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# Comparison of epidermal growth factor receptor (EGFR) and cyclooxygenase-2 (COX-2) immunohistochemical expression and outcomes in canine nasal carcinomas treated with radiation therapy

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**ABSTRACT.** The expression of epidermal growth factor receptor (EGFR) and cyclooxygenase-2 (COX-2) has been reported in human nasopharyngeal and canine nasal carcinomas. The present study measured EGFR and COX-2 expression and calculated correlations between these proteins and clinical variables and outcomes in dogs with nasal carcinoma treated with radiation therapy. Before treatment, the immunohistochemistry of EGFR and COX-2 was performed in 67 biopsied tissues from canine nasal carcinomas. The correlations between these protein levels, clinical variables, and outcomes were evaluated. EGFR and COX-2 were detected in 88.1% and 82.1% of our samples, respectively. Neither EGFR nor COX-2 was associated with T stage and cribriform plate destruction. Dogs with low EGFR levels had a significantly longer survival time than dogs with high EGFR expression ($P$=0.043). The COX-2 expression level was not significantly associated with survival times after radiation therapy ($P$=0.653). Overexpression of EGFR is negatively correlated with survival in dogs with nasal carcinoma. Future studies should identify tumor biomarkers to develop therapeutic targets for effective treatments for canine nasal carcinomas.

**KEYWORDS:** cyclooxygenase-2, dog, epidermal growth factor receptor, nasal tumor, radiation therapy

Epidermal growth factor receptor (EGFR) is a transmembrane protein of the ErbB family of receptor tyrosine kinases activated by binding of EGF or EGF-like growth factor and triggers signal transduction cascades. EGFR regulates the cell cycle, differentiation, angiogenesis, and survival [13, 31]. EGFR overexpression has been associated with poor outcomes in human nasopharyngeal carcinomas (NPC) [8, 10, 20, 33]. It is also expressed in some canine epithelial tumors, including nasal [17, 31], mammary gland [14], bladder [16], and lung [27]. EGFR expression was detected in over 70% of human NPC and over 50% of canine nasal carcinomas [10, 17, 25, 31]. The correlation between overexpression of EGFR and poor survival have been shown in some human and veterinary tumors [4, 13, 26, 27], but which in canine nasal carcinomas has not been studied.

Cyclooxygenase-2 (COX-2) is an inducible isoform involved in prostaglandin-endoperoxide synthesis. It is typically induced by proinflammatory cytokines, growth factors, and tumor promoters [6, 21] and is associated with apoptosis inhibition, metastasis, and angiogenesis [25, 32]. In human NPC, overexpression of COX-2 is associated with poor outcomes [9, 24]. Although COX-2 is expressed in 71–90% of canine nasal carcinomas [3, 6, 18, 21], the relationship between COX-2 and radiation therapy (RT) outcome has rarely studied in canine nasal carcinomas [3].

Epithelial tumors, including adenocarcinomas (ADC), squamous cell carcinomas (SCC), transitional cell carcinomas (TCC), and undifferentiated carcinomas (CA), comprise 60–75% of canine nasal neoplasia [2, 22]. Nasal carcinomas are locally invasive and often die of progressive local disease. Radiation therapy (RT) is the mainstay of local control. The median survival time (MST) of dogs with nasal carcinomas treated with RT ranges from 7 to 13 months [2, 22, 24].

The first objective of the present study was to determine the relationship between protein markers (EGFR and COX-2) and clinical

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variables in canine nasal carcinoma tissue samples. The second objective was to determine whether correlations exist between these proteins and outcomes after radiation therapy.

## MATERIALS AND METHODS

### *Biopsy samples and clinical data*

Sixty-seven biopsy samples were collected from dogs diagnosed with nasal carcinomas before treatment at Rakuno Gakuen University Veterinary Teaching Hospital (RGU-VTH) between 2004 and 2013. All tissue samples were fixed in 10% neutral buffered formalin and embedded in paraffin. The histopathological diagnoses were made by veterinarians in the Department of Veterinary Pathology at RGU.

