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นายเศกรินทร์ พลอยเพ็ชร์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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Saliva Transferrin Level in Dogs with Malignant Oronasal Tumors

Mr. Sekkarin Ploypetch

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By	Mr. Sekkarin Ploypetch
Field of Study	Veterinary Surgery
Thesis Advisor	Associate Professor Chanin Kalpravidh, D.V.M., M.Sc.
Thesis Co-Advisor	Prapruddee Piyaviriyakul, D.V.M., M.Sc., Ph. D.
	Associate Professor Anudep Rungsipipat, D.V.M., M.Sc., Ph.D.

Accepted by the Faculty of Veterinary Science, Chulalongkorn University in Partial Fulfillment of the Requirements for the Master's Degree

--Dean of the Faculty of Veterinary Science

(Professor Roongroje Thanawongnuwech, D.V.M., M.Sc., Ph.D.)

THESIS COMMITTEE

--Chairman

(Professor Marissak Kalpravidh, D.V.M., M.Sc., Ph.D.)

--Thesis Advisor

(Associate Professor Chanin Kalpravidh, D.V.M., M.Sc.)

--Thesis Co-Advisor

(Prapruddee Piyaviriyakul, D.V.M., M.Sc., Ph. D.)

--Thesis Co-Advisor

(Associate Professor Anudep Rungsipipat, D.V.M., M.Sc., Ph.D.)

--Examiner

(Nan Choisunirachon, D.V.M., M.Sc., Ph.D.)

--External Examiner

(Tassanee Charoensong, D.V.M., M.Sc., Ph.D.)

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Saliva collection is non-invasive, easy, non-stressful and reduces animal transportation. Therefore, the saliva is a good source for cancer biomarkers. In human, saliva transferrin is correlated with stages of oral squamous cell carcinoma. Thus, this study was interested in using in oro-nasal cancer dogs. Saliva transferrin might be used for early detection in oro-nasal cancer dogs, this protein does not usually present in the early stage of tumor but is found of the late stage. Saliva samples from fifteen dogs with oro-nasal cancers (experimental group) and nine dogs with chronic hyperplastic gingivitis (control group) were analyzed by ELISA assays. The result showed that a mean level of saliva transferrin in the experimental group before surgical treatment (3.040 ± 0.113 µg/ml) was in significantly ($p > 0.05$) higher than the control group (2.698 ± 0.765 µg/ml), significant difference could not be detected in statistic test. The concentration of the postoperative saliva transferrin was also not significantly differentfrom the control group. The quantity of the preoperative saliva transferrin was not significantly different from that of the postoperative saliva transferrin. Dogs with clinical stage I had the highest saliva transferrin level compared with the level of other stages. This agreed with a previous research in human. However, there was only one dog with clinical stage I in this study, which was not enough for statistical analysis. In this study, transferrin receptor expression by immunohistochemistry in oro-nasal cancer dogs was significantly higher than in normal dogs ($p < 0.001$) by immunohistochemistry. Therefore, the level of saliva transferrin is not suitable for early detection in canine oro-nasal cancers because there are many environmental factors affecting saliva transferrin concentrations more samples are needed for analysis. Transferrin receptor might be more suitable than the saliva transferrin in monitoring and prognos is of marker canine oral tumors.

CONTENTS

LIST OF ABBREATIONS

$^{\circ}$C	=	degree Celsius
µm	=	micrometer
µl	=	microliter
ALP	=	alkaline phosphatase
ALT	=	Alanine transaminase
BUN	=	blood urea nitrogen
CA125	=	cancer antigen 125
CD44	=	complete defense 44
CD59	=	complete defense 59
cm	=	centimeter
cm^2	=	square centimeter
CRP	=	C-reactive protein
CT	=	Computer tomography
CycD1	=	Cyclin D1
Cyfra 21-1	=	cytokeratin 19 fragments
DNA	=	deoxyribonucleic acid
ELISA	=	enzyme-linked immunosorbent assay
HCl	=	Hydrochloric acid

HGB	=	high-grade B-cell
HGT	=	high-grade T-cell
IgG	=	Immunoglobulin G
IHC	=	immunohistochemistry
kV	=	kilovolts
l	=	liter
LC-Q-TOF	=	liquid chromatography with quadruple time-of-fight
LGB	=	low-grade B-cell
LGT	=	low-grade T-cell
LSAB	=	Labeled Streptavidin-Biotin (immunohistochemistry)
M	=	Molar
M2BP	=	Mac-2 binding protein
mA	=	milliamps
mg	=	milligrams
ml	=	milliliter
MMP-9	=	Matrix metalloproteinase-9
MRI	=	magnetic resonance imaging
MRP14	=	migration inhibitory factor-related protein 14
ng	=	nanograms

nm	=	nanometer
oscc	=	oral squamous cell carcinoma
PBS	=	phosphate buffer saline
SD	=	standard deviation
scc	=	squamous cell carcinoma
TNM	=	The TNM Classification of Malignant Tumours
TfR	=	transferrin receptor
UICC	=	Universal TNM staging system of the International Union against Cancer

INTRODUCTION

Importance and Rationale

Older dogs are prone to be affected by many diseases more than the younger, especially cancer. Oral cancer is accounting for 6% among all of cancers in dogs (North and Banks, 2009). Normally, the physical examination and biopsy are standard procedures for diagnosis in oral diseases. Clinical examination always focuses on an abnormal of head and neck, including to palpate in the oral cavity. However, oral cancer was not usually found in the early stage of tumor until it became too the late stage. In human, Biomarkers were developed as diagnostic tools for example; M2BP, CD59, and catalase which were detected in early stage oral cancer patient. The protein biomarkers in saliva also were used for diagnosis (Dowling et al., 2008). Thus, this method was interested to use in oral cancer in dogs. Collection of saliva sample is quite easy, comfortable and non-stressful including the decreased transportation to the animal hospital (Parra et al., 2005). Previous study show that saliva transferrin was detected the early stage of oral cancer by using ELISA assays, which was a highly accurate method (Jou et al., 2010) and canine saliva has transferrin protein as well as human's saliva (Vaerman and Heremans, 1969). Therefore, this research aims to study a correlation between a levels of saliva transferrin and oronasal cancer dogs, that by comparing the preoperative and postoperative saliva transferrin that could be a tool for both diagnostic in early stage of cancer and treatment prognosis.

Objective of study

1. To compare levels of saliva transferrin between oronasal cancer dogs and the normal dogs.

2. To compare the levels of saliva transferrin before and after surgical treatment.

3. To evaluate the expression of transferrin receptor in oronasal cancer dogs and normal dogs

Hypothesis

1. The saliva transferrin concentration are increased in oronasal cancer dogs compared to the normal dogs.

2. In oronasal cancer dogs, the preoperative transferrin are higher than the postoperative saliva transferrin.

3. Transferrin receptor expression in oronasal cancer dogs are higher than normal dogs.

Keywords: Biomarker Cancer Dogs Oronasal Saliva Transferrin

Advantages of study

The advantage of this study prefers to the levels of saliva transferrin as a marker of early stage in oronasal cancer dogs. The saliva transferrin concentration may be used for both diagnostic in early stage of cancer and prognosis treatment.

CHAPTER II

LITERATURE REVIEW

Oral tumors in dogs are accounted for 6% among all tumors and serve as the fourth of all malignant tumors of dogs and cats. These are found in the older dogs than younger dogs (North and Banks, 2009). The oral tumors can be classified into two types by origin which are an odontogenic and a non-odontogenic tumors (Nemec et al., 2012). Odontogenic tumor is a benign tumors that deals with the odotogenic process and includes a non-neoplastic lesion of the cells or tissues such as cyst, hyperplasia, inflammatory and infection (Boehm et al., 2011). The non-odontogenic tumors are malignant tumor such as melanoma, squaumous cell carcinoma (SCC), and fibrosarcoma an accounting for 30 - 40%, 17 - 25% ,and 8 - 25%, respectively. Previous study reported that the oral squamous cell carcinoma (both of non-tonsillar and tonsillar SCCs) was the most incidence (40%), followed by melanoma (37%), and fibrosarcoma (22%) (Coyle and Garrett, 2009).

Melanoma is the most frequently oral malignancy occurring in dogs. Oral melanoma was commonly found in Scottish terriers, Golden retrievers, Poodles, and Dachshunds (Millanta et al., 2002). Canine oral melanoma is aggressively biological behaviors depending on diversity of factors. These are classified based on anatomical site, size, stage, and histological parameters (Bergman, 2007). Gingiva and buccal mucosa are commonly location. Nevertheless, it can be involve the palate, tongue and labial mucosa. The oral melanoma is aggressive local invaded and extremely metastasized to both at regional lymph nodes, and lungs (Proulx et al., 2003).

Squamous cell carcinoma (SCC) is a malignant, locally invasive oral tumors in dogs. The SCC is often recurred at same location after surgical excision. Although SCC is less metastasis than melanoma (Coyle and Garrett, 2009). However, tonsillar squamous cell carcinoma mostly metastasized to regional lymph nodes and other organs such as lungs, thyroid gland, spleen, and others (Todoroff and Brodey, 1979).

Fibrosarcoma is locally invasive and low distant metastatic. Golden retrievers and other large dogs are affected more than small breed dogs (Ciekot et al., 1994). Primary location of oral fibrosarcoma is commonly found at gingiva which is usually seen at maxilla but can be presented at mandible (Coyle and Garrett, 2009). Although, the tumors are growing slowly, they are very aggressive by invading to adjacent bone and soft tissue.

The basic diagnostic tools of oral tumors consisted of history taking, physical examination, complete blood count, biochemical parameters and urinalysis. (Bergman, 2007) Sometimes, oral examination required general anesthesia which depends on a patient's behavior and location of tumor. The information that could be collected from oral examination is including size, site, mucosal status such as ulceration and necrosis, nearby tissue involvement, and abnormal tooth. By physical examination, regional lymph nodes should be also palpated. Moreover, a cytological examination needs to rule out metastasis. According to the oral melanoma dogs, they were metastasis accounting for 70% (Williams and Packer, 2003). The cytological examination of regional lymph node was 100% sensitivity and 96% specificity. Thus, this process was an important procedure for staging of tumors (Coyle and Garrett, 2009). Thoracic radiograph is a significant diagnostic method for distant metastasis (Bergman, 2007). In 361 canine oral tumors with lung metastasis reported oral melanoma 8/59 (14%), oral fibrosarcoma 4/40 (10%), and 3/59 (5%) of oral squamous cell carcinoma, including tonsillar and non-tonsillar SCCs (Todoroff and Brodey, 1979). At the presence, the diagnostic tools are developed to increase an efficiency of early detection and classification of the tumor staging. Computer tomography (CT) and magnetic resonance imaging (MRI) were applied for more details of tumors and diagnosed an early lung metastasis. Moreover, they facilitated a prognosis and treatment plan for radiotherapy. Nevertheless, the gold standard to classified a type of tumors was histopathology (Nemec et al., 2012).

