentage of c-Kit positive cells was then calculated for each tumor case.

Quantitative c-Kit analyses

Positive cells were quantified in melanoma slides submitted to c-Kit immunohistochemical labeling. The subcellular localization of the receptor, whether in the cytoplasm or the membrane, was also analyzed. For each sample, 1,000 cells were counted in 40X magnification field, with positive and negative cells recorded. The procedure was performed manually in the Image Pro Plus System (version 4.5 of Windows, 2002 edition). The percentage of positive cells and cellular localization of c-Kit were registered for each tumor case, and then the mean and standard deviations were obtained for melanotic and amelanotic groups, respectively.

Statistical Analysis

Statistical analysis was performed with Student's t-test. Continuous variables were expressed as mean ± standard deviation or as median if abnormal. This statistic test was used to verify the existence of difference in c-Kit stained cells and localization in the cells between two groups, melanotic and amelanotic melanomas. A two-tailed P value < 0.05 was considered to express statistical significance. A non-parametric analysis among groups was performed using the Kruskal–Wallis test (Prism version 5, Graphpad Software) for the Kaplan Meyer curve.

Results

Amelanotic melanomas present faster evolution and higher metastasis rate

Old male dogs were preferentially affected by both melanotic and amelanotic melanomas and the gingival area were predominantly affected. (Tables 1 and 4). The amelanotic melanomas presented faster evolution and a higher incidence of metastasis than melanotic tumors (Tables 1 and 4). This study included 34 tissues of primary oral melanomas (19 melanotic

Table 3 – Antibodies used for immunolabeling of canine melanomas – School of Veterinary Medicine and Animal Science of the University of São Paulo – 2011

Antibody	Clone	Supplier	Dilution	Antigen Retrieval
HMB45	HMB45	Dako	1:50	Citrate Buffer (pH 6) 12' 98°C microwave
Melan-A	A103	Dako	1:25	Citrate Buffer (pH 6) 12' 98°C microwave
c-Kit	A4502	Dako polyclonal Rabbit	1:80	Citrate Buffer (pH 6) 12' 98°C microwave

Table 4 – Characterization of patients, with amelanotic melanoma. Clinical and tumor evolution – School of Veterinary Medicine and Animal Science of the University of São Paulo – 2011

Case	Age/years	Gender	Location	Tumor Evolution	Metastasis
1	10	F	Gingiva	Progression in 3 months	No
2	11	M	Gingiva	Progression in 3 months	No
3	7	F	Gingiva	Progression in 4 months	No
4	13	M	Gingiva	Progression in 3 months	No
5	4	F	Gingiva	Progression in 15 days	LymphNode
6	12	M	Gingiva	Progression in 3 months	No
7	8	M	Gingiva	Progression in 2 months	Node
8	10	F	Gingiva	Progression in 2 months	Node
9	1	M	Gingiva	Progression in 1 months	LymphNode / Lung
10	9	M	Gingiva	Progression in 2 months	Node
11	11	F	Gingiva	Progression in 50 days	No
12	4	M	Gingiva	Progression in 1.5 month	No
13	8	M	Lip	Progression in 4 months	No
14	11	F	Lip	Progression in 4 months	No
15	4	M	Lip	Progression in 1.5 month	No

and 15 amelanotic). The diagnosis was confirmed by HMB45 and Melan-A immunostaining.

Amelanotic melanomas show greater number of c-Kit positive cells

The both tumors (melanotic and amelanotic) presented immunopositivity for c-Kit in the cytoplasm and membrane locations, as shown in figure 1. However, amelanotic tumors had the greatest number of positively immunostained cells (Figure 2). Among the melanotic melanomas, seven tumors were negative for c-Kit positivity, seven samples were (+); three tumors presented moderate (++), and two tissues showed strong intensity (+++) (Table 2). Among the amelanotic group, five tumors were negative; only one tumor showed weak staining (+), three melanomas had moderate staining (++), and six tumors had strong staining (+++) (Table 2). Regarding the location of c-Kit labeling in melanotic melanoma cells, the predominant location was the cytoplasm. In contrast, there was no significant difference between cytoplasmic and membrane locations in amelanotic melanoma cells, as shown in table 2.

