

Relationships between transferrin and transferrin receptor (TfR) expression in dogs with malignant oronasal tumors

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Abstract

Malignant cells require a large amount of iron for cell growth. Transferrin transfers iron from blood to cells by reacting with transferrin receptor (TfR). In humans, transferrin and transferrin receptor correlate with oral squamous cell carcinoma (OSCC); however, the association with canine oral tumor has not been studied. This study aimed to investigate saliva and serum transferrin and tissue transferrin receptor in canine OSCC and oral melanoma (OM). Saliva and serum transferrin samples were collected pre- and 14 days post-operation, compared with healthy control and evaluated by a sandwich ELISA assay. Serum transferrin concentration in the experimental group both before and after surgery was significantly lower than that in the normal group ($p < 0.05$). However, no significant difference in serum transferrin levels before and after surgery was observed. Saliva transferrin expression of the dogs with oronasal cancer before surgical treatment tended to be higher than that of the control group. In contrast, at post-operation, they were not significantly different. Dogs with clinical stage I oronasal cancer revealed the highest saliva transferrin level as in humans. In addition, transferrin receptor expression, evaluated by immunohistochemistry, in the dogs with oronasal cancer was higher than that in the control group ($p < 0.001$). The decrease in serum transferrin was probably due to the uptake of transferrin bound iron by cancer cells. In conclusion, this study demonstrated the new role of transferrin and its receptor for iron acquisition in canine OSCC and OM.

Keywords: dog, oronasal cancer, protein expression, transferrin, transferrin receptor

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Introduction

Oral cancer accounts for 6% of all cancers in dogs. In general, physical examination and biopsy are standard diagnostic procedures in oral diseases. However, oral cancer is usually found in a late stage. Surgery is a primary method to treat oral cancer; however, it is not effective for all tumors. Most melanoma-affected dogs die due to metastasis in other visceral organs, particularly the lungs. OM and tonsillar squamous cell carcinoma also have high metastasis and are treated in dogs using chemotherapy (North and Banks, 2009). Therefore, the diagnosis and staging of oral tumors are very important, leading to the decision on appropriate treatment method. Moreover, diagnostic tools have been developed to increase the accuracy of early detection and classification of tumor staging.

In humans, biomarkers have been developed as diagnostic tools, for example M2BP, CD59, and catalase, which can help detect early stage oral cancers. These biomarkers have served as validated immunoassays because they can be found just in OSCC patients (Hu et al., 2008). However, they do not correlate the quantity of proteins and the size, stage and recurrence of cancer. Therefore, this leads to the study of the correlation between stage of tumor and level of proteins in the saliva of OSCC patients. Recent studies have found a transferrin level in saliva of OSCC patients that is significant when compared with normal patients. The level of transferrin correlates with the stage of cancer using the Universal TNM staging system of the International Union against Cancer (UICC) (Jou et al., 2010). This was then confirmed in a study of the rat parotid acinar cell, which could synthesize and secrete a transferrin protein to the salivary gland (Nashida et al., 2009). Therefore, biomarkers derived from saliva are of interest for the diagnosis of oral cancer in dogs. Furthermore, the collection of saliva sample is quite easy, comfortable and unstressful, as it decreases the need for animal transportation to hospital (Parra et al., 2005a). Previous studies have shown that saliva transferrin can be detected in the early stage of oral cancer by using the ELISA assay, which is a highly accurate method (Jou et al., 2010), and canine saliva has transferrin protein the same as human saliva (Vaerman and Heremans, 1969). Transferrin combines with a three plus-poled iron that can protect transportation in a toxic iron pole form. The cell needs iron to support oxidation, replication and DNA synthesis. The protein will then transfer iron in blood to cells by reacting with the transferrin receptor (TfR) (de Jong et al., 1990; Daniels et al., 2006). Cancer cells significantly express the level of TfR more than normal cells. This has been seen to be related to the stage and development of cancer, which correlate with cancer cell need for iron support (Daniels et al., 2012). Therefore, this research aimed to examine saliva and serum transferrin and TfR expression in canine OSCC and OM.

Materials and Methods

Samples in this study were obtained from the Small Animal Teaching Hospital, Faculty of Veterinary Science, Chulalongkorn University and were divided

into two groups: control group and experimental group. Experiments were performed under Laboratory Animal Center of Chulalongkorn University No. 1431029. The control group consisted of nine over 6-year-old healthy dogs that were treated with dental scaling and extraction. Exclusion criteria were no evidence of oral, head or neck tumors and no gastrointestinal, liver or renal disease. The experimental group comprised 15 dogs with oronasal mass appointed for surgical excision. Inclusion criteria were age over 6 years and presence of oronasal mass without previous treatment by either chemotherapy or radiotherapy. An oral examination was done to determine tumor size and location. Regional lymph nodes were examined for size and texture by physical examination and ruling out metastasis by cytological examination. Clinical staging of the affected dogs was performed by measuring tumor size under sedation and skull to abdomen radiographic examination by X-rays (Digital X-rays system, Brivo DR-F®, GE Thailand, Bangkok, Thailand) or CT scan (64-slice helical CT unit, Optima®, GE Thailand, Bangkok, Thailand). 64-slice multiple detector computed tomography (MDCT) was performed at 120 kV and 250 mA. The setting field covered head, neck, chest and abdomen. Then, non-ionic, water soluble, iodinated contrast medium (iohexol, Omnipaque®, USA) was intravenously administered at a dosage of 600 mgI/kg by using an automatic MDCT injector at 2 ml/sec. Post-contrast CT images were subsequently achieved while the contrast medium was presented at the mid cervical jugular vein. CT images showed details of oronasal mass, regional lymph nodes, chest and abdomen. TNM classification for the staging of oronasal cancer was provided by Withrow et al. (2013). Tumor diagnosis was done by cytology and histopathology.

