**Immunohistochemical expression of MDR1-Pgp 170 in canine cutaneous and oral melanomas: pattern of expression and association with tumour location and phenotype**

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**Abstract**

Canine melanoma (CMM) more commonly affects the oral mucosa and the cutis. CMM shares several features with human melanomas (HMM), included resistance to a broad variety of antineoplastic chemotherapy agents. P-glycoprotein 1 (Pgp) expression is a well-recognised feature of multi drug resistance and the purpose of this study was to investigate its expression in treatment naïve CMM. We also investigated Pgp association with tumour location and histological features. Histology records of CMM were retrieved, including patients from 2012-2014. Twenty-five cases of CMM were included in this study. Results revealed that Pgp is expressed in CMM and oral tumours were more likely to have a membranous Pgp expression (100%) than cutaneous tumours (66.6%) (p=0.010). Cytoplasmic and nuclear Pgp expression could be also identified. Results of this study bring useful data that help in understanding one of the possible mechanisms responsible of intrinsic chemotherapy resistance in canine CMM.

**Introduction**

Melanocytic tumours are relatively common in dogs, and occur with higher and similar frequency in the oral mucosa and skin.1 Canine malignant melanoma (CMM) is more often diagnosed than the benign counterpart (melanocytoma) and affects the oral mucosa where is also the most frequent malignant neoplasia; other common sites are the cutis, digits, and the eyes.1-2 CMM shares several clinical and histological features with human melanomas (HMM), representing a relevant preclinical model for people; however, the two diseases do not completely overlap.

As for oral HMM, prognosis for oral CMM is generally poor, with most of the patients presenting with locally invasive tumours that often show a highly metastatic potential during the course of the disease.3-4 Indicators of poor prognosis have been identified in histopathology and immunohistochemistry (IHC), and these are presence of a high mitotic index, nuclear atypia, lymphatics invasion, loss of pigmentation, infiltrative growth pattern, high cyclooxygenase 2 (COX-2) and a high Ki-67 expression.4-5 The most common sites for metastasis are the regional lymph nodes and lungs; however, widespread disease may also occur in certain cases.2 Treatment recommendations include local tumour control with curative intent surgery and/or radiation therapy together with systemic adjunctive therapy.6-9 Control of local tumour recurrence has been fairly successful so far but death due to metastatic disease remains a major issue. Reported systemic adjunctive therapy includes administration of chemotherapy or immune therapy but results are so far controversial and inconsistent.2,6,10-14 Especially concerning high dose chemotherapy, there is no evidence that this reduces the risk of metastasis or extends patients’ survival.2,6,14

The precise causes that underlie this therapeutic resistance are not well understood but they are likely to be mediated by multiple mechanisms: increased DNA repair, over expression of anti-apoptotic proteins, altered expression of oncogenes or tumour suppression genes, gene methylation-mediated silencing and increased levels of endogenous nitric oxide.15-18 Besides these mechanisms, one of the most incriminated is the adenosine triphosphate (ATP)-binding cassette (ABC) transporter system.19 This also represents the most common cause of multidrug resistance (MDR) in human cancer.20 Within these, the sub-family B member 1 (ABCB1) also known as P-glycoprotein 1 (Pgp) is one of the most investigated.20 Pgp overexpression has been associated with intrinsic or acquired chemotherapy resistance also in canine neoplasias.21

Besides being associated with intrinsic and acquired MDR phenotype, Pgp overexpression seems to be also associated with a more aggressive tumour cell phenotype in HMM, capable of increased migration and invasive potential.22 Despite the level of knowledge reached in HMM chemotherapy resistance related pathways, no investigations have been performed in the field of CMM. We hypothesised that Pgp could play a role in CMM, conferring an intrinsic MDR phenotype; therefore, the aim of this study was to investigate Pgp immunoreactivity in oral and cutaneous CMM, describing its pattern of expression and its association with tumour location and histological phenotype.

