Study of Prognostic Utilities in Canine Nasal Carcinoma Treated with Radiation Therapy and Radiosensitizing Effects *in vitro*

Rakuno Gakuen University
Graduate School of Veterinary Medicine
Doctoral Course
Laboratory of Veterinary Clinical Oncology

Dah-Renn Fu

Advising Professor
Laboratory of Veterinary Clinical Oncology
Tsuyoshi Kadosawa

2014

犬の鼻腔内癌における放射線治療

予後因子と増感効果に関する研究

酪農学園大学大学院
獣医学研究科
獣医学専攻博士課程

傅　大任

獣医臨床腫瘍学
指導教員　教授　廉澤剛

2014 年度

Study of Prognostic Utilities in Canine Nasal Carcinoma Treated with Radiation Therapy and Radiosensitizing Effects *in vitro*

犬の鼻腔内癌における放射線治療予後因子と増感効果に関する研究

Dah-Renn Fu

傅　大任

CONTENTS

ABBREVIATIONS

ADC	Adenocarcinoma
AI	Apoptotic index
CA	Undifferentiated carcinoma, Carcinoma
COX-2	Cyclooxygenase-2
CR	Complete response
cSurvivin	Cytoplasmic survivin
CT	Computed tomography
DAB	3-3'-Diaminobenzidine
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
FBS	Fetal bovine serum
Gy	Gray
HE	Hematoxylin-Eosin
HIF-1α	Hypoxia-inducible factor-1 alpha
HPF	High power field
IAP	Inhibitors of apoptosis protein
IHC	Immunohistochemistry
MST	Median survival time
NPC	Nasopharyngeal carcinoma
nSurvivin	Nuclear survivin
OD	Optical density
PBS	Phosphate buffered saline
PD	Progressive disease
PDGF	Platelet-derived growth factor
PE	Plating efficiency
PFS	Progression free survival

PR	Partial response
RECIST	Response evaluation criteria in solid tumors
RT	Radiation therapy
SCC	Squamous cell carcinoma
SD	Stable disease
SF	Survival fraction
SF_2	Survival fraction in 2 Gy
TCC	Transitional cell carcinoma
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end-labeling
VEGF	Vascular endothelial growth factor

PREFACE

Surgery, chemotherapy and radiation therapy are three well-established treatment modalities for animals with cancer disease in veterinary medicine. Radiation therapy is a form of disease treatment by using ionizing radiation. The discovery of X-rays by Wilhelm Conrad Röntgen in 1895 followed by Richard Eberlein, a German physician and veterinarian, began to use radiation for tumor therapy in animals in 1907. Alois Pommer, an Austrian veterinarian, published widely on irradiation of diseases and established a radiation therapy protocol in 1958 [28, 53]. Radiation therapy is becoming increasingly available and in demand in companion animals with cancers. Recently, there has been an increase in information on its effectiveness in the treatment of a number of different tumor types [59, 60].

Radiation therapy is a form of medical treatment using high-energy waves or particles to destroy or damage cancer cells. The common radiation sources include beams of x-rays, gamma rays or electrons delivered by either orthovoltage or megavoltage radiation therapy equipment in veterinary medicine [60]. Ionizing radiation may kill cells causing damage of DNA either directly or indirectly within tissue. Unlike with direct action, most the time ionizing radiation interacts with water molecules near the DNA hence creating free radicals, which damage the DNA [28, 62]. Cells may undergo apoptosis when the DNA damage is irreparable. Cells that are in M and in G2 phase during the cell cycle are the most sensitive to radiation damage. The cells become more resistant as they proceed through G1. They even become the most resistant as they reach the S phase [62].

Nasal tumors develop in several tissue types that are found in the nasal and sinus

cavities within the head. However, they are uncommon and only account for less 2% of all canine tumors [97]. Most dogs presenting nasal tumors are middle to old age. Dolichocephalic breeds may be at increased risk for developing nasal tumor. Clinical signs in dogs with nasal tumor include epistaxis, sneezing, mucopurulent discharge, facial deformity, exophthalmos, and occasionally neurologic abnormalities. Nasal tumors are commonly very aggressive to the surrounding tissues. A cross-sectional imaging allows to accurate determination of the localization and extension of tumor. Metastatic rate is generally considered low at the time of diagnosis [23, 103]. Biopsy and histology of the tumor are needed for definitive diagnosis, even though historical information and diagnostic imaging are able to be highly suggestive. In dogs, nasal tumors are nearly all malignant. Approximately two thirds are epithelial in origin (carcinoma). The remaining tumors are mesenchymal in origin (sarcoma) [1, 23, 50, 74]. Other types of tumor, such as transmissible venereal tumor, melanoma, and lymphoma, have been also reported previously [32, 42, 71].

A variety of treatments for nasal tumors have been reported, including surgery, chemotherapy, radiation therapy, cryosurgery, or a combination of these modalities. Recently, the preferred treatment of nasal tumor is radiation therapy, which is the most effective therapy for this disease due to the nature behavior of nasal tumors [23, 31, 47]. Dogs with nasal tumors that remain untreated have a median survival time (MST) of 1.5 to 3 months [74, 108]. On the other hand, MSTs after radiation therapy of nasal tumors vary significantly depending on clinical stage, histological type, and metastasis, but generally range from 7 to 23 months in previous studies [1, 50, 68, 97].

Immunohistochemistry studies have been broadly used to evaluate molecular markers as

well to search valuable biomarkers associated with prognosis in human medicine. Recently, a high number of biomolecular tumor markers such as EGFR, VEGF, survivin, COX-2, LMP1, Ki-67, cyclin-D1, and p63 [16, 18, 48, 55, 69, 93, 100, 106, 107, 112] have been investigated for prognosis and/or therapeutic targets in human nasopharyngeal carcinomas (NPC). These studies have attempted to elucidate the molecular mechanisms associated with NPC carcinogenesis. Few researches were performed in canine nasal tumors. In one study that Gamblin *et al*. published, nuclear *p53* protein accumulation was detected in 11 of 19 nasal adenocarcinomas. This suggested that overexpression of a mutated *p53* tumor suppressor gene protein might be a reliable form of prognostic predictor [27]. COX-2 expression has been detected in most canine nasal epithelial tumors (71-90%) [6, 13, 45, 36]. Expression of EGFR and VEGF were detected in over 50% and 90% of 24 nasal carcinoma samples, respectively [83]. In addition, activity of matrix metalloproteinase-2 (MMP-2) was detected in one-thirds of nasal adenocarcinomas (4 of 12 samples) [66]. However, few number of studies have been investigated the association between the biomarker and outcome for canine nasal tumors.

Enhanced radiosensitivity of the tumor cells might to be an optimal strategy for improving tumor response to radiation therapy [84]. The mechanisms of cellular malignant transformation include the regulation of signal transduction, cell differentiation, apoptosis, DNA repair, cell cycle progression, and angiogenesis. Applications of molecular radiobiology in recent years have altered the understanding of tumor radioresistance and the cellular response to ionizing radiation [57]. Some biological agents designed to target these molecular progresses have shown both radiosensitizing and antiproliferative activities in preclinical models of human cancers [14, 30, 39, 57, 101, 105, 110]. Several studies have

used various ways to enhance such radiosensitivity. These include *p53* transfection, COX-2 antagonist (celecoxib), multi-targeted receptor tyrosine kinase inhibitor (sunitinib), survivin suppressant (YM155), EGFR inhibitors (gefitinib, cetuximab), and some chemotherapeutic agents [39, 54, 84, 110].

This thesis is composed of three chapters. In Chapter I, we evaluated the outcomes for dogs with nasal carcinoma with clinical variables and to compare survival times for dogs treated with radiation therapy in a decade in RGU. Chapter II is to emphasize on evaluation of tumor markers (Ki-67, survivin, EGFR, VEGF, and COX-2) expression and apoptotic index in canine nasal carcinoma through an immunohistochemical staining and TUNEL assay. We also seek correlations among these tumor markers, clinical variables, and the outcomes after radiation therapy. In Chapter III, a cell line of canine nasal squamous cell carcinoma and a radioresistant subclone cell line are established. This chapter then compares differences between the radiosensitive and radioresistant cell line. Additionally, base on the protein expression in canine nasal carcinomas in the results of Chapter II, we also evaluate the radiosensitizing effect of molecular targeted therapeutic agents in the nasal SCC cell line.

CHAPTER I.

Retrospective Study of Prognostic Indicators in Dogs with Nasal Carcinoma Treated with Radiation Therapy in RGU: 2004-2013

1. INTRODUCTION

Canine nasal tumors are uncommon, accounting for 1% to 2% of all neoplasms in dogs [97]. However, they are nearly all malignant. Approximately 60% to 75% of canine nasal tumors are carcinomas, epithelial-origin. The subtypes of nasal carcinomas include adenocarcinomas, squamous cell carcinomas, transitional cell carcinomas, and undifferentiated carcinomas [1, 47, 50, 74]. Adenocarcinoma is the most common tumor type of all nasal tumors in dogs [1, 31, 47].

Most canine nasal tumors are very invasive to the surrounding tissue but are less likely to metastasize. The rate of metastasis is reported to be from 10% to 14% of all nasal tumor cases at the time of diagnosis in previous reports [15, 31, 50, 58]. There is a study reporting a higher metastatic rate (24%) in dogs with nasal carcinomas [74].

Several studies have reported the negative prognostic factors in canine nasal tumor, including age greater than 10 years [50], presence of epistaxis at time of diagnosis [50], regional lymph nodes or lung metastasis [31, 50], cribriform plate destruction on computed tomography (CT) images [1], and tumor histological subtype [1, 97]. Previous reports trended to show shorter survival time for dogs with carcinoma than those with sarcoma. Some studies reported better prognosis for dogs with adenocarcinoma comparing to those with squamous cell carcinoma or undifferentiated carcinoma [1, 2, 97]. The median survival time (MST) of dogs with nasal carcinomas had a range of 7-13 months [1, 50, 68, 97].

Nasopharyngeal tumor staging in humans has commonly been utilized with CT images. Some clinical staging methods based on CT images also have been used for canine nasal tumor staging [1, 2, 47, 50, 97]. A WHO three-tiered staging method was performed with

radiographic or CT findings but prognostic significance was not found [2, 47]. Likewise, the WHO staging system was modified to two-tiered staging method and applied to CT findings, which be observed no prognostic significance [47]. A CT-based four-tiered staging method, modified from human nasopharyngeal carcinoma staging method, published by Adams *et al.* in 2009 and has recently been used in several investigations [1, 58, 90].

A variety of treatments for nasal tumors have been reported, including surgery, chemotherapy, radiation therapy, cryosurgery, or a combination of these modalities. Although chemotherapy has been attempted as primary therapy [52], as an adjuvant therapy [51], or as a radiosensitizer [54], chemotherapy has not improved survival of dogs with nasal tumors. Surgery has also been attempted for local therapy as a sole treatment [31, 90] or with an adjuvant radiation [2, 68]. Due to the complex surrounding anatomical structure, nasal tumors are difficult to treat with surgery alone to prolong survival time. Radiation therapy is considered an effective as well as relatively noninvasive therapy for local control of canine nasal tumors.

The goals of this chapter were to describe the outcomes for dogs with nasal carcinoma according to clinical variables. We also compared the survivals for dogs treated with radiation therapy in a decade in our university veterinary teaching hospital.

2. MATERIALS AND METHODS

(1) Patients data

The medical records of 84 dogs diagnosed with nasal carcinomas in the Rakuno Gakuen University Veterinary Teaching Hospital (RGU-VTH) between April 2004 and January 2014 were reviewed. All dogs had biopsy taken before treatment and had been diagnosed with carcinomas by histopathology. Data collected from the medical records, including age, sex, body weight, breed and clinical signs. Pretreatment evaluation included a complete blood count, serum chemistry, three-view thoracic radiographs, CT scans, and a fine-needle biopsy of regional lymph nodes if enlarged. Follow-up information was obtained from the medical records or by contacting the referring veterinarians. Endpoints were assigned as follow: alive if the dog was known to be alive at the time of data collection, lost to follow-up if survival status was unknown at the time of data collection, or dead if the dog had died before data collection.

(2) CT images evaluation

All the dogs enrolled in this chapter had taken CT scans before biopsy and treatment. All CT images of the archived dogs at the time of initial presentation were reviewed. CT images evaluation was evaluated for the absence or presence of lesions, location of the lesions (unilateral or bilateral), osteolysis (nasal septum, frontal sinus, hard palate, cribriform plate), and invasiveness (frontal sinus, orbit cavity, subcutis, oral cavity, nasopharynx or cranial cavity). Clinical staging was based on CT imaging using Adams' modified staging system [1] (shown in Table 1-1).

Table 1-1. Adams' proposed staging system for canine nasal tumors [1]

Stage	Tumor Characteristics
T1	Confined to one nasal passage or frontal sinus, with no bony involvement
T2	Bony involvement, without evidence of orbital, subcutaneous, or submucosal mass
T3	Orbit involved, or nasopharynx, or a subcutaneous, or submucosal mass
T4	Tumor causing destruction of the cribriform plate

(3) Radiation therapy and response assessment

Radiation therapy was performed by use of an orthovoltage X-ray machine (TITAN-450S, GE) with a half-value layer of 4.8 mm of Cu at 450 KV and 10 mA. The exposure rate was 1.68 Gy/min with a filter of 1.0 mm of Al, 0.3 mm of Cu and 0.5 mm of Sn. The distance from the X-ray source to the skin was 60 cm. Treatment protocols including dose per fraction, number of fractions, treatment schedule, total radiation dose, and field sizes were recorded.

The response to treatment was assessed based on the RECIST system with CT images around one month after completion of radiation. Contrast medium was used to distinguish contrast-enhanced tissues from nasal discharge. Complete response (CR) was determined if the tumor disappeared from CT images. Partial response (PR) was defined as a decrease of 30% or greater in the sum of the longest diameters. Stable disease (SD) was defined as between a decrease of 30% and an increase of 20% in the sum of the longest diameter. Progressive disease (PD) was defined as an increase of 20% or greater in the sum of the longest diameters [98].

(4) Statistical analysis

The overall survival time of a patient was calculated from the starting date of radiation therapy to the time of death or the last follow-up. The progression-free survival (PFS) was defined from the starting date of radiation therapy to the day of disease progression or the last follow-up. The correlation between the survival and prognostic factors was estimated using Kaplan-Meier survival curves, and statistical differences between survival curves were calculated using a log-rank method. P values of < 0.05 were considered statistical significant. The commercial software StatMate III (ATMS Co., Ltd.) was used to perform the statistical analysis.

3. RESULTS

(1) Patient and tumor characteristics

The information of 84 dogs is summarized in Table 1-2. The mean and median ages for sampled dogs were 10.9 years and 11 years (range from 6 to 16 years of age), respectively. There were 15 intact females, 26 spayed females, 19 intact males and 24 neutered males. There were 72 pure-breed dogs and 11 mixed breed dogs. Golden retriever (n = 13), Pembroke Welsh corgi (n = 12) and miniature dachshund (n = 7) were the most three pure-breeds.

Histopathological examination revealed 36 dogs (42.8%) diagnosed with adenocarcinomas (ADC), 23 dogs (27.4%) diagnosed with undifferentiated carcinomas (CA), 13 (15.5%) dogs diagnosed with squamous cell carcinomas (SCC) and 12 dogs (14.3%) diagnosed with transitional cell carcinomas (TCC). Ten dogs had regional lymph nodes metastasis, one dog had lung metastasis, and one dog had regional lymph nodes and lung metastasis at the time of initial presentation. The rate of metastasis was 14.3% in 84 dogs.

The clinical signs revealed epistaxis in 56 dogs (66.7%), nasal discharge in 43 dogs (51.2%), sneezing in 31 dogs (36.9%), facial deformity in 28 dogs (33.3%), exophthalmos in 15 dogs (17.9%), and neurological abnormalities in 9 dogs (7.1%). The clinical signs and tumor subtypes were summarized in Table 1-3. Compared with the other histological subtypes, facial deformity (19.4%) and exophthalmos (5.6%) were present significantly less in dogs with ADC (P = 0.018 and 0.026, respectively). Nasal discharge (15.4%) was seen significantly less in dogs with SCC (P = 0.035). The presences of epistaxis, sneezing and

neurological abnormalities were not significantly associated with tumor subtype.

On CT evaluation, 59 dogs had unilateral disease (33 dogs were left; 26 dogs were right lateral) and 25 had bilateral disease. The CT findings showed frontal sinus involvement in 28 dogs (33.3%), orbital involvement in 32 dogs (38.1%), subcutaneous involvement in 38 dogs (45.2%), oral involvement in 14 dogs (16.7%), nasopharyngeal involvement 13 dogs (15.5%), and cribriform plate destruction in 39 dogs (46.4%). The CT findings and tumor subtype were summarized in Table 1-4. Involvement of oral cavity (25%) and nasopharynx (25%) were trended to see more often in dogs with ADC, but they were not significant (P = 0.076 and 0.074, respectively). Dogs diagnosed with CA occurred most commonly destruction of cribriform plate (65.2%) (P = 0.033). Nasopharyngeal involvement (0%) was detected significantly less in dogs with SCC (P = 0.046). The presences of frontal sinus, orbital, and subcutaneous involvement were not significantly associated with tumor subtype. According to the Adams' proposed stage classification, thirteen dogs were staged as T1 disease (15.5%), 8 dogs as T2 disease (9.5%), 24 dogs as T3 disease (28.6%), and 39 dogs as T4 disease (46.4%).

Table 1-2. Characteristics of dogs

	Cases (n = 84)
Range of age, year (mean, median)	6-16 (10.9, 11)
Range of weight, kg (mean, median)	2.5-50.2 (15.1, 12.2)
Sex	
Intact Female	15
Spayed Female	26
Intact Male	19
Neutered Male	24
Breed	
Golden Retriever	13
Pembroke Welsh Corgi	12
Miniature Dachshunt	7
Beagle	6
Shetland Sheepdog	6
Shiba	6
Miniature Schnauzer	5
Papilon	3
Siberian Husky	3
Hokkaido	2
Labrador Retriever	2
Shih Tzu	2
Bullmastiff	1
Chihuahua	1
Maltese	1
Pomeranian	1
Pug	1
Western Highland White Terrier	1
Mix	11

Table 1-3 . Clinical findings for dogs with nasal carcinomas, by histologic type

Clinical findings	All carcinomas	ADC	CA	SCC	TCC
	n = 84	n = 36	n = 23	n = 13	n = 12
Epistaxis	56 (66.7%)	27 (75%)	15 (65.2%)	7 (53.8%)	7 (58.3%)
Nasal discharge	43 (51.2%)	23 (63.9%)	11 (47.8%)	2 (15.4%)	7 (58.3%)
Sneezing	31 (36.9%)	15 (41.7%)	7 (30.4%)	5 (38.5%)	4 (33.3%)
Facial deformity	28 (33.3%)	7 (19.4%)	8 (34.8%)	7 (53.8%)	6 (50%)
Exophthalmos	15 (17.9%)	2 (5.6%)	7 (30.4%)	3 (23.1%)	3 (25%)
Neurological abnormalities	6 (7.1%)	2 (5.6%)	2 (8.7%)	1 (7.7%)	1 (8.3%)

ADC: Adenocarcinoma. CA: Undifferentiated carcinoma. SCC: Squamous cell carcinoma.
TCC: Transitional cell carcinoma.

Table 1-4 . CT findings for dogs with nasal carcinomas, by histologic type

CT findings	All carcinomas	ADC	CA	SCC	TCC
	n = 84	n = 36	n = 23	n = 13	n = 12
Frontal sinus	28 (33.3%)	10 (27.8%)	7 (30.4%)	5 (38.5%)	6 (50%)
Orbital involvement	32 (38.1%)	12 (33.3%)	9 (39.1%)	6 (46.2%)	5 (41.7%)
Subcutaneous involvement	38 (45.2%)	12 (33.3%)	13 (56.5%)	7 (53.8%)	6 (50%)
Oral involvement	14 (16.7%)	9 (25%)	2 (8.7%)	2 (15.4%)	1 (8.3%)
Nasopharyngeal involvement	13 (15.5%)	9 (25%)	2 (8.7%)	0 (0%)	2 (16.7%)
Cribriform plate destruction	39 (46.4%)	16 (44.4%)	15 (65.2%)	5 (38.5%)	3 (25%)

ADC: Adenocarcinoma. CA: Undifferentiated carcinoma. SCC: Squamous cell carcinoma.
TCC: Transitional cell carcinoma.

.

(2) Treatment methods

Fifty-nine of the 84 dogs received radiation therapy. There were 28 dogs with ADC, 16 dogs with CA, 10 dogs with SCC, and 5 dogs with TCC. The median of the irradiation dose/fraction was 4 Gy (range of 3-6 Gy). One or two directions were carried out for each fraction. Radiation was delivered via a dorsal portal directed to the tip line of the nose to the top of the head with/without an additional ventral portal directed from oral side. The irradiated fields were assessed by CT images. Treated tumor volume included a 1-2 cm margin of normal tissue. Two to three fractions weekly (Monday-Friday schedule or Monday-Wednesday-Friday schedule) were performed. Dogs received a total skin surface dose of 30 to 48 Gy (mean of 41.2 Gy, median of 40 Gy). Treatment duration ranged from 21 to 46 days (mean of 32 days, median of 30 days). One dog also received chemotherapy with carboplatin and cyclophosphamide, and two other dogs received zoledronic acid (Zometa®) during treatment. One dogs received toceranib phosphate (Palladia®) after radiation therapy. Ten dogs were treated with laser therapy by rhinoscopy, and one dog was treated with photodynamic therapy after the end of radiation therapy. Two dogs with regional lymph node metastasis was also received irradiation with the mandibular lymph nodes.

Fifty-five of 59 dogs had CT scan taken after radiation therapy and were available to determine the response by radiation therapy. Thirty-five dogs were determined to have a partial response (PR) (19 ADC, 11 CA, 2 SCC and 3 TCC), and twenty dogs had a stable disease (SD) in response to radiation therapy (7 ADC, 4 CA, 8 SCC and 1 TCC). The overall response rate was 63.6% (35/55) in dogs with nasal carcinomas; the response rates of dogs with ADC, CA, SCC and TCC were 73.1% (19/26), 73.3% (11/15), 20% (2/10) and

75% (3/4), respectively.

Twenty-two of 84 dogs did not receive treatment (8 ADC, 7 CA and 7 TCC). Besides, two dogs diagnosed with SCC were treated with surgery alone. One dogs with SCC received photodynamic therapy in another university.

(3) Follow-up and survival analysis

Of 59 dogs that received radiation therapy, 48 dogs were dead (29 dogs due to tumor progression, two dogs due to euthanasia, seven dogs due to other diseases and ten dogs unknown), seven dogs were lost to follow-up and four dogs were still alive at the time of data analysis. Fourteen of 22 dogs that were not treated died due to tumor progression, two dogs were lost follow-up at 90 and 258 days, and six dogs were lost follow-up after the time of initial presentation. One dogs treated with photodynamic therapy survived 761 days. Of two dogs received surgery alone, one dog survived 1765 days and one dog was lost to follow-up at 139 days.

The median overall survival time (MST) for 59 dogs that treated with radiation therapy was 241 days (ranged from 40 to 1979 days). The 16 dogs that received no treatment had a MST of 95 days (ranged from 9 to 206 days) (Figure 1-1). The median of progression-free survival (PFS) for 59 dogs that treated with radiation therapy was 176 days (ranged from 30 to 1979 days) (Figure 1-2).

The survival curves of dogs diagnosed with each histological subtype show in Figure 1-3. The dogs diagnosed with nasal ADC had MST and median PFS of 242 days and 221 days. The overall MST and median PFS for dogs with nasal CAs was 254 days and 90 days, respectively. The nasal SCC dogs had MST and median PFS of 178 days and 148 days. And,

the dogs diagnosed with nasal TCC had MST and median PFS of 407 days and 186 days. The MSTs for nasal ADC, CA and TCC dogs that did not receive treatment were 103 days, 104 days and 79 days, respectively. No significant differences were observed among histological tumor types.

Median PFSs and MSTs for tumor stages, for all carcinomas and each subtype carcinomas were summarized in Table 1-5. Dogs with high-stage were showed poor prognosis in all type of nasal carcinomas. The MST for dogs with T1, T2, T3 and T4 was 461 days, 407 days, 262 days and 170 days, respectively (Figure 1-4). Six dogs with T4 disease diagnosed metastasis at initial presentation were excluded from T4 group in survival analysis.

The median PFSs and MSTs according to different clinical variables were summarized in Table 1-6, 1-7 and 1-8. In clinical signs (Table 1-6), nasal discharge, facial deformity and exophthalmos were significantly associated with PFS ($P < 0.001$, $= 0.034$ and < 0.001, respectively). Nasal discharge, exophthalmos and neurological abnormalities were closely correlated with overall survival time ($P = 0.005$, 0.014 and 0.007, respectively). Presence of nasal discharge was however a positive prognostic factor in dogs with nasal carcinoma. Neither epistaxis nor sneezing was significantly associated with survival.

In CT findings (Table 1-7), the dogs that detected orbital, subcutaneous involvement and cribriform plate destruction had significantly shorter PFS than those that did not detect ($P =$ 0.012, 0.005 and < 0.001, respectively). Furthermore, the dogs with orbital involvement and cribriform plate destruction were also closely correlated with shorter overall survival times ($P = 0.007$ and < 0.001, respectively). However, frontal sinus, oral and nasopharyngeal involvements did not significantly affect the survivals.

Body weight, sex and irradiation dose were not found significant association with survivals. Dogs with metastasis had a significantly shorter PFS than those without metastasis ($P = 0.002$). Dogs with a PR were significantly correlated with a longer PFS than those with an SD ($P = 0.016$). Additionally, dogs with an age less than 11 years had longer survival than dogs with an age greater than 11 years ($P = 0.033$). Metastasis and RT response also had significant correlations with survival times ($P = 0.006$ and 0.007, respectively) (Table 1-8).

