**Immunohistochemical expression and prognostic significance of MAGE-A in canine oral melanoma**

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**Summary**

Canine oral malignant melanoma (COMM) is considered a chemoresistant cancer with a poor long-term prognosis. The melanoma-associated antigen A (*MAGE-A*) genes, which belong to the cancer-testis antigen family, are expressed in several different canine cancers but not in normal somatic tissue. This study evaluates the expression of MAGE-A proteins and their prognostic role in COMM.

The study was conducted in 2 parts. During the first part, biopsies from oral malignant melanomas from 43 dogs were examined and immunohistochemically assessed for expression of MAGE-A proteins. For the second part, the association between MAGE-A expression and outcome was assessed using follow-up data which was available for 20 dogs whose primary tumour had been controlled with surgery +/- radiation therapy.

MAGE-A proteins were expressed in 88.4% (38/43) of oral malignant melanomas and had a predominantly cytoplasmic expression pattern. Immunopositivity was observed in more than 50% of the cells in 21 dogs (48.8%). Immunostaining intensity was classified as weak, moderate and intense in 16 (37%), 16 (37%) and 6 (14%) cases, respectively. No staining for MAGE-A was seen in 5 dogs (11%). Dogs whose COMM had weak MAGE-A staining intensity had a median survival time (MST) of 320 days while this was 129 days for dogs with moderate and intense immunostaining (p=0.161). Dogs whose COMM had >50% of positive staining neoplastic cells had a MST of 141 days and dogs with a staining <50% had a MST of 320 days (p=0.164). MAGE-A expression did not influence survival in our cohort.

**Key words**: immunohistochemistry, MAGE-A, melanoma, cancer testis antigens

**Abbreviations:** COMM: canine oral malignant melanoma; MAGE: melanoma-associated antigen; TAA: tumour associated antigen; CT: computed tomography; WHO: world health organization; IHC: immunohistochemistry; TMA: tissue microarray; MST: median survival time; MI: mitotic index; HPF: high power field.

**Introduction**

Canine oral malignant melanoma (COMM) accounts for 30-40% of all malignant oral neoplasia (Smith et al., 2002; Liptak and Withrow, 2007) and represents a significant clinical problem because of its aggressive and invasive local behaviour and propensity to rapid metastasis. Although loco-regional control of COMM using combined surgeryand radiotherapy may initially be successful(Proulx et al., 2003), the prevention and treatment of metastatic disease using chemotherapy has been considered unrewarding with a 1-year survival rate <30% even after adjuvant treatment (Bergman, 2007; Bostock, 1979).

Although platinum drugs have shown modest activity on gross disease (Rassnick et al., 2001), there is little evidence that chemotherapy provides a survival benefit in these patients (Murphy et al., 2001; Bergmane et al., 2013; Cancedda et al., 2016).

There is therefore a clear need for more effective anti-metastatic treatments. Melanoma is considered an immunogenic tumour type and among all the immunotherapy strategies that have been investigated in dogs (MacEwen et al., 1986; Grosenbaugh et al., 2011; Bergman et al., 2003; Alexander et al., 2005; Westberg et al., 2013; Quintin-Colonna et al., 1996), the one that has garnered the most attention and resulted in a commercial product is the Oncept™ tyrosinase targeting plasmid DNA vaccine. However, overall efficacy and which patient groups benefit the most from the vaccine remains controversial due to discordant results of published evaluations (Almela & Anson, 2019; Vail, 2013; Ottnod et al., 2013).

Cancer testis antigens (CTAs) are of great interest as a target for anti-cancer immunotherapy (Esfandiary & Ghafouri-Fard, 2015). Attractive characteristics include expression in a broad range of tumours and limited or no expression in adult somatic tissue (Weon and Potts, 2015; Chen et al., 2013). The melanoma-associated antigen (MAGE) family of CTAs was the first discovered and has been widely investigated. The MAGE protein family is subdivided into two categories: type I and type II MAGEs. Type-I MAGEs, which include MAGE-A, -B and –C sub-families, are found on the X-chromosome and are selectively expressed in adult reproductive tissues (testis, ovarium and placenta). Type-II MAGEs include MAGE-D, -E, -F, -G, -H, -L subfamilies and necdin, these genes are not restricted to the X chromosome and are expressed in many tissues in the body (Weon and Potts, 2015).