Medical records and computed tomography (CT) images at the time of diagnosis were reviewed. Clinical staging was evaluated using CT imaging and a previously described staging system (Table 1) [2]. Fine-needle aspiration of regional lymph nodes was performed if nodes were enlarged.

### *Immunohistochemistry and protein expression scoring*

Serial sections were prepared from paraffin-embedded tissues for immunohistochemical (IHC) examinations. Antigen retrieval was performed using proteinase K (20 µg/mL, Dako, Glostrup, Denmark) or by autoclave using 0.1 M citrate buffer (pH 6.0). IHC was performed using an avidin-biotin-peroxidase complex procedure using a commercial kit (Vectastain ABC Kit, Vector Laboratories, Burling, CA, USA). Endogenous peroxidase activity was blocked by 3% hydrogen peroxide for 10 min. Table 2 summarizes information on primary antibodies against human EGFR and COX-2, including dilutions, incubation times, and pretreatment conditions for antigen retrieval. Staining was developed using 3-3′-diaminobenzidine (Kanto Chemical Co., Tokyo, Japan) and counterstained with Mayer's hematoxylin. Negative controls were incubated with isotype-matched, irrelevant monoclonal or irrelevant polyclonal IgG instead of the specific primary antibody.

EGFR and COX-2 immunostaining was scored to semi-quantitatively estimate the degree of protein expression in tumor tissues, based on a previous report [3, 31]. The IHC score was determined from the percentage of positively labeled tumor cells and their average staining intensity. The score was assessed at ×200 magnification, and the score was given as an average of five fields of view (Table 3). The total IHC score was calculated by multiplying the scores for the percentage and intensity to give a total score from 0 to 12. Tumor samples with an IHC score ≥2 were defined as having positive expression. An IHC score of 4 was defined as the threshold separating tumors with high expression from those with low expression.

### *RT and treatment procedure*

Dogs were treated with radiation using an orthovoltage X-ray machine (TITAN-450S, GE, Tokyo, Japan) with a filter of 1.0 mm of aluminum, 0.3 mm of copper, and 0.5 mm of tin. The exposure rate was 1.68 Gy/min. The distance from the X-ray source to the skin was 60 cm. The half-value layer was 4.8 mm copper at 450 kV and 10 mA. A single dorsal portal field was delivered. The radiation field included the tip line of the nose to the top of the head, which was assessed using CT images. The planning target volume included a 1–2 cm margin of normal tissue. The dogs were treated with ten fractions of 4 Gy on alternate days (Monday, Wednesday, and Friday) for a total dose of 40 Gy to evaluate the relationship between IHC score and outcomes.

### *Statistical analysis*

Statistical significance of differences was analyzed using the chi-squared ($\chi^2$) test for the relationship between protein expression and clinical variables (sex, age, clinical stage, and cribriform plate destruction). Dogs were monitored and followed up every three

**Table 1.** Adams' proposed staging system for canine nasal tumors [2]

| 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 destroying the cribriform plate |

**Table 2.** Primary antibodies and staining conditions

| Antibody | Clonality | Isotype | Supplier | Dilution | Incubation time (hr) | Antigen retrieval method |
|---|---|---|---|---|---|---|
| EGFR | Monoclonal 31G7 | Mouse IgG | Zymed (South San Francisco, CA, USA) | 1:50 | 16 | Proteinase K (20 µg/mL, 2 min) |
| COX-2 | Monoclonal 33 | Mouse IgG | BD (Heidelberg, Germany) | 1:50 | 16 | Pressurized heating (121°C, 15 min) |

COX-2: cyclooxygenase-2; EGFR: epidermal growth factor receptor.