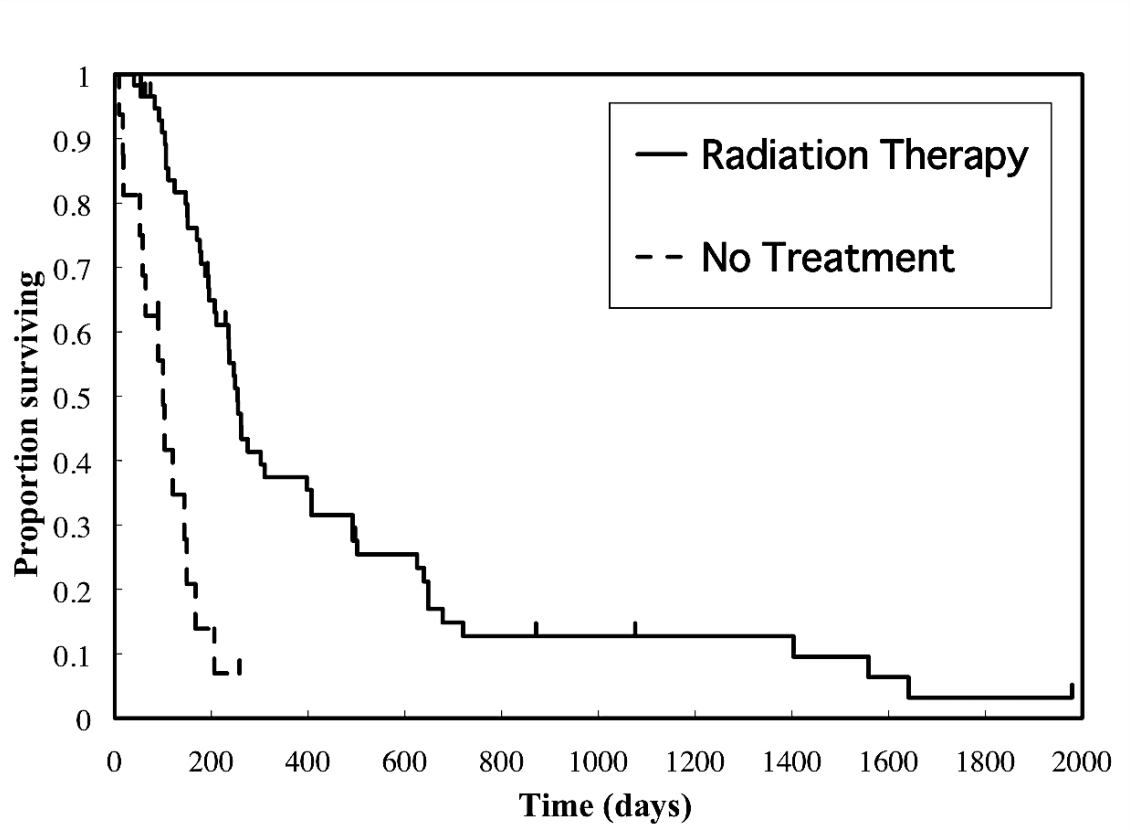


Figure 1-1. Kaplan-Meier survival curves for the overall survival time of dogs with nasal carcinoma treated with radiation therapy (n = 59) and without any treatment (n = 16). The MST of the dogs treated with RT and without treatment was 241 days (range: 40 to 1979 days) and 95 days (range: 9 to 206 days), respectively.

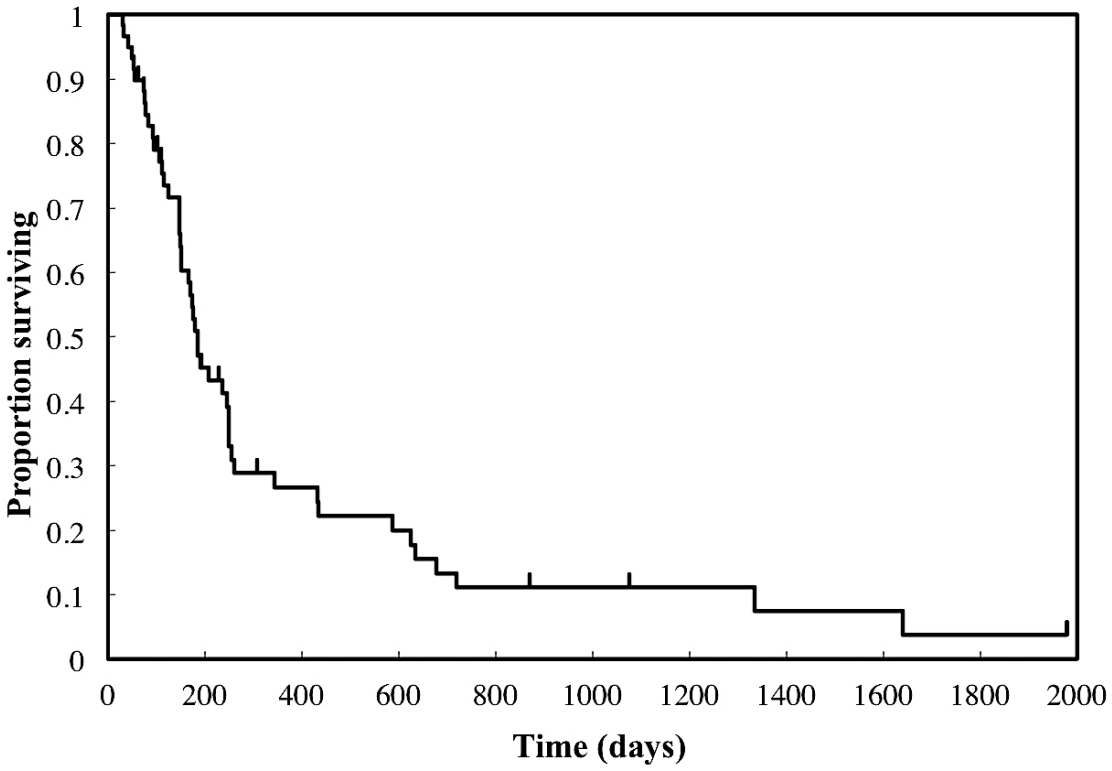


Figure 1-2. Kaplan-Meier survival curve for the progression-free survival (PFS) of dogs (n = 59) with nasal carcinoma treated with radiation therapy. The median PFS was 176 days (range: 30 to 1979 days).

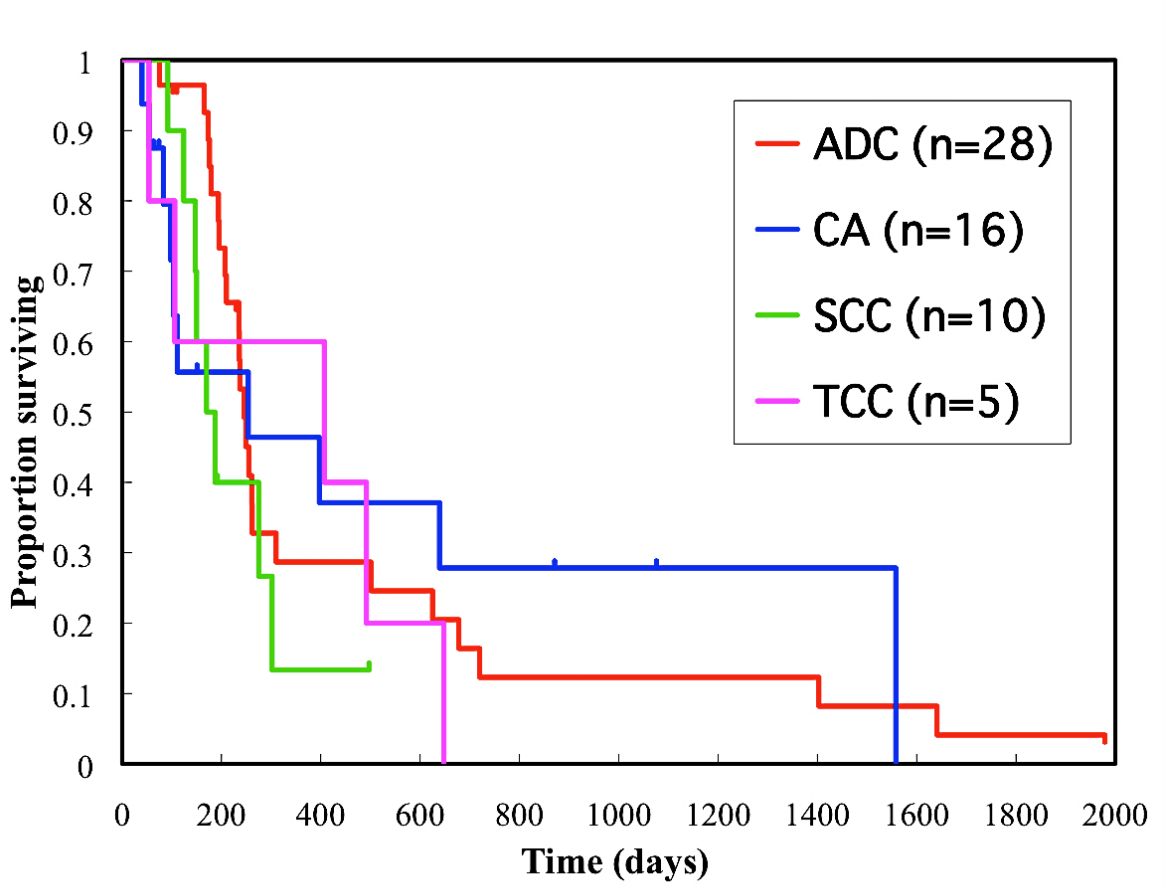


Figure 1-3. Kaplan-Meier survival curves for the overall survival time of dogs with nasal carcinoma treated with RT according to tumor histological subtype. The MST of the dogs diagnosed with adenocarcinoma (ADC; n = 28), undifferentiated carcinoma (CA; n = 28), squamous cell carcinoma (SCC; n = 10) and transitional cell carcinoma (TCC; n = 5) was 242 days (range: 79 to 1979 days), 254 days (range: 40 to 1558 days), 178 days (range: 92 to 497 days) and 407days (range: 54 to 648 days), respectively.

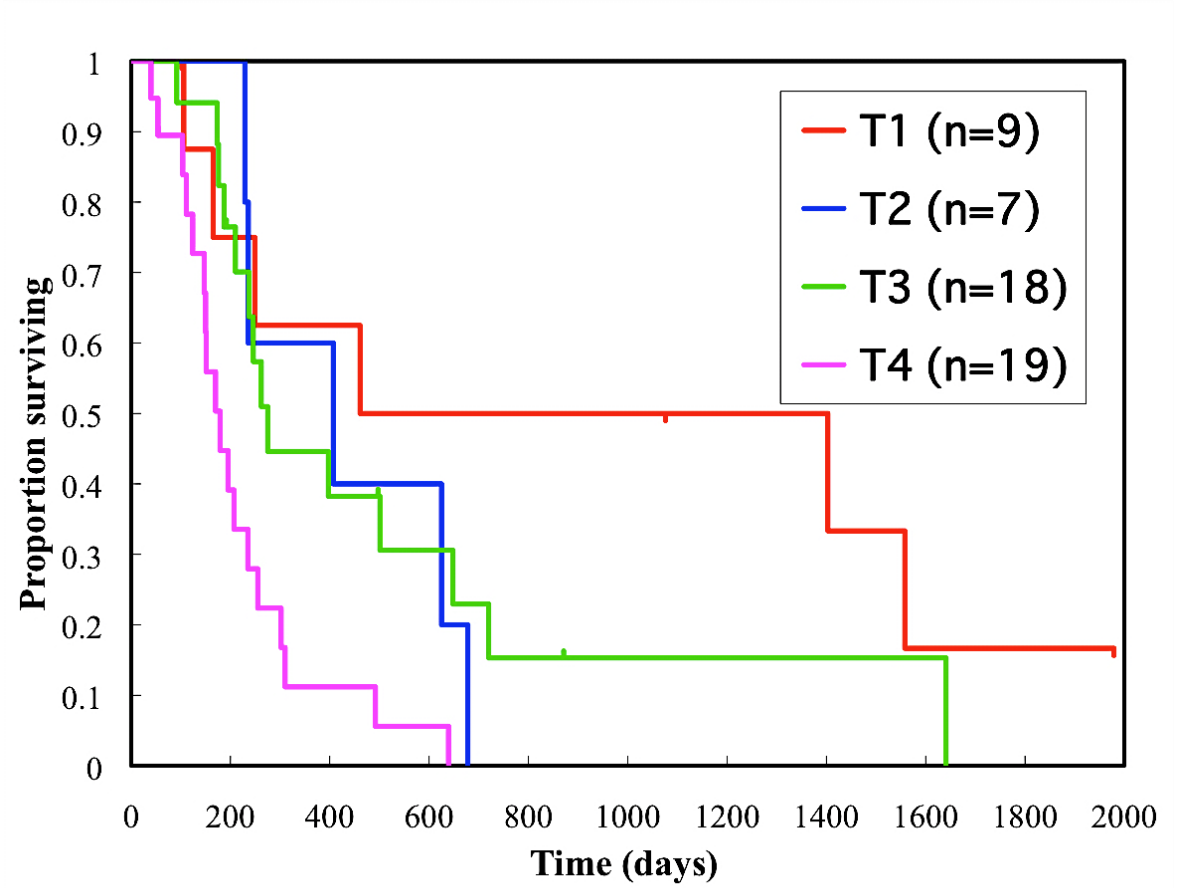


Figure 1-4. Kaplan-Meier survival curves for the overall survival time of dogs with nasal carcinoma treated with RT according to tumor clinical stage. The MST of the dogs staged as T1 (n = 9), T2 (n = 7), T3 (n = 18) and T4 (n = 19) was 461 days (range: 106 to 1979 days), 407 days (range: 236 to 678 days), 262 days (range: 92 to 1641 days) and 170 days (range: 40 to 639 days), respectively.

Table 1-5. Median survival times and computed tomography tumor stages for dogs treated with orthovoltage radiation therapy for nasal carcinomas, by histologic type in RGU-VTH

Histologic type	Tumor stage	Number of cases	Median PFS, days	Median survival time, days
All carcinomas	T1 + T 2	16	255	434
	T3	18	179	262
	T4	19	148	170
Adenocarcinomas	T1 + T 2	10	261	443
	T3	9	170	246
	T4	6	199	221
Undifferentiated carcinomas	T1 + T 2	4	434	575
	T3	2	308	397
	T4	8	81	111
Squamous cell carcinomas	T1 + T 2	0	N/A	N/A
	T3	5	185	192
	T4	4	148	149

PFS: Progression-free survival.

Table 1-6. Median progression-free survival, overall survival time and clinical signs for dogs treated with RT for nasal carcinomas

	Number of cases	PFS median, days	*P* value	Survival time median, days	*P* value
Epistaxis					
Absence	16	185	$P = 0.822$	249	$P = 0.449$
Presence	43	173		236	
Nasal discharge					
Absence	24	147	**$P < 0.001$**	173	**$P = 0.005$**
Presence	35	249		261	
Sneezing					
Absence	34	175	$P = 0.458$	254	$P = 0.878$
Presence	25	179		207	
Facial deformity					
Absence	40	236	**$P = 0.034$**	246	$P = 0.388$
Presence	19	148		210	
Exophthalmos					
Absence	48	236	**$P < 0.001$**	249	**$P = 0.014$**
Presence	11	115		151	
Neurological abnormalities					
Absence	55	179	$P = 0.112$	249	**$P = 0.007$**
Presence	4	147		147	

PFS: Progression-free survival.

Table 1-7. Median progression-free survival, overall survival time and CT findings for dogs treated with RT for nasal carcinomas

	Number of cases	PFS median, days	*P* value	Survival time median, days	*P* value
Frontal sinus involvement					
Absence	38	175	$P = 0.158$	236	$P = 0.153$
Presence	21	185		249	
Orbital involvement					
Absence	39	207	**$P = 0.012$**	254	**$P = 0.007$**
Presence	20	149		170	
Subcutaneous involvement					
Absence	32	246	**$P = 0.005$**	249	$P = 0.061$
Presence	27	148		187	
Oral involvement					
Absence	50	175	$P = 0.945$	246	$P = 0.651$
Presence	9	236		236	
Nasopharyngeal involvement					
Absence	49	179	$P = 0.370$	210	$P = 0.362$
Presence	10	243		262	
Cribriform plate destruction					
Absence	34	261	**$P < 0.001$**	397	**$P < 0.001$**
Presence	25	147		170	

PFS: Progression-free survival.

Table 1-8. Median progression-free survival, overall survival time and clinical characteristics for dogs treated with RT for nasal carcinomas

	Number of cases	PFS median, days	*P* value	Survival time median, days	*P* value
Age					
< 11y	23	249	$P = 0.281$	310	**$P = 0.033$**
≥ 11y	36	170		210	
Body weight					
≤ 15 Kg	40	207	$P = 0.522$	235	$P = 0.594$
> 15 Kg	19	166		236	
Sex					
Female	31	191	$P = 0.164$	249	$P = 0.153$
Male	28	149		210	
Dose of RT					
< 40 Gy	18	191	$P = 0.895$	236	$P = 0.899$
≥ 40 Gy	41	175		237	
Metastasis					
Absence	53	191	**$P = 0.002$**	237	**$P = 0.006$**
Presence	6	77		98	
Response to RT					
PR	35	236	**$P = 0.016$**	254	**$P = 0.007$**
SD	20	148		173	

PFS: Progression-free survival. RT: Radiation therapy. PR: Partial response. SD: Stable disease.

4. DISCUSSION

Canine nasal tumors of epithelial origin are more common than those of mesenchymal origin. And, dogs with nasal carcinoma trended to show worse prognosis than those with nasal sarcoma [1, 47, 97]. Few studies have evaluated outcome of dogs treated for various histological subtypes of nasal carcinomas [31, 74]. It would be helpful to evaluate the tumor characteristics and to seek the prognostic utilities in dogs with nasal carcinoma.

Clinical signs in dogs with nasal tumor include epistaxis, sneezing, mucopurulent discharge, facial deformity, exophthalmos, and occasionally neurologic abnormalities [103]. The clinical signs among tumor histologic subtypes were compared in this chapter. Facial deformity and exophthalmos were not likely showed in dogs with ADC. Nasal discharge was not likely to seen in dogs with SCC. These results indicated the different tumor clinical features among canine nasal carcinoma subtypes.

Cross-sectional imaging has been commonly preformed to investigate the sinonasal diseases. CT image allows to accurate determination of the extent and behavior of tumor in sinonasal cavities [1, 2, 47, 50, 97]. Nasal tumors are commonly very aggressive to the surrounding tissues including orbit, oral cavity, subcutis, nasopharynx or brain. Thirty-nine of 84 (46.4%) dogs with nasal carcinomas showed osteolysis of the cribriform plate and extent to the brain. Cribriform plate destruction (65.2%) on CT images in dogs with CA was detected frequently. Oral and nasopharyngeal involvement were most likely to be ADC. Nasopharyngeal involvement was however detected infrequently in dogs with SCC in this chapter.

The MST for all patients with nasal carcinoma treated with radiation therapy in this

chapter was approximately 8 months (241 days). The dogs with nasal carcinoma that did not receive treatment had a MST of 3.1 months (95 days) in our study. It is similar to the previous report [74]. Regarding histological subtypes, some studies reported better prognosis for dogs with ADC compared to those with SCC or CA [1, 2, 97]. In our study, the rate of response to treatment in dogs with SCC was lower than in dogs with ADC, CA or TCC (20% versus 73.1%, 73.3% and 75%). The dogs with SCC showed the worst response rate of all nasal carcinomas and had the shortest MST of 178 days comparing with ADC's MST of 242 days, CA's MST of 254 or TCC's MST of 407 days. Although no significant difference in survival time was observed among tumor subtypes, nasal SCC was suggested to be the most radioresistant tumor type in canine nasal carcinomas due to the worst response rate to radiation therapy.

Epistaxis has been the most common clinical sign in nasal tumors at the time of diagnosis [47, 68, 74]. It was similar in our study. Two-thirds of dogs were present nasal hemorrhage. One published study has reported that the dogs with epistaxis had worse prognosis than those without epistaxis [74]. However, no significant differences were found in this chapter. Surprisingly, longer median PFS and MST for dogs with nasal discharge than those without nasal discharge were found in our study. When nasal cavity is filled with tumor tissues, nasal discharge may not be present due to an obstructed nasal cavity. It is thinkable to show a worse prognosis for dogs without nasal discharge. The presence of facial deformity or exophthalmos was a negative prognostic factor for PFS. Facial deformity has been reported as a negative prognostic indicator [68]. Dogs with facial deformity and exophthalmos would be thought to have more invasive tumors resulting more difficult local control. Additionally, a nasal tumor that destroyed cribriform plate and then

invaded to the brain would occur neurological signs. Neurological abnormalities were present in dogs with T4 disease. Therefore, the presence of neurological abnormality was also showed to be a prognostic significance in nasal carcinomas.

The prognostic significances have been evaluated according to CT finding, with the finding that response to therapy is significantly related to specific location of tumor invasion [1, 47, 50, 97]. Dogs with subcutaneous involvement (148 days) had significantly shorter PFS than those that did not involve subcutis (246 days). Involvement of orbit and destruction of cribriform plate were highly associated with both short PFS and overall survival time. It is indicated that more aggressive tumors were more difficult to local control resulting poor prognosis. Like nasopharyngeal tumor staging in humans, CT images have commonly been attempted to utilize for canine nasal tumors [1, 2, 47, 50, 97]. Adams' modified staging system that published in 2009 was performed in our study (Table 1-1). It has recently been used in several reports [1, 58, 90]. In our study, sixty-three of 84 (75%) dogs were classified as T stage of T3 to T4 (late stage) with 39 (46.4%) in stage T4. Treating with radiation therapy, stage T3- and T4-dogs had worse prognosis than early stages (T1 and T2). Among histological subtypes, the T4 stage of dogs diagnosed with CA had the shortest median PFS (81 days) and MST (111 days) (TCCs were excluded form statistical analysis due to small numbers). The T4 stage of ADC dogs had better survival comparing with of CA and SCC dogs (Table 1-5). However, additional study with a larger cohort of canine nasal carcinomas may still be necessary.

There were 12 cases of metastasis to regional lymph nodes and/or lung at the time of diagnosis. This finding was similar to a previously reported a metastatic rate of 10-14% for nasal tumors [15, 47, 50, 90]. The dogs with metastatic lesions had significantly shorter PFS

(77 days) and MST (98 days) than dogs without metastasis. Metastatic status has been also previously described for dogs with nasal tumor [47, 50]. Due to using of an orthovoltage machine, lower energy of radiation, in our study, radiation could not deliver enough to the deeper tissues. Large-breed dogs may not be superior to small-breed dogs. However, significant differences of survival were not observed between the dogs with weight of > 15 kg and those with weight of < 15 kg. On the other hand, although the treatment schemes were heterogeneous in our study and the total dose of irradiation also affected survival in an earlier study [90], the dogs that received radiation dose of > 40 Gy were not found to have significant influence for survivals in this chapter. Radiosensitivity of tumors is associated with the outcome of treating with radiation. Dogs with a PR were found longer PFS and overall survival time than dogs with an SD. This result suggested that to grasp the mechanisms of radiosensitivity is necessary in canine nasal carcinomas.

Despite the limitations of nature of a retrospective study, some prognostic significances of canine nasal carcinoma were interpreted in this chapter. In the next chapter, we would evaluate the expression of tumor markers in canine nasal carcinoma and seek correlations between tumor markers and clinical variables.

5. SUMMARY

One of the goals of this chapter was to identify prognostic indicators for survival in dogs with nasal carcinoma treated with radiation therapy. Dogs diagnosed with SCC had the lowest response rate (20%) in all carcinoma types. The dogs with SCC also showed the shortest survival times in this chapter. Additional negative prognostic factors for PFSs or overall survival times were observed in this chapter, including presence of facial deformity, exophthalmos and neurologic abnormalities and absence of nasal discharge at initial presentation, orbital and subcutaneous involvement, cribriform plate destruction on CT images at time of diagnosis, age greater than 11 years, regional lymph nodes or lung metastasis, and the response to treatment.

It would be useful to grasp the mechanisms of radiosensitivity in canine nasal carcinomas. The mechanisms of cellular malignant transformation include the regulation of cell cycle, cell differentiation, apoptosis, and angiogenesis. Applications of molecular radiobiology in recent years have altered the understanding of tumor radioresistance and the cellular response to ionizing radiation. We would put emphasis on expression of tumor markers via imuunohistochemistry staining in canine nasal carcinoma. We would also seek the correlations between tumor markers and prognostic significances in the next chapter.

CHAPTER II.

Immunohistochemical Characterization of Canine Nasal Carcinomas

1. INTRODUCTION

In recent years, despite a large number of studies for prognostic factors of radiation therapy in human medicine, very little research has been performed in veterinary study. Chapter II was to put emphasis on evaluation of tumor markers expression in dogs with nasal carcinoma. This chapter is also to seek a correlation between tumor markers and clinical variables. It is important to search valuable biomarkers involving canine nasal carcinomas for therapeutic targets that may lead to more effective treatment of dogs with nasal carcinoma.

Ki-67 is a nuclear protein that is widely used to detect proliferative cells. It is present in proliferating cells during the G1, S, G2, and M phases of the cell cycle however it is absent during the resting phase (G0). A high positive rate of Ki-67 has been generally considered to be an unfavorable prognostic factor in several human and veterinary studies [7, 67, 81, 88].

Survivin is a small protein that belongs to the inhibitors of apoptosis protein (IAP) family. It is not expressed in normal differentiated tissue but highly expressed in fetal tissues and several cancers [41]. The expression of survivin is associated with poor prognosis in several human cancers [46, 55]. In humans, 60-78.6% of nasopharyngeal carcinomas (NPC) are positive for survivin expression [55, 107, 111]. In veterinary medicine, the expression of survivin also has been detected in several tumors in dogs such as mast cell tumors [81], lymphomas [75], urinary bladder transitional cell carcinomas [73], squamous cell carcinomas [8], osteosarcomas [86], skin tumors [9, 11], and mammary tumors [10]. Survivin expression is correlated with malignancy in these tumors.

Epithelial growth factor receptor (EGFR) is a transmembrane protein of ErbB family of

receptor tyrosine kinase (RTK). Binding of epithelial growth factor (EGF) or EGF-like growth factors activates the receptor and then triggers the cascades of signal transduction. EGFR plays a key role in regulation of cell cycle, angiogenesis, differentiation and survival [18, 25, 83] Overexpression of EGFR has been correlated with poor prognosis in human cervical [25] and nasopharyngeal cancers [16, 18, 93]. The inhibition of EGFR improves the treatment response of radiation therapy or chemotherapy in human head and neck carcinomas [18, 34]. EGFR also expresses in several canine tumors, including nasal carcinomas [83], mammary gland tumors [26], and lung tumors [79]. Over 70% of human nasopharyngeal carcinomas and 54.2% of canine nasal carcinomas were detected EGFR expression [18, 69, 83].