MAGE-A protein expression has been associated with malignant behaviours such as increased tumour growth, presence of metastases, increased tumour recurrence after therapy and ultimately worse prognosis in several human cancers (Rastgoosalami et al., 2016; Li et al., 2015). The mechanism by which MAGE-A proteins lead to these effects are still being unravelled. Broadly, MAGE proteins interact with E3 ring ubiquitin ligases to form MAGE-RING ligases (MRLs). As MRLs they act as regulators of ubiquitination by modulating subcellular location and substrate specificity as well as ligase activity (Lee & Potts, 2017). Consequently, their actions are highly context dependent (and far from fully understood) but functions relevant to the hallmarks of cancer have been identified for many of the MAGE-A proteins. One frequent feature relevant to their oncogenic activity is regulation of p53 in collaboration with TRIM28 (Lee and Potts, 2017; Weon and Potts, 2015).

Type I MAGE protein expression has been documented in a wide variety of cancer types in humans including synovial sarcoma, colon, lung adenocarcinoma, melanoma, glioma, breast, multiple myeloma and prostate (Iussich et al., 2017; Sang et al., 2016; Barrow et al., 2006; Li et al., 2015). In humans, expression of MAGE-A was found in 16-20% of primary cutaneous melanomas and 48-51% of metastatic lesions (Brasseur et al., 1995). Similarly, MAGE-A4 expression occurs in around 60% of human mucosal oral head and neck melanomas (Prasad et al., 2004).

Expression of MAGE-A proteins has been evaluated in dogs (Chen et al., 2013; Nemec et al., 2019). Immunopositivity was demonstrated in COMM (75%), nasal tumours (68.7%), round cell tumours (52.5%) and soft tissue sarcomas (40.5%). In contrast, oral squamous cell carcinomas, multicentric lymphomas, extraosseous osteosarcomas and a variety of normal tissues (except spermatozoids and oocytes) showed no expression (Chen et al., 2013).

The primary goal of this study was to evaluate MAGE-A protein expression in dogs with COMM. As survival data was available for a subset of these COMM patients with local disease control, we also took the opportunity to assess whether expression of MAGE-A protein was associated with outcome. Our hypothesis was that MAGE-A expression would be inversely associated with survival.

**Material and methods**

This study was conducted in two parts: the first part consisted of a descriptive immunohistochemical study of MAGE-A expression in tissue samples from dogs diagnosed with COMM and the second part was a preliminary investigation of the possibility of an association between MAGE-A immunopositivity, immunostaining, and clinical outcomes. Ethical approval was granted by the Veterinary Research Ethics Committee (University of Liverpool, School of Veterinary Science, Neston) prior initiation of the study (VREC 300).

*Sample collection*

The samples used for assessment of expression of MAGE-A protein were derived from tissues submitted to the clinical services at Bridge Pathology and the University of Liverpool. Tissue specimens had been sent by first opinion practices and referral clinics in the UK. The subset of material from the University of Liverpool was then used for the second part.

*Clinical data collection*

For the second part of the study, patients histologically diagnosed with COMM at the University of Liverpool Small Animal Teaching Hospital between 2015 and 2018 were prospectively enrolled.

Data pertaining to signalment, tumour location, tumour size, disease stage, treatment protocol, adverse effects, and survival time was obtained from medical records and by telephone follow-up if needed.

Staging work-up for all dogs included physical examination, total blood cell count and complete biochemistry profile, computed tomography (CT) of head, thorax and abdomen, tumour measurement, fine needle aspirates of regional lymph nodes regardless of their size and ultrasound-guided fine needle aspirates of any suspicious lesion identified in CT scan images to confirm or rule out metastasis. All dogs were staged according to the World Health Organization (WHO) clinical staging system (Owen, 1980).

*Treatment*

All patients enrolled in the second part of the study underwent either surgical resection of the primary tumour and of the metastatic lymph nodes when these could be surgically excised limiting morbidity for the patients, radiotherapy or both. Immunotherapy was also offered and performed depending on owner’s preferences. Radiotherapy was delivered using a linear accelerator (Clinac 2100 or Varian VitalBeam, Varian Medical Systems, Palo Alto, CA, USA). Patients received 4 x 8Gy or 9Gy (one fraction per week for 4 consecutive weeks). Photon energies were 6MV and 10MV, and electron energies 9MeV and 12MeV. For planning CT and treatment delivery, patients were anaesthetised, and immobilized using a thermoplastic mask and a customised head support (Qfix, Avondale, USA), secured to a plastic head-frame with four points of fixation, as appropriate. Computerised plans were generated from Computed Tomography ([CT] images using Pinnacle version 8/9 (Pinnacle, Philips Radiation Oncology Systems, Philips Healthcare, N.A., USA) with the intent to include at least 95% of the planning treatment volume in the 95-105% isodose. Manual plans included the scar or gross tumour plus a 3-5cm margin when possible.