**Table 3.** Scoring percentage range and intensity of positively labeled tumor cells for epidermal growth factor receptor (EGFR) and cyclooxygenase-2 (COX-2)

| IHC score | EGFR | COX-2 |
|---|---|---|
| 0 | Negative | Negative |
| 1 | <10% positive | <1% positive |
| 2 | 10–30% positive | 1–9% positive |
| 3 | 31–60% positive | 10–50% positive |
| 4 | >60% positive | >50% positive |
| 0 | No immunostaining | |
| 1 | Weak immunostaining | |
| 2 | Moderate immunostaining | |
| 3 | Strong immunostaining | |

IHC: immunohistochemical.

months after RT. Follow-up information was collected from medical records or by contacting the referring veterinarians. Endpoints were: alive if the dog was known to be alive at the time of data collection; lost to follow-up, if the survival status was unknown at the time of data collection; or dead if the dog had died before data collection. The progression-free interval (PFI) was defined as the time between the first day of RT and the day of disease progression (local recurrence, metastatic disease, or death). The overall survival time was calculated from the start day of RT to the time of death or the final follow-up. The correlation between survival and protein marker (EGFR or COX-2) level was estimated using Kaplan-Meier survival curves, and statistical differences between survival curves were calculated using the log-rank method. *P*-values of <0.05 were considered significant. StatMate III commercial software (ATMS Co., Ltd., Tokyo, Japan) was used to perform the statistical analysis.

## RESULTS

### *Biopsy samples and clinical data*

Of the 67 tissue samples, 29 were ADCs, 17 were CAs, 12 were SCCs, and nine were TCCs (Table 4). All dogs underwent skull CT scans before biopsy. The CT images at the time of diagnosis were reviewed, and clinical staging was evaluated. Twelve dogs (17.9%) had T1 disease, five (7.5%) had T2 disease, 17 (25.4%) had T3 disease, and 33 (49.2%) had T4 disease. Fine-needle aspiration of regional lymph nodes was performed on 39 dogs. Five dogs had evidence of regional lymph node metastasis.

### *EGFR and COX-2 protein expression*

EGFR staining was predominantly membranous and occasionally cytoplasmic (Fig. 1). Fifty-nine samples (88.1%) were positive. Positive EGFR expression was observed in 24 of 29 ADC (82.8%), 14 of 17 CA (82.4%), all 12 SCC (100%), and all nine TCC (100%) samples. The total IHC score for EGFR expression ranged from 0 to 12. The median total IHC score was 6, and the mean was 6.9, while mean IHC scores for ADC, CA, SCC, and TCC were 5.7, 6.3, 9.9, and 8.2, respectively. Low EGFR expression was found in 20 samples (11 ADCs, six CAs, one SCC, and two TCCs) and high in 47 samples (18 ADCs, 11 CAs, 11 SCCs, and seven TCCs).

COX-2 staining was predominantly cytoplasmic and perinuclear (Fig. 2). Fifty-five samples (82.1%) were positive. Positive COX-2

**Table 4.** Characteristics of dogs included in the present study

| | Cases (n=67) | | Cases (n=67) |
|---|---|---|---|
| Sex | | Breed | |
| Intact female | 11 | Golden Retriever | 10 |
| Spayed female | 25 | Pembroke Welsh Corgi | 9 |
| Intact male | 15 | Miniature Dachshund | 7 |
| Neutered male | 16 | Shetland Sheepdog | 6 |
| Clinical signs | | Shiba | 4 |
| Epistaxis | 45 | Beagle | 3 |
| Nasal discharge | 34 | Miniature Schnauzer | 3 |
| Sneezing | 25 | Siberian Husky | 3 |
| Facial deformity | 22 | Hokkaido | 2 |
| Exophthalmos | 12 | Labrador Retriever | 2 |
| Neurological abnormalities | 5 | Shih Tzu | 2 |
| Tumor type | | Bullmastiff | 1 |
| Adenocarcinoma | 29 | Chihuahua | 1 |
| Undifferentiated carcinoma | 17 | Maltese | 1 |
| Squamous cell carcinoma | 12 | Papillon | 1 |
| Transitional cell carcinoma | 9 | Pug | 1 |
| Clinical stage | | Mix | 11 |
| T1 | 12 | | |
| T2 | 5 | | |
| T3 | 17 | | |
| T4 | 33 | | |

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).