Vascular endothelial growth factor (VEGF) belongs to the platelet-derived growth factor (PDGF) family. It is a dimeric glycoprotein and is secreted by macrophage, vascular smooth muscle cells, and tumor cells to stimulate endothelial migration and proliferation [29, 33]. VEGF regulates angiogenesis by binding to a number of vascular endothelial growth factor receptors (VEGFRs) [4]. The growth of a solid tumor requires an adequate blood supply, which is enabled by vascular formation (angiogenesis) [29]. Tumor angiogenesis is related to tumor development, progression, and metastasis [33]. VEGF production can be induced in cells that lack oxygen. In human NPC, overexpression of VEGF is correlated with the presence of metastasis and recurrence [55]. There is a link between VEGF expression and poor outcome post radiotherapy in NPC [35]. In canine nasal carcinomas, positive rate for VEGF staining was 91.7% [83].

Cyclooxygenase (COX) is a rate-limiting enzyme in prostaglandin endoperoxide synthesis from arachidonic acid. COX-2, an inducible isoform of cyclooxygenase, is

generally undetectable in normal tissues but is induced by some pro-inflammatory cytokines, growth factors and tumor promoters [6, 13, 45]. Expression of COX-2 is related to apoptosis inhibition, tumor metastasis and angiogenesis [69, 91]. Increased COX-2 expression was associated with poor prognosis in NPC [17, 70]. A high positive rate of 71-90% of nasal carcinomas in dogs expressed COX-2 [6, 13, 36, 45]. Expression of COX-2 has been reported to use to predict the prognosis of canine nasal carcinomas that were treated with hypofractionated radiotherapy. However there was no significant correlation between survival and COX-2 score [6].

Apoptosis is the process of eliminating old and damaged cells in tissues. It is mediated through a number of proteins involved in the activation of caspases cascades [102]. The biochemical level of changes lead to cell morphology, which include membrane blebbing, cell shrinkage, chromatin condensation, and DNA fragmentation. Radiation will induce apoptosis in neoplastic cells [78, 102]. A correlation between spontaneous and radiation-induced apoptosis rates has been observed in several studies [78, 82]. These studies revealed that patients with an increased spontaneous apoptotic rate had a good prognosis in human bladder [77], cervical [82] and rectal [3, 76, 94] cancers treated with chemoradiation/radiation therapy.

The primary goal of this chapter was to evaluate the expression of Ki-67, survivin, EGFR, VEGF, and COX-2 in canine nasal carcinomas through immunohistochemical (IHC) staining in tumor tissues. Secondary goal was to seek a correlation between these markers and prognosis after radiation therapy.

2. MATERIALS AND METHODS

(1) Biopsy samples and patient data

Biopsy samples obtained from 67 dogs that were diagnosed with nasal carcinomas in Rakuno Gakuen University Veterinary Teaching Hospital (RGU-VTH) between April 2004 and January 2014. The specimens were collected from dogs before treatment. The histopathological diagnosis of each case was evaluated and diagnosed by the veterinarians in the Department of Veterinary Pathology in RGU. All tissue samples were fixed in 10% neutral buffered formalin and embedded in paraffin.

The medical records of the 67 dogs were reviewed. Pretreatment clinical examinations including a complete blood count, serum chemistry, three-view thoracic radiographs, CT scans, and a fine-needle biopsy of regional lymph nodes if enlarged were performed. The medical records of dogs with nasal carcinomas were reviewed. Clinical staging was evaluated using CT imaging with Adams' staging system (Table 1-1).

(2) Immunohistochemistry staining

Sections of 4-μm thickness were cut from all formalin-fixed paraffin-embedded tissue blocks and mounted on glass slides, then were deparaffinized in xylene and rehydrated in a graded series ethanol. All sections were pretreated for antigen retrieval. Antigen retrieval was carried out by proteinase K (20 μg/ml, Dako) for 2 minutes or by autoclave at 121 ºC for 15 minutes with 0.1 M citrate buffer (pH 6.0). Endogenous peroxidase activity was blocked by rinsing for 10 minutes with 3% hydrogen peroxide (H_2O_2) and then nonspecific binding was blocked with 1% horse serum in phosphate-buffered saline (PBS: NaCl 80 g,

KCl 2 g, Na_2HPO_4 14.4 g and KH_2PO_4 2.4 g dissolving in 10 L distilled water) for 20 minutes. The sections were then incubated with a primary antibody against Ki-67, survivin, EGFR, VEGF, and COX-2. Information on primary antibodies including clonality, dilution and incubation time is summarized in Table 2-1. The sections were subsequently incubated with a secondary antibody and then with an avidin-biotin complex solution (VECTASTAIN® ABC Kit, Vector Labs) for 30 minutes each at room temperature. 3-3'-diaminobenzidine (DAB, Kanto Chemical Co.) solution was used for color development, and Mayer's hematoxylin was used for counterstaining. Negative control data were obtained by replacing the primary antibodies with PBS.

Table 2-1. Primary antibodies and staining conditions

Antibody	Clonality	Isotype	Supplier	Dilution	Incubation time (h)	Antigen retrieval method
Ki-67	Monoclonal MIB-1	Mouse IgG	Dako	1:100	1	Pressurized heating (121ºC, 15 min)
Survivin	Polyclonal NB500-201	Rabbit IgG	Novus Biological	1:600	16	Pressurized heating (121ºC, 15 min)
EGFR	Monoclonal 31G7	Mouse IgG	Invitrogen	1:50	16	Proteinase K (20μg/ml, 2 min)
VEGF	Polyclonal A-20	Rabbit IgG	Santa Cruz	1:100	16	Pressurized heating (121ºC, 15 min)
COX-2	Monoclonal 33	Mouse IgG	BD Transduction Lab	1:50	16	Pressurized heating (121ºC, 15 min)

(3) Immunohistochemistry scoring

The Ki-67 index was determined as the percentage of Ki-67-positive tumor cells in a total of 1000 tumor cells. A low expression of Ki-67 was defined as a Ki-67 index below 25%; a high expression was defined as a Ki-67 index above 25%.

After immunostaining the proteins survivin, EGFR, VEGF and COX-2, scoring of the

labeling was performed to estimate the degree of the protein expression in tumor tissues. A semi-quantitative immunohistochemical scoring system was applied similar to those previous reports [6, 83, 111]. An IHC score of protein was based on the percentage of positive labeling tumor cells and their average intensity. Percentage of positive labeling tumor cells was assessed at ×200 magnification and the score was given an average of 5 fields. Scoring of percentage of positive labeling tumor cells for each protein is summarized in Table 2-2. The intensity of labeling tumor cells was estimated for the whole section when viewed at ×400 magnification. Intensity was graded as 0 for no immunostaining, 1 for weak immunostaining, 2 for moderate immunostaining and 3 for strong immunostaining. The total IHC score was calculated by multiplying the scores for the percentage and intensity, it obtained a total score of 0 to 12. Tumor samples with an IHC score at least 2 were defined as positive. An IHC score of 3 was defined as the threshold of the survivin level that separated tumors with high expression from those with low expression. The threshold of IHC scores for EGFR, VEGF and COX-2 staining were 4, 6 and 4, respectively.

Table 2-2. Scoring of percentage range and intensity of positive labeling tumor cells for survivin, EGFR, VEGF and COX-2

IHC score	Survivin	EGFR VEGF	COX-2
1	< 25% positive	< 10% positive	< 1% positive
2	25-50% positive	10-30% positive	1-10% positive
3	50-75% positive	30-60% positive	10-50% positive
4	> 75% positive	> 60% positive	> 50% positive
1	Weak immunostaining		
2	Moderate immunostaining		
3	Strong immunostaining		

A rate of survivin expression ≥ 25% in the nucleus was considered nuclear positive, and an expression rate of < 25% was considered nuclear negative; a rate of survivin expression ≥ 25% in the cytoplasm was considered cytoplasmic positive, and an expression rate of < 25% was considered cytoplasmic negative.

(4) TUNEL assay and assessment of apoptotic index

A TUNEL assay was carried out using an ApopTag® Peroxidase In Situ Apoptosis Detection Kit (S7100, Chemicon Merck Millipore) to detect spontaneous apoptotic cells in the 47 samples that taken from the dogs before receiving radiation therapy. All section samples were sliced, deparaffinized and rehydrated with the same way in the IHC staining. The samples were pretreated with proteinase K (20 μg/ml, Dako) for 10 minutes at room temperature. Endogenous peroxidase activity was blocked by rinsing with 3% H_2O_2. Equilibration buffer was used to cover the slide for 15 seconds, and then working-strength TdT solution was added; the slide was then incubated in a humidified chamber at 37 ºC for 1 hour. The reaction was stopped by addition of working-strength Wash/Stop buffer for 10 minutes. Anti-digoxigenin peroxidase was applied and the sections were then incubated for 30 minutes at room temperature. DAB solution and Mayer's hematoxylin was used for color development and counterstaining. A canine lymphoma tissue section was used as a positive control.

Apoptotic-positive tumor cells in a total of 1000 tumor cells were counted by microscopic examination in ×1000 magnification fields as the apoptotic index (AI). A low AI was defined as an AI at and below 0.3%; a high AI was defined as an AI above 0.3%.

(5) Radiation therapy and response assessment

Radiation therapy and the assessment of response for treatment were carried out as described in Chapter I.

(6) Statistical analysis

Statistical significance of differences was analyzed with chi-squared (χ^2) test for the relationship between the tumor markers and clinical features. Both spearman's correlation method and chi-squared test were used to analyze the correlation among these tumor markers. The overall survival time of a patient was calculated from the starting date of radiation therapy to the time of death or the last follow-up. The progression-free survival (PFS) was defined from the starting date of radiation therapy to the day of disease progression or the last follow-up. The correlation between the survival and prognostic factors was estimated using Kaplan-Meier curves, and statistical differences between survival curves were calculated using a log-rank method. P values of < 0.05 were considered statistical significant. The commercial software StatMate III (ATMS Co., Ltd.) was used to perform the statistical analysis.

3. RESULTS

(1) Immunohistochemical characterization in canine nasal carcinomas

The information of 67 dogs is summarized in Table 2-3. The mean and median ages for sampled dogs were 11.1 years and 11 years (range from 7 to 16 years of age), respectively. There were 11 intact females, 25 spayed females, 15 intact males and 16 neutered males. There were 56 pure-breed dogs and 11 mixed breed dogs. Golden retriever (n = 10), Pembroke Welsh corgi (n = 9) and miniature dachshund (n = 7) were the most three pure-breeds. Tumor samples were consisted of 29 ADCs, 17 CAs, 12 SCCs and 9 TCCs. Samples from all 67 dogs were evaluated for expression of Ki-67, survivin, EGFR, VEGF, COX-2, and AI.

1) Ki-67 expression

Ki-67 expresses strongly in the nucleus (Figure 2-1). The percentage of Ki-67-positive tumor cells in all samples ranged from 0.8 to 92.3%. The median and mean Ki-67 index was 28.5% and 34.1%. The mean Ki-67 index for ADC, CA, SCC and TCC was 29.2%, 38.2%, 36.3% and 39.3%, respectively. Low Ki-67 expression (Ki-67 index < 25%) was observed in 32 samples, whereas high Ki-67 expression (Ki-67 index > 25%) was observed in 35 samples.

2) Survivin expression

Survivin staining was localized in the nucleus and/or cytoplasm of tumor cells (Figure 2-2). Forty samples (59.7%) were survivin positive in the nucleus, and 48 samples (71.6%)

were positive in the cytoplasm. In addition, 60 samples (89.6%) were positive for survivin in the nucleus or cytoplasm or both. The relationship between nasal tumor types and survivin expression location is shown in Table 2-4. Twenty-five of the 29 ADC (25/29), fifteen of the 17 CA (15/17), all of the 12 SCC (12/12), and eight of the 9 TCC (8/9) were positive expression in survivin. The total IHC score for survivin expression ranged from 0 to 12. The median and mean for the total survivin IHC score were 4 and 5.5. The mean survivin IHC score for ADC, CA, SCC and TCC was 5.5, 5.4, 5.9 and 5, respectively. Low survivin expression (survivin IHC score $\leq$ 3) was observed in 27 samples, whereas high survivin expression (survivin IHC score > 3) in 40 samples.

3) EGFR expression

EGFR staining was predominantly membranous and occasionally cytoplasmic (Figure 2-3). Fifty-nine samples (88.1%) were positive for EGFR expression. In addition, 24 of the 29 ADC (24/29), fourteen of the 17 CA (14/17), all of the 12 SCC (12/12), and all of the 9 TCC (9/9) were positive expression in EGFR. The total IHC score for EGFR expression ranged from 0 to 12. The median and mean for the total EGFR IHC score were 6 and 6.9. The mean EGFR IHC score for ADC, CA, SCC and TCC was 5.7, 6.3, 9.9 and 8.2, respectively. Low EGFR expression (EGFR IHC score $\leq$ 4) was observed in 20 samples, whereas high EGFR expression (EGFR IHC score > 4) was observed in 47 samples.

4) VEGF expression

VEGF staining was observed in cytoplasm and membrane of tumor cells (Figure 2-4). Sixty-five samples (97%) were positive for VEGF expression. In addition, all of the 29

ADC (29/29), sixteen of the 17 CA (16/17), eleven of the 12 SCC (11/12), and all of the 9 TCC (9/9) were positive expression in VEGF. The total IHC score for VEGF expression ranged from 0 to 12. The median and mean for the total VEGF IHC score were 8 and 8.1. The mean VEGF IHC score for ADC, CA, SCC and TCC was 8.3, 7.8, 7.4 and 9.3, respectively. Low VEGF expression (VEGF IHC score ≤ 6) was observed in 21 samples, whereas high VEGF expression (VEGF IHC score > 6) was observed in 46 samples.

5) COX-2 expression

COX-2 staining was predominantly cytoplasmic and perinuclear region (Figure 2-5). Fifty-five samples (82.1%) were positive for COX-2 expression. In addition, 24 of the 29 ADC (24/29), thirteen of the 17 CA (13/17), ten of the 12 SCC (10/12), and eight of the 9 TCC (8/9) were positive expression in COX-2. The total IHC score for COX-2 expression ranged from 0 to 12. The median and mean for the total COX-2 IHC score were 6 and 5.3. The mean EGFR IHC score for ADC, CA, SCC and TCC was 4.7, 5.6, 6.1 and 5.7, respectively. Low COX-2 expression (COX-2 IHC score ≤ 4) was observed in 32 samples, whereas high COX-2 expression (COX-2 IHC score > 4) was observed in 35 samples.

6) Apoptotic index (TUNEL assay)

TUNEL assays were performed for detecting apoptotic index (AI) in 47 paraffin-embedded samples. AI was ranged from 0 to 1.2% in all samples. Mean and median of AI was 0.31% and 0.3%. Low-AI (AI ≤ 0.3%) was observed in 27 samples, whereas high-AI (AI > 0.3%) was observed in 20 samples.

7) Association among the expression of Ki-67, survivin, EGFR, VEGF, COX-2 and AI in canine nasal carcinomas

Using chi-squared method, significant associations between the rate of Ki-67 and EGFR expression, survivin expression and AI were observed. In spearman's correlation method, significant correlations were found between EGFR score and Ki-67 rate, EGFR score and survivin score, AI and Ki-67 rate, as well AI and survivin score (Table 2-5).

Table 2-3. Characteristics of dogs

	Cases (n = 67)
Range of age, year (mean, median)	7-16 (11.1, 11)
Range of weight, kg (mean, median)	3.3-50.2 (16.8, 13.3)
Sex	
Intact Female	11
Spayed Female	25
Intact Male	15
Neutered Male	16
Breed	
Golden Retriever	10
Pembroke Welsh Corgi	9
Miniature Dachshunt	7
Shetland Sheepdog	6
Shiba	4
Beagle	3
Miniature Schnauzer	3
Siberian Husky	3
Hokkaido	2
Labrador Retriever	2
Shih Tzu	2
Bullmastiff	1
Chihuahua	1
Maltese	1
Papillon	1
Pug	1
Mix	11

Table 2-4. The location of survivin expression in different tumor type of canine nasal carcinomas

Histologic Type	nSurvivin (+) cSurvivin (+)	nSurvivin (+) cSurvivin (−)	nSurvivin (−) cSurvivin (+)	nSurvivin (−) cSurvivin (−)
ADC	10	1	14	4
CA	7	7	1	2
SCC	7	1	4	0
TCC	4	3	1	1

(+): positive cells >25%. ADC: Adenocarcinoma. CA: Undifferentiated carcinoma. SCC: Squamous cell carcinoma. TCC: Transitional cell carcinoma.

Table 2-5. Relationship between the expression of Ki-67, survivin, EGFR, VEGF and COX-2 in canine nasal carcinomas (*P values*)

	Ki-67	Survivin	EGFR	VEGF	COX-2	AI
Ki-67		0.194[b]	**0.007**[b]	0.116[b]	0.249[b]	**0.048**[b]
Survivin	0.655[a]		**0.048**[b]	0.088[b]	0.156[b]	**0.028**[b]
EGFR	**0.004**[a]	0.291[a]		0.188[b]	0.194[b]	0.456[b]
VEGF	0.811[a]	0.609[a]	0.250[a]		0.764[b]	0.929[b]
COX-2	0.889[a]	0.582[a]	0.407[a]	0.285[a]		0.683[b]
AI	0.309[a]	**0.005**[a]	0.157[a]	0.556[a]	0.556[a]	

a: chi-squared test

b: spearman's correlation method

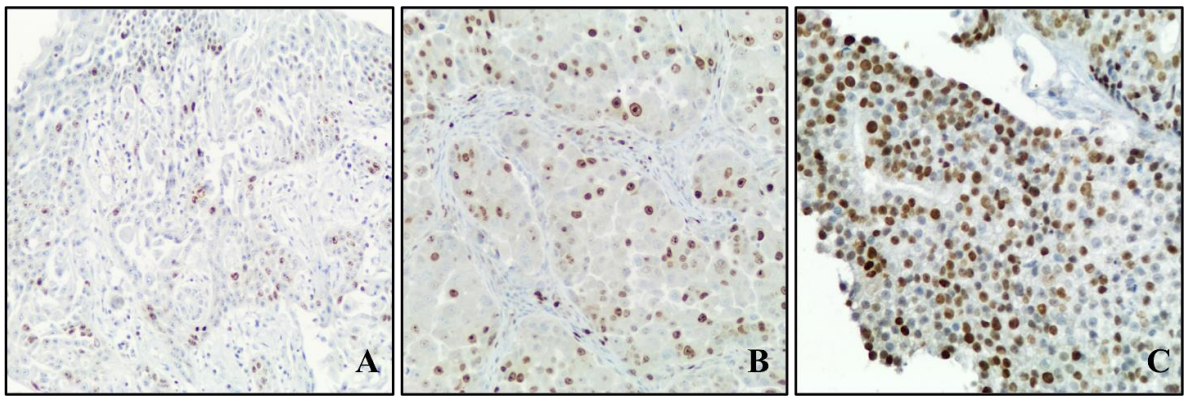


Figure 2-1. Examples of immunohistochemical staining of Ki-67 in canine nasal carcinoma. (**A**) Squamous cell carcinoma, Ki-67 positive rate was 17.6%. (**B**) Adenocarcinoma, Ki-67 positive rate was 36.2%. (**C**) Undifferentiated carcinoma, Ki-67 positive rate was 72.7%. (Original magnification ×100)

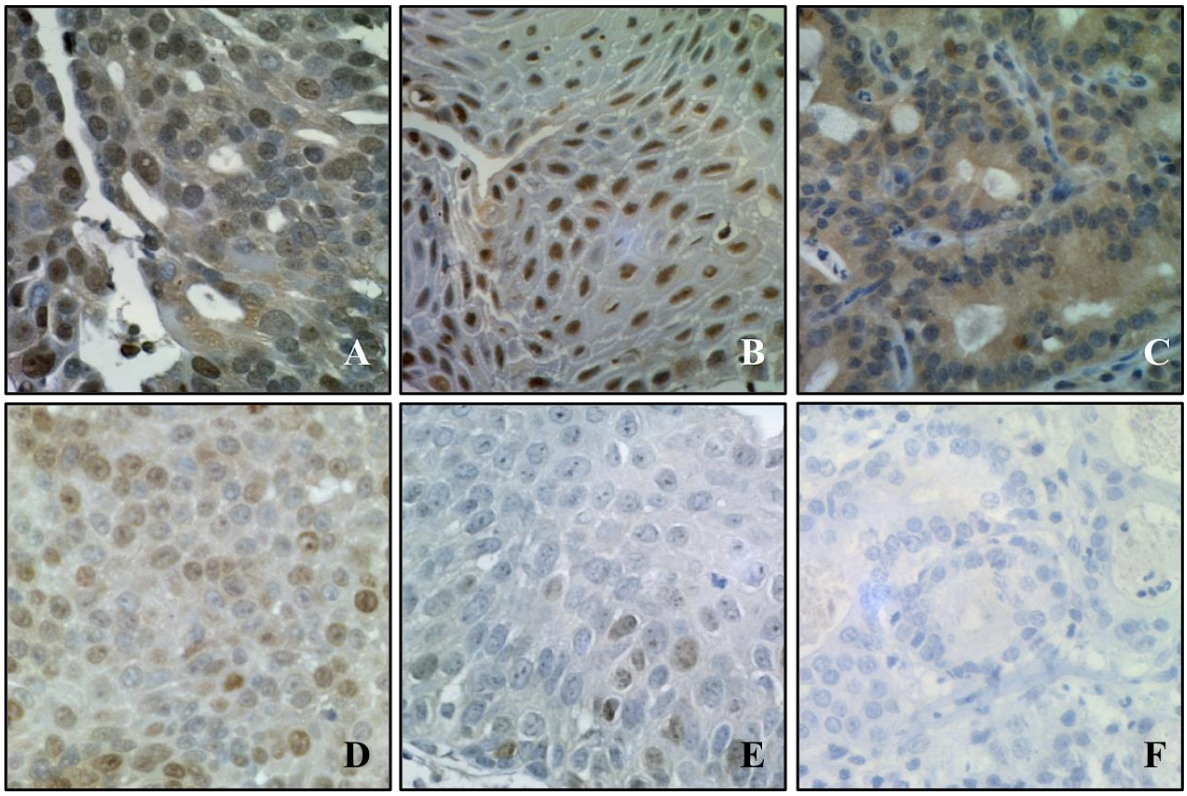


Figure 2-2. Examples of immunohistochemical staining of survivin in canine nasal carcinoma. (**A**) Adenocarcinoma, nuclear and cytoplasmic expression, IHC score of 12. (**B**) Squamous cell carcinoma, nuclear expression, IHC score of 12. (**C**) Adenocarcinoma, cytoplasmic expression, IHC score of 12. (**D**) Undifferentiated carcinoma, nuclear and cytoplasmic expression, IHC score of 8. (**E**) Squamous cell carcinoma, nuclear expression, IHC score of 2. (**F**) Adenocarcinoma, negative expression, IHC score of 0. (Original magnification ×400)

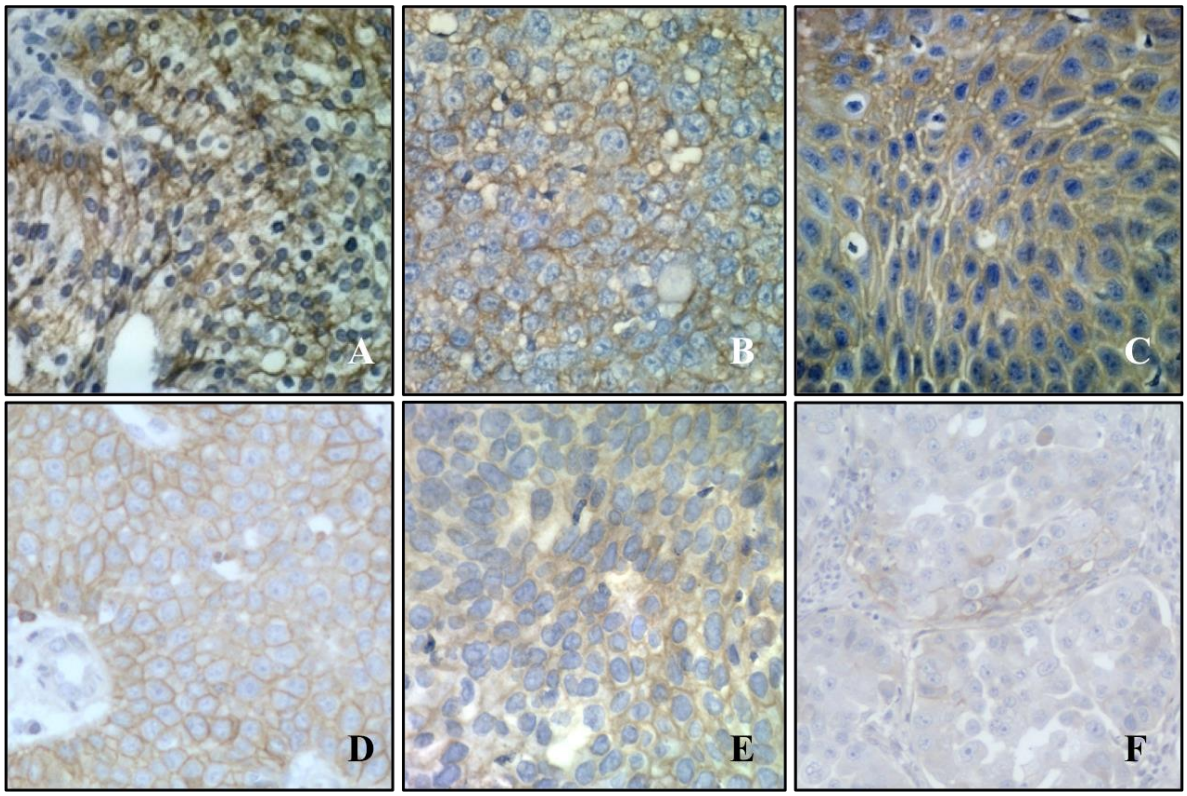


Figure 2-3. Examples of immunohistochemical staining of EGFR in canine nasal carcinoma. (**A**) Adenocarcinoma, IHC score of 12. (**B**) Undifferentiated carcinoma, IHC score of 12. (**C**) Squamous cell carcinoma, IHC score of 12. (**D**) Transitional cell carcinoma, IHC score of 8. (**E**) Squamous cell carcinoma, IHC score of 6. (**F**) Adenocarcinoma, IHC score of 1. (Original magnification ×400)

