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# MicroRNA expression in formalin-fixed-paraffin-embedded samples of canine cutaneous and oral melanoma by RT-qPCR

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## Abstract

MicroRNAs (miRNAs) are a class of small, non-coding RNA that post-transcriptionally regulate protein expression. miRNAs are emerging as clinical biomarkers of many diseases including tumors. The aim of this study was to investigate whether miRNA expression could vary in melanoma samples derived from formalin-fixed-paraffin-embedded (FFPE) tissues.The study included four groups: a) 9 samples of oral canine malignant melanoma; b) 10 samples of cutaneous malignant melanoma; c) 5 samples of healthy oral mucosa; d) 7 samples of healthy skin. The expression levels of six miRNAs–miR-145, miR-146a, miR-425-5p, miR-223, miR-365 and miR-134–were detected and assessed by RT-qPCR using TaqMan probes. Cutaneous canine malignant melanoma showed a decrease of the expression level of miR-145 and miR-365 and an increase of miR-146a and miR-425-5p compared to control samples. MiR-145 was also down-regulated in oral canine malignant melanoma. The miRNAs with decreased expression may regulate genes involved in RAS, Rap1 and TGF-beta signaling pathways, and up-regulated genes associated with phosphatidylinositol signaling system, adherens junction and RAS signalling pathways. In conclusion, miR-145, miR-365, miR-146a and miR-425-5p were differentially expressed in canine malignant melanoma and healthy FFPE samples, suggesting that they may play a role in canine malignant melanoma pathogenesis.



**Key words**: canine, cutaneous melanoma, dog, microRNAs, oral melanoma, RT-qPCR.

Melanocytic tumors are relatively common in dogs and can arise at different sites, although they mainly affect the oral mucosa and the dermis. [17,28] Among melanocytic neoplasms, malignant melanoma prevails in the oral cavity of dogs, accounting also for the most common oral canine malignant tumor.[42] Oral canine malignant melanoma has often an aggressive biological behavior, characterized by rapid invasion of neighboring structures, high propensity for regional and distant metastasis, and is therefore associated with a poor long-term prognosis.[28,42] Oral canine malignant melanoma resembles human malignant mucosal melanomas of the head and neck, which represent a fatal malignancy.[28] Conventional treatment for oral canine malignant melanoma involves surgical resection and/or radiation of the primary tumor often resulting in efficient local tumor control,[5,32] while treatment of metastatic disease has shown little promise.[34, 45, 6]

Cutaneous malignant melanoma is the third most common malignant skin tumor in dogs[49] representing 27% of all canine malignant melanomas.[15] Cutaneous malignant melanoma is thought to have a less aggressive behaviour than those of humans, although metastases are reported in up to 30-75% of the cases, potentially resulting in a poor prognosis. [7, 43] Surgical excision with wide margins represent the treatment of choice.[8]

Previous studies have investigated the correlation between patient survival and the clinicopathological variables, and suggested that clinical tumor staging, location, completeness of excision, adjunctive treatment, mitotic index, Ki-67 index, level of infiltration and cell pigmentation have a prognostic impact; however, results have been sometimes conflicting.[37,43,41,9,48,18,40] The molecular profile of canine melanoma has been only recently investigated. Comparison of the transcriptome profiles of canine cutaneous melanocytoma and melanoma identifyied 60 differentially expressed genes involved in collagen metabolism and extracellular matrix remodelling. [10]

MicroRNAs (miRNAs) are a group of small RNAs, with around 19 to 25 nucleotides, resulting from cleavage of larger non-coding RNAs. They act as post-transcriptional regulators of gene expression.[4] MiRNAs have been associated with several molecular pathways including modulation of proliferation, apoptosis, differentiation, and cell cycle regulation; thus dysregulation of their expression may contribute to a variety of diseases, and disrupt pathways of fundamental importance in development of neoplasia.[11] During tumorigenesis, some miRNAs that negatively regulate oncoproteins (i.e. tumor-suppressor miRNAs) are down-regulated, while those negatively regulating tumor suppressor genes (i.e. oncogenic miRNAs, or oncomiRNAs) are up-regulated.[27,46] The involvement of miRNAs in melanoma pathogenesis has been demonstrated in both humans [1,13,19,39] and dogs.[30,31,29,44] The identification of miRNAs associated with oral and uveal canine malignant melanoma has been investigated by Noguchi and colleagues[29] and Starkey and colleagues,[44] respectively, using a microarray hybridization approach. To the best of authors' knowledge, few data are available on the expression patterns of miRNA in oral and cutaneous canine malignant melanoma when compared to healthy controls. The aims of the present study were 1) to screen by RT-qPCR the expression of a panel of miRNAs, previously demonstrated to be related to melanoma (miR-425-5p, miR-134, miR-145, miR-146a, miR-223-3p and miR-365),[30,39,2,22,51,36,26,35] in a cohort of oral and cutaneous malignant melanoma; 2) to carry out functional enrichment analysis of target genes and functional interaction network analysis, to identify pathways affected by the differentially expressed miRNAs.