The ipsilateral submandibular lymph nodes were routinely irradiated, and retropharyngeal and contralateral nodes irradiated when deemed clinically indicated. Margins around lymph nodes were 1cm (combined clinical target volume and planned target volume).

*Tissue fixation and preparation*

All tissues were fixed in 10% neutral buffered formalin and subsequently paraffin wax embedded following routine protocols. All haematoxylin-eosin stained specimens were reviewed by a board-certified pathologist (LR) and a diagnosis of COMM was made based on the histological criteria described by Smedley et al. (2011). Diagnosis was further confirmed with immunohistochemical analysis of Melan-A and PNL-2 as previously described (Giudice et al., 2010). For the tumour samples from dogs included in the part II, the mitotic index (MI) was calculated as the total number of mitotic figures in 10, tumour representative (microscopic 400×; ocular FN: 22; objective 40x/0.65) high-power fields (HPFs) (~~randomly chosen in cutaneous melanomas,~~ chosen within areas with the highest mitotic rate in oral melanomas), microscopic ×400 (Ocular FN: 22; Objective ×40/0.65) high-power fields (HPFs). A MI cut off was not used as a criteria for inclusion in this study.

*Microarray construction and immunohistochemical staining*

Tissue in wax blocks, previously diagnosed as melanomas, were labelled with a marker pen to identify the area of interest of the original tumour (peripheral areas or necrotic areas were avoided), which corresponded on the same area on the histological slide. Three core areas from each tumour were selected and were then inserted in a recipient paraffin block in a precisely spaced, array pattern 4-micron sections from the recipient block were cut using a microtome and mounted on a microscope slide. For immunohistochemistry (IHC), sections were dewaxed and subjected to antigen retrieval in Dako PT buffer high/low pH (Agilent Technologies Ltd, Stockport, UK) using a computer controlled antigen retrieval workstation (PT Link; Agilent Technologies Ltd) for 20 min at 98°C. Sections were then stained in an automated immunostainer (Link 48; Agilent Technologies Ltd), using primary antibodies against MAGE-A (mouse anti-human MAGE-A monoclonal antibody, 6C1 1:500). This antibody has been previously validated in dogs via Western blot analysis by Chen et al. (2013). This was followed by a 30 min incubation at room temperature with the secondary antibody and polymer peroxidase-based detection system (Anti Mouse/Rabbit Envision Flex+, Agilent Technologies Ltd). The reaction was visualised with both diaminobenzidine (DAB- Agilent Technologies Ltd) and LPR (Liquid Permanent Red - Agilent Technologies Ltd). Consecutive sections incubated with murine subclass-matched unrelated monoclonal antibody served as negative controls. The positive reaction was represented by a distinct brown or red cytoplasmic or nuclear reaction, as previously described (Chen et al., 2013). Since bleaching of sections was not performed a test negative control (all the IHC procedure except from primary antibody on case slides) was run for each tumour slide in order to maximize the stain detection in comparison with the test slide as previously performed (Finotello et al., 2017). Dog testis served as a positive control.

*Immunohistochemical interpretation*

Semiquantitative analysis of the slides was performed by assessing the percentage of MAGE-A positive tumour cells and intensity of the staining for each case. Positivity of tumour cells was assessed by two oncology specialist trainees (AG and KS) and reviewed by a board-certified anatomic pathologist (LR) at ×100 magnification and punctuation was given as a percentage as follows: 0%, <25%, 26–50%, 51-75% and >75%. This parameter was named “immunopositivity”. Tumours were considered positive for ~~CTAs~~ MAGE-A if weak immunostaining was present in at least 1–25% of tumour cells. The intensity of tumour cells staining was assessed at 100× magnification and scored from 0 to 3. A score of 0 represented no staining; 1, weak staining (barely visible ~~brown~~ staining); 2, moderate staining (a staining in between weak and intense) and 3, intense (strong as positive control) staining. This parameter was named “immunostaining intensity”. When specimens showed a range of intensity, the ~~median~~ prevalent intensity was recorded. All evaluations were performed on each core (3 cores per tumour) and results averaged.