To measure saliva transferrin levels, the animals were subjected to mouth cleaning and fasting at least 12 hr before saliva collection without mechanical or chemical stimulation. Samples were collected at the initial visit and 14 days after operation in the experimental group. Saliva of the control group was not collected once 4 weeks after operation. The samples were centrifuged at 12,000 rpm for 10 min at 4°C. The supernatants were placed into a new plain eppendorf, approximately 300 µl (Jou et al., 2010), and kept at -20°C until assay (Parra et al., 2005a). Four ml of whole blood was collected from the cephalic or saphenous veins in both groups. For the experimental group it was collected at the first day of visit and 14 days after operation. Serum of the control group was not collected once 4 weeks after operation. Blood profiles, liver and renal functions were determined by an automated machine. To measure serum levels of transferrin, 3 ml of the collected blood was centrifuged at 7,000 rpm for serum and stored at -20°C until analysis (Parra et al., 2005b).

To evaluate TfR expression, a tumor in the experimental group was removed by surgical treatment. Tumor size was measured by using a vernier caliper. The control group was requested to have dental scaling and tooth extraction, while gingival tissue at the extraction area was collected using punch biopsy. Canine placenta was used as a positive control TfR expression (Priest et al., 2011). All

tissue samples were randomly collected about 1 cm² each and fixed in 10% neutral buffered formalin for 24 hr. Then, the samples were routinely histologically processed, embedded in paraffin blocks, sectioned at 4 µm thickness and placed on silane-coated slides. The tissue samples were stained with Hematoxylin and Eosin (H&E) for histopathological diagnosis.

Serum and saliva transferrin determination by Enzyme-linked immunosorbent assay (ELISA):

Quantitative examination of transferrin in saliva and serum was performed by Transferrin Dog ELISA kit (ab157704, eBioscience, Abcam, Cambridge, UK). Saliva and serum transferrin in the samples reacted with the anti-Transferrin antibodies, which were adsorbed to the surface of polystyrene microtiter wells. One hundred µl of each 1:50,000 dilution of serum sample and 1:10 of saliva sample was pipetted into designated wells, then duplicated in another well. Next, the samples were incubated at room temperature for 30 min. One hundred µl of secondary anti-Transferrin antibodies, conjugate with horseradish peroxidase (HRP), were added into the prepared samples in each well, incubated at room temperature for 30 min and then covered with a plate to prevent exposure to light. Following another washing, the amount of enzyme bound in the complex was measured by the addition of a chromogenic substrate, 3,3',5,5'-tetramethylbenzidine (TMB), for 10 min in the dark. Then, 0.3 M sulfuric acid was added to stop the reaction and absorbance (450 nm) of the samples was determined in each well by an ELISA plate reader. Results were measured for quantity of transferrin by interpolation from the standard curve.

Histopathology: The staging of oronasal cancer was provided by Withrow et al. (2013), which depends on size and metastasis of cancer. Immunohistochemical staining of transferrin receptor in canine oronasal tumor mass and gingival tissues was performed by LSAB method. Antigen retrieval was performed by heating with a Tris-HCl buffer (pH 8.0) using a microwave (700 W medium-high) for 20 min. Endogenous peroxidase enzymes were blocked with a 3% solution of hydrogen peroxide in methanol for 10 min, and non-specific protein was blocked with 1% bovine serum albumin at 37°C for 20 min. The slides

were incubated overnight at 4°C with monoclonal mouse anti-human TfR1 antibody (clone 68.4, Zymed Laboratories, San Francisco, CA, USA), at a dilution of 1:500. Primary antibody was tagged with secondary antibody using an Envision polymer kit (Dako, Carpinteria, CA, USA) at 37°C for 45 min, and 3,3'-diaminobenzidine was used as a chromogen substrate. Mayer's hematoxylin was counterstained for those slides. Positive control was trophoblast cells in canine placenta tissue. Image Proplus Analysis (version 6.0, Media Cybernetics, Bethesda, MD, USA) was applied to describe in detail total captured area, photographed and evaluated as percentage of expression in all samples (Priest et al., 2011).

Statistical and data analyses: Student T-test and Mann-Whitney U test were used to assess difference in transferrin levels and receptor between the oronasal cancer and control groups. Paired T-test and Wilcoxon Signed-Rank test were used to determine difference in transferrin levels before and after surgical treatments. Pearson correlation and Spearman's rank correlation tests were used to demonstrate correlation between saliva transferrin, serum transferrin and TfR expression. Significant differences were determined at $p < 0.05$.