**Material and methods**

***Study Population***

Histology records of cutaneous (excluded digital) and oral mucosal CMM, submitted to the Laboratorio de Diagnóstico Histopatológico Histovet (Barcelona, Spain), were retrieved through database search, including dogs from January 2012 to December 2014. Cases were considered eligible for the study only if there was record of a positive immunohistochemistry staining for anti-Melan-A and if paraffin-embedded (FFPE) tissues were available for review. Patient’s signalment, history and clinical data were retrieved both from the pathology submission forms and via telephone calls to the referring veterinarians. When available, clinical staging was summarised according to the TNM and WHO systems.2 Dogs that had received steroids and/or antineoplastic chemotherapy agents within a month prior to the tissue collection were excluded from the study. The FFPEs that satisfied the mentioned criteria were enrolled and were subsequently divided into two groups: *Cutaneous CMM* and *Oral CMM*.

***Histopathology and immunohistochemistry***

All histological slides were originally diagnosed by three pathologists (MVC, JAP, MRR) and reviewed by a veterinary pathologist (LR) and a veterinary pathology resident (JMMR). PgP expression was scored by a pathologist (LR). The slides were originally prepared from FFPEs ﬁxed in 10% neutral buffered formalin, were routinely stained with haematoxylin and eosin (HE) and were observed under a bright ﬁeld upright microscope. HE examination served to establish a morphological characterisation of the tumours (epithelioid, spindloid or round cell type according to the predominant pattern) and for the semi-quantitative evaluation of anisocytosis (mild: no difference in cell size between melanocytes; marked: evident difference in cell size with cells two times larger than neighbour neoplastic cells; moderate: intermediate level between mild and marked), anisokaryosis (mild: no difference in nuclear size between melanocytes; marked: difference in nuclear size with some nuclei two times larger than the nuclei of other cells; moderate: intermediate level between mild and marked) and granularity (mild: granules absent or barely visible melanin granules in the majority of the cells; marked: large numbers of melanin pigmented granules within the cytoplasm of the cells, obscuring the majority of the cytoplasmic features; moderate: intermediate level between mild and marked).

 The mitotic index (MI) was calculated as the total number of mitotic figures in 10, tumour representative (randomly chosen in cutaneous melanomas*,* chosen within areas with the highest mitotic rate in oral melanomas), microscopic 400× (Ocular FN: 22; Objective 40x/0.65) high-power fields (HPFs). A MI of 3 or 4 was used as a cut off for cutaneous and oral melanomas respectively; the median MI was also calculated for both groups.4 Correlation between the MI chosen cut-offs (3, 4 or the median) and PgP expression was investigated.

Representative sections of the lesions were selected for immunohistochemistry (IHC), which was performed at the Section of Veterinary Pathology of the University of Liverpool (UK). All sections were deparaffinised in xylene and hydrated with graded ethanol concentration until distilled water. Antigen retrieval was performed by calibrated water bath capable of maintaining the epitope retrieval solution in 10mM sodium citrate buffer (pH 6.0) at 97°C for 30 minutes. The sections were allowed to cooldown to room temperature for 20 minutes. Endogenous peroxidase was blocked using 100µl Dako REALTM peroxidase blocking solution for 10 minutes (DAKO, Carpinteria, CA). Tumours sections were incubated with the primary antibody anti-PgP (C219 MoAb; BioLegend, London, UK) at 1:50 dilution, overnight at 4°C. The bound antibody was evaluated by peroxidase conjugated polymer (EnVision plus Detection KIT; Dako) for 30 minutes and diaminobenzidine tetrahydrochloride was used as detection system (DAB; Fisher Scientific, UK). Upon completion of the immunostaining, sections were counterstained with Mayer’s haematoxylin. Canine liver tissue was used as a positive control.23 Within tumour’s slides endothelial cells were considered as an internal positive control. The negative control consisted of substitution of the primary antibody with isotype matched murine immunoglobulin. Since bleaching of sections was not performed as previously reported5, a test negative control (all the IHC procedure except from primary antibody on case slides) was run for each tumour slide in order to maximise the stain detection in comparison with the test slide.