*Statistical analysis*

Patient baseline characteristics and immunohistochemistry scores were summarised and compared using the IBM SPSS statistics (version 26.0, IBM Corp, Armonk, NY, USA). The nonparametric Mann-Whitney *U* test was used to compare tumour size, mitotic index, nodal and distant metastasis and immunostaining intensity scores; whereas the Kruskal-Wallis test was used to compare tumour location with immunostaining intensity scores. Comparison of the clinicopathological parameters and immunopositivity was performed using the χ² test. If counts were lower than 5, Fisher exact tests were used. Overall survival was calculated from the date of diagnosis to the date of death and the Kaplan-Meier method was used to report the probability of survival. Patients alive at the completion of the study were censored from the survival analysis. The relationship between MAGE-A expression parameters (immunopositivity and intensity scores) and survival was compared with the log-rank test. For categorical variables a P value of ≤0.05 was considered significant.

**Results**

*Part I: Descriptive analysis of MAGE-A staining in canine malignant oral melanoma*

Tissue samples obtained from COMMs affecting 43 dogs were available for immunohistochemistry. MAGE-A immunopositivity was observed in 38 cases (88.4%): staining was present in <25% of the cancer cells in 8 samples (18.6%), between 25-50% in 9 samples (20.9%), between 51-75% in 8 samples (18.6%) and >75% in 13 samples (30.2%). Immunostaining intensity was scored as 0 in 5 dogs (11.6%), 1 in 16 dogs (37.2%), 2 in 16 dogs (37.2%) and 3 in 6 dogs (13.9%). The staining was predominantly cytoplasmic, however nuclear stain was also detected. These results are summarised in Table 1.

From the 20 COMM samples with available patient follow-up, the median mitotic index was 6 in 10 HPF (range: 0-30). Eighteen (90%) had positive immunostaining for MAGE-A protein (Figure 1 and 2). Immunopositivity was found in <25% of cancer cells in 4 samples (22.2%), between 26–50% in 2 samples (11.1%), between 51-75% in 1 sample (5.5%) and >75% in 11 samples (61.1%). The immunostaining intensity was scored as 0 in 2 dogs (10%), 1 in 5 dogs (25%), 2 in 9 dogs (45%) and 3 in 4 dogs (20%).

*Part II: Study population and correlation with outcome*

Twenty dogs were prospectively enrolled. There were 7 male neutered (35%), 7 male entire (35%) and 6 female entire (30%). Median age at diagnosis was 10 years (range: 5-14). Breeds included: Golden retriever (5), mixed breed dogs (4), Labrador retriever (4), Bull terrier (1), American bulldog (1), Weimaraner (1), English springer spaniel (1), German shepherd dog (1), Bouvier de Flanders (1) and a Cocker spaniel (1).

The tumour was located in the mandible in 6 dogs (30%), in the maxilla in 8 dogs (40%) and in the upper or lower lip in 6 dogs (30%). Median longest dimension was 2.8 cm (range: 0.8 - 5cm).

According to the WHO classification, 6 dogs were stage I, 6 dogs were stage II, 6 dogs were stage III and 2 dogs stage IV.

Fifteen (75%) dogs underwent surgical excision of the primary tumour, achieving complete excision in 4 dogs, incomplete excision in 9 dogs and in 2 dogs margins assessment was not reported. Sixteen dogs (80%) were treated with radiotherapy: 5 dogs received treatment on macroscopic disease and 11 dogs on microscopic disease post-surgery. Fifteen dogs received 32 Gy of radiation in four 8 Gy fractions and 1 dog 36 Gy in four 9 Gy fractions. Eight dogs (30%) received treatment with immunotherapy with the Oncept® melanoma vaccine (Merial, Duluth, GA, USA). Two dogs (10%) received metronomic cyclophosphamide (15mg/m2/day rounded to the nearest available tablet size). Two dogs (10%) did not receive adjuvant treatment after surgery (Table 2).