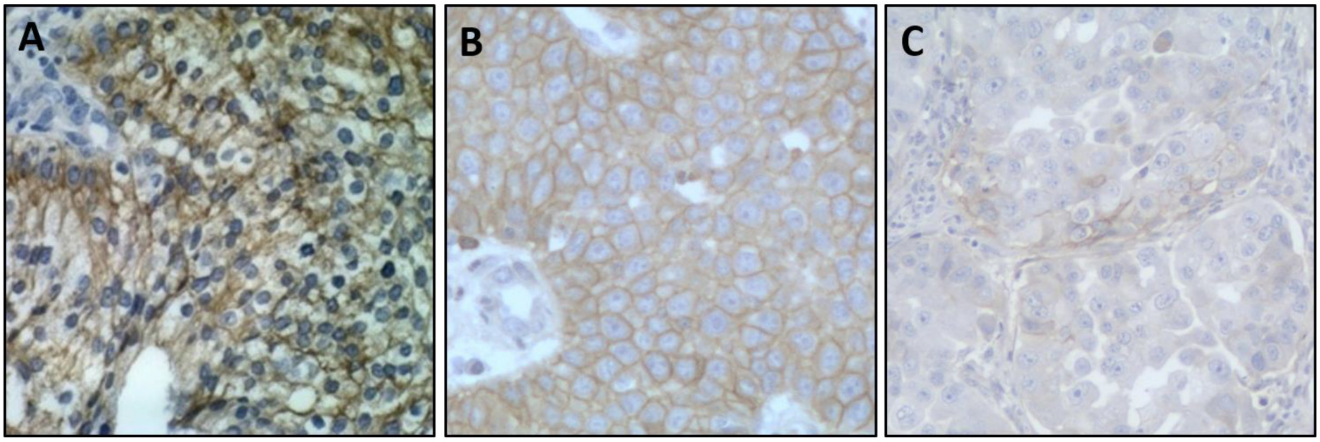


**Fig. 1.** Immunohistochemical (IHC) staining of epidermal growth factor receptor (EGFR) in canine nasal carcinomas. (**A**) Adenocarcinoma, IHC score of 12. (**B**) Transitional cell carcinoma, IHC score of 8. (**C**) Adenocarcinoma, IHC score of 1 (Original magnification ×400).

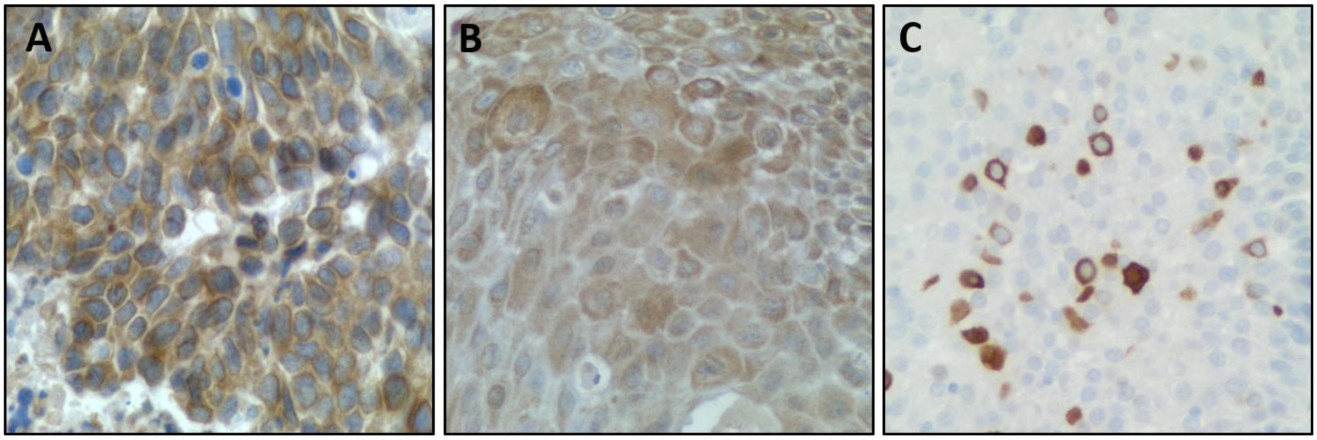


**Fig. 2.** Immunohistochemical (IHC) staining of cyclooxygenase-2 (COX-2) in canine nasal carcinomas (**A**) undifferentiated carcinomas, IHC score of 12. (**B**) Squamous cell carcinoma, IHC score of 8. (**C**) Transitional cell carcinoma, IHC score of 6 (Original magnification ×400).