Surgery is a primary method to treat an oral cancer. However, it is not curable in all tumors. Most affected dog with melanoma were dead due to metastasis to

other visceral organs; particularly in lung (Coyle and Garrett, 2009). Previous studies reported in surgical treated oral melanoma dogs in different clinical stages. They found oral melanoma stage I and II group which had a 20 months survival period whereas stage III and IV group had a 6 months survival period (Kosovsky et al, 1991). Likewise, the dogs, had an oral melanoma smaller than 2 cm and had no metastasis to regional lymph node, had a median survival time approximately 17 months. The median survival time reduced to 5.5 months in dogs that had a mass larger than 2 cm with a metastasis to regional lymph node (MacEwen et al., 1986; MacEwen et al., 1999). According to literature reviews, early stage oral tumors dogs would stay longer alive after surgery. Surgical treatment could reduce a metastasis in oral squamous cell carcinoma better than oral melanoma (Coyle and Garrett, 2009). Oral squamous cell carcinoma dogs could be able to live more than 1 year with 16 - 26 months survival time after surgery (Kosovsky et al 1991). Previous study reported that 16 oral squamous cell carcinoma dogs in stage II and III had a survival time approximately 7 - 9 months after mandibulectomy and 19.2 months treated with hemimaxillectomy (Schwarz et al., 1991); (Wallace et al., 1992). Surgery is a first option for treatment an oral fibrosarcoma in dogs. Ten dogs with partial mandibulectomy did not recur in 10.6 months and accounting for 50% of that had a survival time more than 1 year (Kosovsky et al., 1991). Oral fibrosarcoma was treated by maxillectomy, which had a median survival time about 9.5 - 12.2 months. 17/29 dogs (59%) were dead from cancer and 14/29 dogs (48%) had a recurrence mass (Schwarz et al., 1991); (Wallace et al., 1992). In some studies revealed no statistical significance between surgery and surgery with radiation (Frazier et al., 2012). However, radiation therapy could be used an early treatment with oral melanoma and other oral tumors as well as enlarged mass that could not be treated by surgical resection (Coyle and Garrett, 2009). The radiation therapy has been used for palliative clinical signs, reduction of metastasis and improvement a quality of life (North and Banks, 2009). Even though the surgical resection and radiation therapy was treated an oral cancer, they might controlled a local site. Thus, both methods did not suitable to control a metastasis. Normally, oral melanoma and tonsillar squamous cell carcinoma have highly metastasis, chemotherapy was applied for treating dogs with metastasis. On the other hand, the

oral melanoma was less responsive to treat with chemotherapy but it can reduce clinical signs and increase overall survival time (Coyle and Garrett, 2009).

Last but not least, diagnosis and staging of oral tumors are very important. If we could detect to cancer at an early stage, we would have a chance to treatment. For example, a small cancer was resected by surgery, that could reduce a metastasis, especially in oral melanoma. Routine evaluation of mucosal lesion is clinical sign and biopsy. Although the oral examination used for observing in the dog mouth, it could not detect until to late stage of cancer. Biopsy was a gold standard for evaluating the oral lesion, yet it often could not identify the cancer or pre-cancer stage. Other techniques were possibly used in clinic to illustrate to an interesting tissue staining with toluidine blue. Cytology or molecular analysis of an exfoliation cell have been developed to early detection for malignant cancer. However, both methods had inconstantly false positive and false negative rates. Thus those methods might not be suitable for early recognition of neoplasm completely. Lately, the identification of tumor biomarkers is an importantly primary for early detection in a malignant oral cancer, especially at-risk dogs. Protein in human disease was accepted to detect a disease as biomarkers (Jou et al., 2010).

Recently, development of proteomics techniques was an early detection and monitoring for oral squamous cell carcinoma in human. Early detection and diagnosis of oral tumors lead to increase a survival rate in canine oral cancer. Advance stage of malignant tumors still have been treated with combination, however; they did not respond in cure and no improve a clinical sign. Many researches suggested that human tissue and biological fluid were compounded of many proteins. The protein was a small molecule hence it had an efficiency to be a biomarker, which related to blood such as plasma, serum and urine. Therefore, the novel biomarker in dogs is interesting a saliva for related with disease same blood sample (Dowling et al., 2008).

Therefore, the oral squamous cell carcinoma (oscc) is the most of oral cancer in human. Thus, many researches study a biomarker from serum, plasma, urine and saliva. Discovered proteins such as beta fibrin, S100 calcium binding protein, transferrin, immunoglobulin heavy chain constant region gamma, and cofilin-1 were leveled up in the saliva of oscc patient. Moreover, novel protein, transthyratin, was significantly decreased in the oscc patient (Dowling et al., 2008). Other studies found a high level of CD44 and some significant proteins such as; insulin growth factor 1, metalloproteinase MMP-9, carbonyls, and CyclinD1:CycD1 (Franzmann et al., 2005); (Shpitzer et al., 2009). Moreover, saliva samples from oscc patient were studied with proteomics analysis using capillary reversed-phase liquid chromatography with quadruple time-of-fight (LC-Q-TOF) mass spectrometry. These methods discovered five special biomarkers for oscc that were called M2BP, MRP14, CD59, profiling, and catalase. These biomarkers were prosperously validated immunoassays because they can be found just in oscc patient (Hu et al., 2008). Nevertheless, they did not correlate between the quantity of proteins and size, stage, and recurrence of cancer. Thus, that leads to study a correlation between the stage of tumor and a level of proteins in saliva's oscc patient. Recent study found a transferrin level in saliva of oscc patient wassignificantly compared with normal human. The level of transferrin was correlated with stage of cancer by using the Universal TNM staging system of the International Union against Cancer (UICC). Moreover, quantity of transferrin was not significantly changed in blood between an oscc patient and normal human. There was not related to stage of cancer (Jou et al., 2010). Then, there was confirmed by a study about the rat parotid acinar cell, which could synthesis and secrete a transferrin protein to salivary gland (Nashida et al., 2009). The transferrin in rat parotid acinar cell that was circardianly secreted from both inside and outside of acini. Transferrin is synthesized from parotid acinar cell synthesis as well as amylase. It has been stimulated by beta-adrenergic or muscarinic reagents. The secretion of protein from outside of the parotid acinar cell, which was passed a basolateral parotid acinar cell and transferred to an apical side by transcytosis. (Nashida et al., 2009) Futhermore, the level of transferrin protein in saliva of oscc patient is higher than normal. The researchers mentioned that the transferin was increased as 91%, 88%

and 84% in stage I, stage II and stage III&IV osscs, respectively. The enzyme-linked immunosorbent assay (ELISA) is 100% specificity and sensitivity in oscc patient stage 1; however, it was specificity 100% and sensitivity 95% in all stages (Jou et al., 2010).

In human, the diagnostic methods have been developed for early detection in oral cancer patient. Because this can reduce a cancer metastasis and increase a survival rate. Discovered salivary as a biomarkers have been evaluated a human's disease by proteomic identification. In animals, salivary sample has been used to analyse cortisol level (Vincent and Michell, 1992; Geverink et al., 1999). The collection of salivary sample was easy, non-stressful, comfortable. In addition, this method can facilitate a disease diagnosis in patients whom unsuitably brough to animal hospitals (Schaaij-Visser et al., 2010). Furthermore Immunological chemokines such as IgGs, (Kugler et al., 1992), immune competence (German et al., 1998), rabies antigen (Krasteva and Kisselova, 2011), and drug levels were evaluated using the saliva samples (Dunnett et al., 2002). Moreover, recent study comparably challenged C-reactive protein's (CRP) in serum and saliva dogs which the result found significantly different level of CRP in saliva and serum between normal and inflammation dogs (Parra et al., 2005a).

At the present, many diseases have been recognized along with older age, especially cancer. The saliva biomarker was developed in early recognition of oral cancer patient (Dowling et al., 2008). Therefore, there was interested in saliva biomarker which could use to diagnose oral cancer in dog. Moreover, the salivary was easy, non-stressful, and comfortable (Schaaij-Visser et al., 2010). Dog saliva has a transferrin protein same as human saliva. The levels of transferrin in saliva could be evaluated stage of OSCC patient (Jou et al., 2010).

Normally, Transferrin protein combines with an three plus-poled iron for protection a transportation in toxic iron pole form. The cell needs an iron for supporting oxidative, replication, and DNA synthesis. This protein transfers an iron in the blood to the cell by reacting with transferrin receptor (TfR) (de Jong et al., 1990; Daniels et al., 2006). The transferrin receptor receives an iron into cell and controls a cell development. Some cells and tissues have more transferrin receptors than

others, such as immature red blood cell, placenta, and rapidly dividing cells. Furthermore, the quantitative of TfR is a relative with cell development (Ponka and Lok, 1999). In the previous study, cancer cell had many TfR which resulted of the cancer cell needs an iron support for division. The iron is a cofactor of ribonucleotide reductase enzyme, which enhanced cell division (Daniels et al., 2006). The study about mammary gland tumors compared with mammary gland carcinoma. That showed mammary gland carcinoma expressed TfR 4-5 times than mammary gland tumors (Walker and Day, 1986a). Moreover, expression of TfR was related with level and stage of some types of cancer, such as transitional cell carcinomas, mammary gland carcinoma, gliomas, lung cancer, chronic leukemia and non-Hodgkin's lymphoma (Daniels et al., 2006). Canine lymphoma showed TfR expression depended on stage and cancer cell division (Priest et al., 2011). Cancer cell significantly expresses level of TfR more than normal cell. That had been related with a stage and development of cancer, which correlated a cancer cell need an iron for supporting (Daniels et al., 2012).

This research aimed to develop ELISA method for detection of saliva and plasma transferrin in oronasal tumor affected dogs compared to normal dogs and to demonstrate TfR expression in canine oronasal tumor.

CHAPTER III

Materials and Methods

Sample collection

Samples in this study were divided into two groups; control group and experimental group. This experiment was done under Laboratory Animal Center of Chulalongkorn University No. 1431029.

Control group: nine dogs presented at Small Animal Teaching Hospital, Faculty of Veterinary Science, Chulalongkorn University for dental scaling and extraction. They were over 6-years-old and healthy. Physical examination was performed and complete blood count, kidney function test (BUN and creatinine) and liver enzymes (ALT, ALP) were within normal range. Exclusion criteria was done by intestinal, liver or renal diseases, no evidence of oral, head and neck tumors.

Experimental group: Fifteen dogs had oronasal mass that were appointed for surgical excision at the Small Animal Teaching Hospital, Faculty of Veterinary Science, Chulalongkorn University during November, 2013 to July, 2014. Inclusion criteria were included age over 6 years, presence of oronasal mass without previous treatment either chemotherapy or radiotherapy.