*MAGE-A expression in relation to patient’s clinico-pathological parameters*

The relationship between patients’ clinical and pathological parameters and tumour cell MAGE-A immunostaining positivity and intensity scores is shown in Table 3. MAGE-A immunostaining intensity in oral melanoma tissues was not associated with patient’s age, tumour size, tumour location, nodal and distant metastasis or mitotic index. Similarly, the proportion of immunopositive tumour cells was not associated with clinical and pathological variables, including stage and mitotic index. However, both dogs with stage IV disease had 100% of their cells immunolabelled.

*MAGE-A expression in relation to prognosis*

Median time of follow up for all dogs was 298 days. Two dogs were still alive at the end of the study (751 and 799 days).

Overall median survival time for all dogs was 226 days (range: 35-899). Median survival time for dogs with stage I was 692 days, for stage II was 218 days, for stage III was 123 days and for stage IV 224 days. One of the dogs was classified as stage IV based on the presence of a single pulmonary nodule (1.2cm in maximum diameter) on CT scan images and lived 373 days; though given the prolonged survival this nodule may not have represented metastatic disease.

In order to further investigate the prognostic value of MAGE-A expression in canine melanoma, the log-rank analysis was performed to correlate the MAGE-A expression intensity and proportion of immunolabelled cells in cancer tissues to survival data.

Median survival time for dogs with intensity staining score 1, 2 and 3 was 320 days, 141 days, and 159 days, respectively (Figure 3). ~~The MST~~ For dogs with weak MAGE-A expression (score 1) the MST was 320 days and for dogs with moderate and intense (score 2 and 3) ~~MAGE-A expression MST~~ was 159 days (p= 0.141).

Median survival time for dogs with >50% of positive staining neoplastic cells was 141 days while dogs with a staining <50% had a MST of 320 days (Figure 4) (p= 0.164).

**Discussion**

Prediction and prevention of metastatic disease in canine oral malignant melanoma remains a significant clinical problem. Despite novel molecular therapies the outcome for these patients overall remains poor, emphasising the need for new therapeutic strategies (Almela et al., 2019).

MAGE-A proteins have many of the characteristics which are considered ideal as target tumour antigens for active immunotherapy approaches, and human MAGE-A3 is considered one of the most attractive targets for immunotherapy in human oncology (Cheever et al., 2009; Esfandiary & Ghafouri-Fard, 2015; Salmaninejad et al., 2016). This study characterized the immunohistochemical expression of MAGE-A protein in canine oral malignant melanoma. In this cohort of dogs, MAGE-A was expressed in 88.4% of cases with a predominant cytoplasmic location, ~~however~~ with nuclear stain ~~was~~ also detected. A previous study evaluated MAGE-A IHC expression in a variety of canine neoplasia and this was positive in 81.1% of COMM and the majority of samples had >70% of positively stained cells (Chen et al., 2013). In humans with cutaneous melanoma, the expression of MAGE-A was found in 16-20% of primary tumours and 48-51% of the metastatic lesions (Brasseur et al., 1995).

Aberrant expression of MAGE-A in human tumour cells is associated with alterations in cellular processes and signalling pathways that contribute to tumorigenesis (Weon and Potts, 2015). Moreover, this expression results in more aggressive behaviours and a poorer outcome in a number of tumour types (Rastgosalaami et al., 2016; Li et al., 2015, Tarnowsk et al., 2016). One small study evaluated MAGE-A4 expression in human head and neck mucosal melanoma (Prasad et al., 2004): whilst the results did not reach statistical significance there was a trend towards decreased survival time in patients with positive MAGE-A4 expression. In another study, MAGE-C knockdown delayed tumour formation of metastatic melanoma in vivo and in another study MAGE-B knockdown suppressed the growth of melanoma cells in a syngeneic mouse tumour model (Weon and Potts, 2015).

The overall median survival in our population was 226 days despite a predominance of stage I and II disease. This is lower than in Ottnod et al.’s study where a multimodal approach including immunotherapy was used to treat dogs with COMM achieving a MST of 477 days but similar to other studies that included radiotherapy as part of their treatment approach (Proulx et al. 2003).