Positive Pgp signal was considered as a brown specific cellular stain. Pgp expression was graded according to the number of PgP positive cells and intensity of the PgP stain in 10 consecutive, tumour representative HPFs. The *quantity* of positive cells was expressed as the percentage of Pgp positive cells, which was semi-quantitatively evaluated in each of the 10 HPFs. The percentage of cells exhibiting membranous, cytoplasmic and/or nuclear stain was also recorded for each field.

The *intensity* of Pgp expression was scored as the predominant intensity in each HPF, for each cell compartment (membrane, cytoplasm, nucleus) as mild (barely discernible stain), moderate (intermediate intensity between mild and marked) marked (staining intensity comparable with external positive control or higher). A *quantity score* (QS: average of the percentage of positive cells in 10 HPF) and an *intensity score* (IS: average of the stain intensity in 10 HPF) was then obtained. The *Pgp score* in each compartment was obtained multiplying the *Pgp quantity score* with the *intensity score*. Therefore, the *Pgp membranous score* (Pgp-m) was the result of the product of percentage of cells expressing membranous Pgp signal and the intensity of expression, *Pgp cytoplasmic score* (Pgp-c) and *Pgp nuclear score* (Pgp-n) were calculated accordingly. Finally a *Pgp total score* (Pgp-t) was calculated for each tumour, taking into account the staining of all of the compartments and was calculated as follows: Pgp-m + Pgp-c + Pgp-n = Pgp-t. A summative formula to obtain the final score is the following:

*Pgp-t* = [*Pgp-m* = (mQS in 10 HPFs) \* (IS in 10 HPFs)] + [*Pgp-s* = (QS in 10 HPFs) \* (IS in 10 HPFs)] + [*Pgp-n* = (QS in 10 HPFs) \* (IS in 10 HPFs)]

***Statistical analysis***

Statistical association between Pgp expression and morphological parameters or clinical data was investigated using Mann-Whitney statistical tests, with or without Bonferroni adjustment, depending on group numbers. Statistical analysis was performed using SPSS 13 Software (SPSS 13.0, SPSS Inc, IBM Chicago, IL, USA).

**Results**

***Study Population***

Twenty-five FFPE samples of CMM were included in the study and there was a FFPE sample for each patient: 12 melanomas were assigned to the *Oral CMM* group and 13 to the *Cutaneous CMM* group.

In the *Oral CMM* group there were 3 Cocker Spaniel, 2 Standard Wire-Haired Dachshunds, 2 cross breed, and one each of a Bull Dog, Golden Retriever, Kerry Blue Terrier, Standard Poodle and Shar Pei. There were 7 intact males and 5 intact females; median age was 12 years (range 8-14 years). Information on the clinical staging work-up was available in 3 out of 12 patients: one dog had stage II, one stage III and one stage IV disease. In the *Cutaneous CMM* group there were 4 cross breed, 2 Rottweiler, and one each of a Basset Hound, Boxer, Giant Schnauzer, Golden Retriever, Labrador Retriever, Scottish Terrier and West Highland White Terrier. There were 9 intact males and 4 intact females; median age was 7 years (range 3-13 years). Information on the clinical staging work-up was available in 6 out of 13 patients and these were all T1N0M0.

***Histopathology***

In the *Oral CMM* group 83.3% of the cases (n=10) had a spindloid phenotype, whereas 16.7% were epithelioid type; no round cell type melanomas were observed. Anisocytosis was classified as mild, moderate or marked in 33.3% (n=4), 50% (n=6) and 16.7% (n=2) of the cases respectively. Anisokaryosis was classified as mild, moderate or marked in 8.4% (n=1), 58.3% (n=7) and 33.3% (n=4) of the cases respectively. Presence of pigment was detected in all the cases and it was classified as mild, moderate or marked in 50% (n=6), 33.3% (n=4) and 16.7% (n=2) of the samples respectively.

Concerning the MI, this could be assessed in all but one case due to marked pigmentation. The median MI was 5.4 (1.2-11.4) and 54.5% of the cases (n=6) had a mitotic index > 4, whereas 45.5% (n=5) had a mitotic index < 4.