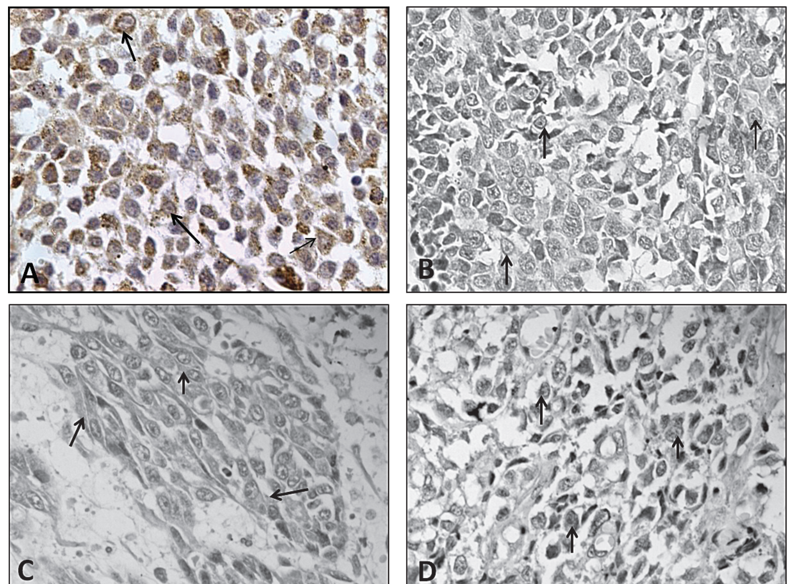

Figure 1 – Immunohistochemical labeling for c-Kit (original magnification X 40). (A) Strong cytoplasmic immunostaining in melanocytic melanoma. (B) Membrane and cytoplasmic c-kit immunostaining in melanotic melanoma. (C) Weak cytoplasmic c-kit immunostaining in amelanotic melanoma. (D) Strong cytoplasmic c-kit immunostaining in amelanotic melanoma
Source: (TEIXEIRA, 2011)

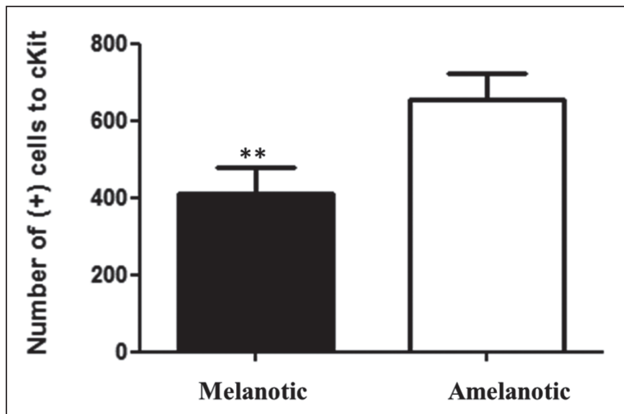

Figure 2 – Statistical analysis was performed with Student's t-test to compare the number of positive cells of c-Kit between melanotic and amelanotic tumors. Amelanotic melanoma has shown significantly higher number of c-Kit positive cells. (P = 0,0177)
Source: (TEIXEIRA, 2011)

The c-Kit expression has no influence on survival time

The survival time curves for each melanotic and amelanotic tumors including the c-Kit expression were compared in figure 3. The statistical analysis for the curves revealed no significant difference between the groups.

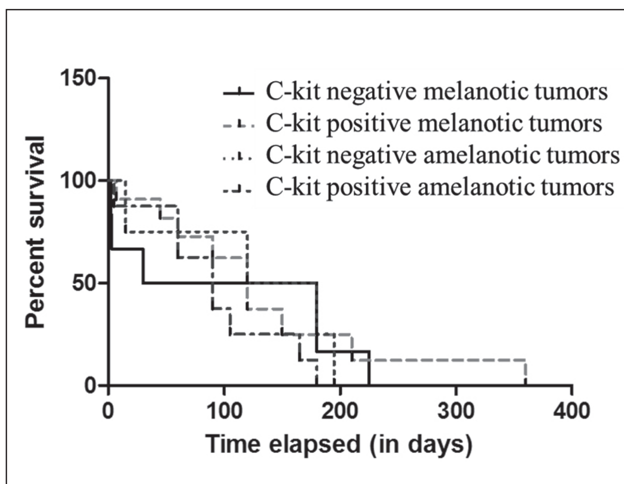

Figure 3 – The Kaplan Meyer curve for each melanotic and amelanotic tumor including the c-Kit expression were compared. Statistical analysis for the curves revealed no significant difference between groups
Source: (TEIXEIRA, 2011)

