Prognostic Utility of Apoptosis Index, Ki-67 and Survivin Expression in Dogs with Nasal Carcinoma Treated with Orthovoltage Radiation Therapy

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ABSTRACT. Apoptosis, Ki-67 and survivin expression have been reported as prognostic values in human cancer treated with radiation therapy. The aim of this study was to evaluate the correlation between the outcome of canine nasal carcinomas treated with radiation therapy and these cancer markers. The apoptotic index (AI) was evaluated with TUNEL assays, and an immunohistochemical evaluation was performed on Ki-67 and survivin in 33 biopsy samples taken before treatment. Median survival times were estimated using Kaplan-Meier curves and the log-rank method. The AI ranged from 0 to 0.7%, and the percentage of Ki-67-positive cells defined as the proliferative index (PI) ranged from 0.8 to 77% in all samples. Neither the AI nor the PI had a significant relationship with survival time (P=0.056 and 0.211). Survivin expression was detected in 84.9% of samples of canine nasal carcinoma. Dogs with high survivin expression were associated with poorer response to treatment and had shorter survival times (P=0.017 and 0.031). Advanced-stage tumors were also significantly associated with a high level of survivin (P=0.026). Overexpression of survivin was shown to be an unfavorable prognostic factor in dogs with nasal carcinomas treated with radiation therapy.

KEY WORDS: apoptosis, canine nasal carcinoma, Ki-67, prognosis, survivin

Canine nasal tumors are uncommon, accounting for less than 2% of all neoplasms in dogs [31]. Approximately 60 to 75% of canine nasal tumors are of epithelial origin (carcinomas) [1, 13, 21]. Most canine nasal tumors are very aggressive to the surrounding tissue, but are less likely to metastasize. Due to the complex surrounding anatomical structure, these tumors are difficult to treat with surgery alone to prolong survival time. Radiation therapy is an effective and relatively noninvasive treatment for local control of canine nasal tumors [7]. In previous studies, the median survival time (MST) of dogs with nasal tumors treated by radiation therapy had a wide range, 7 to 23 months [1, 13, 19, 31]. Apoptosis is the process of eliminating old and damaged cells in tissues. It is mediated by a complex balance of signal transduction pathways and is regulated through a number of proteins involved in the activation of caspase cascades [33]. Radiation kills cancer by inducing cell apoptosis [23, 33]. A correlation between the rate of spontaneous apoptosis and radiation-induced apoptosis has been demonstrated in several studies [23, 27]. These studies revealed that cases with an increased spontaneous apoptotic rate had a good prognosis in human bladder [25], cervical [27] and rectal [2, 24, 30] cancers treated with chemoradiation/radiation therapy.

Survivin is a small protein that belongs to the inhibitors of apoptosis protein (IAP) family. It is not expressed in normal differentiated tissue, but is highly expressed in fetal tissues and several cancers [9]. The expression of survivin is associated with a poor prognosis in several human cancers [11, 15]. In humans, 60–78.6% of nasopharyngeal carcinomas are positive for survivin expression [15, 35, 36]. In veterinary medicine, the expression of survivin also has been detected in several tumors in dogs, including mast cell tumors [26], lymphomas [22], urinary bladder transitional cell carcinomas [20], cutaneous squamous cell carcinomas [5] and osteosarcomas [28]. Survivin expression is correlated with malignancy and poor prognosis in these tumors. Ki-67 is a nuclear protein that is generally used to detect proliferative cells. It is present in proliferating cells during the G1-M phases of the cell cycle. Low Ki-67 levels in tumors are believed to reflect hypoxia and radioresistance [10]. Oral and laryngeal cancers in humans with high Ki-67 levels respond better to radiation therapy than those with low Ki-67 levels [6, 12]. In cats with squamous cell carcinomas treated with electron beam radiation therapy, a high level of Ki-67 has been correlated with a longer disease-free interval [16]. However, a high positive rate of Ki-67 has been generally considered an unfavorable prognostic factor in several human and veterinary medicine studies [4, 18, 26, 29].