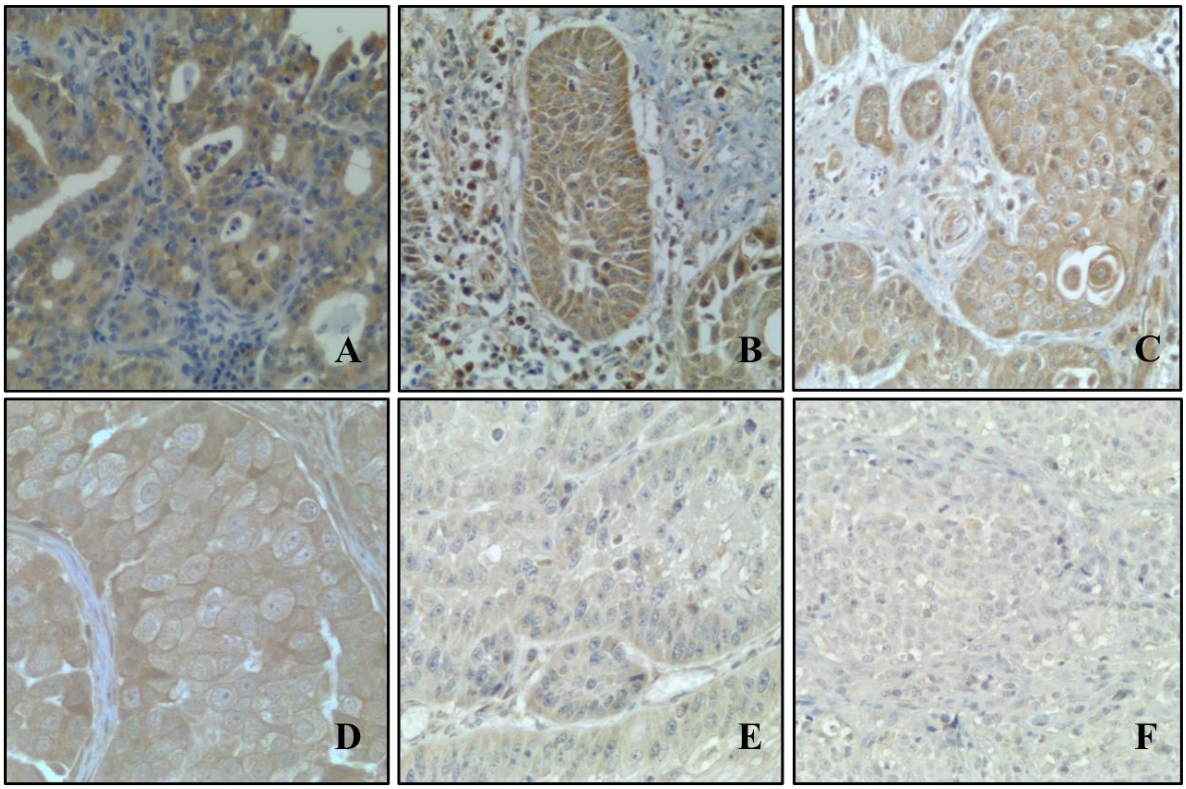


Figure 2-4. Examples of immunohistochemical staining of VEGF in canine nasal carcinoma. (**A**) Adenocarcinoma, IHC score of 12. (**B**) Squamous cell carcinoma, IHC score of 12. (**C**) Squamous cell carcinoma, IHC score of 12. (**D**) Adenocarcinoma, IHC score of 8. (**E**) Squamous cell carcinoma, IHC score of 4. (**F**) Transitional cell carcinoma, IHC score of 2. (Original magnification ×200)

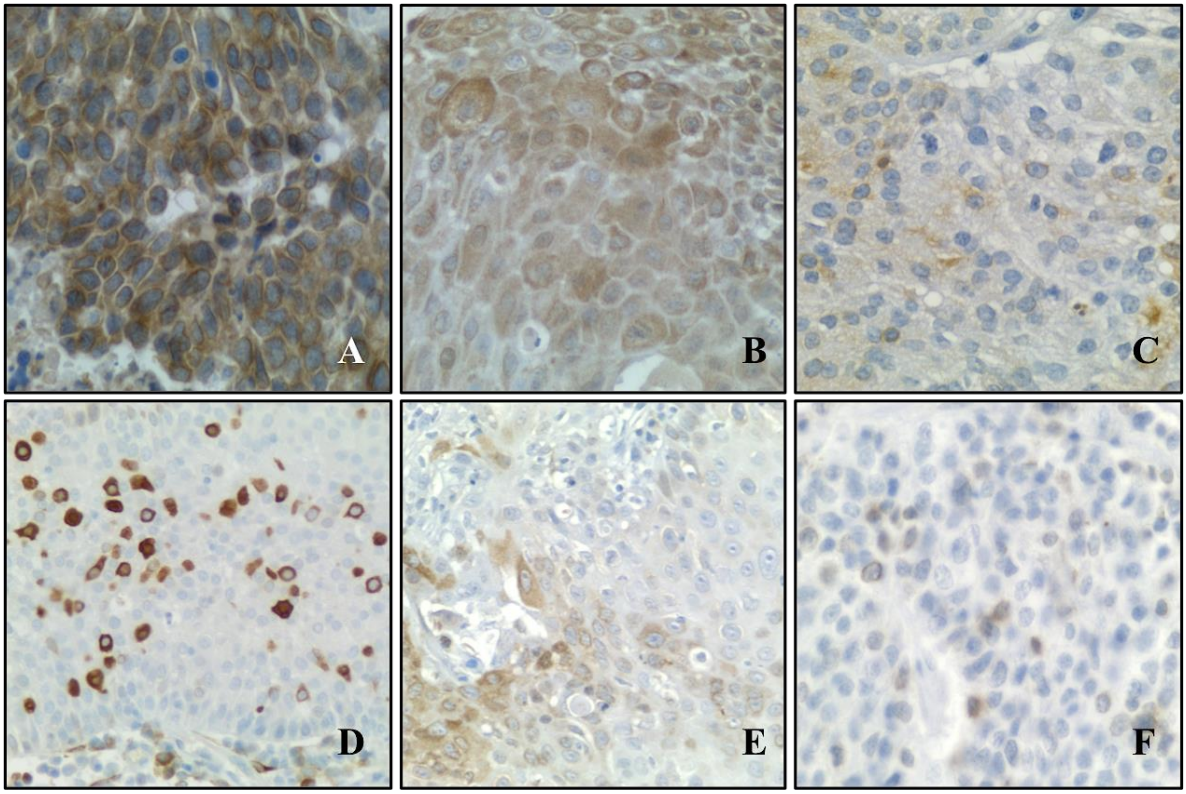


Figure 2-5. Examples of immunohistochemical staining of COX-2 in canine nasal carcinoma. (**A**) Undifferentiated carcinoma, IHC score of 12. (**B**) Squamous cell carcinoma IHC score of 8. (**C**) Adenocarcinoma, IHC score of 4. (**D**) Transitional cell carcinoma, IHC score of 6. (**E**) Adenocarcinoma, IHC score of 6. (**F**) Undifferentiated carcinoma, IHC score of 2. (Original magnification ×200)

(2) Association between expression of tumor markers and clinical features in dogs with nasal carcinoma

Regarding the T stage of 67 dogs, there were 12 dogs (17.9%) with T1 disease, 5 dogs (7.5%) with T2 disease, 17 dogs (25.4%) with T3 disease, and 33 dogs (45.2%) with T4 disease. Cribriform plate destruction was detected on CT images in 33 dogs.

Ki-67 expression was significantly associated with the T stage. High-Ki-67 expression was significantly associated with advanced-tumor dog (T3+T4) (P = 0.029). High-Ki-67 expression was also significantly correlated with the dogs with cribriform plate destruction (P = 0.020). No significant association between Ki-67 expression and sex or age was observed (Table 2-6a).

Survivin expression was significantly correlated with the T stage. High expression of survivin was associated with advanced-tumor (T3+T4) dog significantly (P = 0.018). No significant association between survivin expression and cribriform plate destruction, sex or age was observed (Table 2-6a).

EGFR expression was not highly associated with the clinical findings, including T stage, cribriform plate destruction, sex or age (Table 2-6b).

VEGF expression was significantly associated with the T stage. High VEGF expression was highly correlated with advanced-tumor dog (T3+T4) (P = 0.026). No significant association between VEGF expression and cribriform plate destruction, sex or age was observed (Table 2-6b).

COX-2 expression was not closely related with the clinical features, which included T stage, cribriform plate destruction, sex or age (Table 2-6b).

Table 2-6a. Association between Ki-67, survivin, expression and clinical features in dogs with nasal carcinoma

		Ki-67		**Survivin**	
Clinical characteristics	*N*	Low	High	Low	High
Tumor Stage					
T1+T2	17	**12** (70.6%)	**5** (29.4%)	**11** (64.7%)	**6** (35.3%)
T3+T4	50	**20** (40.0%)	**30** (60.0%)	**16** (32.0%)	**34** (68.0%)
P value		**0.029**		**0.018**	
Destruction of cribriform plate					
Absence	34	**21** (61.8%)	**13** (38.2%)	**16** (47.1%)	**18** (62.9%)
Presence	33	**11** (33.3%)	**22** (66.7%)	**11** (33.3%)	**22** (66.7%)
P value		**0.020**		0.252	
Sex					
Female	36	**19** (52.8%)	**17** (47.2%)	**15** (41.7%)	**21** (68.3%)
Male	31	**13** (41.9%)	**18** (68.1%)	**12** (38.7%)	**19** (61.3%)
P value		0.376		0.806	
Age					
≤ 11 y	37	**17** (46.0%)	**20** (54.0%)	**16** (43.2%)	**21** (56.8%)
> 11 y	30	**15** (50.0%)	**15** (50.0%)	**11** (36.7%)	**19** (63.3%)
P value		0.741		0.585	

Table 2-6b. Association between EGFR, VEGF, COX-2 expression and clinical features in dogs with nasal carcinoma

		EGFR		**VEGF**		**COX-2**	
Clinical characteristics	*N*	Low	High	Low	High	Low	High
Tumor Stage							
T1+T2	17	**6** (35.3%)	**11** (64.7%)	**9** (52.9%)	**8** (47.1%)	**7** (41.2%)	**10** (58.8%)
T3+T4	50	**14** (28.0%)	**36** (72.0%)	**12** (24.0%)	**38** (76.0%)	**25** (50.0%)	**25** (50.0%)
P value		0.570		**0.026**		0.529	
Destruction of cribriform plate							
Absence	34	**10** (29.4%)	**24** (70.6%)	**13** (38.2%)	**21** (61.8%)	**15** (44.1%)	**19** (55.9%)
Presence	33	**10** (30.3%)	**23** (69.7%)	**8** (24.2%)	**25** (75.8%)	**17** (51.5%)	**16** (48.5%)
P value		0.936		0.217		0.544	
Sex							
Female	36	**9** (25.0%)	**27** (75.0%)	**11** (30.6%)	**25** (69.4%)	**20** (55.6%)	**16** (44.4%)
Male	31	**11** (35.5%)	**20** (64.5%)	**10** (32.3%)	**21** (67.7%)	**12** (38.7%)	**19** (61.3%)
P value		0.349		0.881		0.169	
Age							
≤ 11 y	37	**10** (27.0%)	**27** (73.0%)	**15** (40.5%)	**22** (59.5%)	**18** (48.6%)	**19** (51.4%)
> 11 y	30	**10** (33.3%)	**20** (66.7%)	**6** (20.0%)	**24** (80.0%)	**14** (46.7%)	**16** (53.3%)
P value		0.575		0.072		0.872	

(3) Association between expression of tumor markers and the response to RT in dogs with nasal carcinoma

Forty-seven dogs received radiation therapy for treatment. There were 22 dogs with ADC, 13 dogs with CA, 9 dogs with SCC and 3 dogs with TCC. The mean and median ages for sampled dogs were 11.2 years and 11 years (ranged from 7 to 16 years of age), respectively. There were 9 intact females, 19 spayed females, 10 intact males and 9 neutered males. There were 41 pure-breed dogs and 6 mixed breed dogs. Golden retriever (n = 9) and Pembroke Welsh corgi (n = 8) were the most two pure-breeds. Of 47 dogs, 43 dogs were able to evaluate the response of radiation therapy. Twenty-six dogs were determined to have partial response (PR) (14 ADC, 10 CA, 1 SCC, 1 TCC), and seventeen dogs had a stable disease (SD) in response to radiation therapy (6 ADC, 2 CA, 8 SCC, 1 TCC).

Analyzing the associations between the markers and treatment response, only survivin expression was significantly associated with the response to RT (P = 0.039). Regarding Ki-67, EGFR, VEGF and COX-2 expression, no significant associations were observed between the response of treatment and these markers (Table 2-7). TUNEL assay resulted that the group of PR samples had significantly higher AI than the group of SD samples did (mean ± SD of the AI: 0.38 ± 0.25% versus 0.23 ± 0.19%; P = 0.036).

Table 2-7. Association between tumor markers and radiation therapy response in nasal carcinomas

	RT response		*P value*
	PR n = 26	**SD** n = 17	
Ki-67			
Low	**15** (57.7%)	**11** (42.3%)	0.646
High	**11** (64.7%)	**6** (35.3%)	
Survivin			
Low	**14** (82.4%)	**3** (17.6%)	**0.039**
High	**12** (46.2%)	**14** (53.8%)	
EGFR			
Low	**11** (84.6%)	**2** (15.4%)	0.073
High	**15** (50.0%)	**15** (50.0%)	
VEGF			
Low	**9** (69.2%)	**4** (30.8%)	0.439
High	**17** (56.7%)	**13** (43.3%)	
COX-2			
Low	**16** (72.7%)	**6** (27.3%)	0.092
High	**10** (47.6%)	**11** (52.4%)	
AI			
Low	**11** (45.8%)	**13** (54.2%)	0.058
High	**15** (78.9%)	**4** (21.1%)	

PR: Partial response. SD: Stable disease.

(4) Correlation between expression of tumor markers and survival in dogs with nasal carcinoma treated with RT

To investigate the correlation between protein expression and clinical outcome, PFS or overall survival times were compared between the low and high expression groups of the 47 nasal carcinoma dogs. The median of PFSs and survival times are summarized in Table 2-8. High-Ki-67 dogs were highly related with shorter PFS in nasal carcinomas (Median PFS of 124 days versus 236 days; $P = 0.037$) but no significant correlation was observed between PFSs and expression of survivin, EGFR, VEGF, COX-2 or AI. Dogs with a high-Ki-67 revealed significantly shorter survival times than that with a low-Ki-67 ($P = 0.004$), with MST of 150 days and 261 days, respectively. The respective MSTs for high-survivin and low-survivin dogs were 176 days and 261 days, the difference was significant ($P = 0.042$). The dogs with a low expression for EGFR had a better survival time than the dogs with a high EGFR expression (MST of 275 days versus 176 days; $P = 0.006$). However, neither expression of VEGF nor COX-2 in canine nasal carcinoma was found significant correlation with survival times after radiation therapy (246 days versus 187 days for VEGF; 236 days versus 255 days for COX-2). High-AI dogs were tended to relate with longer MST in nasal carcinomas but it was insignificant (Median MST of 249 days versus 176 days; $P = 0.076$).

Table 2-8. Median progression-free survival, overall survival time and tumor markers for dogs treated with RT for nasal carcinomas

	Number of cases	PFS median, days	*P* value	Survival time median, days	*P* value
Ki-67					
< 25%	26	236	**$P = 0.037$**	261	**$P = 0.004$**
> 25%	21	124		150	
Survivin					
Low	18	191	$P = 0.237$	261	**$P = 0.042$**
High	29	148		176	
EGFR					
Low	14	250	$P = 0.064$	275	**$P = 0.006$**
High	33	151		176	
VEGF					
Low	16	167	$P = 0.978$	187	$P = 0.830$
High	31	166		246	
COX-2					
Low	26	185	$P = 0.452$	255	$P = 0.305$
High	21	149		236	
Apoptotic index					
Low	27	147	$P = 0.129$	176	$P = 0.079$
High	20	236		249	

PFS: Progression-free survival.

4. DISCUSSION

IHC staining has not been widely used to predict the outcome to radiation therapy in veterinary medicine. Only two reports have been published recently [6, 61]. Therefore one aim of this chapter was to assess the outcome of radiation therapy using IHC.

Ki-67 is a proliferation marker generally used to assess malignancy and prognosis in several tumors in both humans and dogs [7, 67, 81, 88]. High-Ki-67 tumors have been considered malignant and aggressive in previous human NPC studies [106, 112]. A significant correlation was found between Ki-67 expression and clinical T stage in dogs with nasal carcinoma. High level of Ki-67 was correlated with the advanced-stage tumor (T3+T4), cribriform plate destruction and shorter survivals after radiation therapy. Highly proliferative tumors showed aggressive behaviors in canine nasal carcinomas. Regarding Ki-67 and tumor radiosensitivity, low levels of Ki-67 have been believed to reflect hypoxia and radioresistance. This is because in a hypoxic environment, cells are preferentially in the G0 phase, the most radioresistant phase of the cell cycle [43]. Oral and laryngeal cancers in humans with a high-Ki-67 tend to respond better to radiation therapy than those with a low-Ki-67 [19, 24, 49]. In cats with squamous cell carcinomas that were treated with electron beam radiation therapy, high-Ki-67 cats were found to have a longer disease-free interval [61]. However, no significant difference was observed in Ki-67 expression level between good and poor responses to treatment in this chapter. High Ki-67 expression was suggested a negative prognostic significance for survival times in canine nasal carcinomas.

Survivin expresses in two different locations, the nucleus and the cytoplasm, in tumor cells. Additionally, the different locations of survivin are related to different functions [41,

111]. Expression of survivin in the nucleus is associated with cell proliferation. Expression in the cytoplasm however, is correlated with apoptosis inhibition and the control of cellular survival. Survivin binds to the microtubules of the mitotic spindle during the G2/M phase of the cell cycle in the nucleus. Survivin inhibits apoptosis via either a direct blockage of caspase-3, -7 and -9 or by indirectly blocking activity of the pro-apoptotic protein Smac/DIABLO, preventing binding to other IAPs such as XIAP in the cytoplasm [41]. In our study, 59.7% of the dogs with nasal carcinomas were found to be positive for the expression of nuclear survivin, and 71.4% of the dogs were positive for cytoplasmic survivin. However neither nuclear expression of survivin nor cytoplasmic expression of survivin was found to have any association with survival time in the 47 dogs with nasal carcinoma treated with a radiation therapy. High IHC score of survivin was observed in the advanced clinical stage and poor response to radiation in dogs with nasal carcinoma. Therefore, survivin overexpression is strongly suggested to be an unfavorable prognostic factor for predicting the outcome of treatment with radiation.

EGFR is a transmembrane protein of receptor tyrosine kinase. Being activated the downstream of signal transduction cascade, EGFR play an important role in tumor proliferation, angiogenesis, differentiation, and survival [18, 25]. In canine nasal carcinomas, EGFR expression was not significantly associated with the clinical T stage or cribriform plate destruction. Moreover, EGFR trended to show a relationship with the treatment response in the 43 dogs that treated with radiation therapy despite its insignificance. Overexpression of EGFR has been reported to have negative prognosis for survival in several tumors in human and veterinary studies [7, 25, 70, 79]. Our results suggested that high-EGFR dogs showed significantly shorter overall survival than low-EGFR dogs (MST:

176 days versus 275 days). A shorter PFS was also observed in high-EGFR dogs but it was not significant (151 days versus 250 days; $P = 0.064$). 88.1% of 67 canine nasal carcinoma samples were tested positive for EGFR expression in our results. Only one report that published previously from Shiomitsu *et al.* was about the EGFR expression in 24 dogs with nasal carcinomas. Although the positive rate in our study was higher than that in the previous report (54.2%) performed a same IHC scoring, the mean EGFR IHC score was similar [83].

In this study, 97% of canine nasal carcinoma samples were found to be positive for VEGF staining. VEGF is produced by tumors to stimulate endothelial migration and proliferation. There was a significant correlation between increased microvessel count and VEGF expression [29, 100]. Hypoxia is a regulator of VEGF expression in NPC cells [92]. Expression of VEGF in NPC tumor biopsy samples has been associated with hypoxia markers (e.g. HIF-1α) expression, and with poor outcome post radiation therapy [35]. Overexpression of VEGF was significantly correlated with advanced clinical stage, local recurrence and distant metastasis. This also showed poor survival rate in NPC [55, 100]. A significant association was found between high-VEGF dogs and advanced-tumor dogs (T3+T4) in nasal carcinoma. When a tumor grows, an adequate blood supply is necessary. It is possible to produce VEGF to stimulate vascular formation in a high-stage tumor in canine nasal carcinomas.

In previous studies, a high percentage (71-90%) of canine nasal carcinomas have been reported to express COX-2 [6, 13, 36, 45]. Similarly, positive staining for COX-2 was expressed in 82.1% of canine nasal carcinomas in this chapter results. COX-2 is a key enzyme of prostaglandin synthesis, which has been related to apoptosis inhibition,

metastasis and angiogenesis in tumors [21, 69, 91]. An association has been observed between COX-2 expression and prognosis in NPC [17, 44, 70]. However, only few reports have documented an association between COX-2 expression and prognosis in dogs with cancers [21]. One report currently published by Belshaw *et al*. that no association was found between COX-2 expression and survival in dogs with nasal carcinomas treated with a hypofractionated radiation therapy [6]. The expression of COX-2 was not associated with the clinical variables including T stages (T3+T4 versus T1+T2), presence of cribriform plate destruction, treatment response or survivals in our results. Even though the role of COX-2 in carcinogenesis appears clear, the prognostic significance of COX-2 expression in canine cancer has not been clearly established. COX-2 expression as measured by IHC might not be indicative of the actual enzymatic activity [21].

The relationship between apoptosis and the outcome of radiation has been studied in several cancers in human. High level of apoptosis has a strong association with good outcome in patients that treated with radiation therapy [3, 77, 102]. In our study, the dogs with a good response to radiation therapy appeared to have a higher AI (P = 0.036), although longer survival was not found statistically (P = 0.079). Small biopsy sample that is taken from a large tumor is not a good representation of the whole tumor. This is due to the fact that specimen may come from areas that is highly apoptotic or low. It has also been suggested in previous studies [6, 19].

Most canine nasal carcinomas were overexpressed survivin (89.6%), EGFR (88.1%), VEGF (97%), and COX-2 (82.1%) in the results. Study of the radiation therapy efficacy combined with molecular inhibitors in veterinary medicine is needed, especially in advanced-stage tumors and those that often have a poor response to radiation therapy.

5. SUMMARY

Immunohistochemistry is simple to perform and to evaluate. It is also cheap, fast, and can be used readily on the same small biopsies that are used to evaluate canine nasal carcinomas.

A significant correlation was found between Ki-67 expression and clinical T stage in dogs with nasal carcinoma. High Ki-67 was closely correlated with aggressive tumor. Moreover, high-Ki67 dogs were observed to have shorter survival and progression-free survival after treating with radiation.

Overexpression of survivin was observed in the advanced clinical stage, poor response to radiation and shorter survival after radiation treatment. Therefore, survivin is strongly suggested a valuable prognostic factor for predicting the outcome of treatment with radiation in canine nasal carcinomas.

EGFR expression was not closely correlated with the clinical T stage or presence of cribriform plate destruction. Although EGFR trended to show a relationship with the treatment response in the 43 dogs that treated with radiation, it was not significant. However, high-EGFR dogs showed significantly shorter overall survival than low-EGFR dogs.

High-VEGF dogs were strongly associated with an advanced-stage in nasal carcinomas but they were not related with survivals. On the other hand, COX-2 expression was not associated with the clinical features, treatment response and even survivals in our results.

Survivin, EGFR, VEGF and COX-2 were detected in 60 (89.6%), 59 (88.1%), 65 (97%) and 55 (82.1%) canine nasal carcinoma samples, respectively. It is also important to search valuable biomarkers involving canine nasal carcinomas for therapeutic targets that may lead

to more effective treatment of dogs with nasal carcinoma. We would evaluate the radiosensitizing effect of molecular targeted agents in a nasal SCC cell line in the next chapter.

Part of this chapter was published as "**Fu, D.R., Kato, D., Watabe, A., Endo, Y. and Kadosawa, T. 2014. Prognostic utility of apoptosis index, Ki-67 and survivin expression in dogs with nasal carcinoma treated with orthovoltage radiation therapy. *J. Vet. Med. Sci.* 76: 1505-1512.**"

CHAPTER III.

Establishment and characterization of a canine nasal squamous cell carcinoma cell line, and radiosensitizing effect in the cell line

1. INTRODUCTION

Radiation therapy has become increasingly available and in demand in companion animals with cancers. Recently, there has been an increase in information about its effectiveness in the treatment of a number of different tumor types [59, 60]. Canine nasal tumors are considered one of tumor types usually treated with radiation therapy [1, 50, 68, 97]. Nasal squamous cell carcinoma showed a worse response rate comparing the other nasal carcinomas in Chapter I. Squamous cell carcinomas were clinically suggested a radioresistant nasal tumor type. Hence, it is important to grasp the mechanisms of radioresistance to develop more specific treatment for canine nasal tumor and improve patient prognosis.

The establishment of growing cell lines from solid tumors provides a useful tool for research on various facets of tumor cell biology [95]. Canine tumor cell lines have been used to characterize the tumor cells [5, 37, 63] and to investigate novel therapy *in vitro* and *in vivo* [38, 105]. In spite of this usefulness of cell lines, few canine squamous cell carcinoma cell line has been developed previously.