Results

Saliva and serum transferrin and TfR expression in canine OSCC and OM: Saliva, serum and tissue samples were collected from 24 dogs. The control group was composed of 6 males (Male (M) = 3 and Male castrated (Mc) = 3) and 3 females (Females (F) = 3) with chronic hyperplastic gingivitis. The experimental group consisted of 10 males (M = 9, Mc = 1) and 5 females (F = 1, Female spayed (Fs) = 4) with malignant melanoma (n = 12) and squamous cell carcinoma (SCC) (n = 3). The average age of the experimental group was 10.6 years old (10.6 ± 1.76). In this group, 4 dogs had regional lymph node metastasis (Table 1). Sample data were divided into 4 categories: histology, lymph node involvement, TNM stage and survival time. The saliva sample test provided non-parametric data expressed as µg/ml while the serum sample test provided parametric data expressed as mg/ml (Table 2).

Table 1 Signalment data, TNM stage, and histopathology of the experimental group

No.	Breed	Sex	Age (years)	Tumor size (cm)	Lymph node metastasis	Lungs metastasis	Clinical stage	Histopathological diagnosis
1	LR	M	14	4x6	no	no	III	amelanotic melanoma
2	LR	Fs	9	3x5	no	no	III	amelanotic melanoma
3	GR	M	9	5x5	no	no	III	amelanotic melanoma
4	GR	M	10	5x5	yes	no	IV	amelanotic melanoma
5	Pug	M	11	2.5x3.5	no	no	II	amelanotic melanoma
6	Mixed	Fs	10	4x4	no	no	III	melanoma
7	Mixed	Mc	9	3x4	no	no	III	melanoma
8	Mixed	M	11	4.5x5	no	no	III	melanoma
9	GR	Fs	9	1	no	no	I	melanoma
10	Poodle	M	10	3x2	yes	no	IV	melanoma
11	Poodle	M	13	1.5x2	yes	no	IV	melanoma
12	Poodle	F	14	5x5	yes	no	IV	melanoma
13	Poodle	Fs	11	3x5	no	no	III	SCC
14	ShihTzu	M	9	4x5	no	no	III	SCC
15	Mixed	M	10	4x6	no	no	III	SCC

GR: Golden retriever, LR: Labrador retriever M: Male, F: Female, s: Spayed, c: Castrated SCC: Squamous cell carcinoma

Table 2 Correlation between saliva and serum transferrin concentration before and after surgical treatment in the experimental group with histology, lymph node involvement, TNM stage and survival time (Mean $\pm$ SD, Median; 25th and 75th percentile)

	Saliva transferrin (μ g/ml)		Serum transferrin (mg/ml)		Statistical test: correlation		
	Before	After	Before	After	Group	r	P-value
Group					Before ¹	0.3	0.28
- Experimental	3.06 $\pm$ 0.10	2.99; 2.78-3.08	4.71 $\pm$ 0.98 ^a	4.77 $\pm$ 1.82 ^a	After ²	0.05	0.86
- Control	2.70 $\pm$ 0.77	2.70 $\pm$ 0.77	6.15 $\pm$ 0.29	6.15 $\pm$ 0.29	Control ²	-0.27	0.49
Histopathological Dx							
- Melanoma (n = 12)	3.03 $\pm$ 0.12	2.99; 2.76-3.08	4.70 $\pm$ 1.02	4.94 $\pm$ 1.85	Before ^a /after ^b	0.29/-0.09	0.36/0.78
- SCC (n = 3)	3.07 $\pm$ 0.08	3.01 $\pm$ 0.20	4.31 $\pm$ 0.88	4.11 $\pm$ 1.87	Before ^a /after ^a	0.83/1.00*	0.38/0.03
Lymph node involvement							
- N0 (n = 11)	3.05 $\pm$ 0.08	2.97; 2.78-3.08	4.76 $\pm$ 0.94	4.91; 3.24-5.82	Before ^a /after ^b	0.50/0.12	0.11/0.73
- N1 (n = 4)	3.00 $\pm$ 0.19	3.04; 2.78-3.13	4.59 $\pm$ 1.24	4.43; 2.96-5.98	Before ^a /after ^b	0.09/0.32	0.92/0.68
TNM stage							
- I (n = 1)	3.091	3.053	5.208	5.823	-	-	-
- II (n = 1)	3.049	2.986	6.192	4.912	-	-	-
- III (n = 9)	3.05; 2.96-3.14 ^d	2.94; 2.49-3.07	4.41; 3.82-5.59	5.18; 3.34-6.33	Before ^b /after ^b	0.41/0.00	0.32/1.00
- IV (n = 4)	3.01; 2.81-3.13	3.10; 3.02-3.14	4.42; 3.32-5.32	3.15; 2.99-5.31	Before ^b /after ^b	0.00/0.80	1.00/0.20
Survival time							
- < 3 month (n = 2)	3.05 $\pm$ 0.21 ^c	2.85 $\pm$ 0.21	5.60 $\pm$ 0.13	4.48 $\pm$ 1.99	Before ^a /after ^a	1.00**/1.00**	-/-
- > 3 month (n = 13)	3.04 $\pm$ 0.11	3.05; 2.86-3.10	4.58 $\pm$ 0.99	4.82 $\pm$ 1.87	Before ^a /after ^b	0.33/0.14	0.27/0.64

^aSignificant correlation was assessed by Pearson correlation at $p < 0.05^*$, $p < 0.001^{**}$.^bSignificant correlation was assessed by Spearman's rank correlation coefficient at $p < 0.05^*$.^cIndicate statistically significant difference by Independent T-test at $p < 0.05^*$.^dIndicate statistically significant difference by Mann Whitney U test at $p < 0.05^*$.