Later, the dogs were evaluated stage of cancer as TMN staging (Withrow et al., 2013). An oral examination was done to determine tumor size and located site. Regional lymph nodes were measured a size and texture by physical examination and required to rule out metastasis by cytological examination. Tumor diagnosis was done by cytology and histopathology.

Clinical staging of the affected dogs were performed by measure tumor size under sedation, skull to abdomen radiographic examination by X-rays (Digital X-rays system, Brivo DR-F®, GE Thailand) or CT-scan(64-slice helical CT unit, Optima®, GE Thailand). The 64-slice multiple detector computed tomography (MDCT) was performed at 120 kV and 250 mA. The setting field was covered to head, neck, chest and abdomen. Then, non-ionic, water soluble, iodinated contrast medium (iohexol, Omnipaque, USA) were intravenous administered at a dosage of 600 mgI/kg by using the automatic MDCT injector at 2 ml/second rate. Post contrasts CT image were subsequently achieved while the contrast medium presented at mid cervical jugular vein. CT images showed details of oronasal mass, regional lymph nodes, chest and abdomen. Then clinical staging was done according to TNM staging in Table 1 (Withrow et al., 2013).

Sample preparation

To measure saliva transferrin levels, the dogs in this study were to clean the mouth and fasting at least 12 hours before saliva collection. Saliva was collected in a morning (between 8 and 10 am.) without mechanical and chemical stimulation. Mouth were washed and flushed with 0.9% sterile normal saline solution and let them to swallow saliva one time for 10 minutes before collection (Jou et al., 2010). Whole saliva was collected for 1-2 minute by sterile gauze about 0.5-3.0 ml and kept in tube at -20°C until analysis (Parra et al., 2005b).

Samples were collected at the visit and 14 days after operation in the experimental group. Saliva of the control group did not collect over 4 weeks after operation. Saliva samples were centrifuged at 12,000 rpm for 10 minute at 4°C. The supernatants were placed into a new plain eppendrof approximately 300 µl (Jou et al., 2010) and kept at -20°C until assay (Parra et al., 2005a). All of these procedures were done by researcher at the same condition.

Four ml of Whole blood was collected from the cephalic or the saphenous vein in both groups, at the first day of visit and 14 days after operation in the experimental group. Serum of the control group did not collect over 4 weeks after operation. Blood profiles, Liver and renal functions were determined by automated machine. To measure serum levels of transferrin, 3 ml collected blood was centrifuged at 7000 rpm for serum and stored at -20°C until analysis (Parra et al., 2005b).

Table 1 Clinical staging (TNM) of oral tumors in dogs and cats (Withrow et al., 2013)

Clinical staging system for oral tumors			
Primary tumor (T)			
Tis		Tumor in situ	
T1		Tumor < 2 cm in diameter at greatest dimension	
T1a		Without evidence of bone invasion	
T1b		With evidence of bone invasion	
T2		Tumor 2-4 cm in diameter at greatest dimension	
T2a		Without evidence of bone invasion	
T2b		With evidence of bone invasion	
T3		Tumor 2-4 cm in diameter at greatest dimension	
T3a		Without evidence of bone invasion	
T3b		With evidence of bone invasion	
Regional lymph nodes (N)			
N0		No regional lymph node metastasis	
N1		Moveable ipsilateral lymph nodes	
N1a		No evidence of lymph node metastasis	
N1b		Evidence of lymph node metastasis	
N2		Moveable contralateral lymph nodes	
N2a		No evidence of lymph node metastasis	
N2b		Evidence of lymph node metastasis	
N3		Fixed lymph nodes	
Distant metastasis (M)			
M0		No distant metastasis	
M1		Distant metastasis [specify site(s)]	
Stage grouping	**Tumor (T)**	**Nodes (N)**	**Metastasis (M)**
I	T1	N0, N1a, N2a	M0
II	T2	N0, N1a, N2a	M0
III	T3	N0, N1a, N2a	M0
	Any T	N1b	M0
IV	Any T	N2b, N3	M0
	Any T	Any N	M1

Serum and saliva Transferrin Determination by Enzyme-linked immunosorbent assay (ELISA)

Quantitative of transferrin in saliva and serum was performed by Transferrin Dog ELISA kit. (ab157704**,** *eBioscience*, Abcam, Cambridge, UK). Saliva and serum transferrin in sample reacted with the anti-Transferrin antibodies, which have been adsorbed to the surface of polystyrene microtiter wells. 100 ul of each 1:50,000 dilution of serum sample and 1:10 for saliva sample, was pipetted into designated wells then duplicated into another well. Then the samples were covered with plate and incubated at room temperature for 30 minutes. After incubation, the wells were washed 4 times with washing buffer. 100 ul of secondary anti-Transferrin antibodies, which conjugated with horseradish peroxidase (HRP), were added into the prepared samples in each well. Later, the samples were incubated at room temperature for 30 minutes and then the covered plate from light condition. Following another washing step, the amount of enzyme bound in complex was measured by the addition of a chromogenic substrate, 3,3',5,5'-tetramethylbenzidine(TMB) for 10 minutes in the dark. 0.3 M sulfuric acid was added to stop the reaction then determined the absorbance (450 nm) of the samples of each wells by ELISA plate reader. The results were measured of the quantity of transferrin by interpolation from the standard curve.

Histopathology

Tissue sample collection and preparation

In experimental groups, 15 dogs with oronasal mass were removed tumor by surgical treatment. The tumor size was measured by using vernier caliper. In control groups, 10 dogs were requested for dental scaling and tooth extraction, while gingival tissue at extraction area were collected using punch biopsy. Canine placenta were a positive control for transferrin receptor expression (Priest et al., 2011). All tissue samples were randomly collected about 1 cm^2 per each and fixed in 10% neutral buffered formalin for 24 hours. Then, the samples were routine histologic processed, embedded in paraffin blocks and sectioned at 4 μm thickness and placed on silane-coated slides. Tissue samples were stained with Hematoxylin and Eosin (H&E) for histopathological diagnosis.

Transferrin receptor expression

Immunohistochemical staining of transferrin receptor in canine oronasal tumor mass and gingival tissues was performed by LSAB method. Deparaffinization and rehydration were performed in xylene and graded ethanol. Antigen retrieve was performed by heated with Tris-HCl buffer (pH 8.0) by microwave (medium-high) for 20 minutes. The slides were washed with phosphate buffer saline (PBS), then endogenous peroxidase enzymes were blocked with a 3% solution of hydrogen peroxide in methanol for 10 minutes. The slides were later washed by PBS and non-specific protein was blocked with 1% bovine serum albumin at $37^{o}C$ for 20 minutes. The slides were incubated overnight at $4^{o}C$ with monoclonal mouse anti-human TfR1 antibody (clone 68.4, Zymed Laboratories, San Francisco, CA), at a dilution 1:500. Following several washed slides with PBS, primary antibody was tagged with secondary antibody in Envision polymer kit (Dako, Carpentaria, CA) at $37^{o}C$ for 45

minutes and 3,3' diaminobenzidine was used as a chromogen substrate. Mayer's hematoxylin was counterstained for those slides. Then, the slides were dehydrated and mounted with Permount. Canine placenta was served as positive control to compare between tissue from oronasal cancer and chronic gingival hyperplasia (Priest et al., 2011). The Image Proplus Analysis ver 6.0 was applied to describe in detail for total captured area, photographed and evaluated as percentage of expression in all samples.

Statistical and data analyses

Student T-test and Mann-Whitney U test were used to assess the differentiation of transferrin levels and its receptor between the oronasal cancer and control groups. Paired T test and Wilcoxon Signed-Rank test were used to determine the differentiation of transferrin levels between the 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 $p < 0.05$.

CHAPTER IV
RESULTS

Saliva, serum, and tissue samples were collected from 24 dogs that were presented at the Small Animal Teaching Hospital, Faculty of Veterinary Science, Chulalongkorn University. The normal group was composed of 6 males (Male (M) = 3 and Male castration (Mc) = 3) and 3 females (Females (F) = 3) with chronic hyperplastic gingivitis.

The experimental; oronasal cancer group consisted of 10 males (M = 9, Mc = 1) and 5 females (F = 1, Female sprayed (Fs) = 4) with malignant melanoma (n = 12) and squamous cell carcinoma (SCC) (n = 3). An average age in the experimental group was 10.6 years old (10.6 ± 1.765). In this group, 4 dogs had regional lymph node metastasis (Table 2).
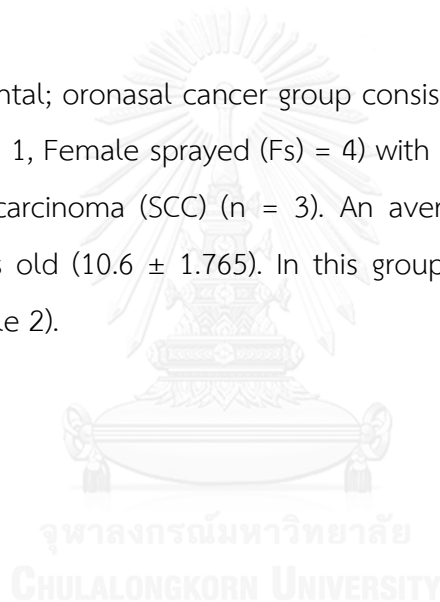
Table 2. Signalment data, TNM stage, and histopathology in the experimental group.

No	Breed	Sex	Age (years)	Tumor Size (cm)	Lymph node Metastasis	Lungs Metastasis	Clinical staging	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: Sprayed, C: Castration

SCC: Squamous Cell Carcinoma

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

Calibration curve was determined from a set of five points of the manufacturer's standard concentration of transferrin. Sensitivity of the ELISA test kit has minimum detectable dose at 4.3054 ng/ml and controlled serum recovery was greater than 85%. Intra-assay and inter-assay coefficient of variance was lower than 10% for canine transferrin ELISA test. A correlation was evaluated between the quantity of transferrin protein and the OD value of the ELISA from 0 to 400 ng/ml of transferrin. The practical measurement range of samples was from 6.25 to 400 ng/ml of protein. Sample data were divided into 5 groups; group, histology, lymph node involvement, TNM stage and survival time. The saliva sample test was non-parametric data and shown as µg/ml in Table 3. The serum sample test was parametric data and expressed as mg/ml in Table 4.

The median of saliva transferrin concentrations in both of before and after surgery experimental groups were 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. Histological analysis was not affected to alternate transferrin in saliva significantly different between melanoma and SCC in before surgery including after surgery. The saliva transferrin level was not statistically significant between the metastasis and non-metastasis to regional lymph node groups in both of before and after surgical treatment. Nevertheless, non-lymph node involvement before surgery was significantly different compared to that of the after surgery ($p < 0.05$). Comparison between clinical staging, TNM stage III and stage IV, stage III were significantly greater than stage IV on statistical analysis in before surgical treatment group ($p < 0.05$) while the after surgical treatment was not significantly different on statistical analysis. The level of

saliva transferrin in dogs had survival times more than 3 months were not significantly different with dogs had survival times less than 3 months after surgical treatment. However, comparison between 2 groups of survival times was significantly different in before surgical treatment ($p < 0.05$). Survival times were not statistically significant different compared between before and after surgical treatment.