Discussion

Since the early 1990s, some evidence has been gathered showing that c-Kit plays an important role in melanogenesis (SULAIMON; KITCHELL; EHRHART, 2002). Recently, the activation of the c-Kit proto-oncogene has been frequently observed in malignant melanomas (WITTE, 1990). Some genetic studies have demonstrated that the natural occurrence of inactivating mutations in the proto-oncogene c-Kit that impair the kinase activity of this receptor, leading to various developmental disorders and resulting in amelanotic congenital patches of white skin (HIDA et al., 2009). Several studies have also shown that c-Kit influences the migration, proliferation and survival of melanocytes (SPRITZ; GEIBEL; HOLMES, 1992; LENNARTSSON et al., 2005). This fact supports the hypothesis that c-Kit plays an important role in melanomas. Based on these statements, the current study aimed to analyze the expression of c-Kit protein in melanotic and amelanotic melanomas from oral cavity of dogs in order to better understand the function of this receptor in tumor aggressiveness.

The results showed faster tumor evolution among animals with amelanotic melanomas and higher metastasis incidence than compared to animals bearing melanotic tumors. Nonetheless, the etiology and pathogenesis of amelanotic tumors has been unclear or not completely understood. Significant differences between melanotic and amelanotic melanomas in c-Kit expression analysis have been observed, with a greater number of c-Kit labeled cells in amelanotic tumors. Unlike amelanotic melanomas that have shown cytoplasmic and membrane location patterns for c-Kit, melanotic tumors have presented a predominant cytoplasmic location for the receptor. It has been proposed that cytoplasmic immunoreactivity for c-Kit might be due to a non-mature form of the protein rather than a proteolytically processed molecule, which would be the active membranous receptor form (GRICHNIK, 2006). In physiologic conditions, the

signaling cascade is initiated by transmembraneous receptor tyrosine kinases (RTK), such as c-Kit, and functions for signal transmission from the cell membrane to the nucleus (TORRES-CABALA et al., 2009). Some studies have indicated that most of the mutations in c-Kit affect the juxta-membrane region of the protein, preventing its positioning in the plasmatic membrane (JIANG et al., 2008; HELD et al., 2011). Although the authors have not analyzed any mutations in the juxta-membrane region of c-Kit in this study, the hypothesis of a disruption in the positioning of c-Kit in the plasmatic membrane leading to inactive forms of the receptors in melanotic melanomas may be considered.

The authors observed a moderate and strong labeling for c-Kit in amelanotic melanomas, disagreeing with the results of Morini et al. (2004), who showed weak to moderate CD117 immunolabeling pattern. A mutational study of c-Kit in human mucosal melanomas demonstrated a correlation between a stronger expression of the protein and the presence of mutations in exon 11 of c-Kit, suggesting a pertinent role for c-Kit mutations in the development of oral mucosal melanoma (RIVERA et al., 2008). According to Satzger et al. (2008), there is a significantly higher immunohistochemical expression of c-Kit in human mucosal melanomas that harbor a potentially activating Kit mutation as compared with tumors without this mutation. The analysis of c-Kit expression and exon 11 mutations in canine oral malignant melanomas was performed and although 20 of 39 cases were positive for c-Kit protein, there was no significant difference between the receptor expression and overall survival. Moreover, polymerase chain reaction amplification and sequencing of c-Kit exon 11 in 17 samples did not detect any mutations (KINOSHITA et al., 2003; MURAKAMI et al., 2011). In this particular case, two aspects should be taken into account. The authors have not classified the melanomas in melanotic or amelanotic categories and have not analyzed mutations in other exons, leaving some points unclear.