The saliva transferrin concentration both before and after surgery of the experimental group was not significantly different from that of the control group. The quantity of saliva transferrin before surgery was not significantly different compared to the quantity of saliva transferrin after surgery. Interestingly, the mean level of saliva transferrin in the experimental group before surgical treatment (3.04 $\pm$ 0.11 μ l) was higher than that of the control group (2.70 $\pm$ 0.77 μ l), but not statistically significant. Histological diagnosis did not affect alternate transferrin in saliva. The saliva transferrin level was not statistically significantly different between the metastasis and non-metastasis to regional lymph node groups both before and after surgical treatment. However, the non-lymph node involvement group before surgery was significantly different compared to after surgery ($p < 0.05$). Using statistical analysis to compare between clinical stages, TNM stage III and stage IV, stage III was significantly greater than stage IV in the before surgical treatment group ($p < 0.05$), while no significant difference after surgical treatment was found. Classification of tumor and lymph node involvement had no effect on saliva transferrin concentrations, even though TNM stage III (3.06 $\pm$ 0.10 μ l) and stage IV (2.99 $\pm$ 0.17 μ l) before surgical treatment had significant differences in saliva transferrin levels ($p < 0.05$). The level of saliva transferrin in dogs having survival times of more than 3 months was not significantly different from dogs having survival times of less than 3 months after surgical treatment. However, comparison between the two groups of survival times was significantly different in the first collection of the experimental group ($p < 0.05$). Still, survival times were not statistically significantly different before and after surgical treatment.

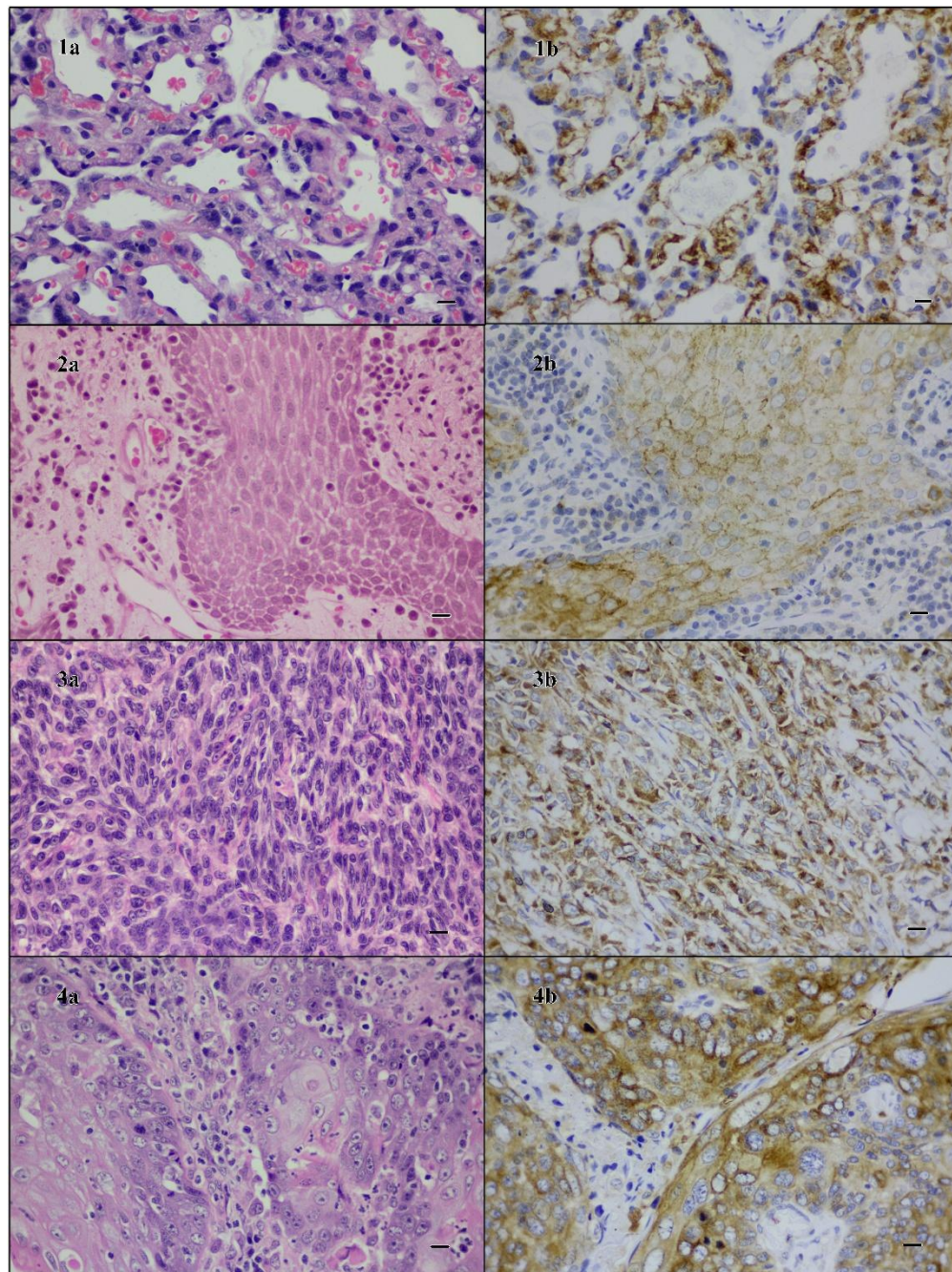
The serum transferrin concentration in the experimental group both before and after surgery was significantly lower than that in the normal group ($p < 0.05$). However, the quantity of serum transferrin before surgery was not significantly different from after surgery. The classification of oral tumor showed no significant difference in the level of transferrin, and the surgical treatment showed no effect on serum transferrin concentration. The serum transferrin of the regional lymph node metastasis group was not significantly different compared to the group without metastasis before and after surgical treatment. For the comparison between clinical stages, TNM stage III and stage IV, there was no significant difference between before and after surgical treatment. The level of serum transferrin in dogs having survival time of more than 3 months was not significantly different from dogs having survival time less than 3 months, including before and after surgical treatment.