The mechanisms of cellular malignant transformation include the regulation of signal transduction, cell differentiation, apoptosis, DNA repair, cell cycle progression, and angiogenesis. Applications of molecular radiobiology in recent years have altered the understanding of tumor radioresistance and the cellular response to ionizing radiation [22, 57, 109]. Immunohistochemical expression of survivin, EGFR, VEGF and COX-2 in nasal carcinomas was investigated in Chapter II. Most canine nasal carcinoma tissues were overexpressed these molecular biomarkers. The result of Chapter II showed that

overexpression of survivin was closely associated with poor prognosis for radiation therapy. Moreover, it was also correlated with the sensitivity to radiation treatment for dogs with nasal carcinoma. Enhancing radiosensitivity of the tumor cells would be an optimal strategy for improving tumor response to radiation therapy [84]. Several biological agents designed to target the molecular progresses have shown both radiosensitizing effects and antiproliferative activities in preclinical models of human cancers [14, 30, 39,101, 105, 110].

Currently, survivin is considered to be a new target for cancer therapy. YM155, a small imidazolium-based compound, was identified a suppressant of survivin. It specifically inhibits the survivin expression of both the mRNA and protein levels and exhibits antitumor activity in preclinical models [65, 104]. Single agent of survivin inhibition has been found to induce apoptosis in tumor cells and in combination with a chemotherapeutic agent, which can enhance the chemotherapeutic effect in human cancers [40, 104]. YM155 also showed to enhance the radiosensitivity of tumor cells *in vitro* and *in vivo* studies [39, 72].

Moreover, celecoxib, a COX-2-selective inhibitor, is a non-steroidal anti-inflammatory drug that has been shown to enhance tumour radiosensitivity in human cancer cell lines, such as NPC, oral SCC and cervical cancers [17, 96, 101, 110]. Toceranib phosphate (Palladia®) is a tyrosine kinase inhibitor (TKI) targeting the VEGFR-2, PDGFR, Kit, and Flt-3. It has demonstrated encouraging single-agent antitumor activity against canine MCT. Toceranib has also been used in canine nasal tumors and frontal sinus SCCs [56, 99]. Some information indicates synergy between the related TKI sunitinib and radiation therapy in human preclinical models. Sunitinib is thought to enhance radiation-induced endothelial damage by inhibition of the PI3K/Akt signaling pathway, which then leads to apoptosis [14,

20]. Gefitinib is an EGFR inhibitor, which interrupt the signaling pathway of EGFR. Gefitinib has enhanced radiosensitivity of human lung cancer cells by inhibiting the activation of the anti-apoptotic and proliferative signal transduction pathways [80].

In Chapter III, we established a cell line of canine nasal squamous cell carcinoma and a radioresistant subclone cell line. This chapter then compared the differences between the radiosensitive and radioresistant cell line. Additionally, according to the protein expression in canine nasal carcinomas in the results of Chapter II, we also attempted to evaluate the radiosensitizing effect of molecular targeted therapeutic agents in the nasal SCC cell line.

2. MATERIALS AND METHODS

(1) Establishment of a cell line and culture

A biopsy of the primary tumor, located in the nasal cavity, was obtained from a 6-year-old neutered female miniature Schnauzer via a plastic cannula at the Oncology Service in Rakuno Gakuen University Veterinary Teaching Hospital (RGU-VTH). Fresh tissue samples from the mass of the nasal cavity were used for primary culture, and the remaining tissues were fixed with 10% neutral buffered formalin for histopathology. The neoplastic mass was diagnosed as a squamous cell carcinoma (SCC).

The tumor tissues were washed with sterile PBS and cut into 1-mm^3 fragments. The fragments were placed into a 100-mm sterile tissue culture dish (TRP) containing 10 ml of RPMI-1640 medium (Sigma) supplemented with 2 mM L-glutamine, 10% heat-inactivated fetal bovine serum (FBS) (Sigma) and antibiotics (50 IU/ml penicillin and 50 μg/ml streptomycin, Sigma). The culture dish was maintained at 37°C humidified atmosphere of 5% carbon dioxide (CO_2) in air. The primary cultured cells were observed every day for any changes.

Cells were allowed to grow to 80% confluence for subsequent passages, and sub-cultured every week. When cells reached confluent, the media was aspirated and cells were washed with PBS solution once, and 1 ml 0.25% trypsin-EDTA (Gibco) was then added to culture dishes for a few minutes. Detached cells were resuspended and 1×10^5 of cells were plated in cell culture dish with 10 ml refreshed medium and incubated at 37°C with 5% CO_2 and 95% air. The cell line, named as CNSC1, was maintained in tissue culture for 20 passages over 6 months. Experiments were carried out with the CNSC1 cells between

20-30 passages.

(2) Evaluation of tumor growth in xenotransplantation

Three 6 week-old male athymic nude mice (BALB/cAJc l-nu (nu/nu), Sankyo Lab Service Co. Sapporo) were used for the xenotransplantation. Mice were housed 3-5 per cage, exposed to 12-hour light dark cycles, and given free access to sterilized pelleted food (FR-1, Funabasi Farm Co.) and sterilized water.

Before transplantaion, the mice were irradiated with a dose of 4 Gy of X-rays using an orthovoltage X-ray machine (TITAN-450S, GE) at 450 KV/10 mA 60 cm X-ray source-cells distance at an exposure rate of 1.96 Gy/min with a filter of 1.0 mm of Al, 0.3 mm of Cu and 0.5 mm of Sn. After 7 days of irradiation, CNSC1 cells (2×10^6 cells resuspended in 0.3 ml of medium) were injected subcutaneously into the right hind leg region of the mice. The size of mass was measured by a caliper and recorded every 5 to 7 days. Tumor volume was calculated from measurement of tumor length (L) and width (W) according to the formula of $0.5\times L\times W^2$. After 8 weeks of transplantation, the mice were killed humanely, and the masses were removed and fixed in 10% neutral buffered formalin for histology. The protocol of experimental animal study was approved by, and in accordance with, the institutional guidelines established by Experimental Animal Ethics Committee, Rakuno Gakuen University.

(3) Establishment of radioresistant CNSC1 subclone cell line

CNSC1 cells were seeded in 100-mm culture dish with a density of $1x10^5$ cells/dish in medium. Cell line was cultured in a 37°C humidified 5% CO_2 incubator. CNSC1 subclone

cell line was established with treating 4 rounds of sublethal ionizing irradiation (12 Gy) by use of an orthovoltage X-ray machine (TITAN-450S, GE) at 200 KV/20 mA 40 cm X-ray source-cells distance at an exposure rate of 2.87 Gy/min with a filter of 2.0 mm of Al. After treatment, the surviving cells were selected and cultured to produce the next generation of the subclone cells. Finishing four rounds of irradiation, the subclone CNSC1 cells were produced, and were defined as a radioresistant subclone cell line and named for CNSC1-IR. Experiments were performed with the CNSC1-IR cells within 10 passages after irradiation. The procedures of established radioresistant subclone cell lines were reported in some published articles [22, 109].

(4) Clonogenic survival assay

Radiosensitivity was measured by a clonogenic survival assay following exposure to irradiation. Cells were seeded in 6-well culture plates (400-2,000 cells/well) for 1 hour and were exposed to radiation doses of 0, 2, 4, 6 and 8 Gy. A 5-cm water-dense pad was placed underneath the plates to absorb X-ray backscatter. Control cells (radiation dose of 0 Gy) were transported along with the other treated cells but remained outside the radiation room during treatments. All cells were returned to the incubator, and the medium was completely changed weekly. The cells were remained in the incubator until the colonies had grown to a size that was sufficient for counting without convergence. The cells were cultured for 7 to 10 days. At the time of counting, each plate was rinsed with PBS, fixed with 70% ethanol, and stained with Wright-Giemsa stain. The surviving colonies were defined as a colony composing 50 or more cells, and the numbers of surviving colonies were recorded. For each of the 3 wells in given cell line. The experiments were performed 3 times, yielding 9

samples for each cell line at each irradiation dose.

Plating efficiency (PE) was calculated as the numbers of colonies the numbers of cells seeded for each plate. The formula for PE is as follows:

$$\mathbf{PE} = \frac{\textbf{(number of surviving colonies)}}{\textbf{(number of cells seeded onto that plate)}}$$

Survival fraction (SF) was calculated as t divided by treated sample divided by the PE of control sample. The formula for SF is as follow:

$$\mathbf{SF} = \frac{\textbf{(PE of treated sample)}}{\textbf{(PE of control sample)}}$$

(5) Cell growth analysis in response to radiation

CNSC1 and CNSC1-IR cells were seeded into a 24-well culture plate (1×10^4 cells/well). After incubation of 18 hours, the cells were treated with or without irradiation of a 6 Gy. Cells growth was monitored and counted at every 24 hour-interval. Cells were collected with trypsinization (0.25% trypsin-EDTA) and centrifugation, were then resuspended in RPMI-1640 and the numbers of survival cells were measured with trypan blue exclusion assay. Trypan blue exclusion assay was applied to distinguish survival and dead cells (dead cells stain dark blue). A cell suspension was mixed with equal volumes of trypan blue solution and 10 μl of the mixed sample was filled into a hemocytometer chamber and counted cell numbers on a ×100 field. The doubling time (DT) of CNSC1 cell line was calculated by a following formula:

$$DT = (T_2 - T_1)\ log2\ /\ (log\ N_2 - log\ N_1)$$

T_1, T_2: The time from cell seeding

N_1: The cell number at T_1

N_2: The cell number at T_2

(6) Detection of apoptotic cells

The effect of X-ray irradiation and/or drugs on the induction of apoptosis was detected with an FITC Annexin-V/Dead cell apoptosis kit (invitrogen). Cells were collected with trypsinization and centrifugation. After harvested, cells were then washed with cold PBS. The washed cells were re-centrifuged and discarded the supernatant, and then resuspended the cells in 1× annexin-binding buffer. The cell density was determined to ~1×10^6 cells/ml, and a volume of 100 μl were prepared for per assay. Five μl of FITC annexin-V solution and 1 μl of the 100 μg/ml propidium iodide (PI) were added to each 100 μl of cell suspension, then the cells were incubated at room temperature for 15 minutes. After the incubation period, additional 400 μl of 1× annexin-binding buffer were added and mixed gently. The stained cells were analyzed by flow cytometry. The population is separated into three groups. Live cells are showed both Annexin-V and PI negative, while cells that are in apoptosis are Annexin-V positive and PI negative, and cells that are in late apoptosis or already dead are showed both Annexin-V and PI positive.

(7) Immunoblot analysis

Western blot analysis was used to compare the level of survivin expression of CNSC1 and of CNSC1-IR cell lines, and assessed the effect of survivin suppressor, YM155 (Selleckchem),

on survivin protein in both cell lines that incubated with in the presence of various concentrations (10, 25, 50 and 100 nM) of YM155 for 48 hours.

Cells were washed twice with ice-cold PBS, then harvested and centrifuged at 4°C for 5 minutes at 1,200 rpm. The cells were then lysed with an extraction buffer containing 50 mM Tris-HCl, 150 mM NaCl, 5 mM EDTA, 1% Triton-X, 0.1% sodium dodecyl sulfate (SDS), 4 mM Pefabloc SC, 5 μg/ml Aprotinin, 5 μg/ml Leupeptin, 10 mM NaF, 2 mM Na_3VO_4 and Phos-stop. The protein concentration of lysates was measured with the BCA Protein Assay Reagent (Thermo).

Equal amounts of protein lysates (20 μg) were loaded into the wells of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of 15 % gel. Standard running buffer (25 mM Tris/HCl, 190 mM glycine and 0.1% SDS) was used as an electrophoresis buffer. The protein samples were separated at 500 V and 10 mA in about 2 hours and the separated proteins were then transferred to a nitrocellulose membrane with continuous buffer (40 mM Tris/HCl, 50 mM glycine, 0.04% SDS and 20% methanol) at 20 V and 500 mA for 45 minutes. The membrane was blocked for 30 minutes with 5% non-fat milk and was incubated overnight at 4°C in primary antibody. Specific antibody from a rabbit against survivin (1:3,000 dilution; Novus biological) was used as the primary antibody. After washing with PBS-Tris, horseradish-peroxidase-labeled goat anti-rabbit IgG (1:10,000 dilution; Abcam) was used as the secondary antibody.

An ECL Plus Western Blotting Detection Reagent (GE Healthcare) was applied for 5 minutes and the optical density of each protein band was captured using a CCD camera-based imager. Image analysis software was used to read the band intensity of the target proteins.

(8) Drugs preparation and cell growth inhibition assay

YM155 (Selleckchem), celecoxib (Sigma), toceranib (Pfizer) and gefitinib (Selleckchem) were dissolved in dimethyl sulfoxide (DMSO, Nacalai tesque) at a stock concentration of 10 mM and stored at -80°C. Drugs were diluted in medium just before addition to cell cultures.

Cell growth inhibition assay were performed with or without drugs. Cells were seeded into 96-well plates (1200 cells/well) and then incubated in RPMI-1640, which was described before, for 18 hours. After the medium was changed, the cells were incubated for 72 hours in the phenol red-free RPMI1640 (100 μl/well) with YM155 (3, 10, 30, 100, 300, 1,000 and 3,000 nM) or celecoxib (0.1, 0.3, 1, 3, 10, 30, 100 and 300 μM) or toceranib phosphate (0.01, 0.03, 0.1, 0.3, 1, 3, 10 and 30 μM) or gefitinib (0.03, 0.1, 0.3, 1, 3, 10, 30 and 100 μM). Cell growth activity was determined by a WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt]-based colorimetric assay with a Cell Counting Kit-8 (CCK-8, Dojin). CCK-8 solution (10 μl) was added in each well containing 100-μl of medium. The plates were incubated for further 3 hours at 37°C. The optical density (OD) was measured at a wavelength of 450 nm with a microplate reader (Ultramark^{TM}, Bio-rad Lab.).

Growth activity was expressed as a percentage of control absorbance values obtained in the absence of drug, which was calculated as $(OD_{drug} - OD_{blank}) / (OD_{vehicle} - OD_{blank}) \times 100\%$. The IC_{50} values of YM155, celecoxib, toceranib and gefitinib based on cellular growth activity were calculated. The IC_{50} value was defined as the drug concentration resulting in 50% of maximal growth inhibition as determined from the dose-response curve.

(9) Statistical analysis

The averages of at least 3 independent experiments were used in each independent study. Data were presented as means ± standard deviation and were compared between groups with unpaired Student's t-test. P values of < 0.05 were considered statistically significant.

3. RESULTS

(1) Establishment and characterization of a canine nasal squamous cell carcinoma cell line

1) Morphology of the cell line, CNSC1

Canine nasal squamous cell carcinoma cell line, CNSC1, cells show a typical epithelial cell morphology with being polygonal to cuboidal in shape, large nuclear to cytoplasmic ratio (N/C ratio) and prominent nucleoli (Figure 3-1). These cells grow in a form of colonies on the culture disk and adherence loosely to each other. The N/C ratio is approximately 1:1. The nuclei commonly often have 2 to 5 large nucleoli. Binucleate cells and "giant forms" are visible. The nuclei and cytoplasm are filled with many tiny vesicles. The mitotic activity is rare.

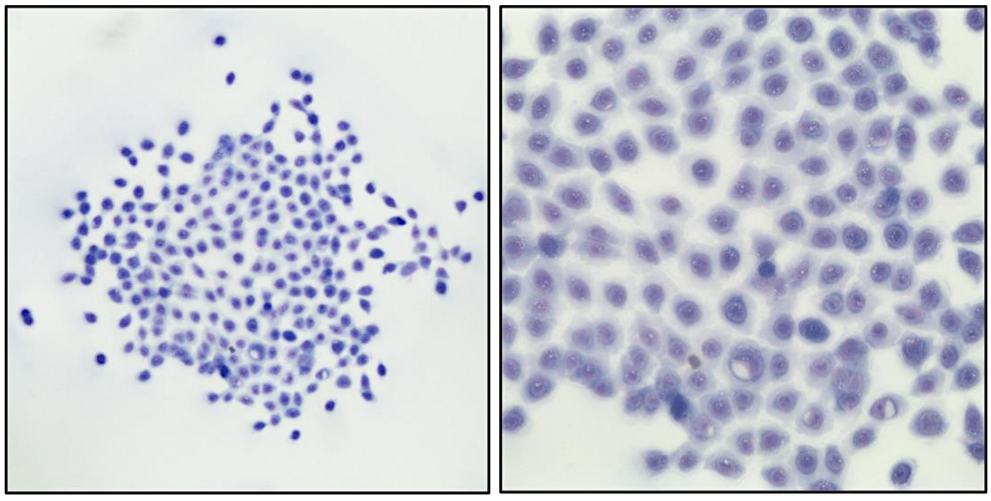


Figure 3-1. Morphology of CNSC1 cells. Cells show a typical epithelial cell morphology with being polygonal to cuboidal in shape, large nuclear to cytoplasmic ratio (N/C ratio) and prominent nucleoli. (Original magnification, Left: ×40; Right: ×200)

2) Growth of the xenotransplanted tumor

The xenotransplanted tumors grew in the right subcutis of hind leg region of nude mice. The growth curve of the xenotransplanted tumors was shown in Figure 3-2.

3) Histological evaluation of the xenotransplanted tumor

Histologically, the xenotransplanted neoplasm was presented multilobular, densely cellular, unencapsulated and poor demarcated in the subcutis. The neoplastic mass was composed of plenty numbers of lobular islands, nests and packets of neoplastic epithelial cells with variable degree of keratinization, and also, accompanied with amounts of remarkably eosinophilic lamellar keratins to form “keratin pearls”. The neoplastic cells showed in pleomorphic shapes and varied sizes, from small basaloid to large polygonal, with distinct cellular borders, abundant eosinophilic cytoplasm, central vesicular nuclei and finely stripped nucleoli. Anisocytosis and anisokaryosis were predominant. Mitotic activity was moderated that ranged from 0-2 per high power field (HPF). Aggressive invasion to muscles and local necrosis mixed by epithelial debris and keratins were present in the neoplastic mass (Figure 3-2).

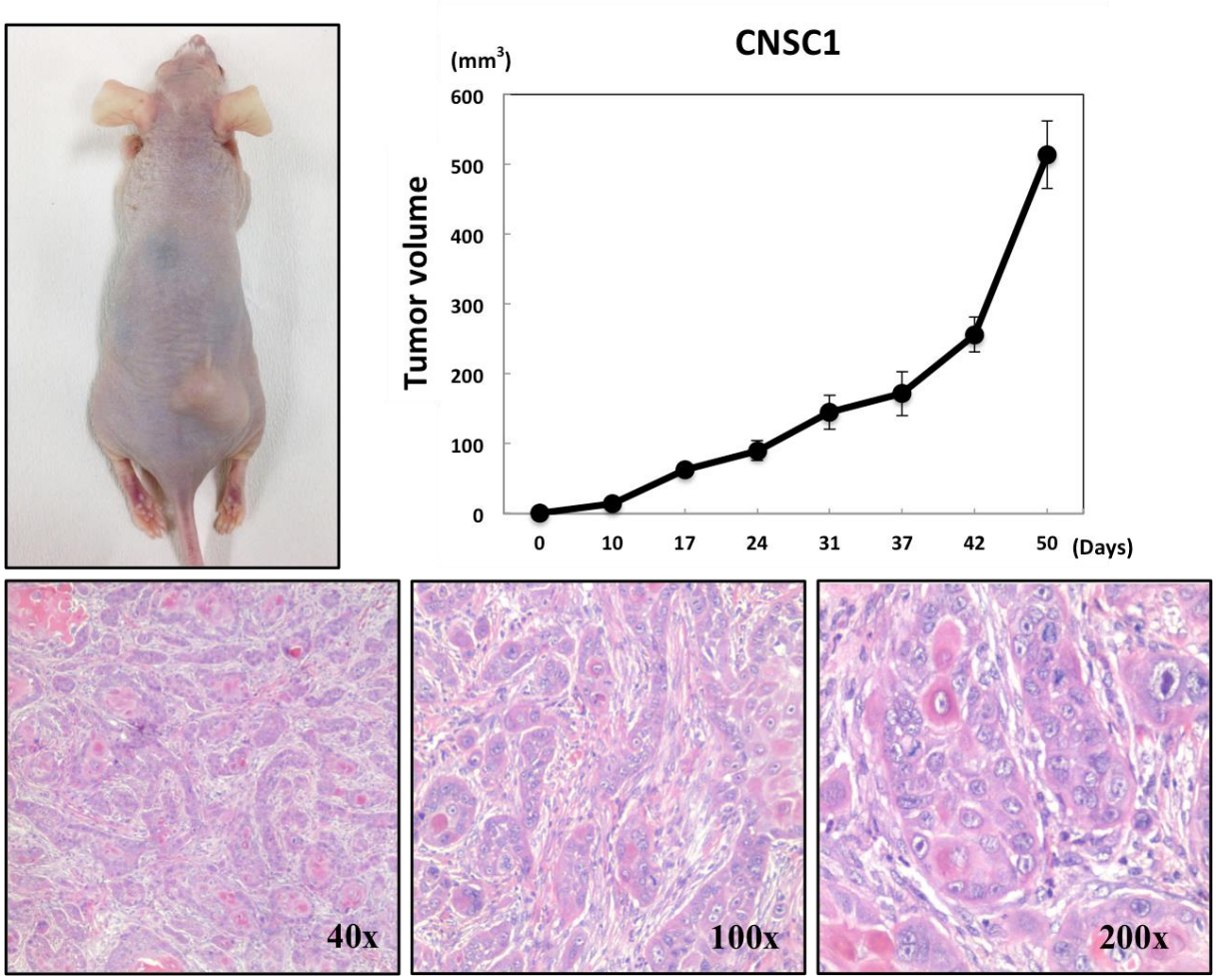


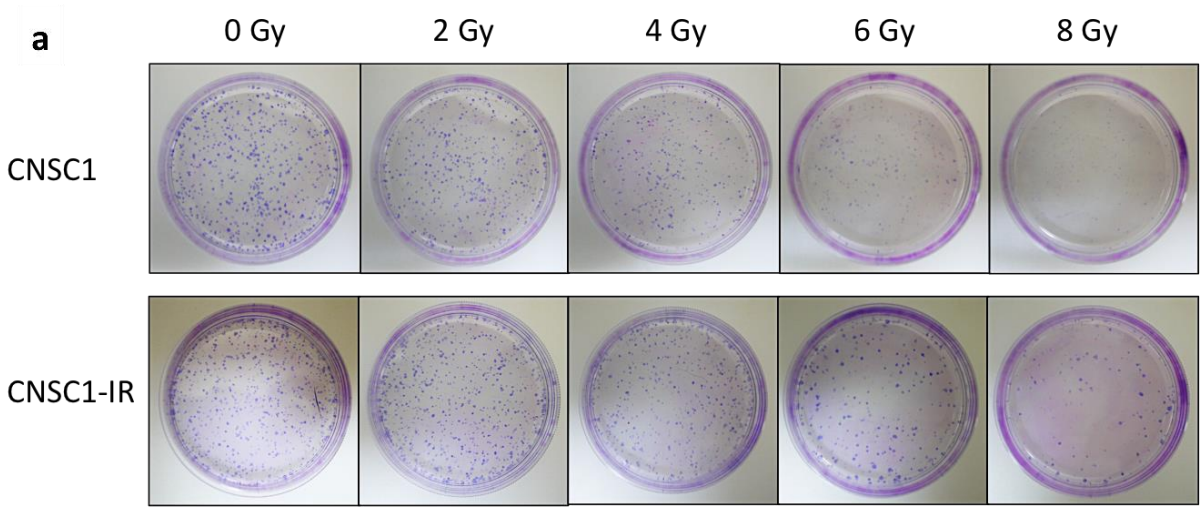
Figure 3-2. The growth curve of CNSC1 xenotransplanted tumors (top right picture) and the histological evaluation of CNSC1 xenotransplanted tumor (bottom pictures)

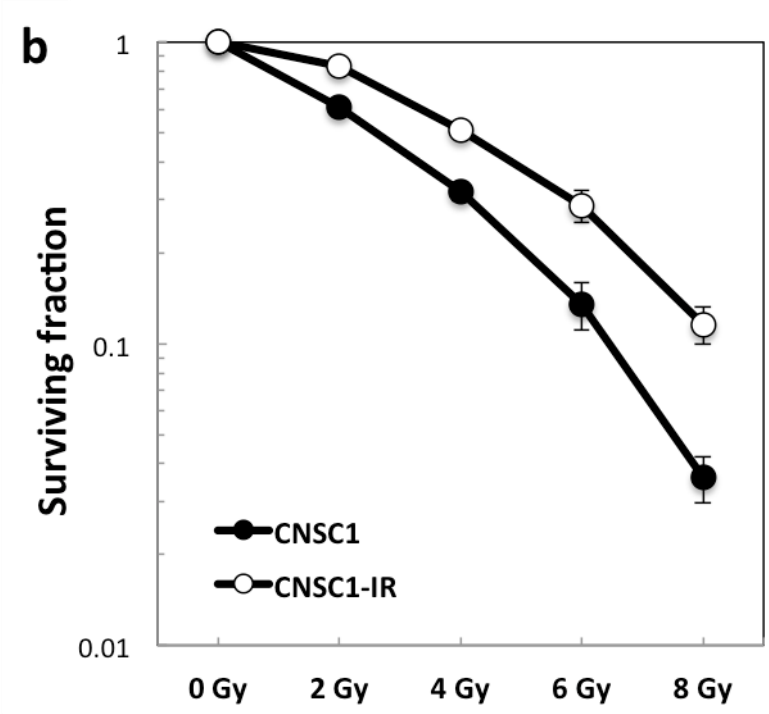
(2) Establishment of a radioresistant CNSC1 subclone cell line (CNSC1-IR)

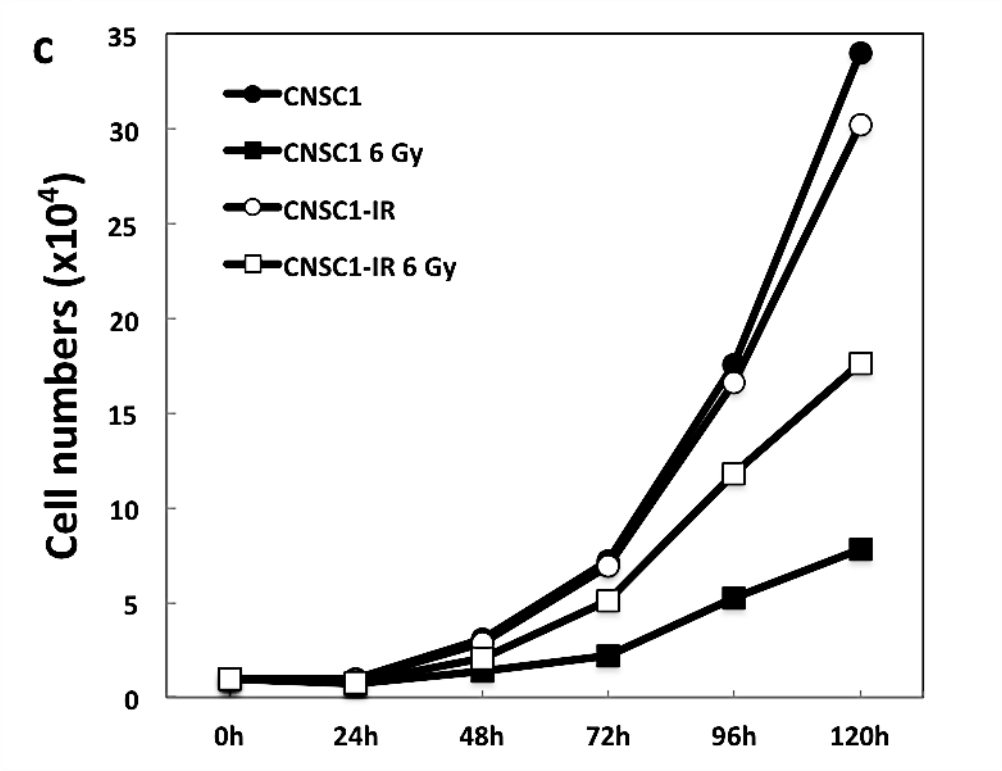
The radioresistant CNSC1 subclone cell line, CNSC1-IR and control CNSC1 cells were irradiated with a range of doses of 2 to 8 Gy, and then examined through a clonogenic survival assay. CNSC1-IR showed decreased radiosensitivity compared with CNSC1 (Figure 3-3a, b). The survival fraction in 2 Gy (SF_2) of CNSC1 and CNSC1-IR was 60% ± 4% and 83% ± 3%, respectively.

