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Immunohistochemical Expression of the Pluripotency Factor OCT4 in Canine Mast Cell Tumours

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Summary

Cancer stem cells (CSCs) are related to malignancy and resistance to chemotherapy in several tumours. OCT4 is a 'pluripotency factor' that is expressed by these cells. The aim of the present study was to investigate OCT4 expression in canine cutaneous mast cell tumours (MCTs) by means of immunohistochemistry. Twenty-eight cases were evaluated and showed variable immunolabelling patterns. The dogs were treated by surgery alone and followed up for a minimum of 180 days. No significant difference was found between histopathological grades and similar results were obtained for mortality due to the disease and post-surgical survival. These preliminary results suggest that OCT4 expression is not a precise prognostic indicator for canine MCT.

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Keywords: cancer stem cell; dog; mast cell tumour; OCT4 protein

Mast cell tumours (MCTs) are important neoplasms in veterinary practice, accounting for approximately 20% of canine tumours and 30% of the malignant neoplasms in this species (Bostock, 1973; Hottendorf and Nielsen, 1967; Strefezzi *et al.*, 2003; Misdorp, 2004). MCT malignancy is evaluated based on cell differentiation levels by histopathological examination, but there is considerable variation among pathologists due to the subjective features that are analysed with the main grading systems (Patnaik *et al.*, 1984; Kiupel *et al.*, 2011).

Several studies have shown the importance of prognostic tools in oncology. The histopathological grading systems of Patnaik *et al.* (1984) and Kiupel *et al.* (2011) are used routinely to predict the behaviour of MCTs. However, additional prognostic markers have been shown to improve prediction. These include mitotic index (Romansik *et al.*, 2007; Strefezzi *et al.*, 2014), Ki67 expression (Abadie *et al.*, 1999; Sakai *et al.*, 2002; Strefezzi *et al.*, 2010), BAX expression (Strefezzi *et al.*, 2012), nuclear morphometry (Strefezzi *et al.*, 2009) and the immunohistochemical patterns of KIT labelling (Zemke *et al.*, 2002; Kiupel *et al.*, 2004).

OCT4 is a transcription factor located in the Pit, Oct and Unc (POU) family and it is highly expressed in pluripotent cells. For this reason, it is considered to be a stem cell marker (Liedtke *et al.*, 2008). Cancer stem cells (CSCs) are believed to initiate and maintain the neoplastic cell population. This cell population is also associated with malignancy and resistance to chemotherapy. Marfels *et al.* (2013) correlated higher OCT4 expression with increased resistance to chemotherapy in human patients with hepatocellular carcinomas. Similar results were obtained by Wen *et al.* (2013) in patients with colorectal

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cancer. Nevertheless, it was demonstrated that OCT4 immunoexpression is indicative of favourable prognosis in cases of rectal cancer after chemoradiotherapy (Saigusa *et al.*, 2009). Raman *et al.* (2006) showed the absence of immunolabelling in renal carcinoma, probably related to low numbers of CSCs. Furthermore, OCT4 expression is increased in human seminomas and breast carcinomas and associated with tumour progression (Ezeh *et al.*, 2005). High OCT4B expression is an independent prognostic tool related to good prognosis in patients with prostate cancer (Resende *et al.*, 2013).

Webster *et al.* (2007) described OCT4 expression in several canine neoplasms, including MCTs, but did not compare OCT4 expression levels with clinical follow-up. OCT4 is expressed weakly by cultured canine prostate carcinoma cells (Moulay *et al.*, 2013). Wilson *et al.* (2008) found OCT4 mRNA expression in canine osteosarcomas, although samples were negative for OCT4 by immunohistochemistry (IHC).

The aim of the present study was to characterize OCT4 immunohistochemical expression in canine cutaneous MCTs, in order to investigate possible associations with histological differentiation grades, mortality and post-surgical survival.