Oronasal cancer tissues were obtained from 15 dogs (experimental group) and gingival tissues were obtained from 9 dogs (control group). Transferrin receptor expression was noted in the cytoplasm of placental epithelial cells (positive control) (Fig. 1), hyperplastic mucocutaneous epithelial cells of chronic hyperplastic lesion (control group) (Fig. 2), squamous cell carcinomas (Fig. 3) and malignant melanoma (Fig. 4). Transferrin receptor expression showed statistically significant difference between the experimental group and control group ($p < 0.01$). The results are shown in Table 3.

There was no correlation between serum and saliva transferrin concentrations in the control and experimental groups tested before and after surgical treatments. Among histopathological diagnosis, lymph node metastasis and clinical staging classified

groups, there was no correlation of statistical difference in serum and saliva transferrin level which were tested before and after surgical treatment. Interestingly, there was a statistically significant

difference in the serum and saliva transferrin levels in dogs having survival times less than 3 months ($p < 0.001$), while the correlation in dogs that had survival times more than 3 months was not found (Table 2).



- Figure 1** Transferrin receptor expression that was detected in the cytoplasm of placental epithelial cells and served as positive control staining was positive in brown color (Fig. 1a H&E stain) (Fig. 1b IHC, counterstained with Mayer's Hematoxylin, Bar = 10 μ m).
- Figure 2** Chronic hyperplastic gingivitis expressed transferrin receptor of proliferative mucosal epithelial cells, particularly basal epithelial cells (H&E stain, Fig. 2a, Bar = 10 μ m) (Fig. 2b IHC, counterstained with Mayer's Hematoxylin, Bar = 10 μ m).
- Figure 3** Malignant melanoma showed solid nests of neoplastic cells; the large spindleoid with large ovoid nuclei contained brown to black intracytoplasmic melanin pigment (Fig. 3a H&E stain), A, Bar = 10 μ m). Transferrin receptor expression was detected in the cytoplasm of melanocytic tumor cells (Fig. 3b IHC, counterstained with Mayer's Hematoxylin, Bar = 10 μ m).
- Figure 4** Oral squamous cell carcinoma showed diffuse islands of squamous cells and growth invading the subcumosal layers. The neoplastic cells were large polyhedral to cuboidal in shape with large round and prominent nuclei (Fig. 4a H&E stain, Bar = 10 μ m). Transferrin receptor expression was detected in the cytoplasm of neoplastic cells (Fig. 4b IHC, counterstained with Mayer's Hematoxylin, Bar = 10 μ m).

In the melanoma, lymph node involvement and clinical staging classified groups, there was no correlation between TfR expression, saliva and serum transferrin concentrations before surgical treatments. However, in the dogs having survival times less than 3 months, a correlation was found between serum and

saliva transferrin and transferrin receptor expression ($p < 0.001$). However, there were only two dogs in this group, which was insufficient for analysis. In contrast, this correlation was not found in the dogs having survival time more than 3 months ($p > 0.05$). These results are presented in Table 3.

Table 3 Correlation between saliva, serum transferrin concentration before surgical treatment and transferrin receptor in the experimental group with histology, lymph node involvement, TNM stage and survival time (Mean $\pm$ SD, Median; 25th and 75th percentile)