As amelanotic melanomas have been associated with a more aggressive type and poor prognosis (RIVERA et al., 2008) and the results have indicated a predominance of strong immunostaining of c-Kit in this type of melanoma in dogs, this could be linked to c-Kit mutation. However, a study conducted by Murakami et al. (2011) has shown no correlation between c-Kit expression and overall survival. Although this study has not classified the tumors into melanotic and amelanotic categories, the data for melanotic and amelanotic tumors in the current study have also not identified any correlation between survival and c-Kit expression. Thus, the aggressiveness of amelanotic melanomas still appears to be related to the lack of melanin and a differentiation issue, rather than to c-Kit expression.

Melanomas are not genetically and histologically homogeneous and may follow different pathways to oncogenic transformation (SMALLEY; SONDAK; WEBER, 2009). One of these pathways may be through the signaling of c-Kit, which appears to play a role in the aggressiveness of oral mucosal melanoma (RIVERA et al., 2008). The answer to this question could be hidden in the lack of melanin in some melanomas. It is probable that the expression of the mutant c-Kit was associated with lower pigmentation of melanocytes, presumably caused by down regulation of the tyrosine gene expression (LUO et al., 1995; ALEXEEV, YOON, 2006). In summary, c-Kit appears to play an important role in melanoma biology. However, further studies are required to confirm this hypothesis.

Conflict of interest statement

None of the authors of this paper has any financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of this paper.

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References

ALEXEEV, V.; YOON, K. Distinctive role of the cKit receptor tyrosine kinase signaling in mammalian melanocytes. Journal of Investigative **Dermatology**, v. 126, p. 1102-1110, 2006. Available from: <http://www.nature.com/jid/journal/v126/n5/full/5700125a.html>. Viewed: 13 Nov. 2015. doi: http://dx.doi.org/10.1038/sj.jid.5700125.

BERGMAN, P. J. Canine oral melanoma. **Clinical Techniques in Small Animal Practice**, v. 22, n. 2, p. 55-60, 2007. Available from: <http://www.sciencedirect.com/science/article/pii/S1096286707000126>. Viewed: 13 Nov. 2015. doi: http://dx.doi.org/10.1053/j.ctsap.2007.03.004.

CHOI, C.; KUSEWITT, D. F. Comparison of tyrosinase related protein-2, S-100 and Melan A immunoreactivity in canine amelanocitic melanomas. **Veterinary Pathology**, v. 40, n. 6, p. 713-718, 2003. Available from: <http://vet.sagepub.com/content/40/6/713.full>. Viewed: 13 Nov. 2015. doi: http://dx.doi.org/10.1354/vp.40-6-713.

GOLDSCHMIDT, M. H.; DUNSTAN, R. W.; STANNARD, A. A.; TOSCHARNER, C. von; WALDER, E. J.; YAGER, J. A. Histological classification of epithelial and melanocytic tumors of skin of domestic animals. In: MEUTEN, D. J. (Ed.). **World Health Organization international histological classification of tumors of domestic animals**. 2nd series. Washington, DC: Armed Forces Institute of Pathology, 1998. v. 3. 106 p.

GRICHNIK, J. M. Kit and melanocyte migration. **Journal of Investigative Dermatology**, v. 126, p. 945-947, 2006. Available from: <http://www.nature.com/jid/journal/v126/n5/full/5700164a.html>. Viewed: 13 Nov. 2015. doi: http://dx.doi.org/10.1038/sj.jid.5700164.

HELD, L.; EIGENTLER, T. K.; MEIER, F.; HELD, M.; RÖCKEN, M.; GARBE, C.; BAUER, J. Oncogenetics of melanoma: basis for molecular diagnostics and therapy. JDDG: **Journal der Deutschen Dermatologischen Gesellschaft**, v. 9, n. 7, p. 510-516, 2011. Available from: <http://onlinelibrary.wiley.com/doi/10.1111/j.1610-0387.2011.07603.x/pdf>. Viewed: 13 Nov. 2015. doi: http://dx.doi.org/10.1111/j.1610-0387.2011.07603.x.