Twenty-eight cases of canine cutaneous MCTs were selected. Dogs were treated in the Veterinary Hospitals at Universidade de São Paulo, Universidade Metodista de São Paulo and Universidade Anhembi-Morumbi. After complete excision, tumour samples were processed routinely for histopathology. Sections (4 μm) were stained with haematoxylin and eosin (HE) (Prophet *et al.*, 1992) and graded, according to the methods of Patnaik *et al.* (1984) and Kiupel *et al.* (2011), by three veterinary pathologists who had no clinical information about the cases. The final histopathological grade was defined by consensus. The criteria for inclusion in the present study were: adequate amount of tissue; complete clinical follow-up (minimum 180 days); treatment by surgery with no adjuvant radiotherapy or chemotherapy. Censored data were deaths not related to the disease and dogs that were alive at the end of the follow-up.

IHC was performed in the Laboratório de Oncologia Comparada e Translacional at Faculdade de Zootecnia e Engenharia de Alimentos, Universidade de São Paulo. Sections were placed onto silane-treated slides, dewaxed in xylene for 30 min and rehydrated in graded alcohols and distilled water. Endogenous peroxidase was blocked by incubation of the slides in H_2O_2 3% in methanol. Antigen retrieval was achieved by heating the slides at 90°C in citrate buffer for 25 min in a steamer. In order to block non-specific interactions, samples were incubated with 5% skimmed milk for 20 min. A primary mouse polyclonal anti-OCT3/4 antibody (clone C-10, code sc-5279, Santa Cruz Biotechnology, Santa Cruz, California, USA) was applied overnight at a 1 in 50 dilution. The sections were incubated with secondary antibody (Advanced HRP, code K406889-2, Dako, Carpinteria, California, USA) for 25 min. Labelling was 'visualized' with 3, 3′-diaminobenzidine and sections were counterstained with Harris' haematoxylin. For negative controls, primary antibody was replaced with normal mouse IgG under the same conditions and the positive control was a section of a human seminoma.

OCT4 labelling was classified by two observers simultaneously as nuclear and/or cytoplasmic or negative. Since OCT4 is a transcription factor, only nuclear labelling was considered positive. OCT4 positivity was compared with histopathological grades (Patnaik *et al.*, 1984; Kiupel *et al.*, 2011) using the ANOVA/Kruskal–Wallis and Mann–Whitney tests. Mortality due to the disease was evaluated with Fisher's exact test. Finally, survival data were analysed by the Kaplan–Meier method, followed by log-rank test. Significance was defined as 5%. Data were analysed with GraphPad Prism software (Version 4.02 for Windows, GraphPad Software).

Thirty-six MCTs from 28 dogs were studied. The mean age was 9.6 years and 22 of the dogs were female (61.2%). Crossbred dogs were the most common (10/28, 29.1%), followed by boxers, dachshunds, Labradors and poodles (3/28, 10.7% each) and Brazilian fila (2/28, 7.1%). The remaining breeds were the American pitbull, doberman pinscher, miniature pinscher and schnauzer. Five dogs had two lesions and one dog had four lesions. In these cases, the disease was classified according to the lesion of highest grade, rendering 10 grade I, 10 grade II (10/28, 35.7% each) and eight grade III MCTs (8/28, 28.6%) when using the three-tier system (Patnaik *et al.*, 1984); or 18 low-grade and 10 high-grade cases, when the two-tier system was applied (Kiupel *et al.*, 2011).