		Saliva transferrin ($\mu\text{g/ml}$)	Serum transferrin (mg/ml)	Transferrin receptor	Statistical test: correlation Transferrin receptor	
		Before	Before	%Area	r	P-value
Group						
-	Experimental	3.06 $\pm$ 0.10	4.71 $\pm$ 0.98	61.05 $\pm$ 16.88 ^c	0.46, -0.11 ^a	0.11, 0.72
-	Control	3.08; 2.21-3.18	6.15 $\pm$ 0.29	0.13; 0.04-1.24	-0.05, -0.46 ^b	0.90, 0.21
Histopathological Dx						
-	Melanoma (n = 12)	3.03 $\pm$ 0.12	4.70 $\pm$ 1.02	57.27 $\pm$ 16.08	-0.52, -0.18 ^a	0.08, 0.57
-	SCC (n = 3)	3.07 $\pm$ 0.08	4.31 $\pm$ 0.88	76.14 $\pm$ 9.82	-1.00*, -0.81 ^a	0.02, 0.40
Lymph node involvement						
-	N0 (n = 11)	3.05 $\pm$ 0.08	4.76 $\pm$ 0.94	58.43 $\pm$ 18.33	0.15, -0.29 ^a	0.67, 0.39
-	N1 (n = 4)	3.00 $\pm$ 0.19	4.59 $\pm$ 1.24	68.23 $\pm$ 10.73	-0.27, 0.63 ^a	0.73, 0.37
TNM stage						
-	I (n = 1)	3.09	5.21	30.86	-	-
-	II (n = 1)	3.05	6.19	60.97	-	-
-	III (n = 9)	3.05; 2.96-3.14	4.41; 3.81-5.59	61.21 $\pm$ 17.76	0.18, 0.25 ^b	0.64, 0.52
-	IV (n = 4)	3.01; 2.81-3.13	4.42; 3.32-5.32	68.23 $\pm$ 10.73	0.20, 0.40 ^b	0.80, 0.60
Survival time						
-	< 3 month (n = 2)	3.05 $\pm$ 0.21	5.60 $\pm$ 0.13	60.93 $\pm$ 10.12	1.00**, 1.00** ^a	-
-	> 3 month (n = 13)	3.04 $\pm$ 0.11	4.58 $\pm$ 0.99	61.07 $\pm$ 17.99	-0.58*, -0.35 ^a	0.04, 0.25

Significant correlation was assessed by Pearson correlation at $p < 0.05^$, $p < 0.001^{**}$.

^bSignificant correlation was assessed by Spearman's rank correlation coefficient at $p < 0.05^*$.

^cIndicate statistically significant difference by Mann-Whitney U test at $p < 0.001^{**}$.

Discussion

Many oral cancer biomarkers have been discovered in human salivary composition (Elashoff et al., 2012). Proteomic analysis of saliva found salivary molecules as potential oral cancer biomarkers. For instance, level of CD44 (Franzmann et al., 2005), salivary Cyfra 21-1, tissue polypeptide anti-gene and CA125 have been proposed as oral cancer markers (Nagler et al., 2006). Previous studies found a high level of some proteins such as insulin growth factor 1, metalloproteinase-9 (MMP-9), carbonyls, lactate dehydrogenase and Cyclin D1 (Shpitzer et al., 2009). Although they discovered many proteins as biomarkers in salivary cancer patients, no molecule had high accuracy for identifying early disease (Elashoff et al., 2012). However, saliva can be affected by many factors such as flow rate, circadian rhythms, exercise, plasma composition, olfaction and disease (Dawes, 1993). Therefore, the dogs in this study underwent food and water fasting at least 12 hr and 6 hr, respectively, and were not allowed to perform mouth washing at least 12 hr before collection of saliva. The collection procedure chosen used unstimulated saliva to reduce factors that could affect saliva composition and flow rate. The aim was to determine the possibility of transferrin as a good prognostic tool in canine oral cancer because oral squamous cell carcinoma patients were found to have a higher level

of transferrin than healthy persons, which correlated well with the stage of cancer. It has been found that in the early stage, saliva transferrin level is higher than in the late stage and possibly could be used for detection and screening of the early stage of cancer (Jou et al., 2010). Therefore, biomarkers derived from saliva are of interest for the diagnosis of oral cancer in dogs. For the above reason, transferrin was measured by the ELISA assay (eBioscience), and it was found that the dogs with clinical stage I oral cancer had a higher saliva transferrin level than the dogs in other stages, which is similar to the findings of a previous research in humans (Jou et al., 2010). It should be noted, however, that only one dog was in clinical stage I, which is not sufficient for analysis. The survival times before surgical treatment were significantly different between the dogs that died within 3 months and those that lived longer than 3 months after surgery ($p < 0.05$). The dogs having survival times less than 3 months had higher levels of saliva transferrin concentration. This is probably due to two dogs having melanoma stage IV, which had more hemorrhage, inflammation and secondary infection than other stages. In general, a dog's oral environment is very hard to control. Dogs in the control group had periodontitis grades III to IV, containing bacterial infection and chronic stomatitis. Free-iron transferrin has an antimicrobial effect (Weinberg, 1977), as illustrated on the competition of bound iron from transferrin and bacterial siderophores

(de Jong et al., 1990). Therefore, the antimicrobial effect might elevate the level of saliva transferrin in the control group. Transferrin from the salivary glands may have bacteriostatic effects because of the free-iron transferrin (not containing Fe^{3+}). Accordingly, lactoferrin, an analogue of transferrin in human saliva, does have antimicrobial properties (Humphrey and Williamson, 2001). In addition, cell proliferation by inflammation might elevate the saliva transferrin (Salonen and Kallajoki, 1986). Therefore, the dogs in the control group had saliva sample collected after dental scaling and extraction up to 4 weeks, so it would not interfere with the normal oral environment.