## MATERIALS AND METHODS

### Study population

Histopathology samples of cutaneous (non-digital) and oral mucosal malignant melanoma was computer searched including records from January 2012 to December 2013. Cases

were considered eligible only if blocks were available for review and tumors had positive staining for Melan A and PNL-2 antibodies with immunohistochemistry (IHC). Patient data were collected both from the pathology submission forms and via telephone calls to the referring veterinarians. The formalin-fixed paraffin embedded samples (FFPEs) that satisfied these criteria (19; 9 oral, 10 cutaneous) were enrolled and were subsequently divided into two groups: Cutaneous malignant melanoma and Oral malignant melanoma. Sample details are described in Supplemental Table S1.

## Histology and Immunohistochemistry

Cases selected were re-examined by a board-certified pathologist (LR) using hematoxylin and eosin stained slides under a brightfield microscope. Tumors were examined for quality of fixation and for areas of necrosis, inflammation or hemorrhage. The mitotic index (MI) was calculated as the total number of mitotic figures in 10, tumor-representative, 400× (Ocular FN: 22; Objective 40x/0.65) high-power fields (HPFs). For IHC, sections were dewaxed and subjected to antigen retrieval in Dako PT buffer high/low pH (Agilent Technologies Ltd) using a computer controlled antigen retrieval workstation (PT Link; Agilent Technologies Ltd) for 20 min at 98°C. Sections were then immunolabelled in an automated immunostainer (Link 48; Agilent Technologies Ltd), using primary antibodies against Melan A (mouse anti human Melan A, clone A103, Santa Cruz Biotechnology Ltd; 1:500), and PNL-2 (mouse anti human Melanoma marker PNL-2), Agilent Technologies Ltd (A4502); 1:400), as previously suggested.[38] This was followed by a 30 min incubation at RT with the secondary antibody and polymer peroxidase-based detection system (Anti Mouse/Rabbit Envision Flex+, Agilent Technologies Ltd). The reaction was visualized with diaminobenzidine (Agilent Technologies Ltd). Consecutive sections were incubated with murine subclass-matched unrelated monoclonal antibody, which served as a negative control. The positive reaction was represented by a distinct brown cytoplasmic reaction. A canine melanoma known to

express Melan A and PNL-2 was used as positive control. Only melanomas with more than 10% of positive cells with either Melan A or PNL-2 were included in the study (Supplemental Figure 1).[41]

## Control population

FFPEs of normal skin and oral mucosa originating from post mortem cases were used as negative controls representative for oral mucosa. Cases with no oral pathology and with non-tumor related cause of death were included.

## MiRNA extraction and real-time quantitative PCR

Upon observation with a brightfield microscope, a 2mm diameter area representative of neoplastic growth, with no areas of necrosis, hemorrhage or inflammation was selected and labelled on the histological slide (Supplemental Figure2a). The same area was then identified in the wax block and labelled (Supplemental Figure 2b). Using a disposable 2mm diameter biopsy punch with plunger (Miltex) the area of interest was sampled and extracted from the block (Supplemental Figure 2c); this tissue core specimen was subsequently placed in an Eppendorf tube (Supplemental Figure 2d) and used to extract small RNA.

Small RNAs were extracted from the core tissue specimens using miRNeasy Kit for FFPE blocks (Qiagen, catalog number 217504). The *Caenorhabditis elegans* miRNA cel-miR-39 (25 fmol final concentration) (Qiagen, catalog number 219610) was used as synthetic spike-in control due to the lack of sequence homology to canine miRNAs. The RNA extraction was then carried out according to the manufacturer's instruction. The reverse transcription was performed using the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, catalog number 4366596) using miRNA-specific stem-loop RT primers, according to the manufacturer's instructions. Reverse transcription reactions were performed in 15 μl volume

reactions containing 1.5 μl 10X miRNA RT buffer, 1 μl MultiScribe reverse transcriptase (50 U/μl), 0.30 μl 100 mM dNTP mix, 0.19 μl RNase Inhibitor (20 U/μl), 6 μl of custom RT primer pool and 3.01 μl of nuclease-free water. The custom RT primer pool was prepared combining 10 μl of each individual 5X RT primer to a final volume of 1000 μl; the final concentration of each primer in the RT primer pool was 0.05X each. Three μl of RNA were added to each RT reaction. Every RT reaction mixture was incubated on ice for 5 min, 16°C for 30 min, 42°C for 30 min and then 85°C for 5 min.

The qPCR experiments were designed following the MIQE guidelines. Small RNA TaqMan assays were performed according to manufacturer's instruction. The selection of miRNAs was based on previous publications in which these miRNAs were correlated to melanoma in dogs or humans. The selected primer/probe assays (Life Technologies) included cel-miR-39-3p (assay ID000200), hsa-miR-425-5p (assay ID001516), mmu-miR-134 (assay ID001186), hsa-miR-145 (assay ID002278), hsa-miR-146a (assay ID000468), hsa-miR-365 (assay ID001020) and hsa-miR-223-3p (assay ID002295).[30,39,2,22,51,36,26,35] Quantitative reactions were performed in duplicate in scaled-down (12 μl) reaction volumes using 6 μl TaqMan 2X Universal Master Mix II (Applied Biosystems, catalog number 4440044), 0.6 μl miRNA specific TaqMan Assay 20X and 1μl of the RT product per reaction on Eco Real Time PCR detection System (Illumina). The standard cycling program was 50°C for 2 min, 95°C for 10 min and 40 cycles of 95°C for 15 sec and 60°C for 60 sec. Data were normalized relative to the expression of cel-miR-39. MiRNAs expression levels were presented in terms of fold change normalized to cel-miR-39 expression using the formula $2^{-\Delta\Delta Cq}$.[24] Predicted targets consisting of significant up- or down-regulated miRNAs were computationally retrieved from the TargetScan (http://www.targetscan.org/vert_71/) and miRWalk (http://mirwalk.umm.uni-heidelberg.de/) databases. The predicted targets of either up- or down-regulated miRNAs identified by both databases were examined using DAVID