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 medicine. The aim of this study was to evaluate the correlation between the outcome of canine nasal carcinomas treated with radiation therapy and these cancer markers in biopsy samples.
MATERIALS AND METHODS

Patient data: The medical records of 43 dogs with nasal carcinomas treated with radiation therapy at the Rakuno Gakuen University Veterinary Teaching Hospital (RGU-VTH) between April 2004 and June 2012 were reviewed. All dogs had received biopsy before radiation therapy and had been diagnosed with carcinomas by histopathology. Pretreatment evaluation included a complete blood count, serum chemistry, three-view thoracic radiographs, a CT scan and a fine-needle biopsy of regional lymph nodes if enlarged. Age, sex, breed, clinical signs and clinical staging were recorded. Clinical staging was based on CT imaging using Adams’ staging system [1] (shown in Table 1). Follow-up information was obtained from the medical records or by contacting the referring veterinarians.

Radiation therapy and response assessment: Radiation therapy was performed by use of an orthovoltage X-ray machine (GE, TITAN-450S) with a half-value layer of 4.8 mm of Al, 0.3 mm of Cu and 0.5 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 Cu. 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 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 [32].

TUNEL assay and assessment of apoptotic index: A terminal deoxynucleotidyl transferase dUTP nick end-labeling (TUNEL) assay was carried out using an ApopTag® Peroxidase In Situ Apoptosis Detection Kit (S7100, Chemicon Merck Millipore, Billerica, MA, U.S.A.) to detect apoptotic cells. All 4 µm sections, which were cut from formalin-fixed paraffin-embedded blocks and mounted on glass slides, were deparaffinized in xylene and rehydrated in 70% ethanol. The samples were pretreated with proteinase K (20 µg/ml, Dako, Glostrup, Denmark) for 10 min at room temperature. Endogenous peroxidase activity was blocked by rinsing with 3% H2O2. Equilibration buffer was used to cover the slide for 15 sec, and then, working-strength TdT solution was added; the slide was then incubated in a humidified chamber at 37°C for 1 hr. The reaction was stopped by addition of working-strength Wash/Stop buffer for 10 min. Anti-digoxigenin peroxidase was applied, and the sections were then incubated for 30 min at room temperature. 3–3’-Diaminobenzidine (DAB, Kanto Chemical Co., Tokyo, Japan) solution was used for color development, and Mayer’s hematoxylin was used for counterstaining. A canine lymphoma tissue section was used as a positive control. Apoptotic-positive tumor cells in a total of 1,000 tumor cells were counted by microscopic examination in 5–10 random fields as the apoptotic index (AI). A low AI was defined as an AI below the median level; a high AI was defined as an AI at or above the median level.

Immunohistochemistry (IHC) staining: All 4 µm section samples were deparaffinized and rehydrated in the same way as in the TUNEL assay. The antigens were retrieved by autoclaving at 121°C for 15 min with 0.1 M citrate buffer (pH 6.0). All sections were rinsed with 3% H2O2 for blocking endogenous peroxidase activity and then blocked by nonspecific binding with 1% horse serum in PBS. The sections were then incubated with a rabbit anti-survivin polyclonal antibody (clone NB500-201, Novus Biologicals, Littleton, CO, U.S.A.) 1:600 PBS dilution overnight at 4°C and a mouse anti-Ki-67 monoclonal antibody (clone MIB-1, Dako) 1:100 PBS dilution for 60 min at room temperature. The sections were subsequently incubated with a secondary antibody and then with an avidin-biotin complex solution (VECTASTAIN® ABC Kit, Vector Labs, Burling, CA, U.S.A.) for 30 min each at room temperature. DAB solution was used as the chromogen, and the sections were then counterstained with Mayer’s hematoxylin. The proliferative index (PI) was determined as the percentage of Ki-67-positive tumor cells in a total of 1,000 tumor cells. A low PI was defined as a PI below the median level; a high PI was defined as a PI at and above the median level. 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. A semiquantitative immunohistochemical scoring system was applied. The IHC score of survivin was based on the percentage of survivin-positive tumor cells and the average intensity. The mean percentage of survivin-positive tumor cells was categorized into 5 groups: 0=<5%, 1=5–25%, 2=25–50%, 3=50–75% and 4=>75% [36]. The intensity of survivin stain was scored as 0 for negative, 1 for weak, 2 for moderate and 3 for strong. The total IHC score was calculated by multiplying the scores for the percentage and intensity and ranged