The mean of serum transferrin concentration in the experimental group both before and after surgery was significantly lower than normal group ($p < 0.05$). However, quantity of serum transferrin before surgery was not significantly different between quantity of serum transferrin after surgery. Classification of oral tumor was not significantly different level of transferrin including surgical treatment was not affect to serum transferrin concentration. Serum transferrin of regional lymph node metastasis group, was not significantly different compared to without metastasis group before and after surgical treatment. Comparison between clinical staging; TNM stage III and stage IV, were not significantly different on statistical analysis same as before and after surgical treatment. The level of serum transferrin in dog had survival time more than 3 months was not significantly different with dog had survival time less than 3 months including before and after surgical treatment.

Table 3. Comparison of saliva transferrin concentration in control and experimental groups with histology, lymph node involvement, TNM stage and survival time (Mean ± SD, Median; 25th and 75th percentile).

	Saliva transferrin (µg/ml)		Statistic test			
	before	after	Independent T test	Pair T test	Mann-Whitney U test	Wilcoxan signed ranks test
Group						
- Experimental	3.055 ± 0.099	2.985; 2.779-3.083	-	-	Before; p>0.05	p>0.05
- Control	2.698 ± 0.765	2.698 ± 0.765	-	-	After; p>0.05	-
Histopathological Dx						
- Melanoma (n=12)	3.031 ± 0.121	2.985; 2.760-3.076	-	-	Before; p>0.05	p>0.05
- SCC (n=3)	3.074 ± 0.084	3.008 ± 0.202	-	-	After; p>0.05	p>0.05
Lymph node involvement						
- N0 (n=11)	3.054 ± 0.079	2.968; 2.779-3.083	Before; p>0.05	-	-	p<0.05*
- N1 (n=4)	2.999 ± 0.189	3.040; 2.776-3.134	-	-	After; p>0.05	p>0.05
TNM stage						
- I (n=1)	3.091	3.053	-	-	-	-
- II (n=1)	3.049	2.986	-	-	-	-
- III (n=9)	3.049; 2.961-3.141	2.935; 2.488-3.073	-	-	Before; p<0.05*	p>0.05
- IV (n=4)	3.010; 2.814-3.132	3.101; 3.021-3.144	-	-	After; p>0.05	p>0.05
Survival time						
- < 3 month (n=2)	3.049 ± 0.205	2.851 ± 0.212	Before; p<0.05*	p>0.05	-	-
- > 3 month (n=13)	3.038 ± 0.107	2.860 ± 0.452	-	p>0.05	After; p>0.05	-

bindicate statistically significant difference at p<0.05*.

Table 4. Comparison of serum transferrin concentration in the control and experimental groups with histology, lymph node involvement, TNM stage and survival time (Mean ± SD, Median; 25th and 75th percentile).

	Serum transferrin (mg/ml)		Statistic test			
	before	after	Independent T test	Pair T test	Mann-Whitney U test	Wilcoxan signed ranks test
Group						
- Experimental	4.713 ± 0.984	4.772 ± 1.818	Before; p<0.05*	p>0.05	-	p>0.05
- Control	6.148 ± 0.285	6.148 ± 0.285	After; p<0.05*	-	-	-
Histopathological Dx						
- Melanoma (n=12)	4.700 ± 1.017	4.937 ± 1.850	Before; p>0.05	p>0.05	-	-
- SCC (n=3)	4.306 ± 0.882	4.113 ± 1.872	After; p>0.05	p>0.05	-	-
Lymph node involvement						
- N0 (n=11)	4.758 ± 0.940	4.912; 3.235-5.823	Before; p>0.05	-		p>0.05
- N1 (n=4)	4.590 ± 1.244	4.425; 2.962-5.978			After; p>0.05	p>0.05
TNM stage						
- I (n=1)	5.208	5.823				
- II (n=1)	6.192	4.912				
- III (n=9)	4.410; 3.815-5.585	5.177; 3.344-6.333	-	-	Before p>0.05	p>0.05
- IV (n=4)	4.415; 3.323-5.320	3.154; 2.989-5.314			After p>0.05	p>0.05
Survival time						
- < 3 month (n=2)	5.604 ± 0.131	4.481 ± 1.991	Before; p>0.05	p>0.05	-	-
- > 3 month (n=13)	4.576 ± 0.988	4.817 ± 1.874	After; p>0.05	p>0.05	-	-

[b] indicate statistically significant difference at p<0.05*.

Estimation of correlation between saliva and serum transferrin. The relation of the percent area of transferrin receptor and transferrin concentrations.

There was no correlation between serum and saliva transferrin concentrations in the control and experimental group tested before and after surgical treatments. Among histopathological diagnosis, lymph node metastasis and clinical staging classified groups, there was no correlation of statistic significantly difference of serum and saliva transferrin level which were tested before and after surgical treatment. Interestingly, there was a statistically significant difference of serum and saliva transferrin level in dogs had survival times less than 3 months ($p < 0.001$). The correlation in dogs had survival times more than 3 months, was not related to serum and saliva transferrin. There were shown in Table 5.

Oronasal cancer tissues were obtained from 15 dogs (experimental group presented at Small Animal Teaching Hospital Faculty of Veterinary Science, Chulalongkorn University. Gingival tissues from 9 dogs (control group) that were extracted and dental scaling, were collected. The result of transferrin receptor expression showed statistically significant difference between experimental group and control group ($p < 0.001$). There was no correlation between transferrin receptors (TfR) expression, saliva and serum transferrin levels before surgical treatments in the control and experimental groups. The melanoma, lymph node involvement and clinical staging classified groups, there was no correlation of statistically significant difference between TfR expression, saliva and serum transferrin concentrations before surgical treatments. Dogs had survival times less than 3 months was correlated between serum and saliva transferrin with transferrin receptor expression ($p < 0.001$). However, only 2 dogs had survival times less than 3 months that was not enough for analysis. The correlation in dogs had survival times more

than 3 months, was not related to serum and saliva transferrin with transferrin receptor expression (p>0.05). These results were demonstrated in table 6.

Table 5. The correlation between saliva and serum transferrin concentration before and after surgical treatment in experimental group with histology, lymph node involvement, TNM stage and survival time (Mean ± SD, Median; 25th and 75th percentile).

	Saliva transferrin (µg/ml)		Serum transferrin (mg/ml)		Statistic test : correlation		
	before	after	before	After	group	r	P-value
Group							
- Experimental	3.055 ± 0.099	2.985; 2.779-3.083	4.713 ± 0.984	4.772 ± 1.818	Before[1]	0.297	0.283
- Control	2.698 ± 0.765	2.698 ± 0.765	6.148 ± 0.285	6.148 ± 0.285	After[2]	0.050	0.860
					Control[2]	-0.268	0.486
Histopathological Dx							
- Melanoma (n=12)	3.031 ± 0.121	2.985; 2.760-3.076	4.700 ± 1.017	4.937 ± 1.850	Before[1]/after[2]	0.290/-0.091	0.361/0.779
- SCC (n=3)	3.074 ± 0.084	3.008 ± 0.202	4.306 ± 0.882	4.113 ± 1.872	Before[1]/after[1]	0.826/0.999*	0.382/0.025
Lymph node involvement							
- N0 (n=11)	3.054 ± 0.079	2.968; 2.779-3.083	4.758 ± 0.940	4.912; 3.235-5.823	Before[1]/after[2]	0.504/0.118	0.114/0.729
- N1 (n=4)	2.999 ± 0.189	3.040; 2.776-3.134	4.590 ± 1.244	4.425; 2.962-5.978	Before[1]/after[2]	0.085/0.316	0.915/0.684
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)	3.049; 2.961-3.141	2.935; 2.488-3.073	4.410; 3.815-5.585	5.177; 3.344-6.333	Before[2]/after[2]	0.405/0.000	0.320/1.000
- IV (n=4)	3.010; 2.814-3.132	3.101; 3.021-3.144	4.415; 3.323-5.320	3.154; 2.989-5.314	Before[2]/after[2]	0.000/0.800	1.000/0.200
Survival time							
- < 3 month (n=2)	3.049 ± 0.205	2.851 ± 0.212	5.604 ± 0.131	4.481 ± 1.991	Before[1]/after[1]	1.000**/1.000***	-/-
- > 3 month (n=13)	3.038 ± 0.107	3.053; 2.857-3.103	4.576 ± 0.988	4.817 ± 1.874	Before[1]/after[2]	0.329/0.143	0.273/0.642

[1]The significant correlation was assessed by Pearson correlation at p < 0.05*, p < 0.001**.

[2]The significant correlation was assessed by Spearman's rho at p < 0.05*

Table 6. The correlation between saliva, serum transferrin concentration before surgical treatment and transferrin receptor in experimental group with histology, lymph node involvement, TNM stage and survival time (Mean ± SD, Median; 25th and 75th percentile).

	Saliva transferrin (µg/ml)	Serum transferrin (mg/ml)	Transferrin receptor	Statistic test : correlation Transferrin receptor	
	before	before	%Area	r	P-value
Group					
- Experimental	3.055 ± 0.099	4.713 ± 0.984	61.047 ± 16.875***	0.461, -0.110[a]	0.113, 0.721
- Control	2.698 ± 0.765	6.148 ± 0.285	0.133; 0.042-1.244	-0.050, -0.460[b]	0.898, 0.213
Histopathological Dx					
- Melanoma (n=12)	3.031 ± 0.121	4.700 ± 1.017	57.274 ± 16.079	-0.522, -0.181[a]	0.082, 0.573
- SCC (n=3)	3.074 ± 0.084	4.306 ± 0.882	76.136 ± 9.816	-0.999*, -0.805[b]	0.023, 0.404
Lymph node involvement					
- N0 (n=11)	3.054 ± 0.079	4.758 ± 0.940	58.434 ± 18.330	0.147, -0.290[a]	0.666, 0.386
- N1 (n=4)	2.999 ± 0.189	4.590 ± 1.244	68.231 ± 10.728	-0.270, 0.633[a]	0.730, 0.367
TNM stage					
- I (n=1)	3.091	5.208	30.864	-	-
- II (n=1)	3.049	6.192	60.970	-	-
- III (n=9)	3.049; 2.961-3.141	4.410; 3.815-5.585	61.214 ± 17.762	0.183, 0.250[b]	0.637, 0.516
- IV (n=4)	3.010; 2.814-3.132	4.415; 3.323-5.320	68.231 ± 10.728	0.200, 0.400[b]	0.800, 0.600
Survival time					
- < 3 month (n=2)	3.049 ± 0.205	5.604 ± 0.131	60.929 ± 10.121	1.000**, 1.000**[a]	-
- > 3 month (n=13)	3.038 ± 0.107	4.576 ± 0.988	61.065 ± 17.992	-0.578*, -0.346[a]	0.039, 0.247

[a] This significant correlation was assessed by Pearson correlation at $p < 0.05$; $p < 0.001$*.