Normally, transferrin in blood is produced by hepatocytes in the liver. Moreover, transferrin synthesis appears in other organs such as the mammary gland, Sertoli cells, parotid acinar cells and others. Transferrin stimulates cell growth and division by binding to specific proliferation-related surface receptors. The level of serum transferrin depends on many abnormal conditions. There are studies that found that the increase or decrease in transferrin level correlated with plasma iron concentration (de Jong et al., 1990). Iron deficiency from anemia, pregnancy and childhood may increase transferrin level in serum according to a rise in protein synthesis. Moreover, acute hepatitis, dietary hemosiderosis and effect of oestrogenic components could elevate transferrin and iron concentration. In contrast, reduction in transferrin concentration results from iron overload, but not secondary iron overload (transfusion or dietary). Atransferrinaemia as well as infections, malnutrition, rheumatoid arthritis, cancer malignancies, trauma and surgery might cause decrease in both serum transferrin and iron concentration (Morgan, 1981). In this study, the decrease in serum transferrin in the experimental group is similar to that found in humans, which might result from malignancy. However, the serum transferrin levels in OM and OSCC did not significantly correlate with malignancy. Malignant cells need iron for cell development and division, and they require iron uptake higher than normal cells. Transferrin bound iron uptake is greater in cancer cells than normal liver cells, which might be the cause of a decrease in serum transferrin. The dogs in this study that had clinical stage III cancer or a survival time of more than 3 months had serum transferrin after surgical treatment higher than those with clinical stage IV or a survival time of less than 3 months. Since the dogs in clinical stage IV or with a survival time of less than 3 months had metastasis, the results were similar to cancer malignancy. However, cancer dogs are often found to be anemic, causing reduction in serum transferrin.

No correlation between the levels of transferrin in saliva and serum was found in this study, similar to a previous study in humans (Jou et al., 2010). The saliva transferrin level in OSCC patients was higher than in free oral cancer patients, and the concentration of saliva transferrin correlated well with the stage of tumors based on an ELISA assay. These results demonstrated that the mean levels of saliva transferrin in OSCC patients was higher by 91% in T1 group, 88% in T2 group and 84% in T3/T4 group, when compared to a healthy person (Jou et al., 2010). In

contrast, the levels of salivary transferrin in oral cancer dogs were not significantly different compared to the control group and showed no association with metastasis of cancer. Serum transferrin in humans and dogs was similarly less in OSCC patients and in canine OM and OSCC, but it was not significantly related to the stage of cancer, metastasis, classification or survival times.

According to the biopsy results of the control group, chronic hyperplastic gingivitis was evident. This study compared the density of transferrin receptor among OM, OSCC and chronic hyperplastic gingivitis in dogs. The results showed that the density of transferrin receptor in the experimental group was significantly higher than that of the control group ($p < 0.05$). Transferrin receptor (TfR) is a type II glycoprotein on cell membrane involving iron uptake and control of cell growth (Neckers and Trepel, 1986). TfR expression depends on intracellular iron levels, showing a low level on normal cells and a higher level on a great proliferative cell (Daniels et al., 2006). Cancer cells have shown elevated levels of TfR greater than normal cells in many studies (Shindelman et al., 1981; Sutherland et al., 1981; Gatter et al., 1983; Walker and Day, 1986; Sciort et al., 1990; Daniels et al., 2006; Prutki et al., 2006). Accordingly, cancer cells require more iron as a cofactor of the ribonucleotide reductase enzyme for dividing cells (Daniels et al., 2006). This study exhibited TfR expression in malignant breast cells 4-5 times more than benign breast cells (Walker and Day, 1986). Another study showed that TfR expression increased in cancer patients and correlated with the stage of tumors and prognosis, including bladder transitional cell carcinomas, breast cancer, gliomas, lung adenocarcinoma, chronic lymphocytic leukemia and non-Hodgkin's lymphoma (Habeshaw et al., 1983; Seymour et al., 1987; Das Gupta and Shah, 1990; Kondo et al., 1990; Prior et al., 1990). Previous research demonstrated transferrin receptor in oral tumor patients using immunohistochemistry and flow cytometry. TfR expression was rarely found in benign oral tumors but often found in the basal and parabasal cell of normal epithelium. Moreover, all malignant samples had staining greater than normal epithelium, and the positive reaction in malignant tumors might indicate the degree of malignancy. TfR expression could be useful as a biomarker for prognosis (Miyamoto et al., 1994). This study found a strong reaction with TfR in canine oral cancer more than chronic hyperplastic gingivitis using standard immunohistochemistry (Gatter et al., 1983; Priest et al., 2011). In chronic hyperplastic gingivitis, weak staining was detected in parabasal and basal layers. In contrast, oral cancer yielded positive stronger staining in cancer cells. TfR expression showed no correlation with metastatic cancer. The levels of serum and saliva transferrin did not correlate with TfR. However, the present study demonstrated that OSCC with survival time less than 3 months correlated with saliva transferrin, serum transferrin and TfR. However, the number of dogs in this study was not sufficient for statistical analysis. The results supported that there was TfR expression in cancer cells, but no relationship between the TfR expression and metastasis of cancer was evident. Several studies in humans have

demonstrated that TfR expression correlates with mitotic rate, Ki67 staining and H-thymidine incorporation. However, this study found the levels of TfR expression in oral cancer higher than that in chronic hyperplastic gingivitis. It should be noted that TfR expression in cancer tissue is very useful for showing a prognostic marker and target receptor for therapeutic purposes against malignant cells.