HIDA, Y.; KUBO, Y.; MIYAJIMA, O.; ARASE, S. Primary dermal melanoma: a case report and molecular characterization. **The Journal of Dermatology**, v. 36, n. 6, p. 346-352, 2009. Available from: <http://onlinelibrary.wiley.com/enhanced/doi/10.1111/j.1346-8138.2009.00650.x>. Viewed: 13 Nov. 2015. doi: http://dx.doi.org/10.1111/j.1346-8138.2009.00650.x.

HSU, S. M.; RAINE, L.; FANGER, H. Use of avidin-biotin-peroxidase complex (ABC) in immunoperoxidase techniques: a comparison between ABC and unlabeled antibody (PAP) procedure. **Journal of Histochemistry and Cytochemistry**, v. 29, n. 4, p. 577-580, 1981. Available from: <http://jhc.sagepub.com/content/29/4/577.abstract>. Viewed: 13 Nov. 2015. doi: http://dx.doi.org/10.1177/29.4.6166661.

HUANG, S.; JEAN, D.; LUCA, M.; TAINSKY, M. A.; BAR-ELI, M. Loss of AP-2 results in downregulation of c-KIT and enhancement of melanoma tumorigenicity and metastasis. **The EMBO Journal**, v. 17, n. 15, p. 4358-4369, 1998. Available from: <http://onlinelibrary.wiley.com/doi/10.1093/emboj/17.15.4358/abstract>. Viewed: 13 Nov. 2015. doi: http://dx.doi.org/10.1093/emboj/17.15.4358.

JIANG, X.; JUN, Z.; YUEN, N. K.; CORLESS, C. L.; HEINRICH, M. C.; FLETCHER, J. A.; DEMETRI, G. D.; WIDLUND, H. R.; FISHER, D. E.; HODI, S. F. Imatinib targeting of KIT-Mutant oncoprotein in melanoma. **Clinical Cancer Research**, v. 14, n. 23, p. 7726-7732, 2008. Available from: <http://clincancerres.aacrjournals.org/content/14/23/7726>. Viewed: 13 Nov. 2015. doi: http://dx.doi.org/10.1158/1078-0432.CCR-08-1144.

KINOSHITA, K.; ISOZAKI, K.; HIROTA, S.; NISHIDA, T.; CHEN, H.; NAKAHARA, M.; NAGASAWA, Y.; OHASHI, A.; SHINOMURA, Y.; KITAMURA, Y.; MATSUZAWA, Y. c-kit gene mutation at exon 17 or 13 is very rare in sporadic gastrointestinal stromal tumors. **Journal of Gastroenterology and Hepatolology**, v. 18, n. 2, p. 147-151, 2003. Available from: <http://onlinelibrary.wiley.com/enhanced/doi/10.1046/j.1440-1746.2003.02911.x>. Viewed: 13 Nov. 2015. doi: http://dx.doi.org/10.1046/j.1440-1746.2003.02911.x.

LENNARTSSON, J.; JELACIC, T.; LINNEKIN, D.; SHIVAKRUPA, R. Normal and oncogenic forms of the receptor tyrosine kinase kit. **Stem Cells**, v. 23, n. 1, p. 16-43, 2005. Available from: <http://onlinelibrary.wiley.com/doi/10.1634/stemcells.2004-0117/full>. Viewed: 13 Nov. 2015. doi: http://dx.doi.org/10.1634/stemcells.2004-0117.

LUO, D.; CHEN, H.; SEARLES, G.; JIMBOW, K. Coordinated mRNA expression of the c-Kit with tyrosinase and TRP-1 in melanin pigmentation of normal and malignant human melanocytes and transient activation of tyrosinase by Kit/SCF-R. **Melanoma Research**, v. 5, n. 5, p. 303-309, 1995.

MORINI, M.; BETTINI, G.; PREZIOSI, R.; MANDRIOLI, L. C-kit gene product (CD117) immunoreactivity in canine and feline paraffin sections. **Journal of Histochemistry and Cytochemistry**, v. 52, n. 5, p. 705-708, 2004. Available from: <http://jhc.sagepub.com/content/52/5/705.long>. Viewed: 13 Nov. 2015. doi: http://dx.doi.org/10.1177/002215540405200515.