bioinformatic tool (https://david.ncifcrf.gov/), in order to perform functional annotation and biological pathway enrichment.

### Statistical analysis

Statistical analysis was performed using XLStat (AddinSoft, Inc., NY, USA). Statistical significance was accepted at $P \leq 0.05$. Data were tested for normality and homogeneity of variance using the Kolmogorov-Smirnov test. As data were not normally distributed, non-parametric statistical tests were applied. Kruskal-Wallis test was used to assess differences in miRNA concentrations between malignant melanoma groups and control groups. *P* values were adjusted using Bonferroni correction. Linear regression was used to investigate any relationship between differentially expressed -miRNAs and age. Spearman's rho test was performed to evaluate possible correlations among miRNA expression levels, mitoses, melanophages and melanocytes rates. Principal component analysis was performed to evaluate single correlations among miRNAs.

## RESULTS

### Study Population and Tumor Histology

The 9 oral and 10 cutaneous malignant melanoma were from dogs with a median age of 9 years (range 6-13), and predominantly of mixed breed (n=4), with Rottweiler (n=2), Cocker Spaniel (n=2) overrepresented among other breeds. Median mitotic index was 2.5 (range: 0.8 – 11.4).

### miRNAs Expression

To characterize the differences between groups, principal components analysis (PCA) on control and malignant melanoma groups was performed (Figure 1). Data points with a higher correlation had a smaller degree of separation within the chart; a probable correlation was predicted if the factors were within 45° of each other. Healthy samples were separated from

malignant melanoma groups in both skin (figure 1a) and oral (figure 1b) samples and were well correlated to each another. Cutaneous malignant melanoma samples were positively correlated; in oral malignant melanoma samples, miR365a and miR-145 were negatively correlated and no correlation was observed between miR-146a and miR-425.

The selected miRNAs were detected in all samples. Among these, miR-145-5p, miR-146a, miR-365 and miR-425-5p exhibited statistically significant differences among the malignant melanoma and control groups (Figure 2). Compared to the corresponding non-neoplastic tissue, miR-145 (figure 2a) was down-regulated in both cutaneous ($P$ = 0.0076; ratio of cutaneous malignant melanoma/healthy skin = -5.7) and oral malignant melanoma ($P$ < 0.0028; ratio of oral malignant melanoma /healthy oral mucosae=-7.6), miR-146a (figure 2b) and miR-425-5p (figure 2d) were up-regulated and miR-365 (figure 2c) was down-regulated in cutaneous malignant melanoma (miR-146a: $P$ = 0.02, ratio of malignant melanoma/healthy skin= 9.6; miR-425-5p: $P$ = 0.04, ratio of malignant melanoma/healthy skin= 4.9; miR-365: $P$ = 0.0087, ratio of malignant melanoma/healthy= -5.9), while there were no differences between oral malignant melanoma and control groups for these miRNAs. The expression levels of miR-223-3p and miR-134 were not different in malignant melanoma compared to healthy skin (figure 2e and f). The levels of differentially expressed miRNAs were not affected by the age of the dog (linear regression, $P$ > 0.05). To test the possible collinearity, Spearman correlation analysis of miRNAs was performed, suggesting that there was a positive correlation among miR-145, miR-146a, miR-365 and miR-425-5p relative concentration (data not shown). No correlation was observed between miRNAs and mitotic count.

## miRNA target prediction and pathway enrichment

To investigate the relevance to tumor development, predicted targets of either significantly up- or down- regulated miRNAs were computationally identified by using TargetScan and

miRWalk databases. The number of predicted targets shared by both databases included 602 for miR-145, 95 for miR-365, 103 for miR-146a and 98 for miR-425-5p. The mRNA enrichment was performed using the DAVID bioinformatic tool. The Gene Ontology analysis was carried out using DAVID at three different levels: molecular function, cellular component and biological process (Figure 3). Most Gene Ontology molecular function items mainly included genes involved in the regulation of transcription and serine/threonine kinase activity for both up- and down-regulated miRNAs. The enriched Gene Ontology terms in cellular component converged on genes associated with the cytosol, nucleus and nucleoplasm for both up- and down-regulated miRNAs. Down-regulated miRNAs may modulate ruffle and focal adhesion, which are involved in cell migration, whereas up-regulated miRNAs may regulate activin responsive complex, which is activated by TGF-beta and acts primarily through SMADs. The biological process items focused on the modulation of protein binding and transcriptional activator activity for both sets of miRNAs. The down-regulated miRNAs influence genes involved in the SMAD binding, RNA polymerase and DNA binding activity; up-regulated miRNAs genes are involved in the regulation of transcription. The KEGG pathway analysis was separately performed on the targets of the two sets of miRNAs. The most significantly enriched pathways for downregulated miRNAs were RAS, Rap1 and TGF-beta signaling pathways; and for upregulated miRNAs were phosphatidylinositol signaling system, adherens junction and RAS signaling pathway (Figure 4).