[b] This significant correlation was assessed by Spearman's rho at $p < 0.05$.

** indicates statistically significant difference at $p < 0.001$. assessed by Mann-Whitney U test at $p < 0.001$

Determination of transferrin receptor between experimental group and control group.

Oronasal cancer tissues were obtained from 15 dogs (experimental group) whom treated at Small Animal Teaching Hospital Faculty of Veterinary Science, Chulalongkorn University. Gingival tissues from 9 dogs (control group) who were extracted and dental scaling, were collected. The result of transferrin receptor expression showed statistically significant difference between experimental group and control group ($p < 0.01$). The result was shown in table 6. The histology of placental and gingival tissues were illustrated by H&E and IHC in fig 1. Oral melanoma and squamous cell carcinoma tissues were demonstrated by H&E and IHC in fig 2.
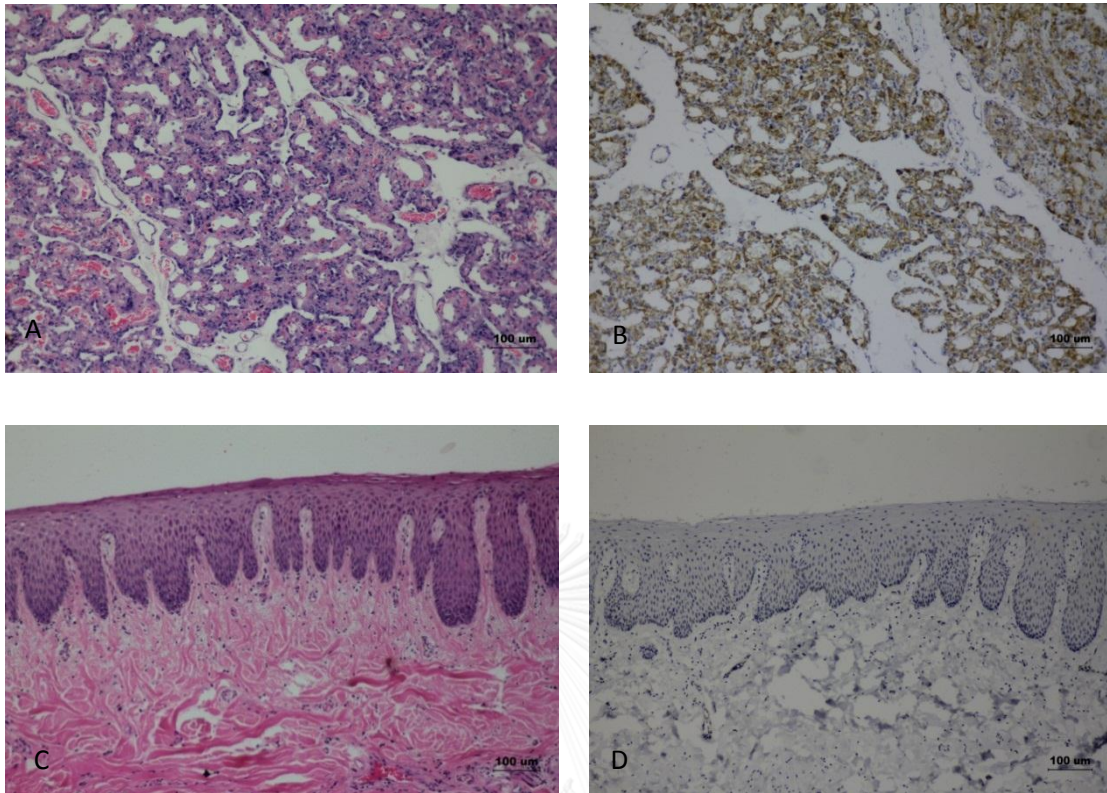
Figure 1. The histopathological of canine placental tissue were observed transferrin receptor of trophoblastic cells (H&E, A, BAR = 100 μm).

Transferrin receptor was positive in brown color in trophoblastic cells (IHC, B, BAR = 100 μm). Chronic hyperplastic gingivitis expressed transferrin receptor of proliferative mucosal epithelial cells (H&E, C, BAR = 100 μm). Transferrin receptor was seen in brown color in basal epithelial cells (IHC, D, BAR = 100 μm).
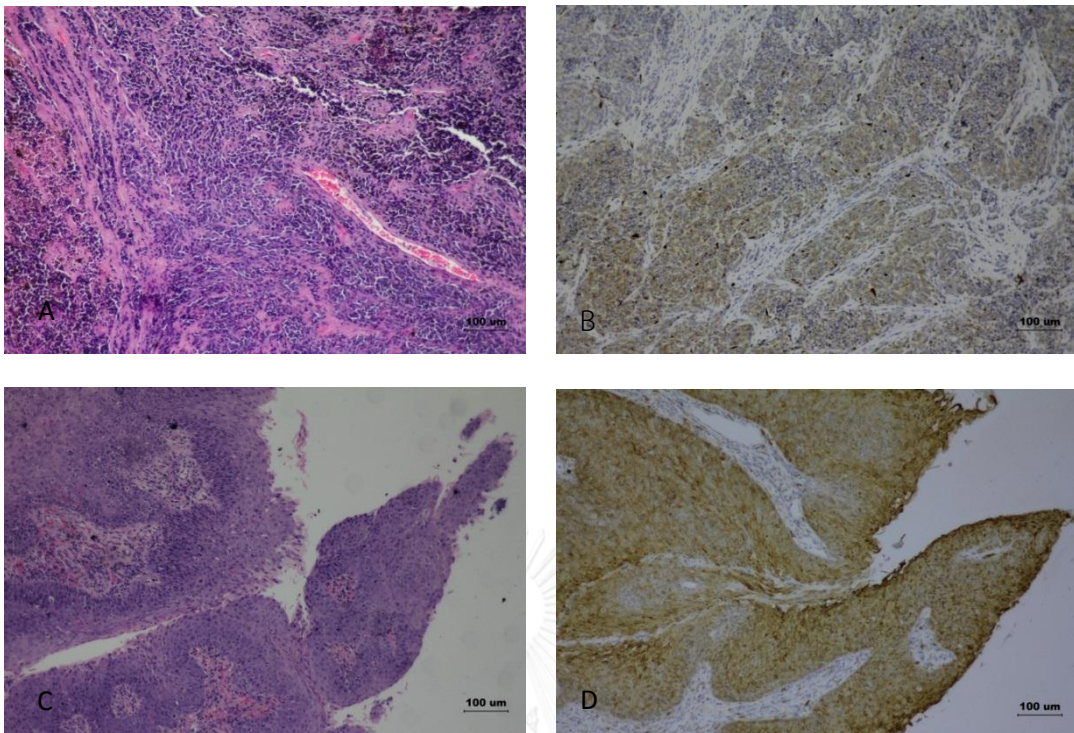
Figure 2. Hematoxylin and eosin section showed multilobulated of neoplastic cells. that large spindloid with large ovoid nuclei and contained brown to black intracytoplasmic pigment interpreted as melanin (H&E, A, BAR = 100 µm). Monoclonal mouse anti-human TfR1 antibody was assessed and the neoplastic cells demonstrated strong cytoplasmic immunoreactivity (IHC, B, BAR = 100 µm). The histopathology from oral mass showed diffuse islands of squamous cells and doward growth invading the subcumosa layers. The neoplastic cells were large polyhedral to cuboidal shape, pleomorphic large round nuclei with prominent nucleoli (H&E, C, BAR = 100 µm). As same previous, Transferrin receptor demonstrated brown color in positive areas by immunohistochemistry using monoclonal mouse anti-human and squamous cell carcinoma (IHC, D, BAR = 100 µm).

For example, this picture illustrated to collection, clinical staging and IHC from tumors

Figure 3. Case No.15 (HN 5613379) presented a large necrotic hemorrhagic mass. (arrow) and loss of teeth with rostral mandibular bone lysis.

Figure 4. The skull-cervical radiographs revealed a mass at rostral mandible with bone reaction (arrow).

Figure 5. Macroscopic lesions were shown as severe chronic focally extensive gingivostomatitis, size 4x6 cm on the rostral mandibular (bar = 1 cm).
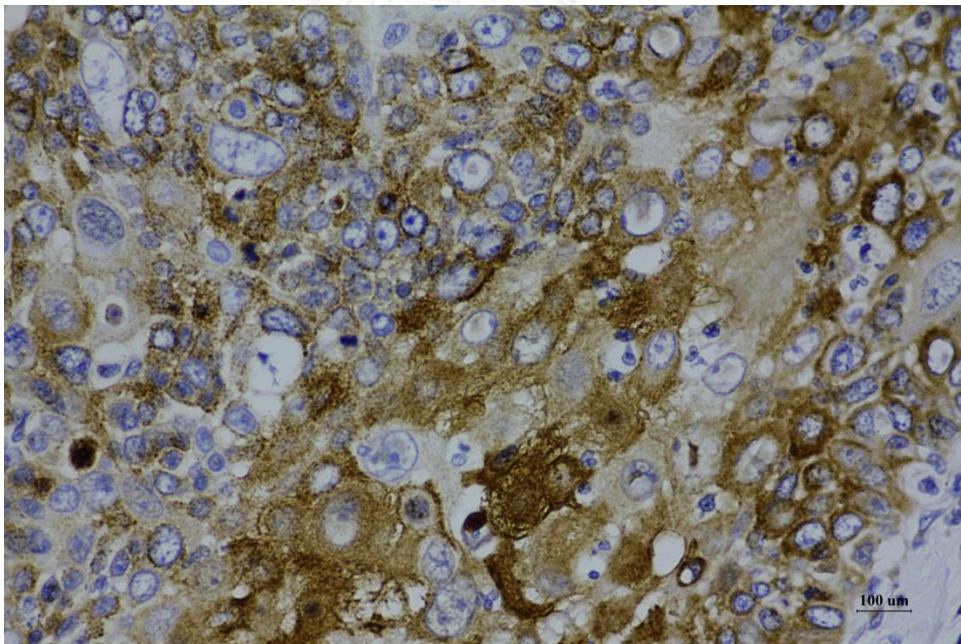
Figure 6. Histopathological diagnosis of this case was a positive immunohistochemistry in cytoplasm of neoplastic cells, that was seen in squamous cell carcinoma.