The doubling time (DT) of control CNSC1 cell line was 18.5 hours. Furthermore, CNSC1-IR and control CNSC1 were subjected to 6 Gy of irradiation to examine the effect on cell growth. As shown in Figure 3-3c, cell growth delay after 6 Gy of irradiation was less for CNSC1-IR than for control CNSC1. For fivefold increase in cell number, the delay was ~72 hours for CNSC1-IR compared with ~94 hours for control CNSC1.

The rates of apoptotic cells were detected with an FITC Annexin-V/PI apoptosis kit for flow cytometry. As shown in Figure 3-3d, CNSC1-IR showed significantly decreased apoptosis cell rate than control CNSC1 at 24 hours, 48 hours and 72 hours post irradiation of 6 Gy. The above results indicated that a CNSC1-radioresistant subclone cell line (CNSC1-IR) was established.


a
0 Gy
2 Gy
4 Gy
6 Gy
8 Gy
CNSC1
CNSC1-IR


b
Surviving fraction
1
0.1
0.01
CNSC1
CNSC1-IR
0 Gy
2 Gy
4 Gy
6 Gy
8 Gy


c
Cell numbers (x10^4)
35
30
25
20
15
10
5
0
CNSC1
CNSC1 6 Gy
CNSC1-IR
CNSC1-IR 6 Gy
0h
24h
48h
72h
96h
120h

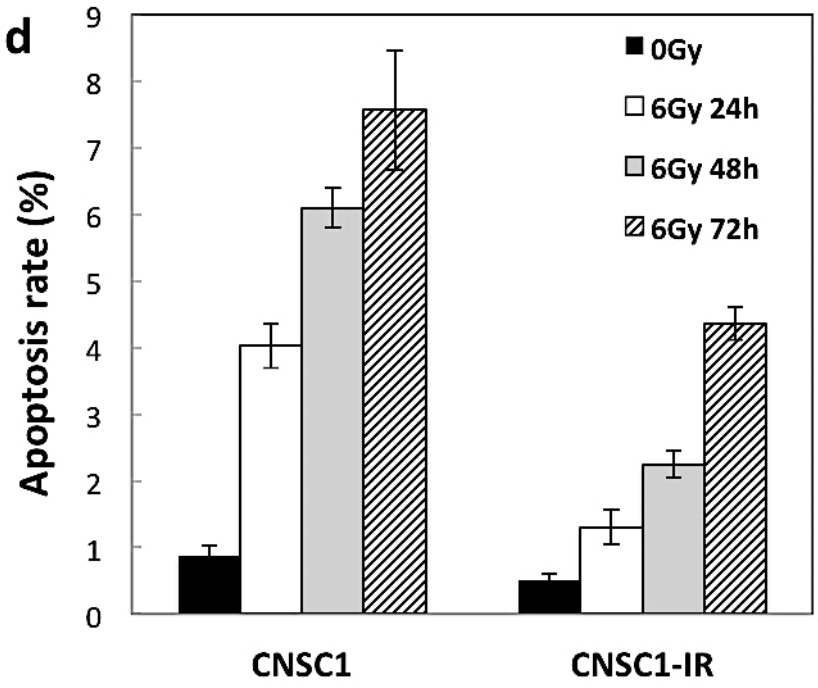

d
Apoptosis rate (%)
9
8
7
6
5
4
3
2
1
0
0Gy
6Gy 24h
6Gy 48h
6Gy 72h
CNSC1
CNSC1-IR

Figure 3-3. Comparison of radiosensitivity of CNSC1 and CNSC1-IR cell line. **(a, b)** Clonogenic survival assay. CNSC1 and CNSC1-IR were irradiated with 2-8 Gy of radiation dose, then were counted the colonies after 7-10 days and calculated the survival fractions. The SF_2 of CNSC1 was 60% ± 4% and SF_2 of CNSC1-IR was 83% ± 3%. **(c)** The proliferation of CNSC1 and CNSC1-IR cell lines with/without irradiation of 6 Gy. Cell numbers were counted at different times. Cell growth delay after irradiation was less for CNSC1-IR than for CNSC1 cell line. **(d)** The apoptotic rates were detected with an FITC Annexin-V/PI apoptosis kit. CNSC1-IR showed significantly decreased apoptotic cell rate than CNSC1 at different times after irradiation (6 Gy).

(3) Level of survivin protein expression and radiosensitizing effect of YM155 in CNSC1 and CNSC1-IR cells

1) Expression of survivin in cell lines

We detected the levels of survivin protein in CNSC1 and CNSC1-IR cells via immunoblot analysis (Figure 3-4a). Survivin expression was examined higher in the radioresistant cell line, CNSC1-IR than in control CNSC1 cells.

2) Inhibition of survivin expression in CNSC1 and CNSC1-IR cells by YM155

We examined the effect of YM155 on survivin expression in CNSC1 and CNSC1-IR cell lines by immunoblot analysis. CNSC1 and CNSC1-IR cells were incubated in the absence (control, 0.1% DMSO) or presence of various concentrations (10, 25, 50, 100 nM) of YM155 for 48 hours. Cell lysates were then prepared and subjected to immunblot analysis with an antibody to survivin. Inhibition of survivin protein level showed with a concentration-dependent manner (Figure 3-4b).

3) Effect of YM155 on radiation-induced apoptosis in CNSC1 and CNSC1-IR cells

We next examined the effect of YM155 on radiation-induced apoptosis in CNSC1 and CNSC1-IR cells with the use of the FITC Annexin-V/PI apoptosis assay for flow cytometry. Combined treatment of both cell lines with YM155 and radiation (6 Gy) resulted in an increase in the number of apoptotic cells after a 48 hour-incubation, which was greater than the sum of the increase induced by YM155 or radiation alone (Figure 3-5).

4) Radiosensitizing effect of YM155 in CNSC1 and CNSC1-IR cells

We examined the radiosenistizing effect of YM155 in CNSC1 and CNSC1-IR cell lines by clonogenic assay. Cells were incubated with YM155 (50 nM) or a vehicle (control, 0.1% DMSO) for 48 hours, exposed to the radiation doses of 2 to 8 Gy, and then incubated in drug-free medium for 7 to 10 days for determination of colony-forming ability (Figure 3-6). The SF_2 of CNSC1 treated with and without YM155 was 52% ± 5% and 60% ± 4%, respectively. And, the SF_2 of CNSC1-IR treated with and without YM155 was 62% ± 2% and 83% ± 3%, respectively. The survival fractions for irradiation decreased in both CNSC1 and CNSC1-IR cells that treated with YM155.

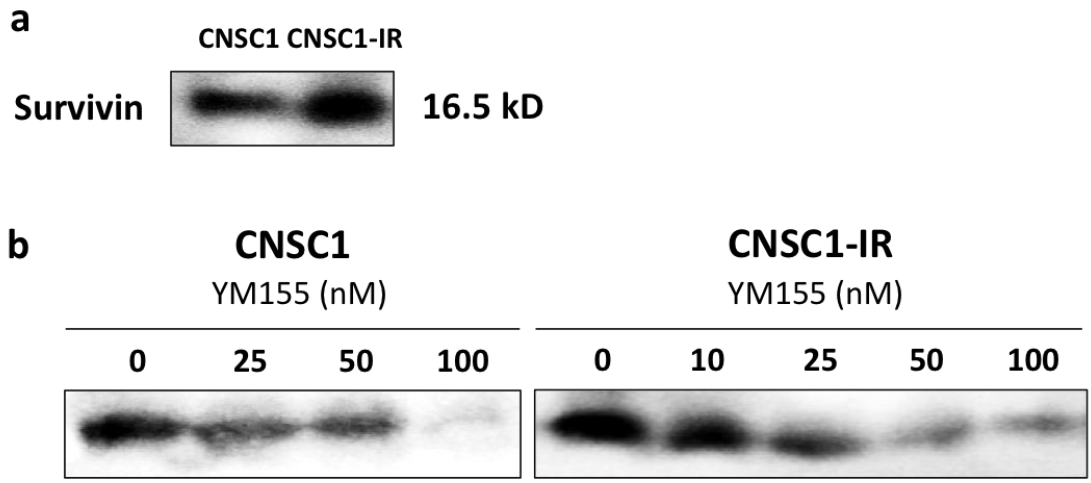


Figure 3-4. (a) Expression of survivin in CNSC1 and CNSC1-IR cells. Survivin expression was higher in the radioresistant cell line, CNSC1-IR than in the control CNSC1 cells. **(b)** Effect of YM155 on survivin expression in CNSC1 and CNSC1-IR cells. CNSC1 and CNSC1-IR cells were treated with a various concentration of YM155 for 48 hours.

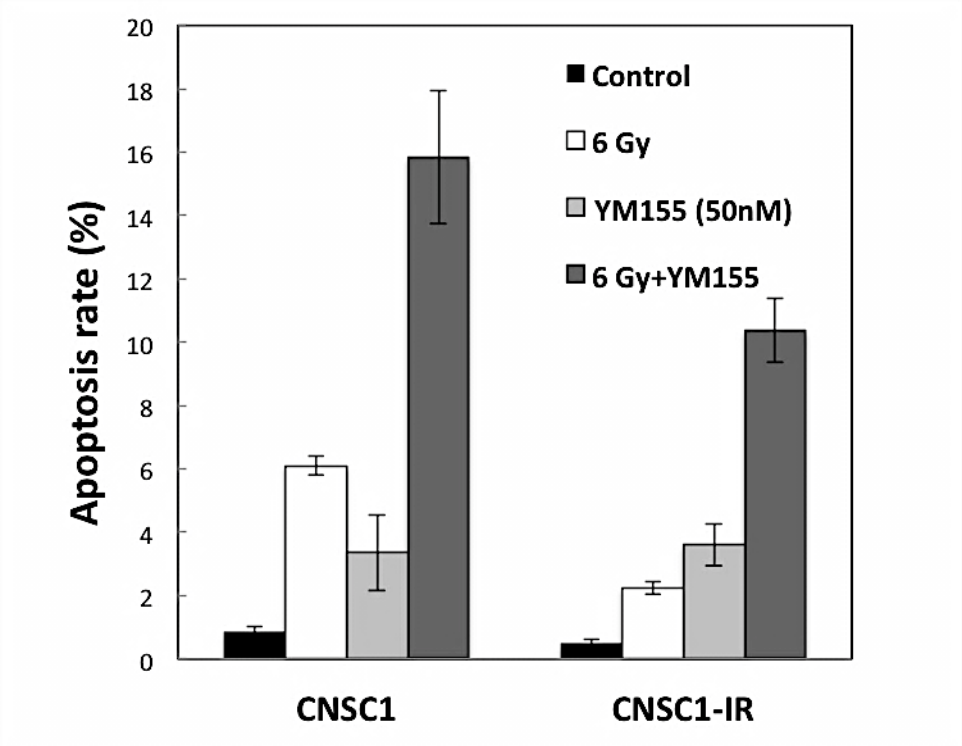


Figure 3-5. Apoptotic rate of CNSC1 and CNSC1-IR cells treated with an irradiation of 6 Gy or YM155 (50 nM) alone or a combined treatment for 48 hours. Apoptosis cells were detected with an FITC Annexin-V/PI apoptosis assay for flow cytometry. Combined treatment of either cell line with YM155 and radiation occurred in an increase in the number of apoptotic cells after a 48 hour-incubation, which was greater than the sum of the increase induced by YM155 or radiation alone.

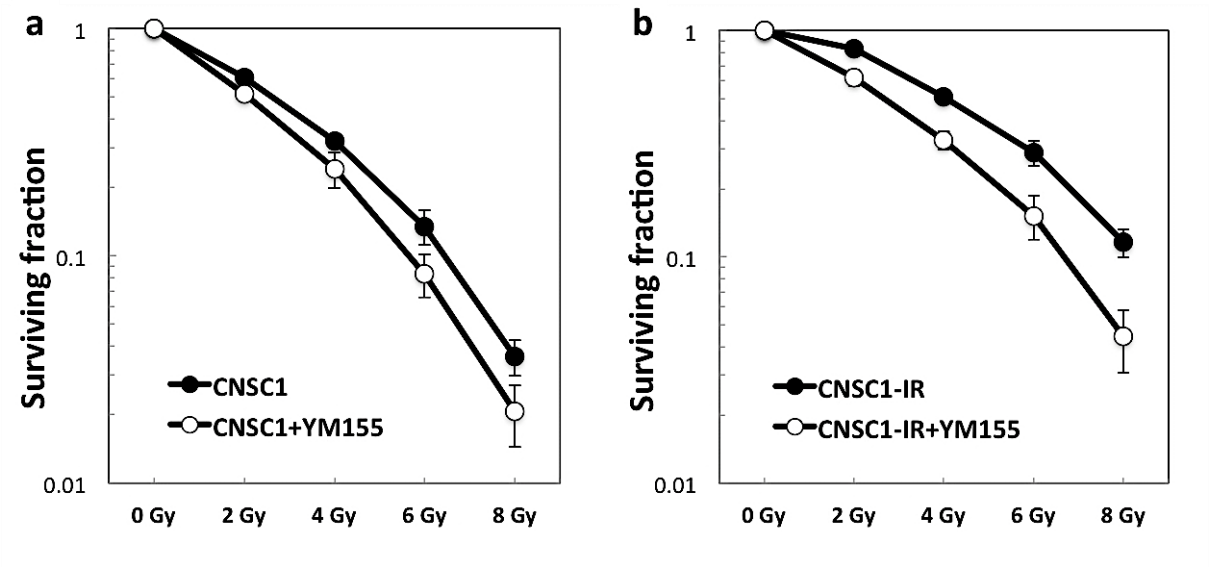


Figure 3-6. Clonogenic survival assay of cell lines that treated with/without YM155. Cells were incubated with YM155 (50 nM) or a vehicle for 48 hours, exposed to the indicated doses of radiation, and then incubated in drug-free medium for 7 to 10 days. **(a)** CNSC1 treated with YM155 decreased SF_2 from 60% ± 4% to 52% ± 5%. **(b)** CNSC1-IR treated with YM155 decreased SF_2 from 83% ± 3% to 62% ± 2%.

(4) Radiosensitizing effect of other molecular targeted agents in the radioresistant canine nasal squamous cell carcinoma cell line (CNSC1-IR)

1) Cell growth inhibition assay of each molecular targeted agent

Cell growth inhibition assay were performed with YM155, celecoxib, toceranib and gefitinib in CNSC1 and CNSC1-IR cells. Cell growth activities were determined by a WST-8 colorimetric assay. The cell growth inhibition curves were shown in Figure 3-7. For each drugs, the IC_{50} value was defined as the drug concentration resulting in 50% of maximal growth inhibition as determined from the dose-response curve. The IC_{50} value of YM155 in CNSC1 and CNSC1-IR was 90.2 nM and 42 nM, respectively (Figure 3-7a). The IC_{50} value of celecoxib in CNSC1 and CNSC1-IR was 48.3 μM and 49.2 μM, respectively (Figure 3-7b). The IC_{50} value of toceranib in CNSC1 and CNSC1-IR was 4.1 μM and 5.4 μM, respectively (Figure 3-7c). Additionally, the IC_{50} value of gefitinib in CNSC1 and CNSC1-IR was 8.3 μM and 6.1 μM, respectively (Figure 3-7d).

2) Effect of the molecular targeted agents on radiation-induced apoptosis in CNSC1-IR cells

We also examined the effect of celecoxib (50 μM), toceranib (1 μM) and gefitinib (5 μM) on radiation-induced apoptosis in the radioresistant cell line, CNSC1-IR, with the use of an FITC Annexin-V/PI apoptosis assay for flow cytometry. Combined treatment with each molecular targeted agent and radiation (6 Gy) also resulted in an increase in the number of apoptotic cells after a 48 hour-incubation. However, the combined treatment of YM155 and radiation occurred the greatest numbers of radiation-induced apoptosis cells than of other

agents and radiation (Figure 3-8).

3) Radiosensitizing effect of each molecular targeted agent in CNSC1-IR cells

We also examined the radiosenistizing effect of celecoxib, toceranib and gefitinib in CNSC1-IR cell line by clonogenic survival assay. Cells were incubate with celecoxib (50 μM), toceranib (1 μM) and gefitinib (5 μM) or a vehicle (control, 0.1% DMSO) for 48 hours, exposed to the radiation doses of 2 to 8 Gy, and then incubated in drug-free medium for 7 to 10 days for determination of colony-forming ability (Figure 3-9). The CNSC1-IR cells that treated with celecoxib decreased the SF_2 of 83% ± 3% to 68% ± 4%. Toceranib and gefitinib also decreased the SF_2 of CNSC1-IR, but they were not significant. YM155 showed the greatest effect of radiosensitizing among these agents for CNSC1-IR cells.

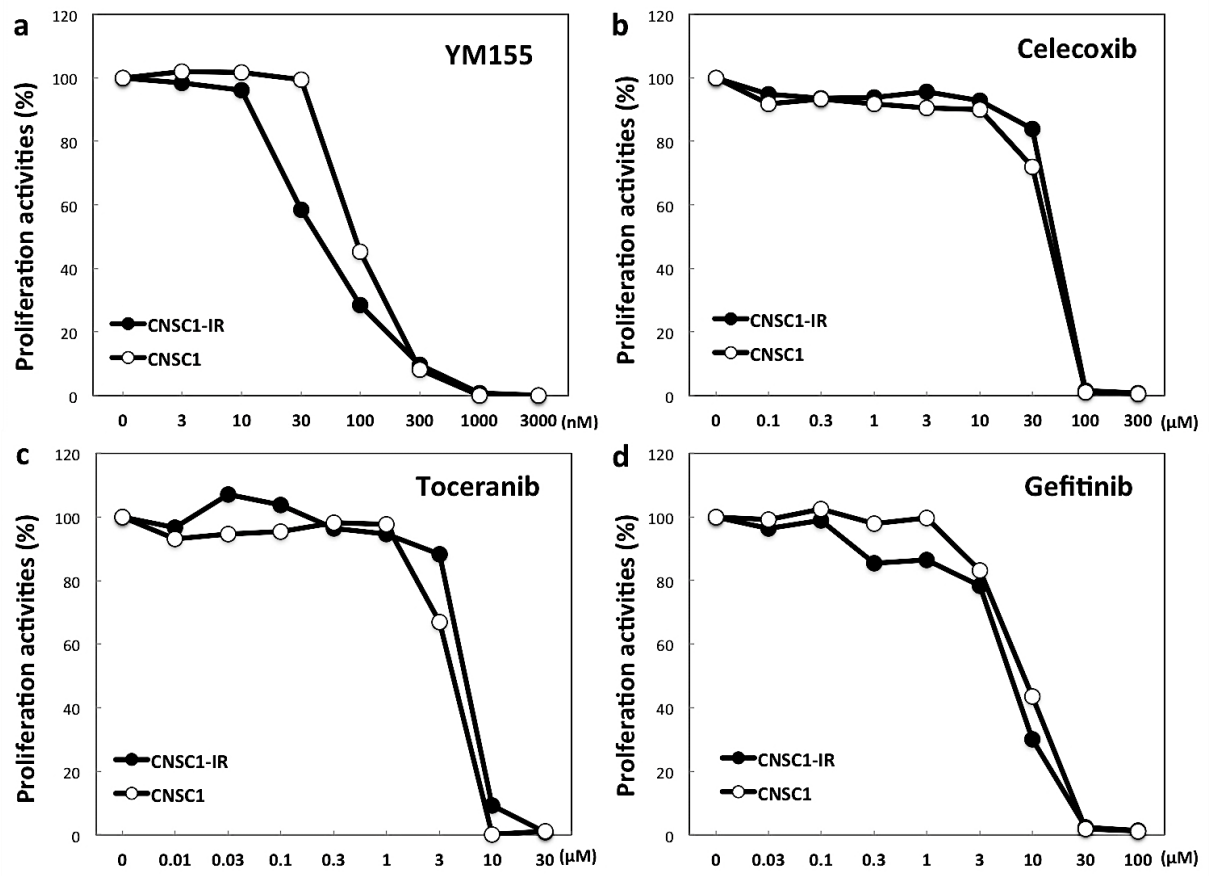


Figure 3-7. Cell growth inhibition assay of YM155, celecoxib, toceranib and gefitinib in CNSC1 and CNSC1-IR cells. Cell growth activities were determined by a WST-8 colorimetric assay. **(a)** YM155, the IC_{50} value of CNSC1 was 90.2 nM; CNSC1-IR was 42 nM. **(b)** Celecoxib, the IC_{50} value of CNSC1 was 48.3 μM; CNSC1-IR was 49.2 μM. **(c)** Toceranib, the IC_{50} value of CNSC1 was 4.1 μM; CNSC1-IR was 5.4 μM. **(d)** Gefitinib, the IC_{50} value of CNSC1 was 8.3 μM; CNSC1-IR was 6.1 μM.

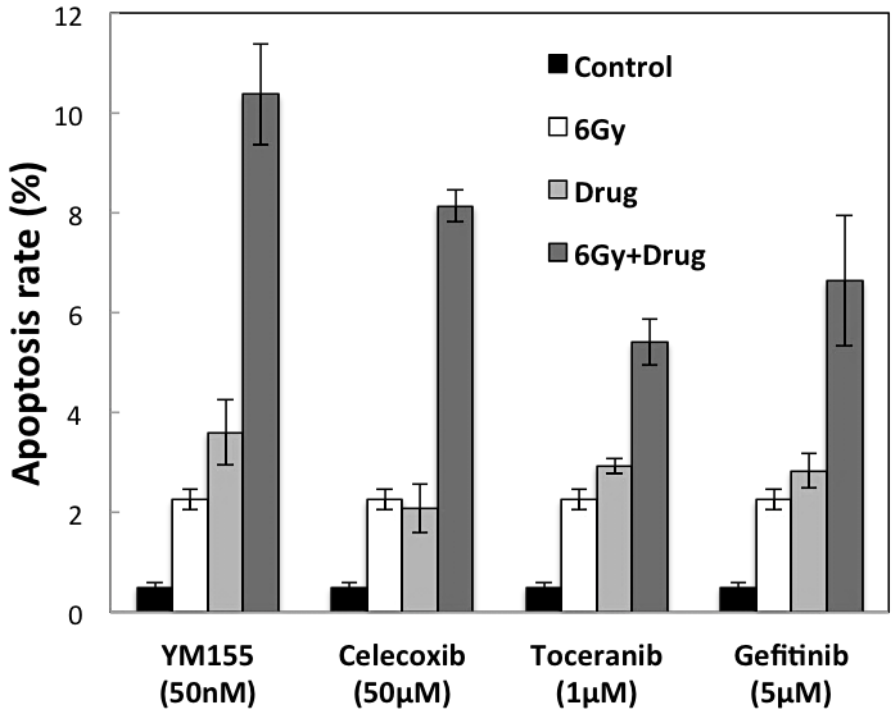


Figure 3-8. Comparison of apoptotic rate of CNSC1-IR cells treated with an irradiation of 6 Gy or molecular targeted agent alone or a combined treatment. Cells were incubated for 48 hours. Apoptosis cells were detected with an FITC Annexin-V/PI apoptosis assay for flow cytometry. Combined treatment with each molecular targeted agent and radiation (6 Gy) resulted in an increase in the number of apoptotic cells. The combined treatment of YM155 and radiation occurred the greatest increased folds of radiation-induced apoptosis cells than of other agents and radiation.