expression was observed in 24 of 29 ADC (82.8%), 13 of 17 CA (76.5%), ten of 12 SCC (83.3%), and eight of nine TCC (88.9%) samples. The total IHC score for COX-2 expression ranged from 0 to 12. The median total IHC score was 6, and the mean was 5.3, while mean IHC scores for ADC, CA, SCC, and TCC was 4.7, 5.6, 6.1, and 5.7, respectively. Low COX-2 expression was found in 32 samples (16 ADCs, seven CAs, five SCCs, and four TCCs) and high in 35 samples (13 ADCs, ten CAs, seven SCCs, and five TCCs).

Neither expression of EGFR nor COX-2 was strongly associated with T staging, cribriform plate destruction, sex, or age (Table 5). There was no relationship between the expression of EGFR and COX-2 ($P$=0.407).

### *Associations between protein expressions and survivals*

Thirty-eight dogs, including 18 ADCs, nine CAs, nine SCCs, and two TCCs, completed RT as their only curative treatment with no evidence of metastatic disease. According to medical records, nineteen dogs did not receive any treatments, three dogs did not complete ten fractions (two received only eight, and one received nine treatments due to skin radiation side effects), and seven dogs received different radiation protocols (3 or 6 Gy per fractions). Of these 38 dogs who completed RT, seven were staged as T1, two as T2, ten as T3, and 19 as T4. Follow-up intervals ranged from 40 to 1,979 days, with a median follow-up interval of 220 days. Thirty-one dogs died during the study and follow-up period, and seven dogs were lost to follow-up. Twenty-one died from tumor progression (fourteen had tumor local progression which was diagnosed by head CT scan, and seven had lymph nodes and/or distant metastatic diseases), two from euthanasia, three from other diseases, and five from unknown causes. Median PFI and overall survival times (MST) were 150 and 236 days, respectively. Kaplan-Meier and log-rank analysis revealed no significant correlations between PFIs and protein levels of EGFR or COX-2 expression ($P$=0.201 and 0.692, respectively). Dogs with low EGFR had longer survival times than those with high EGFR (MST: 262 days versus 187 days, respectively; $P$=0.043) (Fig. 3A). However, COX-2 expression was not correlated with survival (MST: 236 days versus 237 days, respectively; $P$=0.635) (Fig. 3B).

**Table 5.** Association between the expression of epidermal growth factor receptor (EGFR), cyclooxygenase-2 (COX-2) and clinical features in dogs with nasal carcinoma

| Clinical characteristics | *N* | EGFR | | COX-2 | |
|---|---|---|---|---|---|
| | | Low | High | Low | High |
| Tumor stage | | | | | |
| T1+T2 | 17 | 6 (35.3%) | 11 (64.7%) | 7 (41.2%) | 10 (58.8%) |
| T3+T4 | 50 | 14 (28.0%) | 36 (72.0%) | 25 (50.0%) | 25 (50.0%) |
| *P value* | | 0.57 | | 0.529 | |
| Destruction of the cribriform plate | | | | | |
| Absence | 34 | 10 (29.4%) | 24 (70.6%) | 15 (44.1%) | 19 (55.9%) |
| Presence | 33 | 10 (30.3%) | 23 (69.7%) | 17 (51.5%) | 16 (48.5%) |
| *P value* | | 0.936 | | 0.544 | |
| Sex | | | | | |
| Female | 36 | 9 (25.0%) | 27 (75.0%) | 20 (55.6%) | 16 (44.4%) |
| Male | 31 | 11 (35.5%) | 20 (64.5%) | 12 (38.7%) | 19 (61.3%) |
| *P value* | | 0.349 | | 0.169 | |
| Age | | | | | |
| ≤11 y | 37 | 10 (27.0%) | 27 (73.0%) | 18 (48.6%) | 19 (51.4%) |
| >11 y | 30 | 10 (33.3%) | 20 (66.7%) | 14 (46.7%) | 16 (53.3%) |
| *P value* | | 0.575 | | 0.872 | |