CHAPTER V

DISCUSSION

Transferrin in canine serum was estimated by ELISA assay *(eBioscience)*. The result showed the mean level of serum transferrin in the control group was insignificantly ($p > 0.05$) higher than before and after surgical of theexperimental group. Surgical treatment could not significantly changed the quantity of serum transferrin. Type of cancer, stage of cancer, and survival time, did not affect serum transferrin concentrations. However, serum transferrin level after surgical treatment in the clinical stage III was higher than that in the clinical stage IV. In comparing the serum transferrin concentrations between the survival time of > 3 months and survival time of < 3 months after surgery, the concentration was higher in the survival time of more than 3 months. The serum transferrin did not significantly related with cancer metastasis but the level in the metastasis group was lower than that with no metastasis. Normally, the main cells producing transferrin in blood are hepatocytes in the liver. Moreover, the transferrin synthesis appears in other organs such as 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 were studies, that found an increase or decrease in transferrin correlated with plasma iron concentration (de Jong et al., 1990). Iron deficiency from anemia, pregnancy, and childhood may increase transferrin level in serum that is according to a rise in protein synthesis. Moreover, an acute hepatitis, dietary hemosiderosis, and effect of oestrogenic components could elevate transferrin and iron concentration. In contrast, the reduction of transferrin results from iron overload, other than secondary iron overload (transfusional, dietary). The atransferrinaemia and other diseases including infections, malnutrition, rheumatoid arthritis, cancer malignancies, trauma and surgery

were causes of the decrease both of serum transferrin and iron concentration (Morgan, 1981). In this study, The decrease of serum transferrin in experimental group was similar to that found in human which might result from malignancy. Nevertheless, serum transferrin in oronasal cancer was not significantly correlated with the malignancy of tumors. Malignancy cells need iron for cell development and division and require iron uptake higher than the normal cell. A transferrin bound iron uptake is greater in cancer cells than normal liver cells. This might be the cause of a decrease serum transferrin. Dogs used in this study had clinical stage III of oronasal cancer or survival time more than 3 months, which had serum transferrin after surgical treatment higher than clinical stage IV or survival time less than 3 months. Because dogs were clinical stage IV or survival time less than 3 months had a metastasis which shown a result same as the cancer malignancy. However, the cancer dog was often found anemia, which causes of reduction of serum transferrin.

The saliva collection is noninvasive, less stressful for animals, and less complicated than blood, and decreases risk of contamination compared to serum collection. Moreover, the method for collection of saliva was easily done by the owner, reduce cost and transported animals to the hospital (Parra et al., 2005a). On the other hand, The saliva was effected by many factors such as; flow rate, circadian rhythms, exercise, plasma composition, olfaction, and disease (Dawes, 1993). Thus, dogs in this study were prohibited to eat food and mouth washing at least 12 hours and drink water at least 6 hours before collection of saliva. The collection procedure was decide to use unstimulated saliva and reduce factors effected to salivary composition and salivary flow rate. In human, saliva as a diagnostic tool to early detection for clinical disease including previous plan for effective prevention and treatment of disease. Saliva as diagnostic fluid was the diagnostic potentials of salivary biomarkers in monitoring and detecting periodontal disease, Oral and Breast cancers, and SjÖgren's syndrome (Liu and Duan, 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). The others study found a high level of some proteins such as; insulin growth factor 1, metalloproteinase MMP-9, carbonyls, and Cyclin D1:CycD1, too (Franzmann et al., 2005) (Shpitzer et al., 2009). Although, they discovered many protein as biomarkers in salivary cancer patient but no molecule was a high accuracy for identifying early disease (Elashoff et al., 2012). In animals, salivary sample was used to analysis cortisol level (Vincent and Michell, 1992; Geverink et al., 1999). Furthermore, Level of immunity was evaluated from saliva. For example, IgGs (Kugler et al., 1992), immune competence (German et al., 1998), search rabies antigen (Krasteva and Kisselova, 2011), and level drug (Dunnett et al., 2002). Other study, C-reactive protein's (CRP) quantitative in serum compared with CRP in saliva dogs that was stimulated by drug for inflammation. They found significantly different level of CRP in saliva and serum between normal and inflammation dog (Parra et al., 2005a), Transferrin was interested to diagnostic tool in canine oral cancer. Because oral squamous cell carcinoma patient found level of transferrin higher than healthy person which correlated with a stage of cancer. In early stage, saliva transferrin level was higher than late stage that was very interested to use for detection and screening the early stage of cancer (Jou et al., 2010). For above reason, transferrin was used in this research that was estimated by ELISA assay (*eBioscience*). We found a mean level of saliva transferrin in the experimental group before surgical treatment (3.040 ± 0.113 μl) higher than the control group (2.698 ± 0.765 μl) but no significantly in statistic test. After surgery, the concentration of saliva transferrin was not significantly differentiated with control group. Classification of tumor and lymph node involvement, which were not significant effected on saliva transferrin concentrations. We found each of TNM stage before surgical treatment had a mean different of saliva transferrin level; stage I (3.091 μl), stage II (3.049 μl), stage III (3.057 ± 0.096 μl) and stage IV (2.985 ± 0.171 μl) when compared between saliva transferrin in dogs with stage III and IV, that was significantly differentiated in statistic test ($p < 0.05$). Dog with clinical stage I was highest a saliva transferrin more than other stages same as previous research in human (Jou et al., 2010) but only one dog had a clinical stage I, that was not enough for analysis. Survival times before surgical treatment was

significantly different between dogs died in 3 months and still lived over 3 months after surgery ($p < 0.05$). The dogs had a survival times less than 3 months, which were a higher saliva transferrin concentration. This probably because 2 dogs was a melanoma stage IV, which had more hemorrhage, inflammation and secondary infection than other stages. Normally, Dogs are very hard to control the oral environment. Dogs in the control group had a periodontitis grade III-IV that is shown, they was a bacterial infection and chronic stomatitis. Free-iron transferrin was an antimicrobial effect against infection by microorganisms (Weinberg, 1977). It was illustrated to competition bound iron from transferrin and bacterial siderophores, thus bereaving bacteria from a nutrient (de Jong et al., 1990). Therefore, antimicrobial effect might be elevated level of saliva transferrin in control group that was supported by Nishida's research. They found a rat parotid acinar cell which secreted endogenous and exogenous transferrin in saliva (Nashida et al., 2009). Transferrin from salivary glands may have bacteriostatic effects because it may be free-iron transferrin (not contain Fe^{3+}). According to the reason, we known lactoferrin and analogue of transferrin in human saliva which presented antimicrobial properties (Humphrey and Williamson, 2001). Thus, the oral problem in dogs might be elevated the saliva transferrin that might cause a different result in human research. Because, human could control an oral problem by themselves. Another reason, cell proliferation by inflammation that might elevated the saliva transferrin (Salonen and Kallajoki, 1986). Therefore, the dogs in control group were kept the saliva sample after dental scaling and extraction up to 4 weeks. That might close to normal oral environmental as possible.

There was no correlation between level transferrin in saliva and serum found in this study similar to a previous study in human. They found saliva transferrin in oscc patient that was higher than free oral cancer patient and the concentration of saliva transferrin was correlated with stage of tumors by ELISA assay. Their results exhibited that the mean level of saliva transferrin in oscc patients was higher by 91% in T1 group, 88% in T2 group and 84% in T3/T4 group compared with healthy person (Jou et al., 2010). By contrast, the level of salivary transferrin in oral cancer dog was

not significantly different compared with control group and no associated with metastasis of cancer. The serum transferrin in human and dog shown in one direction that decreased in oscc patients and oral cancer dogs, but did not significantly related with stage of cancer, metastasis, classification and survival times.

The age of dogs in this study which average 10.6 years old. According to the biopsy result in control group was a chronic hyperplastic gingivitis. Thus, this study presented to compare a density of transferrin receptor between oral cancer and chronic hyperplastic gingivitis in dogs. The results exhibited a density of transferrin receptor in experimental group was significantly higher than control group ($p < 0.05$). Transferrin receptor (TfR) is a type II glycoprotein on cell membrane that involved in iron uptake and control of cell growth (Neckers and Trepel, 1986). TfR expression is depended on intracellular iron levels that show a low levels on normal cells and a higher level on a great proliferative cell (Daniels et al., 2006). Variety of cellular process need an iron as cofactor such as; metabolism, respiration, and DNA synthesis (Ponka and Lok, 1999). Cancer cells have shown elevated levels of TfR greater than normal cell in many studies (Shindelman et al., 1981; Sutherland et al., 1981; Gatter et al., 1983; Walker and Day, 1986; Sciot et al., 1990; Daniels et al., 2006; Prutki et al., 2006). According to, cancer cells required more iron as a cofactor of the ribonucleotide reductase enzyme for dividing cell which was concerned in DNA synthesis (Daniels et al., 2006). The study exhibited TfR expression in malignant breast cells more 4-5 times than benign breast cells (Walker and Day, 1986) . Another study shown TfR expression was increase in cancer patient that correlated with stage of tumors and prognosis such as; 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). Miyamoto et al. shown transferrin receptor in oral tumor patients by immunohistochemical study and flow cytometry. They found TfR was rarely expression in a benign oral tumors, but strongly in the basal and parabasal cell of normal epithelium. Moreover, All of malignant samples had staining greater than normal epithelium and the positive reaction in malignant tumors might

be indicated the degree of malignancy that TfR expression could be useful a biomarkers for prognosis (TfR in OT). TfR expression was estimated by immunohistochemistry in four subgroup (low-grade B-cell (LGB), high-grade B-cell (HGB), low-grade T-cell (LGT), and high-grade T-cell (HGT)) of canine lymphoma that shown a levels of TfR correlated to tumor grade and mitotic rate. In present study, we found a significant strongly reacted with TfR in canine oral cancer more than chronic hyperplastic gingivitis by standard immunohistrochemistry. We used the mouse anti-human TfR1 antibody H68.4 directed aginst human TfR1 and positive staining in canine placenta and canine lymphoma that used in previous study (Gatter et al., 1983; Priest et al., 2011). In chronic hyperplastic gingivitis, weak staining was detected in parabasal and basal layers same as human. In contrast, the oral cancer was positive stronger stained in cancer cell. There was no correlated a metastatic cancer. The levels of serum and saliva transferrin did not related with TfR. However, the present study demonstrated that scc group and survival time less than 3 months were related between saliva, serum and TfR. Nevertheless, they had not enough dogs for analysis with statistic test. The result supported that TfR over expression in cancer cell, but we no found relationship between the expression of TfR and the metastasis of cancer. To differentiated, several studies in human shown TfR expression correlated with mitotic rate, Ki67 staining and H-thymidine incorporation. We believed in a quantitative of saliva transferrin which depended on a TfR expression on cancer cell. However, the result of this study exhibited they was not related between TfR expression and levels of saliva transferrin.

In conclusion, this study presents a saliva transferrin in canine oronasal cancer that might not be used for early detection of oral cancer that different as previous studied in human (Jou et al., 2010). According to, the dogs have a many oral problem such as: periodontitis, bacterial stomatitis, gingivitis, etc. The factors might be latent effected to elevated levels of saliva transferrin (Morgan, 1981). However, the level of saliva transferrin respectively decreased from stage I to stage IV and was higher than the control group that found in the previous study in human. Nevertheless, the number of samples were not enough for statistical analysis.