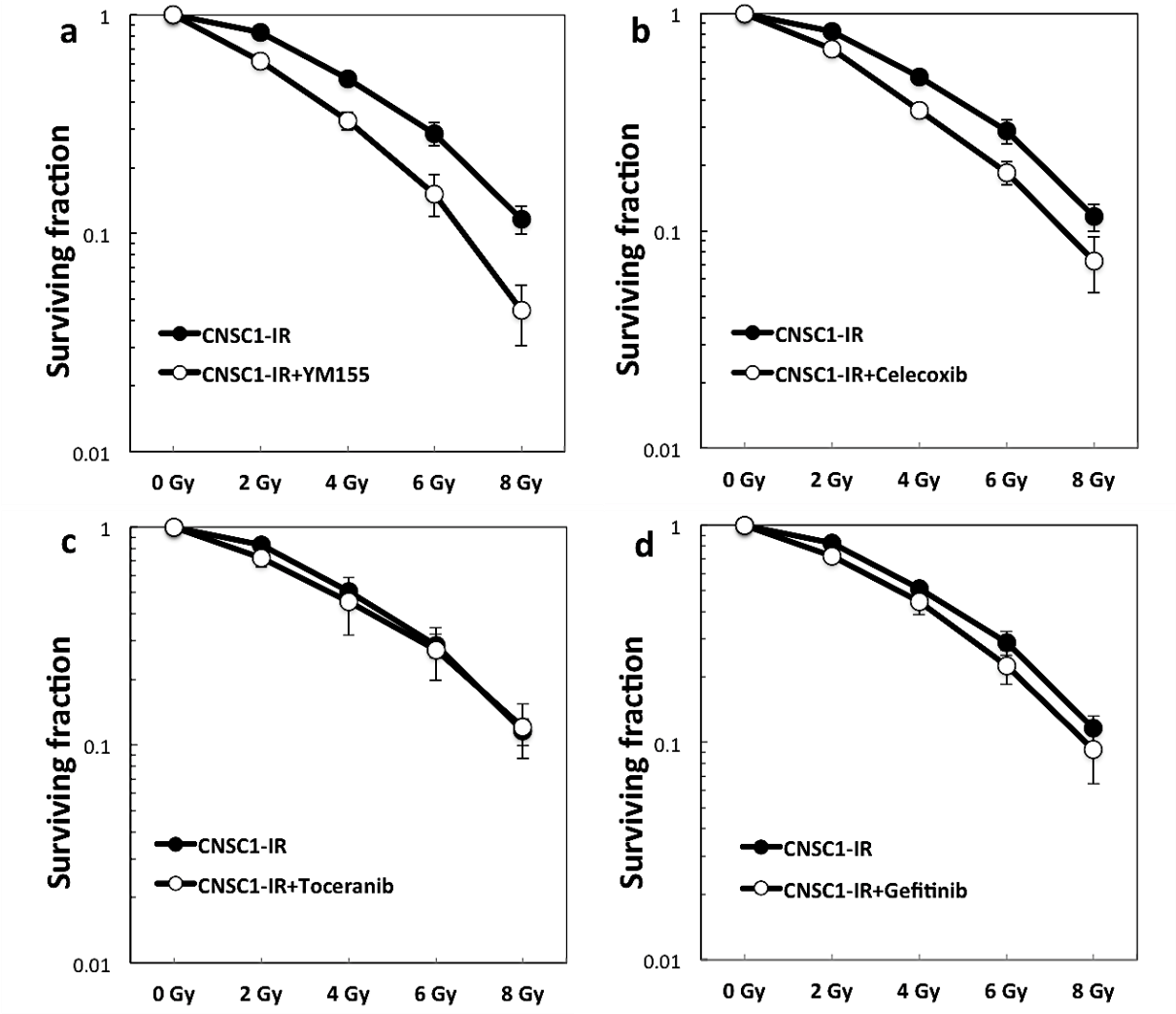


Figure 3-9. Comparison of radiosensitizing effect of YM155, celecoxib, toceranib and gefitinib in CNSC1-IR cells by clonogenic survival assay. Cells were incubated with **(a)** YM155 (50 nM), **(b)** Celecoxib (50 μM), **(c)** Toceranib (1 μM) and **(d)** Gifitinib (5 μM) for 48 hours, exposed to the indicated doses of radiation, and then incubated in drug-free medium for 7 to 10 days.

4. DISCUSSION

The establishment of growing cell lines from solid tumors provides a useful tool for research on various facets of tumor cell biology. Human cancer cell lines have been established for researching frequently [95]. It is important to that using spontaneously occurring canine tumors as translational models of diseases necessitates corresponding tumor cell lines [89]. Canine tumor cell lines have been used to characterize the tumor cells [5, 37, 63] and to study novel therapy *in vitro* and *in vivo* [38, 105]. Squamous cell carcinoma (SCC) is one of common type of tumor in canine malignancies. We found the prognostic factors and radiosensitivity of nasal tumor in each type of nasal carcinomas in Chapter I. Nasal SCC showed a worse response rate and prognosis comparing other types of nasal carcinoma. The establishment of CNSC1 as canine nasal SCC cell line provides one tool for understanding the mechanism of malignancy and of radioresistance of SCC in veterinary research. CNSC1 cell line has been sub-cultured over 70 passages.

Ionizing radiation may cause damage of DNA in cells. They undergo cell death when the DNA damage is irreparable [28, 62]. Radiation-induced cell death is classified into “reproductive death” and “interphase death”. Reproductive death is the loss of the proliferative ability of the cell. Interphase cell death is the death of irradiated cells before they enter in mitosis. It commonly causes cell death through apoptosis [62]. Clonogenic survival assay determines the ability of a cell to proliferate, thereby retaining its reproductive ability to form a colony. FITC Annexin-V staining is commonly used to detect the loss of membrane integrity if the cells undergo death resulting from either apoptotic or necrotic processes. Clonogenic survival assay and FITC Annexin-V staining were

performed to detect cellular radiosensitivity in this chapter.

A radioresistant subclonal cell line (CNSC1-IR) was established for comparing the character with the control cell line (CNSC1). Radiation may cause apoptosis for killing cancer cells [78, 87, 102]. A close correlation between the rate of spontaneous apoptosis and of radiation-induced apoptosis has been proved, and the spontaneous apoptotic rate of untreated tumor cell line also correlated with the radiosensitivity [78]. A higher level of spontaneous apoptosis was detected in the sensitive control cell line, CNSC1, (0.93% ± 0.13%) compared with the radioresistant CNSC1-IR cells (0.5% ± 0.11%). A higher level of radiation-induced apoptosis was also observed in CNSC1 cell line at 24, 48 and 72 hours after treating with radiation. It was suggested that the apoptosis induced by irradiation was altered in the radioresistant CNSC1-IR, which was also consistent with the typical radioresistant phenotype. The different intrinsic radiosensitivities in human lung or colorectal carcinoma cells have also found higher levels of spontaneous apoptosis to show more sensitivity to radiation *in vitro* [78, 87].

Survivin, a member of the IAP family, involves in both inhibition of apoptosis and control of cell division. Its anti-apoptotic function seems to be related to its ability to inhibit caspase-related proteins directly or indirectly. However, it has become increasingly clear that the role of survivin in response to ionizing radiation far exceeds a simple inhibition of apoptotic pathway [41, 78, 72, 111]. A weak but significant negative correlation was also found between the level of survivin expression and spontaneous apoptosis rate in canine nasal carcinoma tissues ($r = -0.312$; $P = 0.028$) in Chapter II. Our *in vitro* study results could be confirmed that CNSC1-IR cells expressed a higher level of survivin protein and also showed a lower rate of spontaneous and radiation-induced apoptosis. And it was

resistant to irradiation as determined performing the clonogenic survival assay (Figure 3-3 and 3-4). These results are in line with a study on human colorectal cancer cells *in vitro*, where an inverse relationship between survivin expression and radiosensitiv was also found [78]. Therefore, survivin expression is strongly suggested to be a predictor for cellular radiosensitivity.

YM155 is a small molecule agent that specifically inhibits survivin expression in various types of tumor cell line *in vitro* [64]. Two previous studies have been reported that YM155 increased the sensitivity of human non-small cell lung cancer and esophageal squamous cell carcinoma cells to radiation *in vitro* and *in vivo* [39, 72]. Iwasa *et al.* indicated that radiosensitization of tumor cells by YM155 was associated with increased activity of caspase-3, suggesting that YM155 sensitized tumor cells to radiation partly by enhancing radiation-induced apoptosis [39]. YM155 is also able to abrogate G2 checkpoint in response to radiation. Shortening of the G2 checkpoint leads to decreased repair of radiation-induced damage before next cell division [72]. Our result revealed that YM155 markedly potentiated the decrease in canine nasal SCC cell survival induced by radiation. Our finding suggested that YM155 suppresses the level of survivin protein and then increases radiation induced-apoptosis to enhance the radiosensitivity of the canine nasal SCC cells.

In a broad study of YM155 antitumor activity in a variety human cell lines, Nakahara *et al.* have found a correlation between survivin levels and IC_{50} values. The sensitivity of cell line to YM155 was related with high level of survivin [64]. Our result also showed that the radioresistant nasal SCC cells, CNSC1-IR, with higher level of survivin protein had lower value of IC_{50} (42 nM) than the lower survivin level cells, CNSC1 (90.2 nM) did. CNSC1-IR

cells revealed more sensitivity to YM155.

Although some studies have been reported about the radiosensitizing effects exerted by COX-2 selective inhibitors on a variety of human cancer cells, the mechanisms underlying these effects have yet to be clearly understood [85, 101, 110]. Celecoxib enhanced radiosensitivity of canine nasal SCC cells and increased the apoptotic cells with a combination of radiation in this chapter. Previous studies have shown that an increase of radiosensitivity induced by celecoxib in an NPC cell line is associated with G2-M phase arrest and apoptosis induction [110]. Furthermore, celecoxib was related to the down-regulation of not only COX-2 but also VEGF in human cervical cancer cells [101]. Irradiation and COX-2 inhibition seemed to have a synergistic inhibitory effect on tumor cell proliferation and tumor angiogenesis.

Toceranib phosphate is a tyrosine kinase inhibitor (TKI) targeting the VEGFR-2, PDGFR, Kit, and Flt-3, which inhibits the PI3K/Akt signaling pathway then induces cell apoptosis [56, 99]. Toceranib did not directly sensitize cytotoxicity in CNSC1 cells (IC_{50} > 1 μM). Hence, it also did not show a radiosensitizing effect in our study. However, toceranib was originally developed as an anti-angiogenic agent as inhibition of VEGFR and PDGFR family members limit angiogenesis in murine tumor models [56]. Anti-angiogenic activity is not able to show in our *in vitro* study. Additional *in vivo* or clinical study is needed. On the other hand, EGFR inhibitor (cetuximab) plus radiotherapy is superior to radiotherapy alone in increasing both the duration of local control and survival in human head and neck cancer [12]. Gefitinib has also found to enhance radiosensitivity of human lung cancer cells by inhibiting the activation of the anti-apoptotic and proliferative signal transduction pathways [80]. However, gefitinib is only effective in cancers with mutated

and overactive EGFR. CNSC1-IR was not analyzed the mutation of EGFR, there is therefore no evidence for enhancing radiosensitivity in our study. Our result still provides a rationale for future preclinical and clinical investigation of therapeutic efficacy of molecular targeted agents in combination with radiation therapy.

5. SUMMARY

We established a nasal squamous carcinoma cell line that called CNSC1 from a dog, and also developed a radioresistant subclonal cell line (CNSC1-IR). They provide a useful tool for research on canine tumor cell radiobiology. Our *in vitro* study showed that CNSC1-IR cells expressed a higher level of survivin protein and also had a lower rate of spontaneous and radiation-induced apoptosis than the control CNSC1 cells. Therefore, survivin in our study is considered to play a role for cellular radiosensitivity.

YM155 suppressed the level of survivin protein and then increased radiation induced-apoptosis to enhance the radiosensitivity of the canine nasal SCC cells. It showed that YM155 sensitized CNSC1 and CNSC1-IR cells to radiation *in vitro*. Our results provided a rationale for future preclinical and clinical investigation of therapeutic efficacy of survivin inhibition in combination with radiation therapy.

On the other hand, COX-2 inhibitor, celecoxib, was also observed a synergy effect of combination with radiation. However, neither multi-targeted TKI (toceranib) nor EGFR inhibitor (gefitinib) showed to alter the radiosensitivity of CNSC1-IR cells *in vitro*.

CONCLUSION

Radiation therapy has become widely available and high in demand in companion animals with cancers. Recently, there has been an increase in information on its effectiveness in the treatment of a number of different tumor types. Canine nasal tumors are considered one of the most tumor types frequently treated with radiation therapy. Nasal tumors are uncommon but they are nearly all malignant. Canine nasal tumors of carcinomas are more common than those of sarcomas. Clinical and immunohistochemical prognostic factors were evaluated in dogs with nasal carcinoma. Moreover, the mechanisms of radioresistance and radiosensitizing effect were also analyzed for a nasal SCC cell line in this thesis.

In Chapter I, we attempted to describe the outcomes for dogs with nasal carcinoma based on clinical variables. We then compared the survivals for dogs that were treated with such radiation therapy in RGU. The median survival time for all patients with nasal carcinoma treated with radiation therapy in our study was approximately 8 months (241 days), whereas the dogs that did not receive treatment only survived 3.1 months (95 days). Dogs that were diagnosed with SCC had a worse response rate (20%) to radiation compare other carcinoma subtypes. The dogs with SCC also found to have a shorter survival times (178 days) in our study. Additional negative prognostic factors for progression-free survivals (PFS) or overall survival times were observed in this chapter. In clinical signs and CT findings, presence of nasal discharge, facial deformity, exophthalmos at initial presentation, orbital or subcutaneous involvement and cribriform plate destruction were significantly related with PFSs. Dogs with metastasis had significantly shorter PFS than

those without metastatic disease. Dogs with a partial response were closely correlated with a longer PFS than those with a stable disease. Furthermore, dogs without nasal discharge or with exophthalmos, neurological abnormalities, orbital involvement and cribriform plate destruction were also highly associated with shorter overall survival times. Additionally, dogs with an age less than 11 years had longer survival than dogs with an age greater than 11 years. Presence of metastasis and RT response also had significant correlations with the survival times.

In Chapter II, we evaluated the expression of tumor markers (Ki-67, survivin, EGFR, VEGF and COX-2) in canine nasal carcinoma through an immunohistochemical staining. Survivin (89.6%), EGFR (88.1%), VEGF (97%), and COX-2 (82.1%) were expressed in canine nasal carcinoma samples that were biopsied before treatment. Overexpression of survivin was observed in the dogs with a late clinical stage and worse response to radiation. It also yielded shorter survival times after radiation therapy. Therefore, survivin in our study is strongly suggested a valuable prognostic factor for predicting the outcome of canine nasal carcinomas with radiation treatment. On the other hand, although EGFR seemed to show a correlation with the treatment response in the dogs that treated with radiation, it was insignificant. However, high-EGFR dogs showed significantly shorter overall survival than low-EGFR dogs. High level of VEGF was strongly associated with an advanced-stage in nasal carcinoma but it was not related with survival. COX-2 expression was not associated with clinical features, treatment response and even survivals in our results. Additionally, high level of Ki-67 was closely correlated with an aggressive tumor. Therefore, high-Ki67 dogs were observed to have shorter survivals after treating with radiation.

In Chapter III, we established a cell line of canine nasal squamous cell carcinoma

(CNSC1) and a radioresistant subclone cell line (CNSC1-IR). In this *in vitro* study, CNSC1-IR cells showed a higher level of survivin protein and a lower rate of spontaneous and radiation-induced apoptosis than the control CNSC1 cells. Therefore, survivin expression is considered to play a role for cellular radiosensitivity. YM155 suppressed the level of survivin protein and then increased radiation induced-apoptosis to enhance the radiosensitivity of the canine nasal SCC cells. It showed that YM155 sensitized CNSC1 and CNSC1-IR cells to radiation *in vitro*. Our results provided a rationale for future preclinical and clinical investigation of therapeutic efficacy of survivin inhibition in combination with radiation therapy. On the other hand, COX-2 inhibitor, celecoxib, also found a synergy effect of combination with radiation. However, neither multi-targeted TKI (toceranib) nor EGFR inhibitor (gefitinib) was shown to alter the radiosensitivity of CNSC1-IR cells *in vitro*.

Based from the results described above, survivin is considered a strong prognostic significance in dogs with nasal carcinoma. However, additional study of the efficacy of radiation therapy combined with survivin inhibition for improving patient prognosis is necessary, especially in advanced-stage tumors and those that often have a poor response to radiation therapy. This thesis also provided a rationale for future preclinical and clinical investigation of therapeutic efficacy of molecular targeted therapeutic agents in a combination with radiation therapy.

ACKNOWLEDGEMENTS

First of all, I would like to express my sincere appreciation to Prof. Tsuyoshi Kadosawa, my supervisor, at the Laboratory of Veterinary Clinical Oncology, School of Veterinary Medicine, Rakuno Gakuen University for offering me the opportunity to study and take training in his laboratory in Japan. I am also very grateful to Dr. Yoshifumi Endo for his fruitfully advice with my projects. I have benefited a great deal from their extraordinary advices and suggestions. I cannot wait to continue my clinical practice and to become specialized in the fields of radiation oncology and surgical oncology in my upcoming journey.

I would like to take this opportunity to sincerely thank the reviewers, Prof. Hiroyuki Taniyama (Department of Veterinary Pathology), Prof. Masanobu Hayashi (Department of Veterinary Basic Radiology) and Prof. Tsuyoshi Uchide (Department of Veterinary Internal Medicine), for their professional suggestions and assistance with my academic writing.

My thanks go to Dr. Kazuko Hirayama, (Department of Veterinary Pathology, Rakuno Gakuen University) and Dr. Yumiko Kagawa (North Lab, Sapporo) for their co-operation in histopathology; and to Prof. Katsuro Hagiwara (Department of Veterinary Virology) and Associate Prof. Hidetomo Iwano (Department of Veterinary Biochemistry) for their co-operation in performing experiment and enhancing the quality of the work.

I also sincerely thank Rakuno Ikueikai (酪農育英会) for financial support during my doctoral study. Last but not the least, a big thank to my family who shared my worries, frustrations and supported me throughout my study. I cannot wait to share the ultimate moment with them upon the completion of my PhD degree.

REFERENCES

1. Adams, W.M., Kleiter, M.M., Thrall, D.E., Klauer, J.M., Forrest, L.J., La, Due T.A. and Havighurst, T.C. 2009. Prognostic significance of tumor histology and computed tomographic staging for radiation treatment response of canine nasal tumors. *Vet. Radiol. Ultrasound* **50**: 330-335.
2. Adams, W.M., Withrow, S.J., Walshaw, R., Turrell, J.M., Evans, S.M., Walker, M.A. and Kurzman, I.D. 1987. Radiotherapy of malignant nasal tumors in 67 dogs. *J. Am. Vet. Med. Assoc.* **191**: 311-315.
3. Adell, G.C., Zhang, H., Evertsson, S., Sun, X.F., Stål, O.H. and Nordenskjöld, B.A. 2009. Apoptosis in rectal carcinoma: prognosis and recurrence after preoperative radiotherapy. *Cancer* **91**: 1870-1875.
4. Al-Dissi, A.N., Haines, D.M., Singh, B. and Kidney, B.A. 2009. Immunohistochemical expression of vascular endothelial growth factor and vascular endothelial growth factor receptor in canine cutaneous fibrosarcomas. *J. Comp. Pathol.* **141**: 229-236.
5. Azakami, D., Bonkobara, M., Washizu, T., Iida, A., Kondo, M., Kato, R., Niikura, Y., Iwaki, S., Tamahara, S., Matsuki, N. and Ono, K. 2006. Establishment and biological characterization of canine histiocytic sarcoma cell lines. *J. Vet. Med. Sci.* **68**: 1343-1346.
6. Belshaw, Z., Constantio-Casas, F., Brearley, M.J., Dunning, M.D., Holmes, M.A. and Dobson, J.M. 2011. COX-2 expression and outcome in canine nasal carcinomas treated with hypofractionated radiotherapy. *Vet. Comp. Oncol.* **9**: 141-148.
7. Bergkvist, G.T., Argyle, D.J., Morrison, L., MacIntyre, N., Hayes, A. and Yool, D.A. 2011. Expression of epidermal growth factor receptor (EGFR) and Ki67 in feline oral

squamous cell carcinomas (FOSCC). *Vet. Comp. Oncol.* **9**: 106-117.

8. Bongiovanni, L., Colombi, I., Fortunato, C. and Della Salda, L. 2009. Survivin expression in canine epidermis and in canine and human cutaneous squamous cell carcinomas. *Vet. Dermatol.* **20**: 369-376.
9. Bongiovanni, L., D'Andrea, A., Romanucci, M., Malatesta, D., Candolini, M., Salda, L.D., Mechelli, L., Sforna, M. and Brachelente, C. 2013. Epithelial-to-mesenchymal transition: immunohistochemical investigation of related molecules in canine cutaneous epithelial tumours. *Vet. Dermatol.* **24**: 195-203.
10. Bongiovanni, L., Romanucci, M., Malatesta, D., D'Andrea, A., Ciccarelli, A. and Della Salda, L. 2014. Survivin and related proteins in canine mammary tumors: immunohistochemical expression. *Vet. Pathol.* pii: 0300985814529312.
11. Bongiovanni, L., Suter, M.M., Malatesta, D., Ordinelli, A., Ciccarelli, A., Romanucci, M., Brenner, O. and Della Salda, L. 2012. Nuclear survivin expression as a potentially useful tool for the diagnosis of canine cutaneous sebaceous lesions. *Vet. Dermatol.* **23**: 394-e73.
12. Bonner, J.A., Harari, P.M., Giralt, J., Azarnia, N., Shin, D.M., Cohen, R.B., Jones, C.U., Sur, R., Raben, D., Jassem, J., Ove, R., Kies, M.S., Baselga, J., Youssoufian, H., Amellal, N., Rowinsky, E.K. and Ang, K.K. 2006. Radiotherapy plus cetuximab for squamous cell carcinoma of the head and neck. *N. Engl. J. Med*. **354**: 567-578.
13. Borzacchiello, G., Paciello, O. and Papparella, S. 2004. Expression of cyclooxygenase-1 and -2 in canine nasal carcinomas. *J. Comp. Pathol.* **131**: 70-76.

14. Brooks, C., Sheu, T., Bridges, K., Mason, K., Kuban, D., Mathew, P. and Meyn, R. 2012. Preclinical evaluation of sunitinib, a multi-tyrosine kinase inhibitor, as a radiosensitizer for human prostate cancer. *Radiat. Oncol.* **7**: 154.

15. Buchholz, J., Hagen, R., Leo, C., Ebling, A., Roos, M., Kaser-Hotz, B. and Bley, C.R. 2009. 3D conformal radiation therapy for palliative treatment of canine nasal tumors. *Vet. Radiol. Ultrasound* **50**: 679-683.

16. Cao, X.J., Hao, J.F., Yang, X.H., Xie, P., Liu, L.P., Yao, C.P. and Xu, J. 2012. Prognostic value of expression of EGFR and nm23 for locoregionally advanced nasopharyngeal carcinoma. *Med. Oncol.* **29**: 263-271.

17. Chen, W.C., McBride, W.H., Chen, S.M., Lee, K.F., Hwang, T.Z., Jung, S.M., Shau, H., Liao, S.K., Hong, J.H. and Chen, M.F. 2005. Prediction of poor survival by cyclooxygenase-2 in patients with T4 nasopharyngeal cancer treated by radiation therapy: clinical and in vitro studies. *Head & Neck* **27**: 503-512.

18. Chua, D.T., Nicholls, J.M., Sham, J.S. and Au, G.K. 2004. Prognostic value of epidermal growth factor receptor expression in patients with advanced stage nasopharyngeal carcinoma treated with induction chemotherapy and radiotherapy. *Int. J. Radiat. Oncol. Biol. Phys.* **59**: 11-20.

19. Couture, C., Raybaud-Diogène, H., Têtu, B., Bairati, I., Murry, D., Allard, J. and Fortin, A. 2002. p53 and Ki-67 as markers of radioresistance in head and neck carcinoma. *Cancer* **94**: 713-722.

20. Cuneo, K.C., Geng, L., Fu, A., Orton, D., Hallahan, D.E. and Chakravarthy, A.B. 2008. SU11248 (sunitinib) sensitizes pancreatic cancer to the cytotoxic effects of ionizing radiation. *Int. J. Radiat. Oncol. Biol. Phys*. **71**: 873-879.

21. Doré, M. Cyclooxygenase-2 expression in animal cancers. 2011. *Vet. Pathol.* **48**: 254-265.

22. Feng, X.P., Yi, H., Li, M.Y., Li, X.H., Yi, B., Zhang, P.F., Tang, C.E., Li, J.L., Chen, Z.C. and Xiao, Z.Q. 2010. Identification of biomarkers for predicting nasopharyngeal carcinoma response to radiotherapy by proteomics. *Cancer Res*. **70**: 3450–3462.

23. Forrest, L.J. 2009. Nasal tumors. pp. 352-353. *In*: Kirk's Current Veterinary Medicine. 14th ed. (Bonagura, J.D. and Twedt, D.C. eds), Elsevier, Saunders.

24. Freudlsperger, C., Freier, K., Hoffmann, J. and Engel, M. 2012. Ki-67 expression predicts radiosensitivity in oral squamous cell carcinoma. *Int. J. Oral Maxillofac. Surg.* **41**: 965-969.

25. Gaffney, D.K., Haslam, D., Tsodikov, A., Hammond, E., Seaman, J., Holden, J., Lee, R.J., Zempolich, K. and Dodson, M. 2003. Epidermal growth factor Receptor (EGFR) and vascular endothelial growth factor (VEGF) negatively affect overall survival in carcinoma of the cervix treated with radiotherapy. *Int. J. Radiat. Oncol. Biol. Phys.* **56**: 922-928.

26. Gama, A., Gärtner, F., Alves, A. and Schmitt, F. 2009. Immunohistochemical expression of epidermal growth factor receptor (EGFR) in canine mammary tissues. *Res. Vet. Sci.* **87**: 432-437.

27. Gamblin, R.M., Sagartz, J.E. and Couto, C.G. 1997. Overexpression of p53 tumor suppressor protein in spontaneously arising neoplasms of dogs. *Am. J. Vet. Res*. **58**: 857-863.

28. Green, E.M. 2009. Radiotherapy: basic principles and indications. pp. 305-319. *In*: Kirk's Current Veterinary Medicine. 14th ed. (Bonagura, J.D. and Twedt, D.C. eds), Elsevier, Saunders.

29. Guang-Wu, H., Sunagawa, M., Jie-En, L., Shimada, S., Gang, Z., Tokeshi, Y. and Kosugi, T. 2000. The relationship between microvessel density, the expression of vascular endothelial growth factor (VEGF), and the extension of nasopharyngeal carcinoma. *Laryngoscope* **110**: 2066-2069.

30. Hainsworth, J.D., Spigel, D.R., Greco, F.A., Shipley, D.L., Peyton, J., Rubin, M., Stipanov, M. and Meluch, A. 2011. Combined modality treatment with chemotherapy, radiation therapy, bevacizumab, and erlotinib in patients with locally advanced squamous carcinoma of the head and neck: a phase II trial of the Sarah Cannon oncology research consortium. *Cancer J.* **17**: 267-272.