In the *Cutaneous CMM* group 69% of the cases (n=9) had a spindloid phenotype, whereas 31% were epithelial type; no round cell type melanomas were observed. Anisocytosis was mild in the majority of the cases (76.9%, n=10) and was classified as moderate or marked just in the remaining 15.3% (n=2) and 7.8% (n=1) respectively. The majority of the samples showed mild (53.8%, n=7) to moderate (38.5%, n=5) anisokaryosis, whereas this was marked just in 1 case (7.7%). Presence of pigment was detected in all the cases and this was classified as mild, moderate or marked in 30.8% (n=4), 38.4% (n=5) and 30.8% (n=4) of the samples respectively. Concerning the MI, this could be assessed in all but one case with marked pigmentation. The median MI was 1.8 (0.4-7.2) and 25% of the cases (n=3) had a mitotic index > 3, whereas 75% (n=9) had a mitotic index < 3.

***PgP Immunohistochemistry***

Pgp staining was detected in external and internal controls as a membranous staining. In tumour samples the stain was variable in terms of number of positive cells and intensity; the staining pattern was membranous and/or nuclear and/or cytoplasmic. In some samples the staining was predominantly membranous (Fig.1A), cytoplasmic (Fig. 1B) or nuclear (Fig.1C), while in other cases the pattern was mixed within the same tumour (Fig. 1D). In the *Oral CMM* group, Pgp membranous staining was detected in all the samples, exhibiting mild (41.6%, n=5), moderate (41.6%, n=5) or marked (16.8%, n=2) immunoreactivity. The median Pgp-m was 35 (5-160). Cytoplasmic staining was detected positively in 83.3% of the cases (n=10), exhibiting mild (75%, n=9) or moderate (8.4%, n=1) immunoreactivity. The median Pgp-c was 50 (0-160). Pgp nuclear staining was detected positively in 58.3% of the cases (n=7), exhibiting mild (16.6%, n=2), moderate (25%, n=3) and marked (16.6%, n=2) immunoreactivity. The median Pgp-n was 30(0-210). The median Pgp-t in this group was 170 (10-220).

In the *Cutaneous CMM* group, one of the cases was excluded from IHC evaluation, as an accurate Pgp score could not be determined due to the strong pigmentation (the same case for which the MI could not be assessed). Within the evaluable cases, membranous staining was detected in 66.6% of the cases (n=8), exhibiting mild (50%, n=6) or moderate (16.6%, n=2) immunoreactivity. The median Pgp-m was 5 (0-40). Pgp cytoplasmic staining was detected positively in 83.3% of the cases (n=10), exhibiting mild (66.6%, n=8) or moderate (16.6%, n=2) immunoreactivity. The median median Pgp-c was 45 (0-160). Pgp nuclear staining was detected in 41.6% of the cases (n=5), exhibiting mild (16.6%, n=2), moderate (16.6%, n=2) and marked (8.4%, n=1) immunoreactivity. The median Pgp-n was 0 (0-180). The median Pgp-t in this group was 70 (0-340).

Subsequently, Pgp-t in each group (*Oral CMM* and *Cutaneous CMM*) was compared to granularity, anisokaryosis, anisocytosis, histological subtype and MI among groups and no statistically significant association was observed with any of the variables listed. When Pgp-m, Pgp-c, Pgp-n and Pgp-t were compared among groups, all the scores did exhibit similar trends and in particular Pgp-m was significantly higher in the *Oral CMM group* compared to the *Cutaneous CMM group* (p=0.010; Figure 2). Due to the small number of patients enrolled in the two groups, statistical analysis was also performed considering the whole study population (*Oral CMM* plus *Cutaneous CMM*); although statistical significance was again not reached, for higher Pgp-t there was a trend towards increase in anisokaryosis, anisocytosis, and loss of granularity (figure 3). Concerning the MI, no correlation or trend was observed with any of the Pgp scores, regardless of the cut-off chosen (3, 4 or the median).