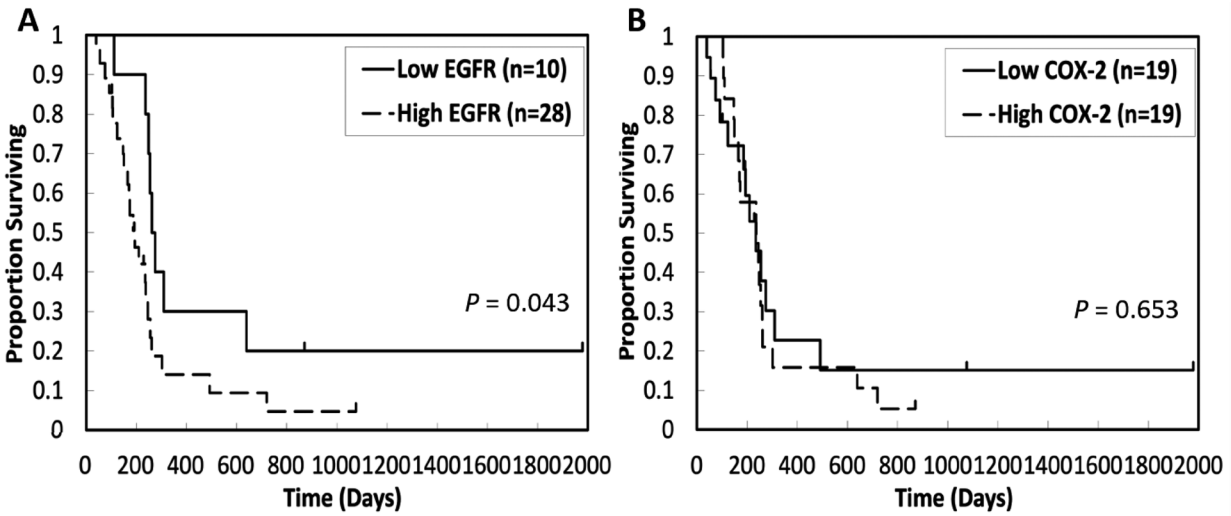


**Fig. 3.** Kaplan-Meier survival curves for 38 dogs with nasal carcinoma treated with RT. According to the scoring criteria, thirty-eight dogs were divided into low- and high-expression groups. Survival times were compared between the groups for epidermal growth factor receptor (EGFR) (A) and cyclooxygenase-2 (COX-2) (B) using Kaplan-Meier survival curves. (**A**) The median survival times (MSTs) in dogs with low and high EGFR expression were 262 and 187 days, respectively ($P$=0.043). (**B**) The MSTs in dogs with low and high COX-2 expression were 236 days and 237 days, respectively ($P$=0.653).

## DISCUSSION

Most canine nasal carcinomas demonstrated expression of EGFR (88.1%) and COX-2 (82.1%). Although previous reports investigated EGFR and COX-2 IHC expression in canine nasal carcinomas [3, 6, 18, 21, 31], one study evaluated the potential correlation between protein markers expression and outcome after RT [3]. Unlike in human medicine, IHC has not been widely used to evaluate outcomes of RT in veterinary studies. Survivin expression was a negative predictor in canine nasal carcinomas treated with RT [12]. Therefore, our study aimed to calculate correlations between IHC and clinical variables and outcomes in canine nasal carcinomas.

EGFR plays a crucial role in regulating the cell cycle, angiogenesis, differentiation, and survival [13, 31]. In a previous study, 54.2% had positive expression in 24 canine nasal carcinomas [31]. In another report, EGFR immunoreactivity was noted in 14 of 16 (87.5%) samples, and messenger RNA for EGFR was detected in 15 of 16 samples. Phosphorylation of EGFR was demonstrated in 10 of 16 samples [17]. In the present study, 67 samples were evaluated, and 59 tested positive. EGFR positivity was 82.8% in ADC, 82.4% in

CA, 100% in SCC, and 100% in TCC. Overexpression of EGFR has been reported to correlate with poor survival in some human and veterinary tumors [4, 13, 26, 27]. However, the correlation between EGFR overexpression and outcomes in dogs with nasal tumors has not been studied. Our result found that EGFR overexpression is associated with shorter overall survival in dogs with nasal carcinoma.