MURAKAMI, A.; MORI, T.; SAKAI, H.; MURAKAMI, M.; YANAI, T.; HOSHINO, Y.; MARUO, K. Analysis of KIT expression and KIT exon 11 mutations in canine oral malignant melanomas. **Veterinary and Comparative Oncology**, v. 9, n. 3, p. 219-224, 2011. Available from: <http://onlinelibrary.wiley.com/doi/10.1111/j.1476-5829.2010.00253.x/pdf>. Viewed: 13 Nov. 2015. doi: http://dx.doi.org/10.1111/j.1476-5829.2010.00253.x.

NATALI, P. G.; NICOTRA, M. R.; WINKLER, A. B.; CAVALIERE, R.; BIGOTTI, A.; ULLRICH, A. Progression of human cutaneous melanoma is associated with loss of expression of c-kit proto-oncogene receptor. **International Journal of Cancer**, v. 52, n. 2, p. 197-201, 1992. Available from: <http://onlinelibrary.wiley.com/doi/10.1002/ijc.2910520207/abstract>. Viewed: 13 Nov. 2015. doi: http://dx.doi.org/10.1002/ijc.2910520207.

NEWMAN, S. J.; JANKOVSKY, J. M.; ROHRBACH, B. W.; LEBLANC, A. K. C-kit expression in canine mucosal melanomas. **Veterinary Pathology**, v. 49, n. 5, p. 760-765. 2012. Available from: <http://vet.sagepub.com/content/49/5/760>. Viewed: 13 Nov. 2015. doi: http://dx.doi.org/10.1177/0300985811414032.

PROULX, D. R.; RUSLANDER, D. M.; DODGE, R. K.; MARLENE, L. H.; WILLIAMS, L. E.; HORN, B.; PRICE, G. S.; THRALL, D. E. A retrospective analysis of 140 dogs with oral melanoma treated with external beam radiation. **Veterinary Radiology & Ultrasound**, v. 44, n. 3, p. 352-359, 2003. Available from: <http://onlinelibrary.wiley.com/doi/10.1111/j.1740-8261.2003.tb00468.x/abstract>. Viewed: 13 Nov. 2015. doi: http://dx.doi.org/10.1111/j.1740-8261.2003.tb00468.x.

RIVERA, R. S.; NAGATSUKA, H.; GUNDUZ, M.; CENGIZ, B.; GUNDUZ, E.; SIAR, D. H.; TSUJIGIWA, H.; TAMAMURA, R.; HAN, K. N.; NAGAI, N. C-kit protein expression correlated with activating mutations in KIT gene in oral mucosal melanoma. **Virchows Archiv**, v. 452, n. 1, p. 27-32, 2008. Available from: <http://link.springer.com/article/10.1007%2Fs00428-007-0524-2>. Viewed: 13 Nov. 2015. doi: http://dx.doi.org/10.1007/s00428-007-0524-2.

SATZGER, I.; SCHAEFER, T.; KUETTER, U.; BROECKER, V.; VOELKER, B.; OSTERTAG, H.; KAPP, A.; GUTZMER, R. Analysis of c-Kit expression and KIT gene mutation in human mucosal melanomas. **British Journal of Cancer**, v. 99, p. 2065-2069, 2008. Available from: <http://www.nature.com/bjc/journal/v99/n12/full/6604791a.html>. Viewed: 13 Nov. 2015. doi: http://dx.doi.org/10.1038/sj.bjc.6604791.

SMALLEY, K. S. M.; SONDAK, V. K.; WEBER, J. S. C-Kit signaling as the driving oncogenic event in sub-groups of melanomas. **Histology and Histopathology**, v. 24, n. 5, p. 643-650, 2009.

SMEDLEY, R. C.; LAMOUREUX, J.; SLEDGE, D. G.; KIUPEL, M. Immunohistochemical diagnosis of canine oral amelanotic melanocytic neoplasms. **Veterinary Pathology**, v. 48, n. 1, p. 32-40, 2011a. Available from: <http://vet.sagepub.com/content/48/1/32.full>. Viewed: 13 Nov. 2015. doi: http://dx.doi.org/10.1177/0300985810387447.