## Discussion

The role of miRNAs in canine malignant melanoma is only beginning to be defined. Using microarray and qPCR, Noguchi and colleagues[30] analyzed the expression pattern of miRNAs in oral canine malignant melanoma and observed that miR-520c-3p was up-regulated and that six other miRNAs (miR-126, miR-200a, miR-203, miR-205, miR-527b

and miR-713) were down-regulated compared to healthy oral mucosa. Moreover, they demonstrated an association between the down-regulation of miR-203 and shorter survival times. A recent study by Starkey and colleagues[44] identified nine miRNAs able to discriminate between metastatic or non- metastatic canine uveal melanomas, therefore suggesting their potential in predicting biological behavior. The present study investigated the expression of six miRNAs in FFPE-samples of oral and cutaneous canine malignant melanoma, and results suggest that specific miRNAs are differentially expressed in neoplastic versus normal tissue samples. Differentially expressed -miRNAs identified herein have previously been implicated in human melanoma and other neoplastic conditions, as molecular regulators of tumor development and progression; a similar mechanism of action is hypothesized from the result of our study. MiR-145 was down-regulated in both oral and cutaneous malignant melanoma, and so was miR-365 in cutaneous malignant melanoma. These were considered as potential onco-suppressor miRNAs in accordance to the knowledge from the human oncology literature. It has been demonstrated that miR-145 and miR-365 modulate tumor cell growth, invasion and metastasis by targeting *NRAS*[23] and *c-MYC,*[30] and *neuropilin1*,[2] respectively. The Gene Ontology and pathway analysis would suggest that miR-145 and miR-365 modulate cell migration and cell growth, influencing the RAS and RAP1 signaling pathways, among others.[3, 23] Liu and co-workers[23] demonstrated that expression level of miR-145 is lower in melanoma tissues than those in the matched adjacent normal tissues; conversely, *NRAS* levels are higher. A previous study also demonstrated that miR-365 influences the development of melanoma by targeting *BCL2* and *Cyclin D1,* which are respectively involved in apoptosis and cell cycle progression.[53] We could speculate that the lack of regulatory miR-145 and miR-365 observed in oral and cutaneous malignant melanoma samples, may be involved in a different aggressive behavior, which can be influenced by other molecular regulators.

MiR-425 and miR-146a were over-expressed in samples of cutaneous malignant melanoma. The Gene Ontology and pathway analysis showed that these miRNAs may target genes associated with cell proliferation, cell-cell adherens junction, TGF-beta signaling pathway and protein ubiquitination, which can contribute to tumor development and progression. In human medicine, miR-425 over-expression is associated with cell migration and invasion by targeting *CYLD*[50] and cell proliferation in gastric cancer,[52] hepatocellular carcinoma[16] and esophageal squamous cell carcinoma.[22] The function of miR-425 in melanoma is still debated; Chen and colleagues[12] demonstrated that the over-expression of miR-425/489 plays a pivotal role in the melanoma progression by activating the PI3K-Akt pathway. On contrary, Liu and co-workers[21] suggested that miR-425 inhibits cell proliferation and metastasis, and promotes apoptosis. The role of miR-146a has mainly been investigated in the context of immune response[47] while its role in human melanoma is still controversial. MiR-146a has been proposed as a negative regulator of immune response activation in melanoma by targeting *STAT1* and *IFN-gamma* in mice models, affecting melanoma migration, proliferation, and mitochondrial function as well as *PD-L1* levels.[25] A recent study reported that miR-146a directly targets *SMAD4*, promoting cell metastasis and invasion;[35] Forloni and colleagues[14] concluded that miR-146a plays a central role in the initiation and progression of melanoma by targeting *NUMB*, a suppressor of Notch signaling. Nonetheless, Raimo and colleagues[36] hypothesized that miR-146a has two synchronous but distinctive functions in human melanoma, the enhancement of tumor growth and the suppression of cell metastasis.

Brachelente and colleagues[10] characterized the transcriptome profiles of canine cutaneous melanocytoma and melanoma using a transcriptomic approach, identifying 60 differentially expressed -genes. The comparison between this list and the list of genes potentially modulated by differentially expressed -miRNAs identified in the present study, showed that four genes (*ADAM metallopeptidase with thrombospondin type 1 motif 2* (*ADAMTS2*),

*Coiled-Coil Domain Containing 80* (*CCDC80*), *Lysyl Oxidase* (*LOX*) and *Cysteine Rich Secretory Protein LCCL Domain Containing 2* (*CRISPLD2*)) may be potentially modulated by miR-145 and miR-365, and one (*SH3 Domain GRB2 Like Endophilin Interacting Protein 1* (*SGIP1*)) by miR-146a and miR-425-5p. The RNA-seq results[10] showed that *ADAMTS2, CCDC80, LOX and CRISPLD2* are up-regulated in cutaneous malignant melanoma, while *SGIP1* is down-expressed; these results are consistent with the reduced or increased expression of molecular modulators, such as miR-145 and miR-365 and miR-146a and 425-5p, respectively.