Moreover, we found no correlated with metastasis of cancer and saliva transferrin similar to serum transferrin and transferrin receptors expression. In contrast, the serum transferrin is reduced in canine oronasal cancer compared the control group, but did not significantly correlate with tumor stage same as discover in human (Jou et al., 2010). This study known the levels of TfR expression in oral cancer higher than chronic hyperplastic gingivitis. Further studies are determine TfR expression on oral cancer cell that correlated mitotic rate or tumor stage same as canine lymphoma (Priest et al., 2011). According to, TfR expression in cancer tissue is very useful that show in various studies in human. TfR1 is a prognostic marker and target receptor for therapeutic purposes against malignant cells (Lai et al., 2009; Nakase et al., 2009; Indira Chandran et al., 2012; Akhtar et al., 2014; Szwed et al., 2014). Saliva sample is very interesting to candidate compare with serum samples in dog. Because it is easy collected, non-stressful, comfortable, and decrease transportation to animal hospital. In this study, the levels of transferrin in salivary is effected many factor. Thus, the recently research about biomarkers in saliva dogs should be avoid a factor from oral environment and use a special or novel technique for detect a novel protein as biomarkers in oral cancer dogs.

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APPENDIX

Appendix A: Saliva and serum transferrin concentration in experimental group.

Dog No.	Saliva transferrin (µg/ml)		Serum transferrin (mg/ml)	
	Before surgery	After surgery	Before surgery	After surgery
1	3.049	2.968	6.192	4.912
2	3.091	3.053	5.208	5.823
3	3.117	3.161	4.165	5.469
4	2.783	3.152	4.085	6.008
5	3.155	1.574	6.415	6.777
6	3.194	2.701	5.696	5.888
7	2.929	2.937	4.654	2.388
8	2.977	2.780	3.504	1.977
9	3.116	3.080	3.069	2.961
10	2.904	3.001	5.512	3.073
11	3.128	3.083	5.250	4.892
12	3.137	3.122	4.746	3.235
13	2.945	2.274	4.092	4.300
14	3.021	2.935	4.385	5.177
15	3.049	3.064	3.723	8.704
Mean ± SD Median; Q1-Q3	3.070; 2.945 - 3.128	2.985; 2.779 - 3.083	4.713 ± 0.984	4.772 ± 1.818

Appendix B: Saliva and serum transferrin concentration in control group.

Dog No.	Saliva transferrin (µg/ml)	Serum transferrin (mg/ml)
1	1.578	6.115
2	2.841	6.546
3	3.134	6.227
4	3.078	6.158
5	3.212	5.696
6	3.204	6.115
7	1.180	5.892
8	2.891	5.996
9	3.161	5.185
Mean ± SD	2.698 ± 0.765	6.148 ± 0.285

Appendix C: Transferrin receptor expression in control and experimental groups.

Experimental group	Positive area (%)	Control group	Positive area (%)
Amelanotic melanoma	58.755	Hyperplastic gingivitis	0.0382862
Amelanotic melanoma	42.857	Hyperplastic gingivitis	0.1806052
Amelanotic melanoma	76.477	Hyperplastic gingivitis	0.0451542
Amelanotic melanoma	56.003	Hyperplastic gingivitis	0.0113356
Amelanotic melanoma	76.281	Hyperplastic gingivitis	0.1201717
Melanoma	68.086	Hyperplastic gingivitis	0.6314374
Melanoma	60.970	Hyperplastic gingivitis	3.3539596
Melanoma	68.690	Hyperplastic gingivitis	0.133424
Melanoma	28.147	Hyperplastic gingivitis	1.8556061
Melanoma	30.864		
Melanoma	53.772		
Melanoma	66.392		
SCC	69.529		
SCC	68.868		
SCC	90.013		
Median; Q1-Q3	66.392; 53.772-69.529		0.133; 0.042-1.244

Appendix D: Bar graph of saliva and serum transferrin concentration.

Saliva transferrin concentration of experimental and control group
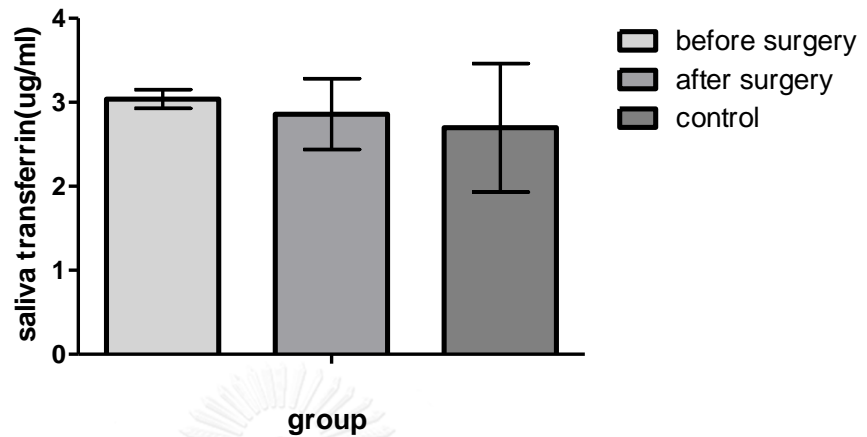
Figure 7: Bar graph of saliva transferrin concentration of each group.

Serum transferrin concentration of experimental and control group
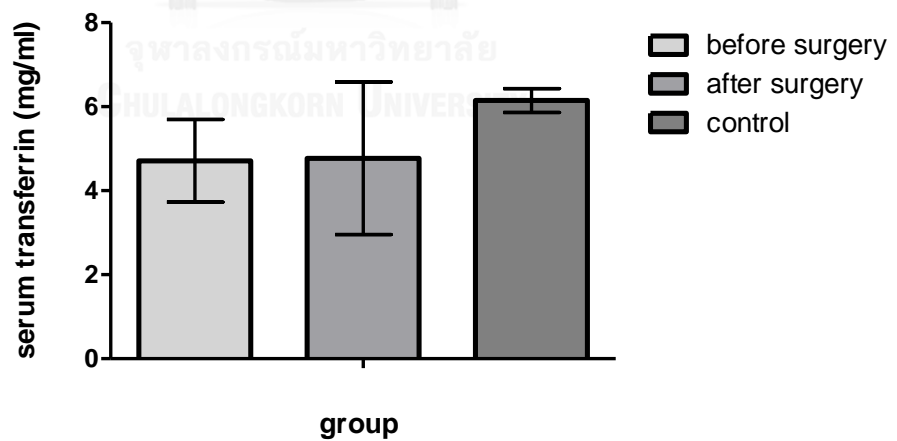
Figure 8: Bar graph of serum transferrin concentration of each group.

Saliva transferrin concentration of melanoma and SCC group
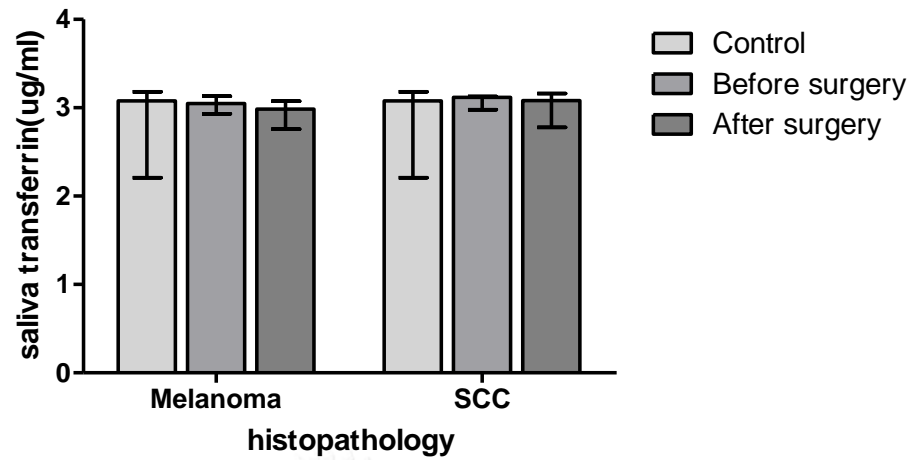
Figure 9: Bar graph of saliva transferrin concentration of histology.

Serum transferrin concentration of melanoma and SCC group
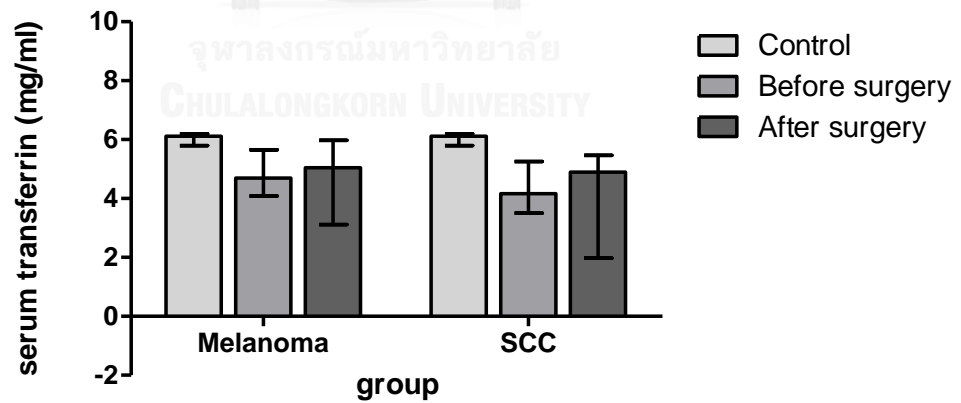
Figure 10: Bar graph of serum transferrin concentration of histology.