31. Henry, C.J., Brewer, W.G. Jr, Tyler, J.W., Brawner, W.R., Henderson, R.A., Hankes, G.H. and Royer, N. 1998. Survival in dogs with nasal adenocarcinoma: 64 cases (1981-1995). *J. Vet. Intern. Med.* **12**: 436-439.

32. Hicks, D.G. and Fidel, J.L. 2006. Intranasal malignant melanoma in a dog. *J. Am. Anim. Hosp. Assoc.* **42**: 472-476.

33. Holmes, K., Roberts, O.L., Thomas, A.M. and Cross, M.J. 2007. Vascular endothelial growth factor receptor-2: Structure, function, intracellular signaling and therapeutic inhibition. *Cell. Signal.* **19**: 2003-2012.

34. Hsu, C.H., Gao, M., Chen, C.L., Yeh, P.Y. and Cheng, A.L. 2005. Inhibitors of epidermoid growth factor receptor suppress cell growth and enhance chemosensitivity of nasopharyngeal cancer cells in vitro. *Oncology* **68**: 538-547.

35. Hui, E.P., Chan, A.T., Pezzella, F., Turley, H., To, K.F., Poon, T.C., Zee, B., Mo, F., Teo, P.M., Huang, D.P., Gatter, K.C., Johnson, P.J. and Harris A.L. 2002. Coexpression of hypoxia-inducible factors 1alpha and 2alpha, carbonic anhydrase IX, and vascular endothelial growth factor in nasopharyngeal carcinoma and relationship to survival. *Clin. Cancer Res*. **8**: 2595-2604.

36. Impellizeri, J.A. and Esplin, D.G. 2008. Expression of cyclooxygenase-2 in canine nasal carcinomas *Vet. J.* **176**: 408-140.

37. Inoue, K., Ohashi, E., Kadosawa, T., Hong, S.H., Matsunaga, S., Mochizuki, M., Nishimura, R. and Sasaki, N. 2004. Establishment and characterization of four canine melanoma cell lines. *J. Vet. Med. Sci.* **66**: 1437-1440.

38. Ito, K., Kobayashi, M., Kuroki, S., Sasaki, Y., Iwata, T., Mori, K., Kuroki, T., Ozawa, Y., Tetsuka, M., Nakagawa, T., Hiroi, T., Yamamoto, H., Ono, K., Washizu, T. and Bonkobara, M. 2013. The proteasome inhibitor bortezomib inhibits the growth of canine malignant melanoma cells in vitro and in vivo. *Vet. J.* **198**: 577-582.

39. Iwasa, T., Okamoto, I., Suzuki, M., Nakahara, T., Yamanaka, K., Hatashita, E., Yamada, Y., Fukuoka, M., Ono, K. and Nakagawa, K. 2008. Radiosensitizing effect of YM155, a novel small-molecule survivin suppressant, in non-small cell lung cancer cell lines. *Clin. Cancer Res.* **14**: 6496-6504.

40. Iwasa, T., Okamoto, I., Takezawa, K., Yamanaka, K., Nakahara, T., Kita, A., Koutoku, H., Sasamata, M., Hatashita, E., Yamada, Y., Kuwata, K., Fukuoka, M. and Nakagawa,

K. 2010. Marked anti-tumour activity of the combination of YM155, a novel survivin suppressant, and platinum-based drugs. *Br. J. Cancer* **103**: 36-42.

41. Johnson, M.E. and Howerth, E.W. 2004. Survivin: a bifunctional inhibitor of apoptosis protein. *Vet. Pathol.* **4**: 599-607.
42. Kaldrymidou, E., Papaioannou, N., Poutahidis, T., Karayannopoulou, M., Gruys, E., Toliou, T. and Tsangaris, T. 2000. Malignant lymphoma in nasal cavity and paranasal sinuses of a dog. *J. Vet. Med. A. Physiol. Pathol. Clin. Med.* **47**: 457-462.
43. Kennedy, A.S., Raleigh, J.A., Perez, G.M., Calkins, D.P., Thrall, D.E., Novotny, D.B. and Varia, M.A. 1997. Proliferation and hypoxia in human squamous cell carcinoma of the cervix: first report of combined immunohistochemical assays. *Int. J. Radiat. Oncol. Biol. Phys.* **37**: 897-905.
44. Kim, T.J., Lee, Y.S., Kang, J.H., Kim, Y.S. and Kang, C.S. 2011. Prognostic significance of expression of VEGF and Cox-2 in nasopharyngeal carcinoma and its association with expression of C-erbB2 and EGFR. *J. Surg. Oncol*. **103**: 46-52.
45. Kleiter, M., Malarkey, D.E., Ruslander, D.E. and Thrall, D.E. 2004. Expression of cyclooxygenase-2 in canine epithelial nasal tumors. *Vet. Radiol. Ultrasound* **45**: 255-260.
46. Knutsen, A., Adell, G. and Sun, X.F. 2004. Survivin expression is an independent prognostic factor in rectal cancer patients with and without preoperative radiotherapy. *Int. J. Radiat. Oncol. Biol. Phys.* **60**: 149-155.
47. Kondo, Y., Matsunaga, S., Mochizuki, M., Kadosawa, T., Nakagawa, T., Nishimura, R. and Sasaki, N. 2008. Prognosis of canine patients with nasal tumors according to modified clinical stages based on computed tomography: a retrospective study. *J. Vet. Med. Sci.* **70**: 207-212.

48. Krikelis, D., Bobos, M., Karayannopoulou, G., Resiga, L., Chrysafi, S., Samantas, E., Andreopoulos, D., Vassiliou, V., Ciuleanu, E. and Fountzilas, G. 2013. Expression profiling of 21 biomolecules in locally advanced nasopharyngeal carcinomas of Caucasian patients. *BMC. Clin. Pathol.* **13**: 1.

49. Kropveld, A., Slootweg, P.J., Blankenstein, M.A., Terhaard, C.H. and Hordijk, G.J. 1998. Ki-67 and p53 in T2 laryngeal cancer. *Laryngoscope* **108**: 1548-1552.

50. LaDue, T.A., Dodge, R., Page, R.L., Price, G.S., Hauck, M.L. and Thrall, D.E. 1999. Factors influencing survival after radiotherapy of nasal tumors in 130 dogs. *Vet. Radiol. Ultrasound* **40**: 312-317.

51. Lana, S.E., Dernell, W.S., Lafferty, M.H., Withrow, S.J. and LaRue, S.M. 2004. Use of radiation and a slow-release cisplatin formulation for treatment of canine nasal tumors. *Vet. Radiol. Ultrasound* **45**: 577–581.

52. Langova, V., Mutsaers, A.J., Phillips, B. and Straw, R. 2004. Treatment of eight dogs with nasal tumours with alternating doses of doxorubicin and carboplatin in conjunction with oral piroxicam. *Aust. Vet. J.* **82**: 676–680.

53. LaRue, S.M. and Gordon, I.K. 2013. Radiation therapy. pp. 180-197. *In*: Small animal clinical oncology. 5th ed. (Withrow, S.J., Vail, D.M. and Page, R.L. eds), Elsevier, Saunders.

54. LeBlanc, A.K., LaDue, T.A., Turrel, J.M. and Klein, M.K. 2004. Unexpected toxicity following use of gemcitabine as a radiosensitizer in head and neck carcinomas: a veterinary radiation therapy oncology group pilot study. *Vet. Radiol. Ultrasound* **45**: 466–470.

55. Li, Y.H., Hu, C.F., Shao, Q., Huang, M.Y., Hou, J.H., Xie, D., Zeng, Y.X. and Shao, J.Y. 2008. Elevated expressions of survivin and VEGF protein are strong independent predictors of survival in advanced nasopharyngeal carcinoma. *J. Transl. Med.* **6**: 1.

56. London, C., Mathie, T., Stingle, N., Clifford, C., Haney, S., Klein, M.K., Beaver, L., Vickery, K., Vail, D.M., Hershey, B., Ettinger, S., Vaughan, A., Alvarez, F., Hillman, L., Kiselow, M., Thamm, D., Higginbotham, M.L., Gauthier, M., Krick, E., Phillips, B., Ladue, T., Jones, P., Bryan, J., Gill, V., Novasad, A., Fulton, L., Carreras, J., McNeill, C., Henry, C. and Gillings, S. 2012. Preliminary evidence for biologic activity of toceranib phosphate (Palladia®) in solid tumours. *Vet. Comp. Oncol*. **10**: 194-205.

57. Ma, B.B., Bristow, R.G., Kim, J. and Siu, L.L. 2003. Combined-modality treatment of solid tumors using radiotherapy and molecular targeted agents. *J. Clin. Oncol.* **21**: 2760-2776.

58. Mason, S.L., Maddox, T.W., Lillis, S.M. and Blackwood, L. 2013. Late presentation of canine nasal tumours in a UK referral hospital and treatment outcomes. *J. Small Anim. Pract.* **54**: 347-353.

59. McEntee, M.C. 2004. A survey of veterinary radiation facilities in the United States during 2001. *Vet. Radiol. Ultrasound* **45**: 476-479.

60. McEntee, M.C. 2006. Veterinary radiation therapy: review and current state of the art. *J. Am. Anim. Hosp. Assoc.* **42**: 94-109.

61. Melzer, K., Guscetti, F., Rohrer Bley, C., Sumova, A., Roos, M. and Kaser-Hotz, B. 2006. Ki67 reactivity in nasal and periocular squamous cell carcinomas in cats treated with electron beam radiation therapy. *J. Vet. Intern. Med.* **20**: 676-681.

62. Moore, A.S. 2002. Radiation therapy for the treatment of tumours in small companion animals. *Vet. J.* **164**: 176-187.

63. Murai, K., Nakagawa, T., Endo, Y., Kamida, A., Yoshida, K., Mochizuki, M., Nishimura, R. and Sasaki, N. 2012. Establishment of a pair of novel cloned tumour cell lines with or without metastatic potential from canine mammary adenocarcinoma. *Res. Vet. Sci.* **93**: 468-472.

64. Nakahara, T., Kita, A., Yamanaka, K., Mori, M., Amino, N., Takeuchi, M., Tominaga, F., Kinoyama, I., Matsuhisa, A., Kudou, M. and Sasamata, M. 2011. Broad spectrum and potent antitumor activities of YM155, a novel small-molecule survivin suppressant, in a wide variety of human cancer cell lines and xenograft models. *Cancer Sci.* **102**: 614-621.

65. Nakahara, T., Takeuchi, M., Kinoyama, I., Minematsu, T., Shirasuna, K., Matsuhisa, A., Kita, A., Tominaga, F., Yamanaka, K., Kudoh, M. and Sasamata, M. 2007. YM155, a novel small- molecule surviving suppressant, induces regression of established human hormone-refractory prostate tumor xenografts. *Cancer Res.* **67**: 8014– 8021.

66. Nakaichi, M., Yunuki, T., Okuda, M., Une, S. and Taura, Y. 2007. Activity of matrix metalloproteinase-2 (MMP-2) in canine oronasal tumors. *Res. Vet. Sci.* **82**: 271-279.

67. Nichols, A.C., Whelan, F., Basmaji, J., Dhaliwal, S., Dowthwaite, S., Chapeskie, C., Read, N., Palma, D.A., Fung, K., Venkatesan, V., Hammond, JA., Franklin, J.H., Siddiqui, I., Wehrli, B., Kwan, K., Koropatnick, J., Mymryk, J.S., Barrett, J.W. and Yoo, J. 2012. Ki-67 expression predicts radiotherapy failure in early glottic cancer. *J. Otolaryngol. Head Neck Surg.* **41**: 124-130.

68. Northrup, N.C., Etue, S.M., Ruslander, D.M., Rassnick, K.M., Hutto, D.L., Bengtson, A., Rand, W. and Moore, A.S. 2001. Retrospective study of orthovoltage radiation therapy for nasal tumors in 42 dogs. *J. Vet. Intern. Med.* **15**: 183-189.

69. Pan, J., Kong, L., Lin, S., Chen, G., Chen, Q. and Lu, J.J. 2008. The clinical significance of coexpression of cyclooxygenases-2, vascular endothelial growth factors, and epidermal growth factor receptor in nasopharyngeal carcinoma. *Laryngoscope* **118**: 1970-1975.

70. Pan, J., Tang, T., Xu, L., Lu, J.J., Lin, S., Qiu, S., Chen, G. and K Tham, I.W. 2013. Prognostic significance of expression of cyclooxygenase-2, vascular endothelial growth factor, and epidermal growth factor receptor in nasopharyngeal carcinoma. *Head & Neck* **35**: 1238-1247.

71. Papazoglou, L.G., Koutinas, A.F., Plevraki, A.G. and Tontis, D. 2001. Primary intranasal transmissible venereal tumour in the dog: a retrospective study of six spontaneous cases. *J. Vet. Med. A. Physiol. Pathol. Clin. Med.* **48**: 391-400.

72. Qin, Q., Cheng, H., Lu, J., Zhan, L., Zheng, J., Cai, J., Yang, X., Xu, L., Zhu, H., Zhang, C., Liu, J., Ma, J., Zhang, X., Dai, S. and Sun, X. 2014. Small-molecule survivin inhibitor YM155 enhances radiosensitization in esophageal squamous cell carcinoma by the abrogation of G2 checkpoint and suppression of homologous recombination repair. *J. Hematol. Oncol.* **7**: 62.

73. Rankin, W.V., Henry, C.J., Turnquist, S.E., Turk, J.R., Beissenherz, M.E., Tyler, J.W., Rucker, E.B., Knapp, D.W., Rodriguez, C.O. and Green, J.A. 2008. Identification of survivin, an inhibitor of apoptosis, in canine urinary bladder transitional cell carcinoma. *Vet. Comp. Oncol.* **6**: 141-150.

74. Rassnick, K.M., Goldkamp, C.E., Erb, H.N., Scrivani, P.V., Njaa, B.L., Gieger, T.L., Turek, M.M., McNiel, E.A., Proulx, D.R., Chun, R., Mauldin, G.E., Phillips, B.S. and Kristal, O. 2006. Evaluation of factors associated with survival in dogs with untreated nasal carcinomas: 139 cases (1993-2003). *J. Am. Vet. Med. Assoc*. **229**: 401-406.

75. Rebhun, R.B., Lana, S.E., Ehrhart, E.J., Charles, J.B. and Thamm, D.H. 2008. Comparative analysis of survivin expression in untreated and relapsed canine lymphoma. *J. Vet. Intern. Med.* **22**: 989-995.

76. Rödel, C., Grabenbauer, G.G., Papadopoulos, T., Bigalke, M., Günther, K., Schick, C., Peters, A., Sauer, R. and Rödel, F. 2002. Apoptosis as a cellular predictor for histopathologic response to neoadjuvant radiochemotherapy in patients with rectal cancer. *Int. J. Radiat. Oncol. Biol. Phys.* **52**: 294-303.

77. Rödel, C., Grabenbauer, G.G., Rödel, F., Birkenhake, S., Kühn, R., Martus, P., Zörcher, T., Fürsich, D., Papadopoulos, T., Dunst, J., Schrott, K.M. and Sauer, R. 2000. Apoptosis, p53, bcl-2, and Ki-67 in invasive bladder carcinoma: possible predictors for response to radiochemotherapy and successful bladder preservation. *Int. J. Radiat. Oncol. Biol. Phys.* **46**: 1213-1221.

78. Rödel, C., Haas, J., Groth, A., Grabenbauer, G.G., Sauer, R. and Rödel, F. 2003. Spontaneous and radiation-induced apoptosis in colorectal carcinoma cells with different intrinsic radiosensitivities: survivin as a radioresistance factor. Int. *J. Radiat. Oncol. Biol. Phys.* **55**: 1341-1347.

79. Sabattini, S., Mancini, F.R., Marconato, L., Bacci, B., Rossi, F., Vignoli, M. and Bettini, G. 2014. EGFR overexpression in canine primary lung cancer: pathogenetic implications and impact on survival. *Vet. Comp. Oncol.* **12**: 237-248.

80. Sato, Y., Ebara, T., Sunaga, N., Takahashi, T. and Nakano, T. 2012. Interaction of radiation and gefitinib on a human lung cancer cell line with mutant EGFR gene in vitro. *Anticancer Res*. **32**: 4877-4881.

81. Scase, T.J., Edwards, D., Miller, J., Henley, W., Smith, K., Blunden, A. and Murphy, S. 2006. Canine mast cell tumors: correlation of apoptosis and proliferation markers with prognosis. *J. Vet. Intern. Med.* **20**: 151-158.

82. Sheridan, M.T., Cooper, R.A. and West, C.M. 1999. A high ratio of apoptosis to proliferation correlates with improved survival after radiotherapy for cervical adenocarcinoma. *Int. J. Radiat. Oncol. Biol. Phys.* **44**: 507-512.

83. Shiomitsu, K., Johnson, C.L., Malarkey, D.E., Pruitt, A.F. and Thrall, D.E. 2009. Expression of epidermal growth factor receptor and vascular endothelial growth factor in malignant canine epithelial nasal tumours. *Vet. Comp. Oncol.* **7**: 106-114.

84. Shiomitsu, K., Sajo, E., Xia, X., Hunley, D.W., Mauldin, G.E., Li, S. and Mauldin, G.N. 2008. Radiosensitivity of canine osteosarcoma cells transfected with wild-type p53 in vitro. *Vet. Comp. Oncol.* **6**: 193-200.

85. Shin, Y.K., Park, J.S., Kim, H.S., Jun, H.J., Kim, G.E., Suh, C.O., Yun, Y.S. and Pyo, H. 2005. Radiosensitivity enhancement by celecoxib, a cyclooxygenase (COX)-2 selective inhibitor, via COX-2-dependent cell cycle regulation on human cancer cells expressing differential COX-2 levels. *Cancer Res*. **65**: 9501-9509.

86. Shoeneman, J.K., Ehrhart, E.J. 3rd, Eickhoff, J.C., Charles, J.B., Powers, B.E. and Thamm, D.H. 2012. Expression and function of survivin in canine osteosarcoma. *Cancer Res.* **72**: 249-259.

87. Sirzén, F., Zhivotovsky, B., Nilsson, A., Bergh, J. and Lewensohn, R. 1998.

Spontaneous and radiation-induced apoptosis in lung carcinoma cells with different intrinsic radiosensitivities. *Anticancer Res*. **18**: 695– 700.

88. Sittel, C., Ruiz, S., Volling, P., Kvasnicka, H.M., Jungehülsing, M. and Eckel, H.E. 1999. Prognostic significance of Ki-67 (MIB1), PCNA and p53 in cancer of the oropharynx and oral cavity. *Oral Oncol.* **35**: 583-589.

89. Snyder, S.A., Linder, K., Hedan, B. and Hauck, M.L. 2011. Establishment and characterization of a canine soft tissue sarcoma cell line. *Vet. Pathol.* **48**: 482-485.

90. Sones, E., Smith, A., Schleis, S., Brawner, W., Almond, G., Taylor, K., Haney, S., Wypij, J., Keyerleber, M., Arthur, J., Hamilton, T., Lawrence, J., Gieger, T., Sellon, R. and Wright, Z. 2013. Survival times for canine intranasal sarcomas treated with radiation therapy: 86 cases (1996-2011). *Vet. Radiol. Ultrasound* **54**: 194-201.

91. Soo, R., Putti, T., Tao, Q., Goh, B.C., Lee, K.H., Kwok-Seng, L., Tan, L. and Hsieh, W.S. 2005. Overexpression of cyclooxygenase-2 in nasopharyngeal carcinoma and association with epidermal growth factor receptor expression. *Arch. Otolaryngol. Head Neck Surg.* **131**: 147-152.

92. Sung, F.L., Hui, E.P., Tao, Q., Li, H., Tsui, N.B., Lo, Y.M., Ma, B.B., To, K.F., Harris, A.L. and Chan, A.T. 2007. Genome-wide expression analysis using microarray identified complex signaling pathways modulated by hypoxia in nasopharyngeal carcinoma. *Cancer Lett*. **253**: 74-88.

93. Taheri-Kadkhoda, Z., Magnusson, B., Svensson, M., Mercke, C. and Björk-Eriksson. T. 2009. Expression modes and clinical manifestations of latent membrane protein 1, Ki-67, cyclin-B1, and epidermal growth factor receptor in nonendemic nasopharyngeal carcinoma. *Head & Neck* **31**: 482-492.

94. Tannapfel, A., Nüsslein, S., Fietkau, R., Katalinic, A., Köckerling, F. and Wittekind, C. 1998. Apoptosis, proliferation, bax, bcl-2 and p53 status prior to and after preoperative radiochemotherapy for locally advanced rectal cancer. *Int. J. Radiat. Oncol. Biol. Phys.* **41**: 585-591.

95. Tatake, R.J., Rajaram, N., Damle, R.N., Balsara, B., Bhisey, A.N. and Gangal, S.G. 1990. Establishment and characterization of four new squamous cell carcinoma cell lines derived from oral tumors. *J. Cancer Res. Clin. Oncol.* **116**: 179-186.

96. Terakado, N., Shintani, S., Yano, J., Chunnan, L., Mihara, M., Nakashiro, K. and Hamakawa, H. 2004. Overexpression of cyclooxygenase-2 is associated with radioresistance in oral squamous cell carcinoma. *Oral Oncol*. **40**: 383-389.

97. Théon, A.P., Madewell, B.R., Harb, M.F. and Dungworth, D.L. 1993. Megavoltage irradiation of neoplasms of the nasal and paranasal cavities in 77 dogs. *J. Am. Vet. Med. Assoc.* **202**: 1469-1475.

98. Therasse, P., Arbuck, S.G., Eisenhauer, E.A., Wanders, J., Kaplan, R.S., Rubinstein, L., Verweij, J., Van Glabbeke, M., Van Oosterom, A.T., Christian, M.C. and Gwyther, S.G. 2000. New guidelines to evaluate the response to treatment in solid tumors. *J. Natl. Cancer Inst.* **92**: 205-216.

99. de Vos, J., Ramos Vega, S., Noorman, E. and de Vos, P. 2012. Primary frontal sinus squamous cell carcinoma in three dogs treated with piroxicam combined with carboplatin or toceranib. *Vet. Comp. Oncol*. **10**: 206-213.

100. Wakisaka, N., Wen, Q.H., Yoshizaki, T., Nishimura, T., Furukawa, M., Kawahara, E., and Nakanishi, I. 1999. Association of vascular endothelial growth factor expression

with angiogenesis and lymph node metastasis in nasopharyngeal carcinoma. *Laryngoscope* **109**: 810-814.

101. Wang, A.H., Tian, X.Y., Yu, J.J., Mi, J.Q., Liu, H. and Wang, R.F. 2012. Celecoxib radiosensitizes the human cervical cancer HeLa cell line via a mechanism dependent on reduced cyclo-oxygenase-2 and vascular endothelial growth factor C expression. *J. Int. Med. Res.* **40**: 56-66.
102. Wheeler, J.A., Stephens, L.C., Tornos, C., Eifel, P.J., Ang, K.K., Milas, L., Allen, P.K. and Meyn, R.E. Jr. 1995. ASTRO Research Fellowship: apoptosis as a predictor of tumor response to radiation in stage IB cervical carcinoma. *Int. J. Radiat. Oncol. Biol. Phys.* **32**: 1487-1493.
103. Withrow, S.J., Turek, M.M. and Lana, S.E. 2013. Tumors of the respiratory system. pp. 432-451. *In*: Small animal clinical oncology. 5th ed. (Withrow, S.J., Vail, D.M. and Page, R.L. eds), Elsevier, Saunders.
104. Yamanaka, K., Nakahara, T., Yamauchi, T., Kita, A., Takeuchi, M., Kiyonaga, F., Kaneko, N. and Sasamata, M. 2011. Antitumor activity of YM155, a selective small-molecule survivin suppressant, alone and in combination with docetaxel in human malignant melanoma models. *Clin. Cancer Res.* **17**: 5423-5431.
105. Yamazaki, H., Takagi, S., Hoshino, Y., Hosoya, K. and Okumura, M. 2013. Inhibition of survivin influences the biological activities of canine histiocytic sarcoma cell lines. *PLoS One* **8**: e79810.
106. Yang, Y., Xuan, J., Yang, Z., Han, A., Xing, L., Yue, J., Hu, M. and Yu, J. 2012. The expression of epidermal growth factor receptor and Ki67 in primary and relapse

nasopharyngeal cancer: a micro-evidence for anti-EGFR targeted maintenance therapy. *Med. Oncol.* **29**: 1448-1455.

107. Yip, K.W., Shi, W., Pintilie, M., Martin, J.D., Mocanu, J.D., Wong, D., MacMillan, C., Gullane, P., O'Sullivan, B., Bastianutto, C. and Liu, F.F. 2006. Prognostic significance of the Epstein-Barr virus, p53, Bcl-2, and survivin in nasopharyngeal cancer. *Clin. Cancer Res.* **12**: 5726-5732.
108. Yoon, J.H., Feeney, D.A., Jessen, C.R. and Walter, P.A. 2008. External-beam Co-60 radiotherapy for canine nasal tumors: a comparison of survival by treatment protocol. *Res. Vet. Sci.* **84**: 140-149.
109. Zhang, B., Qu, J.Q., Xiao, L., Yi, H., Zhang, P.F., Li, M.Y., Hu, R., Wan, X.X., He, Q.Y., Li, J.H., Ye, X., Xiao, Z.Q. and Feng, X.P. 2012. Identification of heat shock protein 27 as a radioresistance-related protein in nasopharyngeal carcinoma cells. *J. Cancer Res. Clin. Oncol.* **138**: 2117-2125.
110. Zhang, S.X., Qiu, Q.H., Chen, W.B., Liang, C.H. and Huang, B. 2014. Celecoxib enhances radiosensitivity via induction of G2-M phase arrest and apoptosis in nasopharyngeal carcinoma. *Cell Physiol. Biochem.* **33**: 1484-1497.
111. Zhang, Y., Huang, D. and Yu, G. 2005. Survivin expression and its relationship with apoptosis and prognosis in nasal and paranasal sinus carcinomas. *Acta. Otolaryngol.* **125**: 1345-1350.
112. Zheng, X., Hu, L., Chen, F. and Christensson, B. 1994. Expression of Ki67 antigen, epidermal growth factor receptor and Epstein-Barr virus-encoded latent membrane protein (LMP1) in nasopharyngeal carcinoma. *Eur. J. Cancer B. Oral Oncol.* **30**: 290-295.