SMEDLEY, R. C.; SPANGLER, W. L.; ESPLIN, D. G.; KITCHELL, B. E.; BERGMAN, P. J.; HO, H. J.; BERGIN, I. L.; KIUPEL, M. Prognostic markers for canine melanocytic neoplasms: a comparative review of the literature and goals for future investigation. **Veterinary Pathology**, v. 48, n. 1, p. 54-72, 2011b. Available from: <http://vet.sagepub.com/content/48/1/54>. Viewed: 13 Nov. 2015. doi: http://dx.doi.org/10.1177/0300985810390717.

SMITH, S. H.; GOLDSCHMIDT, M. H.; McMANUS, P. M. A comparative review of melanocytic neoplasms. **Veterinary Pathology**, v. 39, n. 6, p. 651-678, 2002. Available from: <http://vet.sagepub.com/content/39/6/651>. Viewed: 13 Nov. 2015. doi: http://dx.doi.org/10.1354/vp.39-6-651.

SPANGLER, W. L.; KASS, P. H. The histologic and epidemiologic bases for prognostic considerations in canine melanocytic neoplasia. **Veterinary Pathology**, v. 43, n. 2, p. 136-149, 2006. Available from: <http://vet.sagepub.com/content/43/2/136.full>. Viewed: 13 Nov. 2015. doi: http://dx.doi.org/10.1354/vp.43-2-136.

SPRITZ, R. A.; GEIBEL, L. B.; HOLMES, S. A. Dominant negative and loss of function mutations of the c-kit (mast-stem cell growth factor receptor) proto-oncogene in human piebaldism. **American Journal of Human Genetics**, v. 50, n. 2, p. 261-269, 1992.

SULAIMON, S. S.; KITCHELL, B. E.; EHRHART, E. J. Immunohistochemical detection of melanoma-specific antigens in spontaneous canine melanoma. **Journal of Comparative Pathology**, v. 127, n. 2-3, p. 162-168, 2002. Available from: <http://www.sciencedirect.com/science/article/pii/S0021997502905761>. Viewed: 13 Nov. 2015. doi: http://dx.doi.org/10.1053/jcpa.2002.0576.

TAJIMA, S.; URA-ISHIKO, A.; HAYASHI, A. Melanogenesis, biosynthetic phenotype of fibronectin and collagen, and migrating activity in cloned B16 mouse melanoma cells. **Journal of Dermatological Science**, v. 12, n. 1, p. 24-30, 1996. Available from: <http://www.sciencedirect.com/science/article/pii/0923181195004556>. Viewed: 13 Nov. 2015. doi: http://dx.doi.org/10.1016/0923-1811(95)00455-6.

TEIXEIRA, T. F.; GENTILE, L. B.; SILVA, T. C.; MENNECIER, G.; CHAIBLE, L. M.; COGLIATI, B.; ROMAN, M. A.; GIOSO, M. A.; DAGLI, M. L. Cell proliferation and expression of connexins differ in melanotic and amelanotic canine oral melanomas. **Veterinary Research Communication**, v. 38, n. 1, p. 29-38, 2014. Available from: <http://link.springer.com/article/10.1007%2Fs11259-013-9580-z>. Viewed: 13 Nov. 2015. doi: http://dx.doi.org/10.1007/s11259-013-9580-z.

TORRES-CABALA, C. A.; WANG, W. L.; TRENT, J.; YANG, S.; CHEN, S.; GALBINCEA, J.; KIM, K. B.; WOODMAN, S.; DAVIES, M.; PLAZA, J. A.; NASH, J. W.; PRIETO, V. G.; LAZAR, A. J.; IVAN, D. Correlation between KIT expression and KIT mutation in melanoma: a study of 173 cases with emphasis on the acral-lentiginous mucosal type. **Modern Pathology**, v. 22, p. 1446-1456. 2009. Available from: <http://www.nature.com/modpathol/journal/v22/n11/full/modpathol2009116a.html>. Viewed: 13 Nov. 2015. doi: http://dx.doi.org/10.1038/modpathol.2009.116.

WITTE, O. N. Steel locus defines new multipotent growth factor. **Cell**, v. 63, n. 1, p. 5-6, 1990. Available from: <http://www.sciencedirect.com/science/article/pii/009286749090280R>. Viewed: 13 Nov. 2015. doi: http://dx.doi.org/10.1016/0092-8674(90)90280-R.