**Discussion**

Pgp, is an ATP-dependent efflux pump that is a member of a family of ATP-binding cassette (ABC) transporters.20-21 The normal function of Pgp is not completely known, but it has been found to be physiologically expressed in human and canine tissues and it has been suggested to normally function as a drug efflux pump showing a protective effect against toxic substances.21,24 Conversely, at some stage of several neoplastic diseases, Pgp may become overexpressed on the cell plasma membrane of neoplastic cells; this process is often associated with the occurrence of MDR phenotypes, either spontaneously or after initial chemotherapy.20-21,23 For example, in cell cultures of canine mammary carcinoma and in canine lymphoma patients, Pgp expression increases significantly after administration of chemotherapy compared to base line where this can be undetectable.25-27 It is therefore understandable why a strong Pgp immunoreactivity and/or altered expression of other ABC family transporters, is believed to represent a negative prognostic factor in cancer patients.28 However, this has been broadly investigated in humans but not in veterinary oncology, where it has been demonstrated just for canine lymphoma patients.23,26-27,29

Melanoma is a chemotherapy refractory tumour and its mechanisms of chemotherapy resistance have been widely investigated in human patients; although this appears complex and still need to be fully elucidated, the role of ABC family transporters is for some authors one of the most relevant.19,22

Concerning Pgp, its expression seems to vary significantly depending on melanoma anatomical location. Investigations in cutaneous melanoma found 3 and 4% positivity in primary tumours and metastases, respectively.30 In contrast, Pgp has been found to be far more frequently expressed in non-cutaneous melanomas such as ocular (42%) and uveal (80%), representing a negative prognostic marker.31-32

Results of our study confirmed the hypothesis that Pgp is expressed in CMM and that this is present in treatment naïve patients conferring a peculiar phenotype, with a pattern of protein distribution similar to HMM cells. Conversely, we noted that Pgp was not expressed just in oral tumours but also in all the cutaneous melanomas at least in one of the subcellular compartment. This discrepancy between our results and the results reported by Fuchs and colleagues may highlight a different level of protein expression in cutaneous CMM.30 However some cross reactivity of the MoAB C219 for other ABC family transporters could not be excluded (for example with the highly homologous ABCB5); although this doesn’t seem to be an issue for many authors, we considered this possibility as it has been demonstrated to occur in fishes.33

Pgp is known to be mainly expressed on the plasma cell membrane, however studies have suggested that intracytoplasmic and nuclear localisation are not uncommon among tumours, which support our findings.23,25,27,34-37 Nuclear Pgp is likely to be involved in the active removal of cytotoxic drugs and/or intracellular compounds from deoxyribonucleic acid (DNA), whereas Pgp located in the cytoplasm may play a link role in transport of toxics between the nucleus and the cell surface.38-39 Concerning melanoma, studies have confirmed the wide intracellular distribution of ABC family transporters to vesicles, endosomes, lysosomes and melanosomes and have specifically ascribed the cytoplasmic localisation of Pgp mainly to the Golgi apparatus.19,40-41 Nuclear Pgp localisation has been also described, however studies rarely focus on this cellular compartment and data on prevalence are therefore scarce.19,41 Results of our study highlighted that oral CMM were more likely to have a predominant membranous Pgp expression (100%) than cutaneous tumours (66.6%). Cytoplasmic protein expression was often present in both groups (83.3%) followed by nuclear Pgp expression; although the latter was less common, it could be identified in almost half of the patients in both groups (58.3% vs 41.6%). These IHC results revealed that the MDR phenotype of CMM cells seems as complex and peculiar as the HMM counterpart, highlighting a further similarity between these two species. The reason for such a wide distribution of Pgp has been explained in humans through the melanogenic model, a mechanistic system adopt to link drug resistance and melanogenesis.42 Melanogenesis consists of three main components, the melanosome biogenesis, melanin synthesis and endogenous melanogenic toxicity (EMC) related homeostasis. Melanogenesis depends on melanosome biogenesis and goes through four stages of maturation based on change in melanosome morphology. According to the melanogenic model, melanogenesis is divided into three main phases: phase I (stage I- early stage II), phase II (stage II- early stage IV) and phase III (stage IV and melanin release). At the end of the third phase, integrity of the melanosome is compromised and the maturation process generates the maximal EMC. In normal melanocytes the ABC transporter system regulates the homeostasis by trapping cytotoxic melanin intermediates into subcellular organelles, and then exporting those from the cell. In melanoma cells, the same system is thought to confer this peculiar MDR phenotype. We refer the reader to the review article from Chen *et al* for a more detailed explanation on the melanogenic model of MDR.19