In conclusion, the present study suggests that miR-145, miR-365, miR-146a and miR-425 are abnormally expressed in cutaneous and oral malignant melanoma, potentially modulating pathways involved in cell proliferation. Further studies are needed in order to elucidate the real molecular targets of these miRNAs and to identify candidate targets for molecular therapies in the treatment of canine malignant melanoma

## Conflict of Interest Statement

The authors declare no competing interests.

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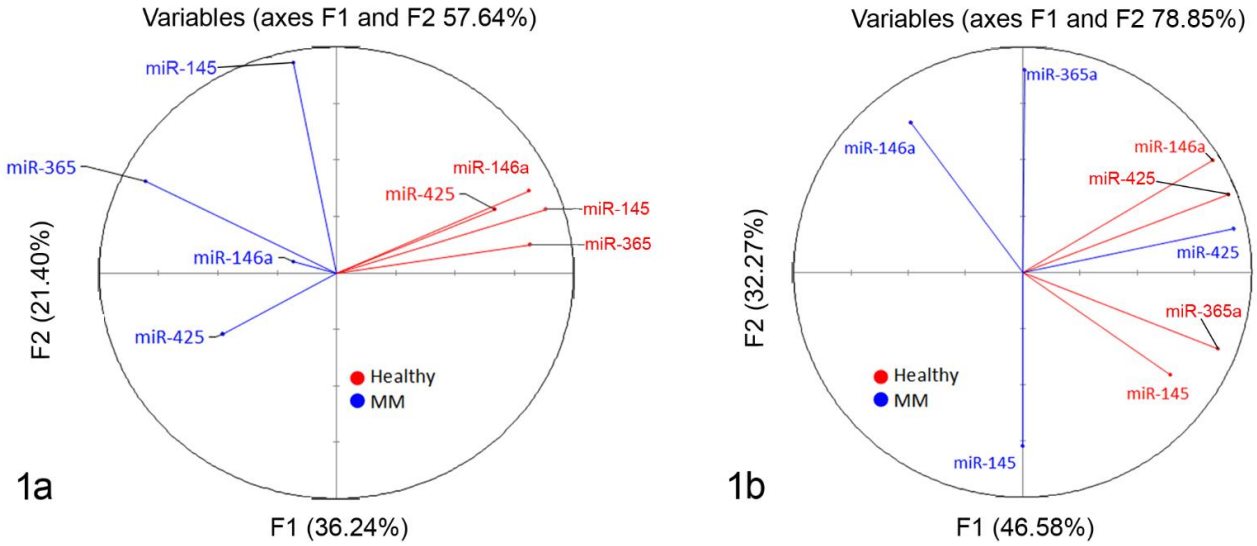
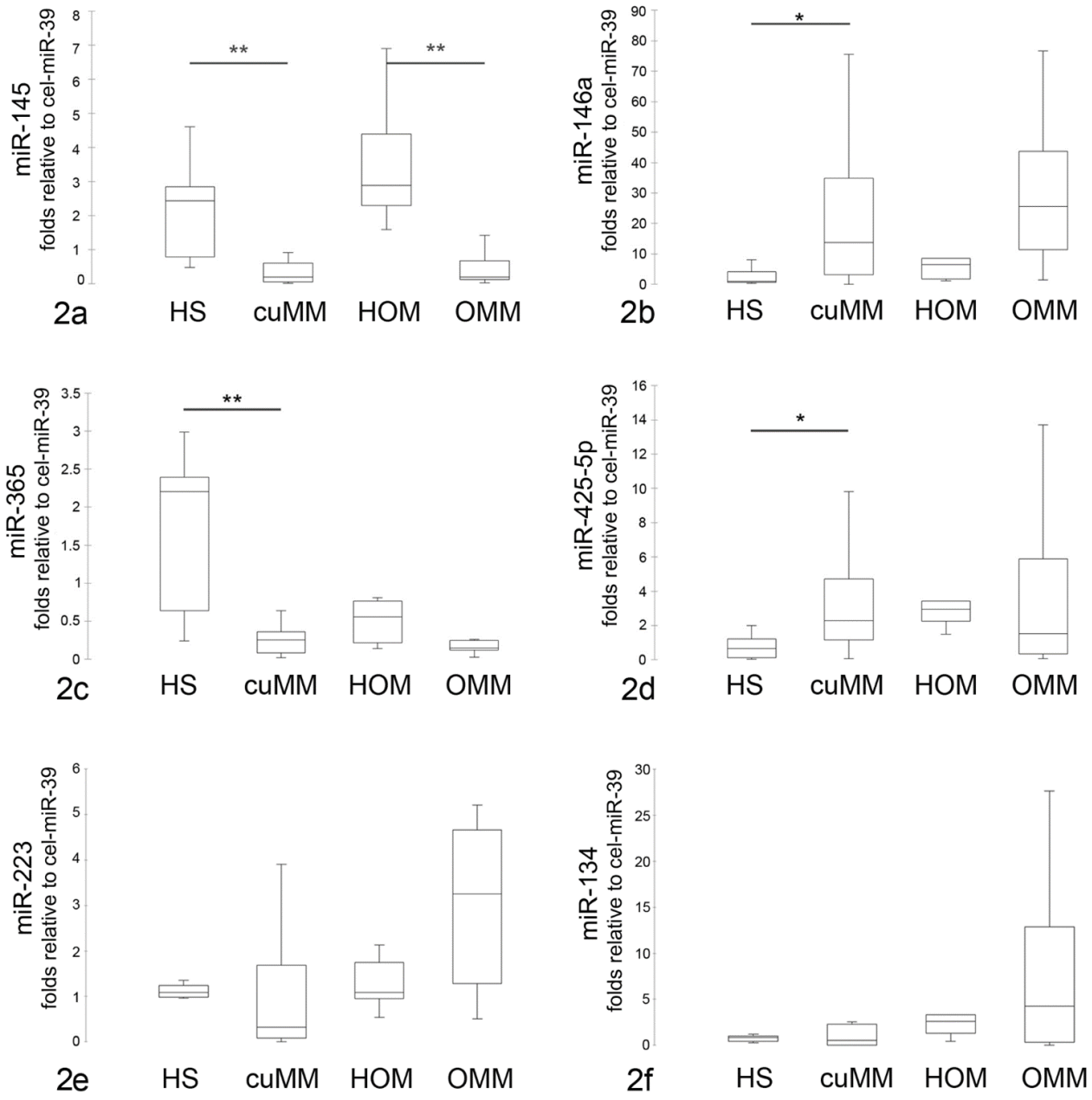
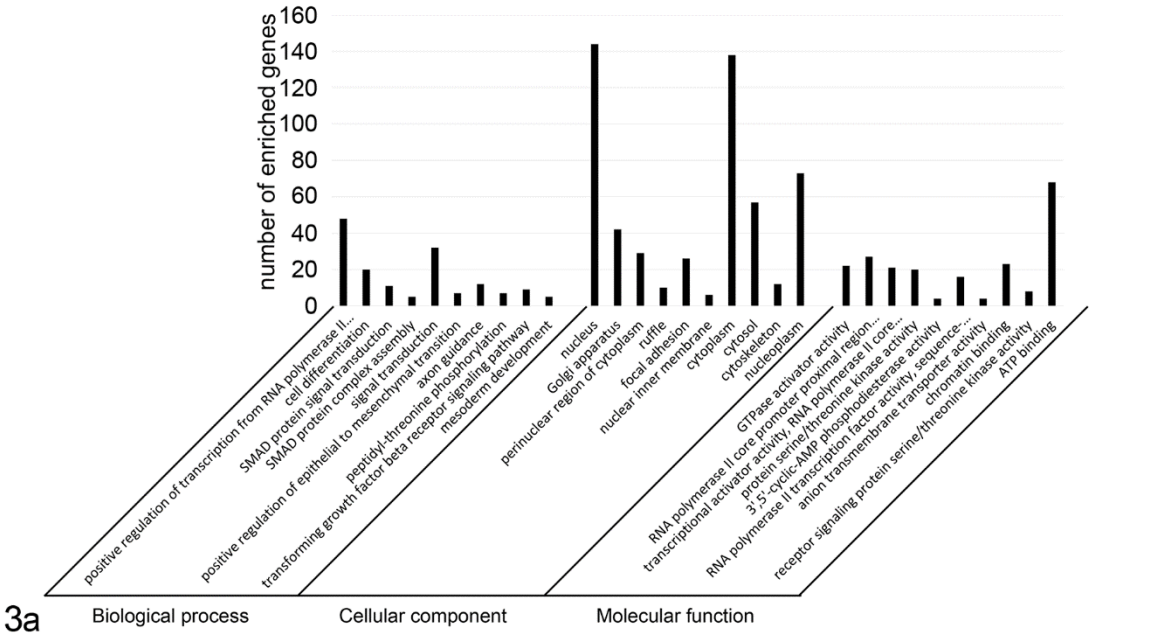