OCT4 immunolabelling varied among the samples, 21 showed cytoplasmic and nuclear labelling (Fig. 1), while in six tumours only the cytoplasm was positive (Fig. 2) and one sample was negative for OCT4. Six grade I (6/10, 60%), nine grade II (9/10, 90%) and six grade III MCTs (6/8, 75%) were positive for OCT4. In the two-tier system, thirteen (13/18, 72.2%) were positive for OCT4 among the low-grade MCTs and eight (8/10, 80%) among the high-grade MCTs. No significant difference was found between histopathological grades using ANOVA/Kruskal–Wallis ($P = 0.3144$) followed by

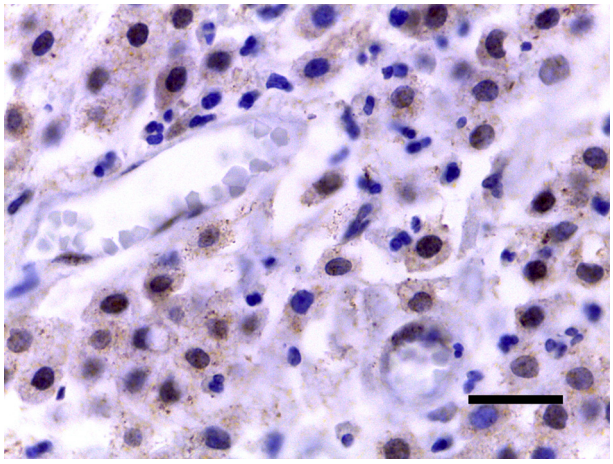

Fig. 1. Mast cell tumour showing nuclear and cytoplasmic expression of OCT4. IHC. Bar, 20 µm.

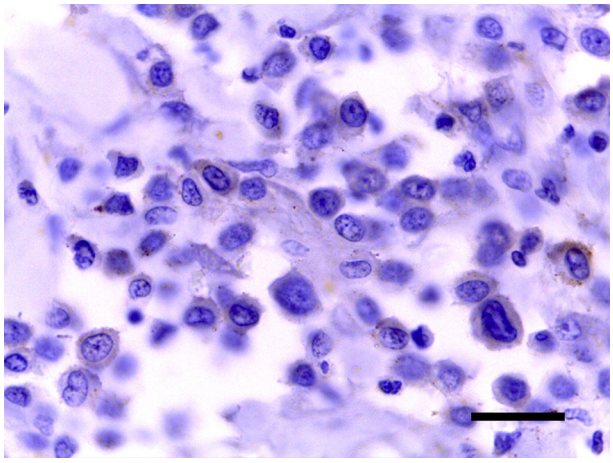

Fig. 2. Mast cell tumour showing cytoplasmic expression of OCT4, with no nuclear labelling. IHC. Bar, 20 µm.

Dunn's multiple comparison post-test ($P > 0.05$) and Mann–Whitney test ($P = 0.7493$). Nine of the 10 dogs (90%) that died due to the MCT and 12 of the 18 (66.7%) that were censored in the survival analysis were positive for OCT4 ($P = 0.3642$, Fisher's exact test, Table 1). Therefore, OCT4 expression by IHC was not a prognostic indicator for post-surgical survival (Chi-square = 1.267, $P = 0.2603$, median survival for OCT4-positive dogs = 751 days; Fig. 3).

Table 1
Distribution of the mortality rates as a function of OCT4 expression

	Mortality		*Alive at the end of the study*
	Due to MCTs	*Other cause*	
$OCT4^+$ ($n = 21$)	9 (42.8%)	3 (14.4%)	9 (42.8%)
$OCT4^-$ ($n = 7$)	1 (14.3%)	4 (57.1%)	2 (28.6%)
Total ($n = 28$)	10 (35.7%)	7 (25.0%)	11 (39.3%)

Fisher's exact test, $P = 0.3642$; sensitivity = 0.90; specificity = 0.33.

OCT4 nuclear labelling was not demonstrated in embryo samples, human primitive germ cell tumours (Lenardo *et al.*, 1989), osteosarcomas (Levings *et al.*, 2009), normal endometrium (Matthai *et al.*, 2006) and thyroid glands (Thomas *et al.*, 2006). Gidekel *et al.* (2003) showed abundant OCT3/4 expression in germ cell tumours, but not in lung, breast, endometrial, prostate, thyroid, skin and brain tumours. In a study with testicular tumours, all seminomas and embryonic carcinomas showed nuclear positivity (De Jong *et al.*, 2005). Mançanares *et al.* (2013) identified OCT4-positive cells in the metanephros, primordial gonad and hepatic parenchyma, as well as in the yolk sac cells of bovine embryos. Pluripotency was successfully induced in canine fibroblasts by transduction of OCT4 and SOX2 transcription factors (Gonçalves *et al.*, 2012). Tai *et al.* (2005) investigated OCT4 immunolabelling in 30 different normal canine tissues and found that only few scattered cells in the basal layer of the epidermis were positive, the normal location for epidermal stem cells.