Analysis of Pgp expression between groups revealed a significantly higher Pgp-m intensity score value in oral CMM compared to cutaneous CMM (p=0.010; Figure 2). The same significant difference could not be documented for the other Pgp categories, although a positive trend was observed in the *Oral CMM* group. The higher Pgp-m score observed in the *Oral CMM* groupmay reflect the aggressive biological behaviour that distinguish oral CMM from the cutaneous counterpart.43 In support of this theory should be also mentioned that high levels of Pgp expression have been associated with a more invasive phenotype an enhanced metastatic potential in HMM cells.22 However this cannot be confirmed from our results due to the lack of sufficient follow up data. When specific tumour morphology features where considered, regardless of tumour location, data analysis failed also to demonstrate a statistically significant association between Pgp-t and features known to be prognostic in CMM. However, we noted that for higher Pgp-t there was a trend towards increase in anisokaryosis, anisocytosis and loss of granularity (Figure 3). Although it is possible that Pgp may not be linked to any specific tumour feature in canine patients, it should also be considered that the low number of patients may have affected results and that a link between Pgp expression and a more aggressive tumour phenotype may exist.

Pgp IHC expression was not homogeneous within the tumours and in addition different staining patterns were demonstrated, including a previously unreported nuclear pattern in dogs. This led to the creation of a, “ad-hoc” rather complex semiquantitative scoring system, which is in line with the complexity of previously published systems of PgP immunohistochemical scoring of other neoplasms.27

Our findings suggest that treatment naïve CMM have a complex pattern of Pgp expression that may be more pronounced in oral tumours compared to cutaneous neoplasias. Chemotherapy resistance is thought to be a multifactorial process, however the results of this study may provide a potential explanation for the lack of efficacy observed in CMM trials.2,6,14-18 Although additional studies would be required to investigate this hypothesis, it is in the authors’ opinion that the use of compounds substrate for Pgp should be considered carefully in the treatment of CMM. This seems to be supported by the human literature where the benefit of chemotherapy is scarce and positive results have been recorded just with methylating agents (not substrate for Pgp).44-46 Further investigations are also necessary to clarify the role of Pgp as a mediator of tumour invasion and therefore it’s potential as a tumour prognostic marker.

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**Captions to figures:**

**Figure 1**: Patterns of PgP immunohistochemical expression among tumours: A: Dog, oral melanoma: predominant PgP membranous stain: cells exhibit predominant stronger membranous stain (circle) in the majority of the cells. B: Dog, oral melanoma: predominant PgP cytoplasmic stain: the stronger PgP stain is diffuse within the cytoplasm (circle) in the majority of the cells. C: Dog, oral melanoma: predominant nuclear stain: the stronger PgP stain is localised within the nucleus (circle) in the majority of the cells. Endothelial cell (internal control) exhibit membranous positive stain (arrow). D: Dog, cutaneous melanoma: PgP mixed expression pattern. Within the neoplasm membranous (black arrow), cytoplasmic (white arrow) and nuclear (arrowhead) are detected. Immunoperoxidase, scale bars = 50 microns.

**Figure 2**: PgP scores among melanoma groups (Oral vs cutaneous). PgP-t: PgP total score (blue bars); PgP-c: PgP cytoplasmic score (green bars); PgP-n: PgP nuclear score (beige bars); PgP-m: PgP membranous score. Black orizontal bars within boxes represent the median value; Asterisk: significant difference between groups. Circles: outlayers.

**Figure 3**: PgP scores in association with morphological criteria: anisokaryosis (A), anisocytosis (B) or granularity (C). PgP-t: PgP total score (blue bars); PgP-c: PgP cytoplasmic score (green bars); PgP-n: PgP nuclear score (beige bars); PgP-m: PgP membranous score. Black orizontal bars within boxes represent the median value; Asterisk: significant difference between groups. Circles: outlayers.