Figure 1. Principal components analysis of miRNA expression in canine malignant melanoma . The correlation circle shows the correlations between the miRNAs in (a) healthy skin and cutaneous canine malignant melanoma (MM) samples, and (b) healthy oral mucosa and oral canine malignant melanoma samples. Variables with a higher correlation have a smaller degree of separation within the chart; a probable correlation can be predicted if the factors are within 45°. If two variables are far from the center and close to each other, they are significantly positively correlated; orthogonal variables are not correlated; and variables on the opposite side of the center line are negatively correlated.




480

481 Figure 2 Expression of miRNAs in canine malignant melanoma. qPCR results were
482 normalized using cel-miR-39 as reference miRNA and the formula $2^{-\Delta\Delta Cq}$. Expression levels
483 of miR-145 (a), miR-146a-5p (b), miR-365 (c), miR-425-5p (d), miR-223 (e) and miR-134 (f)
484 in healthy skin (HS), cutaneous malignant melanoma (cuMM); healthy oral mucosa (HOM),
485 and oral malignant melanoma (OMM). The boxes outline the quartiles, the horizontal line
486 shows the median, and the whiskers show the range. *= $P < 0.05$, **= $P < 0.01$.



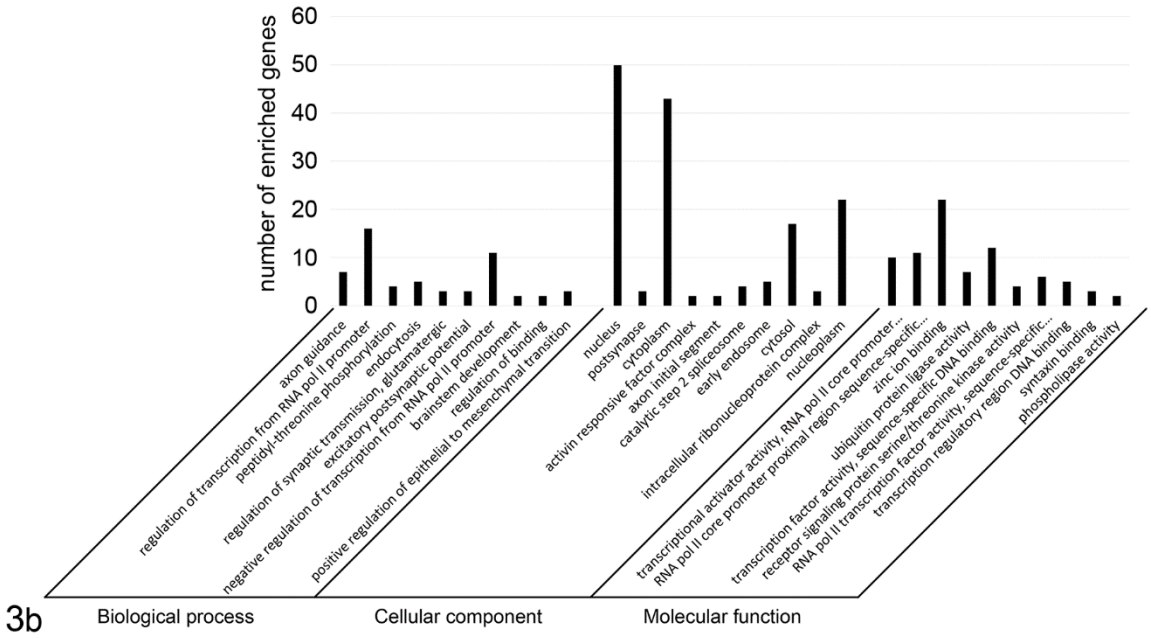
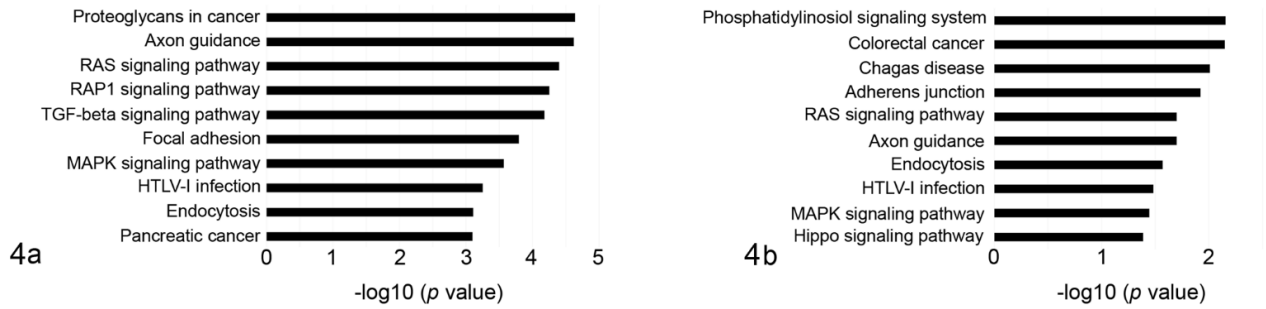