Outcome in canine COMM has not previously been assessed in relation to MAGE-A protein expression. In this pilot study, MST for dogs with weak MAGE-A expression was longer (320 days) compared to dogs with moderate and intense MAGE-A expression (159 days). When analysing MAGE-A immunopositivity, median survival for dogs with >50% of positive staining neoplastic cells was shorter (141 days) compared to dogs with a staining <50% (320 days), although this difference was not statistically significant. Chen *et al.* (2013) also described a level of 100% of cells stained in the 4 dogs with stage IV disease, as observed in our study. Of possible interest, the two dogs alive at the end of the study had a negative MAGE-A expression. The numeric differences did not reach significance in our cohort and therefore an association between MAGE A expression and patient survival was not found.

The *MAGE-A* family is composed of over 15 highly homologous genes. The antibody used in this study is believed to have affinity for at least 6 of the protein products (Chen et al., 2013); as such, given the fact that the function of MAGE proteins in dogs has not been evaluated, it is possible that expression of some proteins are prognostically relevant and others not. Unfortunately, this could not be evaluated as there are no antibodies with affinity limited to a single canine MAGE-A protein currently available and producing such an antibody would be difficult given the extreme homology between the proteins.

This study has some limitations; a combination of treatment modalities including surgery, radiotherapy, chemotherapy and immunotherapy was used. Based on the small number of patients for each subgroup, no statistical analysis was performed to assess the influence of different treatments on survival.

In terms of assessment of expression by IHC heavily pigmented areas of tumours were difficult to score, even though two different stains were used and MAGE-A stained slides were always compared with the negative control. In addition, only tissue microarrays were evaluated and this may not be representative of the whole tumour sample, although multiple cores were evaluated in each case. ~~Although~~ While staining intensity and immunopositivity are standard methods of defining biomarker expression, this is a semi-quantitative assay subject to both preanalytical and interobserver variations with a lack of reference standards and whole slide digital imaging may soon provide a faster and more precise method of antigen quantification. It cannot be excluded that patient or tumour characteristics influence *MAGE-A* expression, but no correlation between MAGE-A immunopositivity or intensity and clinico-pathological parameters (patient’s age, tumour size, tumour location, or nodal and nodal metastasis, mitotic index) was found in this study. Similarly, Chen *et al*. (2013), also investigated MAGE-A expression in relation to patient characteristics but no association was found.

In regard to prognostic assessment, the most significant limitation is the small population of dogs available for prognostic evaluation. This small size limits the effect size that could be identified by this study and increased the risk of type II statistical error. In this context, a prospective study using a larger patient cohort, would allow for a multi-variable analysis and detection of a smaller effect size. Conceptually a small effect size would be of less use as a prognostic marker, but would still provide support for targeting MAGE-A proteins with immunotherapy. The work presented here provides the basis for determining the size of the required patient cohort for such a study.

**Conclusion**

MAGE-A proteins are expressed in the majority of canine oral melanomas providing them an interesting opportunity for further evaluation and development as anti-melanoma therapy. This study supports the need for a larger study to evaluate the possibility of an association between MAGE-A protein expression and patient survival in COMM.

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**Conflict of interest**

The authors declare no conflict of interest.

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**Table 1.** Immunopositivity and intensity scores of MAGE-A immunohistochemistry in 43 canine oral malignant melanomas.

**Table 2.** Clinical presentation and tumour characteristics of the 20 dogs with oral malignant melanoma included in the second part of the study.

**Table 3**. Correlation between clinical and pathological parameters and MAGE-A immunostaining intensity and immunopositivity in 20 dogs with oral melanoma.

**Figure 1**. Examples of TMA stained with MAGE-A antibody and negative control. A: negative control of sample in “B”; B: example of weak stain in 50-75% of cells (same sample as in “A”). C: Example of moderate stain in >75% of cells. D: Example of intense stain in >75% of the cells. Scale Bar = 50 microns. Indirect immunoperoxidase stain.

**Figure 2**. High power microphotographs of examples of TMA stained with MAGE-A antibody (LPR Red Chromogen). A: example of weak stain in 75% of the cells; B: Example of moderate stain in 50-75% of the cells in a sample with pigmented melanocytes; Inset: the difference in stain between a MAGE positive stain (arrow), melanin (black arrowhead), and a negative cell (white arrowhead). C: Example of intense stain in 50-75% of the cells. Scale Bar = 50 microns. Indirect Immunoperoxidase stain (LPR).

**Figure 3**. Kaplan-Meier survival analysis for dogs based on MAGE-A immunohistochemical staining intensity.

**Figure 4**. Kaplan-Meier survival analysis for dogs based on the % of positive cells for the MAGE-A immunostaining.