Saliva transferrin concentration of LN involvement
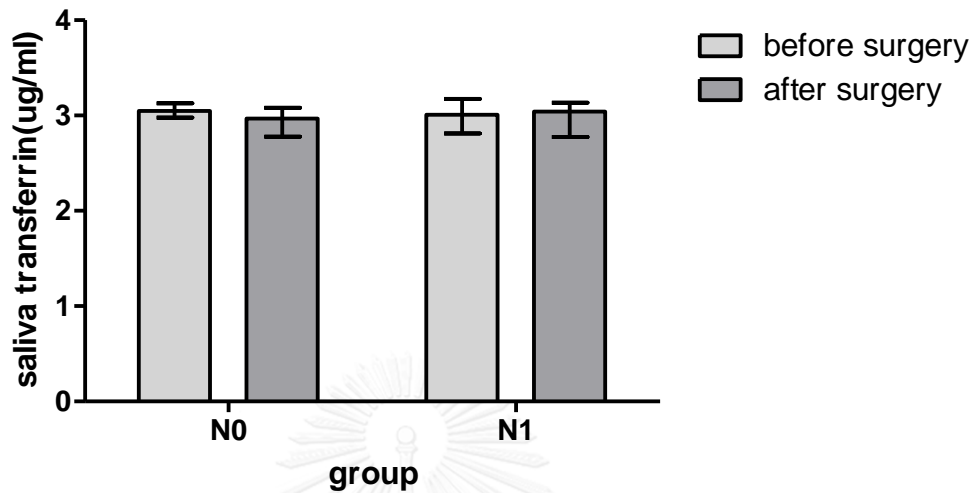
Figure 11: Bar graph of saliva transferrin of lymph node involvement
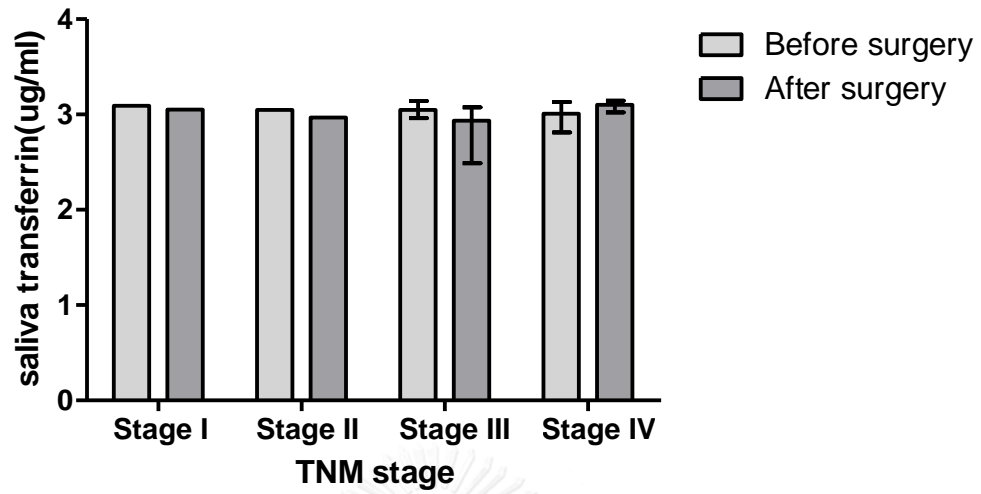
Serum transferrin concentration of LN involvement
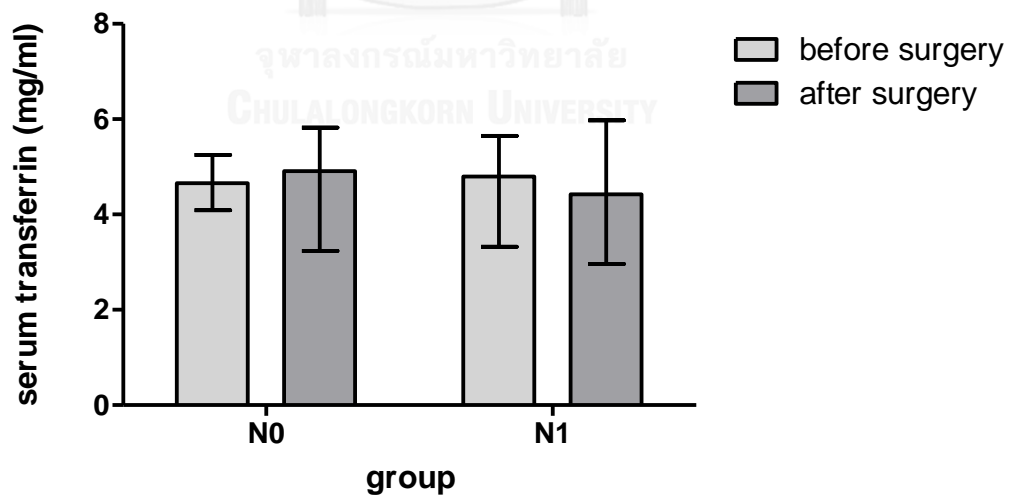
Figure 12: Bar graph of serum transferrin of lymph node involvement.

Saliva transferrin concentration of TNM staging

Figure 13: Bar graph of saliva transferrin of TNM staging.
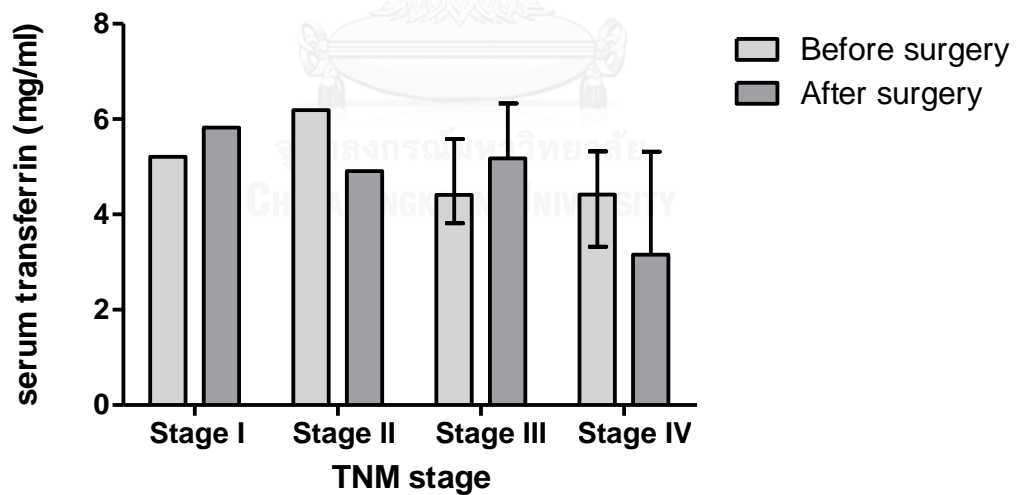
Serum transferrin concentration of TNM staging

Figure 14: Bar graph of serum transferrin of TNM staging.

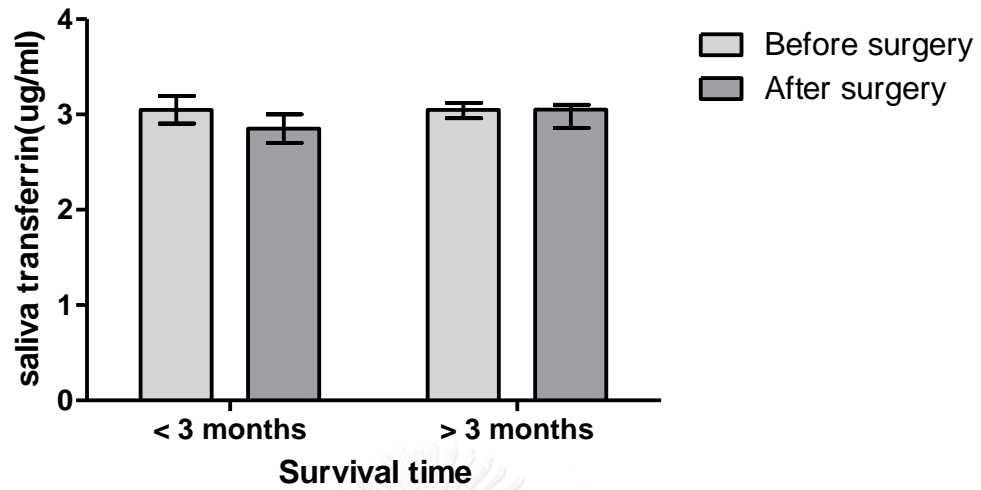**Saliva transferrin concentration of survival time**

Figure 15: Bar graph of saliva transferrin concentration of survival time.

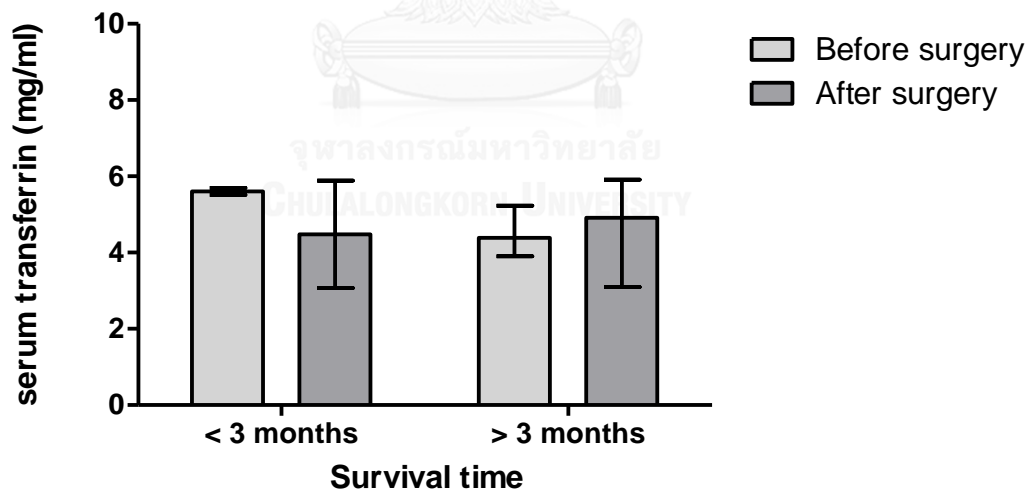**Serum transferrin concentration of survival time**

Figure 16: Bar graph of saliva transferrin concentration of survival time

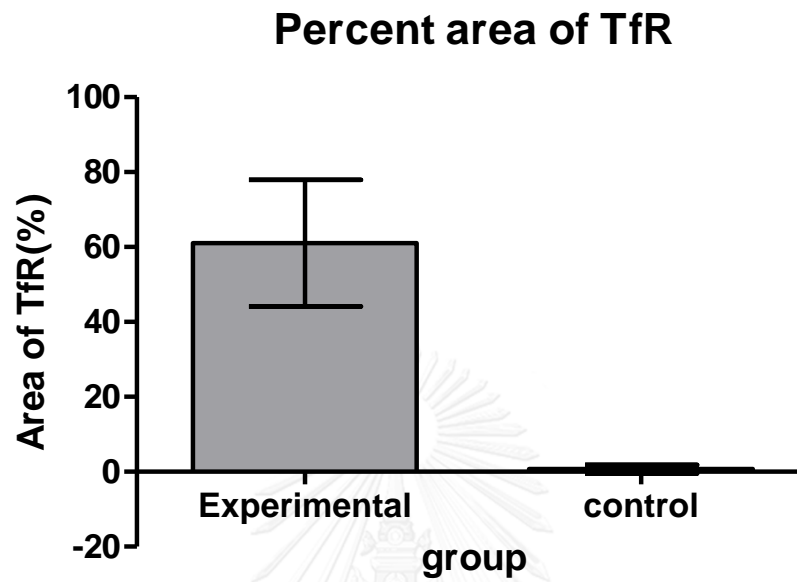**Appendix E**: Bar graph showed comparison of transferrin receptor expression in experimental and control groups.

Figure 17: Bar graph of percent area of transferrin receptor expression.