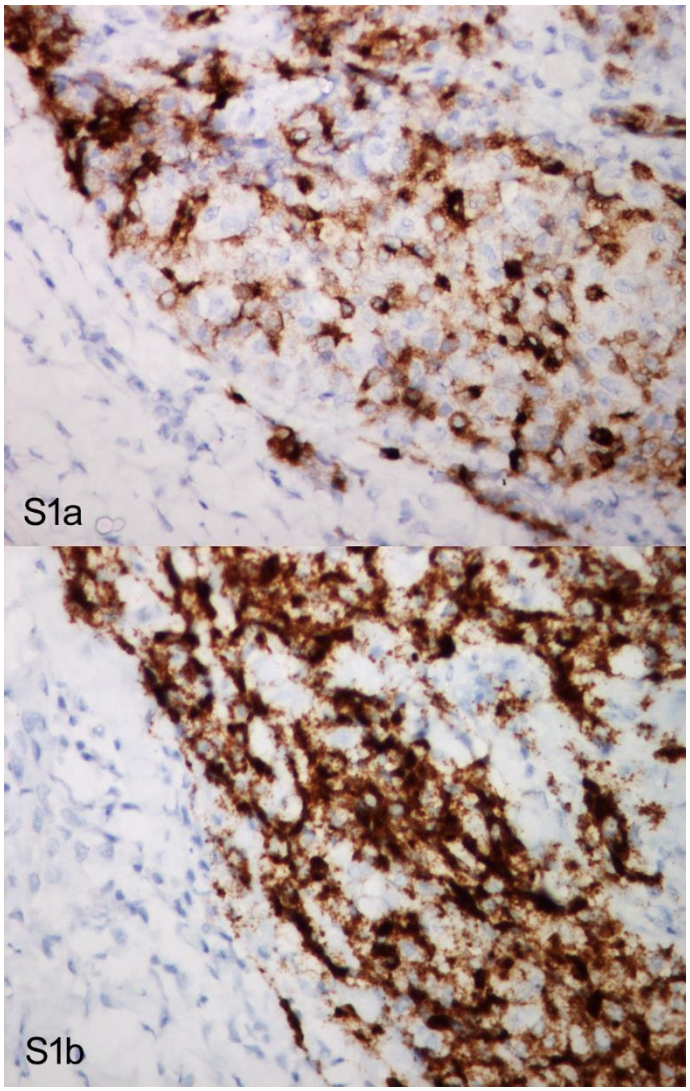

Figure 3. Gene Ontology annotation of genes predicted to be regulated by miRNAs that were found to have significantly lower or higher expression in canine malignant melanoma samples compared to the corresponding healthy control tissues. The targeted genes were annotated by DAVID tool at three levels, including biological process, cellular component and molecular function. (a) Gene Ontology annotation of genes regulated by down-regulated miR-145 and miR-365. (b) Gene Ontology annotation of genes regulated by over-regulated miR-146a-5p and miR-425-5p.



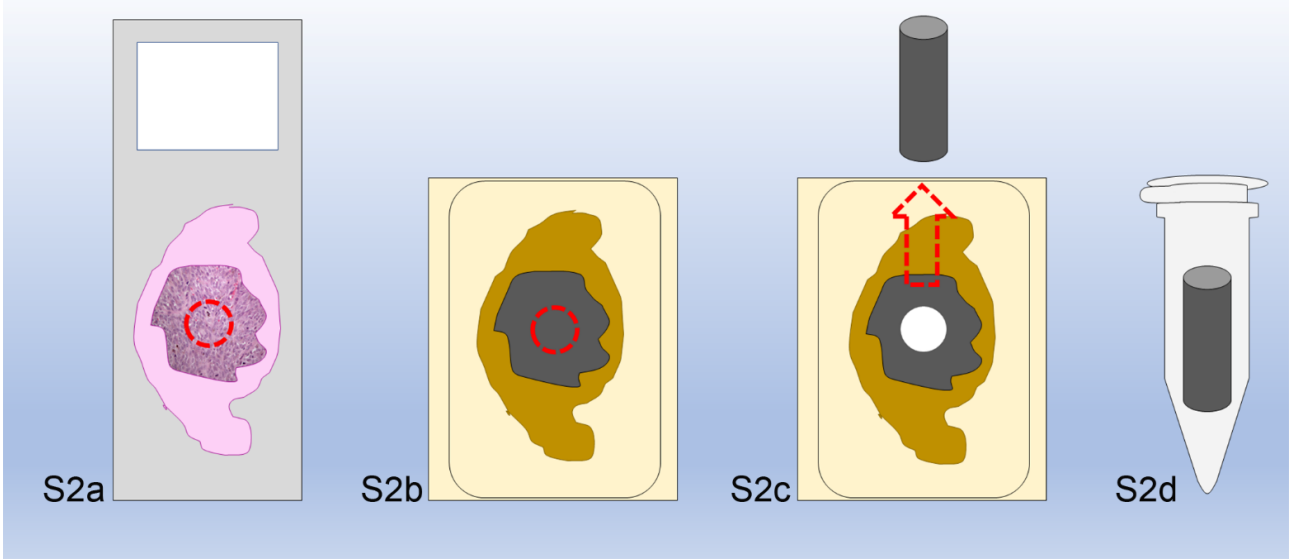

497

498 Figure 4. Pathway enrichment for miRNAs that were found to have significantly lower or
499 higher expression in canine malignant melanoma samples compared to the corresponding
500 healthy control tissues. Genes were retrieved and enriched in KEGG pathway with DAVID
501 tools. (a) Pathway enrichment for genes targeted by down-regulated miR-145 and miR-365.
502 (b) Pathway enrichment for genes targeted by over-regulated miR-146a-5p and miR-425-
503 5p. The statistical significance level shown is the negative of the logarithm (base 10) of the
504 *P* value.

505




**Supplemental Figure S1. Melanoma, oral mucosa, dog.** Cytoplasmic granular multifocal immunolabeling expression of Melan-A (a) and PNL-2 (b) in neoplastic cells.




**Supplemental Figure S2**. Tissue sampling from formalin-fixed-paraffin-embedded samples (FFPEs) schematic drawing. The area of interest (red dotted circle) is identified under the microscope (a) and in the corresponding area of the FFPE block (b). A disposable biopsy punch is used to sample the area (c) subsequently submitted for microRNA extraction (d).