In the present study, almost all MCTs had some OCT4 immunolabelling, nuclear or cytoplasmic, corroborating previous observations by Webster *et al.* (2007). These authors found that, among the 21 different canine tumour types investigated, the highest levels of OCT4 expression were in MCTs. OCT4 expression was investigated at the protein and mRNA levels by others, but the results were not conclusive due to the presence of pseudogenes and different isoforms of the protein (De Jong and Looijenga, 2006). Moulay *et al.* (2013) showed negative OCT4 expression in canine prostate carcinomas by polymerase chain reaction (PCR) and reverse

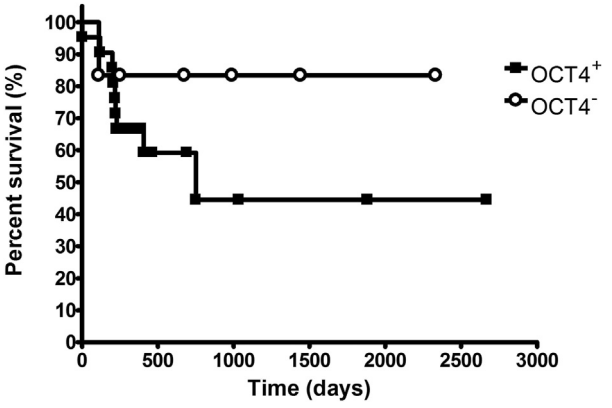

Fig. 3. Survival curves for dogs with cutaneous MCT according to OCT4 expression ($P = 0.2603$, Chi-square = 1.267, median survival for OCT4-positive dogs = 751 days, log-rank test). Points indicate the time at which survival was censored.

transcriptase PCR. Despite the expression of OCT4 in canine osteosarcomas, it was not labelled by IHC (Wilson *et al.*, 2008). In canine mammary tumours, only peripheral cells of cultured spheroids and single cells were positive for OCT4 expression (Ferletta *et al.*, 2011). Lim *et al.* (2012) did not find OCT4 expression in the adult canine brain.

The identical C-terminal structure of the two OCT4 isoforms increases the risk of false-positive results in protein studies (Liedtke *et al.*, 2008). Moreover, variation in the location of OCT4 is also related to the presence of different isoforms. OCT4A, which is believed to sustain stem cell properties, is confined to the nucleus (Cauffman *et al.*, 2006). On other hand, OCT4B shows cytoplasmic localization and is able to activate or repress the transcription of OCT4-responsive genes (Lee *et al.*, 2006). Considering these data and in order to better compare our results, we opted to use the same antibody used by Webster *et al.* (2007), which does not cross-react with the OCT4B isoform and only nuclear labelling was classified as positive in the present study.

Although not statistically significant, an additional interesting result was the high mortality rate observed in the group of dogs with nuclear OCT4 expression: 90% of the dogs died due to the MCTs versus 66% in the group of censored individuals. Due to this high mortality rate in OCT4-positive dogs, we believe that complementary studies with more samples of MCTs are needed to properly evaluate the role of OCT4 in the prognosis of this neoplasm.

To the best of our knowledge, this is the first study that compared OCT4 labelling with the histological grades, disease-related mortality and post-surgical survival in veterinary oncology. In order to confirm these preliminary observations and to better understand the role of OCT4 in canine cutaneous MCTs, ongoing studies aim to evaluate the expression of different spliced isoforms and other stem cell markers.

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