In conclusion, this study demonstrated the new role of transferrin and its receptor for iron acquisition in canine OSCC and OM. It also showed that saliva transferrin in canine oronasal cancer tended to be higher than that in the control group. In contrast, the saliva transferrin of the postoperative treatment group was not significantly different from that of the control group. Moreover, the levels of saliva transferrin respectively decreased from stage I to stage IV and were higher than those of the control group, which is similar to the findings of a previous study in humans (Jou et al., 2010). Therefore, canine transferrin might be useful for the detection and prognosis of canine OM and OSCC.

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บทคัดย่อ

การแสดงออกของความสัมพันธ์ระหว่างโปรตีนทรานเฟอร์รินและตัวรับโปรตีนทรานเฟอร์รินในสุนัขที่เป็นมะเร็งช่องปากและโพรงจมูก

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เซลล์มะเร็งต้องการธาตุเหล็กจำนวนมากในการเจริญเติบโต โดยปกติโปรตีนทรานเฟอร์รินทำหน้าที่ขนส่งธาตุเหล็กจากเลือดสู่เซลล์ผ่านตัวรับโปรตีนทรานเฟอร์รินบริเวณผิวเซลล์ ในมนุษย์นั้นพบว่าโปรตีนทรานเฟอร์รินและตัวรับโปรตีนบริเวณผิวเซลล์มีความสัมพันธ์กับมะเร็งช่องปากชนิดสแควมัสเซลล์ อย่างไรก็ตามความสัมพันธ์ของโปรตีนและตัวรับของโปรตีนชนิดนี้ยังไม่เคยมีรายงานในสุนัขมาก่อน ดังนั้นการศึกษานี้จึงมีวัตถุประสงค์เพื่อวิเคราะห์ระดับของโปรตีนทรานเฟอร์รินในน้ำลายและซีรัม รวมถึงปริมาณตัวรับของโปรตีนทรานเฟอร์รินในสุนัขที่เป็นมะเร็งช่องปากชนิดสแควมัสเซลล์และชนิดเมลาโนมา โดยทำการเก็บตัวอย่างน้ำลายและซีรัมของสุนัขที่เป็นมะเร็งทั้งสองชนิดนี้ 2 ครั้ง คือ ก่อนและหลังการผ่าตัดเป็นเวลา 14 วัน เปรียบเทียบกับสุนัขปกติด้วยการวัดปริมาณโปรตีนทรานเฟอร์รินในน้ำลายและซีรัมโดยใช้วิธีแชนวียอิลสา ผลพบว่าปริมาณโปรตีนทรานเฟอร์รินในซีรัมของสุนัขที่เป็นมะเร็งทั้งก่อนและหลังการผ่าตัดมีค่าลดลงอย่างมีนัยสำคัญทางสถิติเมื่อเทียบกับสุนัขปกติ ($p < 0.05$) แต่ไม่พบความแตกต่างนี้เมื่อเปรียบเทียบระหว่างช่วงก่อนและหลังการผ่าตัด สำหรับการแสดงออกของโปรตีนทรานเฟอร์รินในน้ำลายนั้นพบว่า สุนัขที่เป็นมะเร็งช่องปากทั้ง 2 ชนิด มีแนวโน้มการแสดงออกของโปรตีนทรานเฟอร์รินในน้ำลายสูงกว่าสุนัขปกติ ในขณะที่หลังการผ่าตัดพบว่าปริมาณโปรตีนทรานเฟอร์รินไม่แตกต่างจากสุนัขปกติ และสุนัขที่เป็นมะเร็งระยะที่ 1 นั้น มีปริมาณโปรตีนทรานเฟอร์รินในน้ำลายมากที่สุดเช่นเดียวกับการศึกษาในมนุษย์ นอกจากนี้ยังพบว่าปริมาณตัวรับโปรตีนทรานเฟอร์รินในสุนัขที่เป็นมะเร็งนั้นสูงกว่าสุนัขปกติอย่างมีนัยสำคัญทางสถิติ ($p < 0.001$) ซึ่งการลดลงของโปรตีนทรานเฟอร์รินในซีรัมนั้นอาจเกิดจากเซลล์มะเร็งมีการรับธาตุเหล็กที่จับกับโปรตีนไปมากกว่าปกติ การศึกษานี้จึงเป็นการค้นพบทฤษฎีใหม่ของโปรตีนทรานเฟอร์รินและตัวรับของโปรตีนทรานเฟอร์รินในสุนัขที่เป็นมะเร็งช่องปากชนิดสแควมัสเซลล์และชนิดเมลาโนมา

คำสำคัญ: สุนัข, มะเร็งช่องปากและโพรงจมูก, การแสดงออกของโปรตีน, ทรานเฟอร์ริน, ตัวรับโปรตีนทรานเฟอร์ริน

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