Combined treatment using an EGFR inhibitor (cetuximab) and RT was superior to RT treatment alone. RT + cetuximab increases the duration of local control and survival in human head and neck cancers [5]. Another EGFR inhibitor (gefitinib) inhibited activation of anti-apoptotic and proliferative signal transduction pathways to enhance radiosensitivity in human lung cancer cells [28]. In the present study, nearly all canine nasal carcinomas overexpressed EGFR; this may be considered a similar therapeutic target in dogs with nasal carcinoma.

Previous studies reported that a high percentage (71–90%) of canine nasal carcinoma expressed COX-2 [3, 6, 18, 21]. Similarly, COX-2 was positive in 82.1% of our 67 samples. COX-2 expression inhibits apoptosis and causes tumor metastasis and angiogenesis in human NPC [25, 32]; these phenomena have been associated with outcomes [9, 26]. Few studies reported associations between COX-2 expression and outcomes in dogs with cancers. As in our study, one study found no association between COX-2 expression and survival in dogs with nasal carcinoma treated with hypofractionated RT [3]. Although the role of COX-2 in carcinogenesis appears straightforward, the prognostic value of COX-2 expression in canine cancer is not well understood. COX-2 levels measured by IHC might not indicate actual enzymatic activity [19].

A few studies reported the radiosensitizing effects of COX-2 selective inhibitors in human cancer cells; however, the mechanisms of these effects are poorly understood [11, 30]. Celecoxib (a COX-2 inhibitor) increased radiosensitivity in NPC cells. This result highlighted the relationship between G2-M phase arrest and apoptosis induction [35]. Celecoxib caused the downregulation of COX-2 and vascular endothelial growth factor in human cervical cancer cells. COX-2 inhibition and RT may appear to synergistically inhibit tumor proliferation and angiogenesis [30, 34]. In our result, most of our nasal carcinoma samples (82.1%) expressed positive in COX-2. Although no relationship was revealed between COX-2 level and survival in dogs with nasal carcinoma treated with RT in our and a previous study [3], the combination effect of RT and COX-2 inhibitor has been studied recently [7]. Cancedda *et al.* combined treatment of RT and firocoxib for canine nasal carcinomas. Despite the absence of statistical evidence for survival differences between firocoxib and placebo groups, the quality of life was significantly improved by combining firocoxib and RT [7].

Currently, most of veterinary radiation oncology facility are using megavoltage radiation machine. Megavoltage radiation has greater penetrability and dose distribution and would be expected to be superior to orthovoltage RT for nasal tumors in dogs [29]. This phenomenon is particularly evident in tumors located at greater depths from the skin surface. Orthovoltage radiation was used in recent study because of the limitations of RT units in our institution. We observed median PFI and MST of 5 and 7.9 months, respectively, which is similar to the outcomes described in previous canine nasal tumor studies involving orthovoltage treatment [1, 24]. Computer-based conformal RT planning techniques, including three-dimensional conformal (3DCRT) and intensity-modulated radiation therapy (IMRT), are designed to maximize the tumor dose while sparing the normal organs surrounding the target, in order to minimize radiation side effects [23]. Stereotactic radiation therapy (SRT) is becoming increasingly available at veterinary facility. SRT delivers an ablative high dose of radiation to a well-defined target with a rapid fall-off of dose, allowing adjacent critical organ to be spared. It is treated with a limited number of fractions (1–5 treatments) within a same week [15]. The association with current radiation protocols and molecule markers should be investigated.

The limitations of this study include the retrospective nature of the study, a limited case population, and the use of orthovoltage radiation unit. Despite the limitations of this retrospective study, our results showed that EGFR overexpression could be a prognostic marker in canine nasal carcinoma. Further investigations into the efficacy of RT combined with molecular inhibitors in veterinary medicine are needed, especially in advanced-stage tumors and those with poor response to RT.

CONFLICT OF INTEREST. None of the authors of this paper has any financial or personal relationships that could inappropriately influence or bias the content of the paper.

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