| Stage | Tumor characteristics                          |
|-------|-----------------------------------------------|
| T1    | Confined to one nasal passage or frontal sinus, with no bony involvement |
| T2    | Bony involvement, without evidence of orbit, subcutaneous, or submucosal mass |
| T3    | Orbit or nasopharynx involved, or a subcutaneous, or submucosal mass |
| T4    | Tumor causing destruction of the cribriform plate |
from 0 to 12. 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.

Statistical analysis: The t-test was used to examine the relationships between the tumor markers (AI and PI) and response to treatment. Statistical significance of differences was analyzed with the χ² test for the relationship between the tumor markers and clinical findings (clinical staging and response to treatment). The correlation between the AI and survivin IHC score was analyzed using Spearman’s correlation method. The overall survival time of a patient was defined from the starting date of radiation therapy to the time of death. The correlation between the MST 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 statistically significant. The commercial software StatMate III (ATMS Co., Ltd., Tokyo, Japan) was used to perform the statistical analysis.

RESULTS

Of the 43 dogs with nasal carcinomas, 17 were males, and 26 were females. Eight of the males were neutered, and 16 of the females were spayed. The mean age of the dogs that started treatment with radiation therapy was 11.1 years (range of 7–16 years, median of 11 years). The mean weight was 14.2 kg (range of 2.5–39.4 kg, median of 16.8 kg). Fifteen breeds were represented in the study. Golden retriever was the most common breed (n=8). The other breeds were Pembroke Welsh corgi (n=6), beagle (n=5), miniature dachshund (n=3), Siberian Husky (n=3), Shetland Sheepdog (n=3), Shiha (n=2), Shih Tzu (n=2), Labrador retriever (n=1), Hokkaido (n=1), Papillon (n=1), miniature schnauzer (n=1), Maltese (n=1), Pomeranian (n=1) and mixed-breed dog (n=5). The median and mean for the total survivin IHC score were 4 (range of 0 to 12). 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.

irradiation with the mandibular lymph nodes.

Histopathological examination revealed 22 dogs diagnosed with adenocarcinomas (ADC), 12 dogs diagnosed with undifferentiated carcinomas (CA) and 9 dogs diagnosed with squamous cell carcinomas (SCC). Twenty-seven dogs were determined to have partial response (PR) (15 ADC, 10 CA and 2 SCC), and sixteen dogs had a stable disease (SD) in response to radiation therapy (7 ADC, 2 CA and 7 SCC). The overall response rate was 62.8% (27/43) in dogs with nasal carcinomas; the response rates of dogs with ADC, CA and SCC were 68.2% (15/22), 83.3% (10/12) and 22.2% (2/9), respectively.

Apoptosis assay and Immunohistochemistry staining: Thirty-three paraffin-embedded blocks were available for performing TUNEL assays and immunohistochemistry staining (included 16 ADCs, 10 CAs and 7 SCCs). AI ranged from 0 to 0.7% in all tumors. The median of the AI was 0.25%. In all tumor types, the group of PR samples showed no significant differences in AI from the group of SD samples (mean of the AI: 0.32% versus 0.22%; P=0.142). Regarding ADCs, the patients with a PR to radiation showed a significantly higher AI than those with an SD (mean of the AI: 0.34% versus 0.11%; P=0.012). However, there were no significant differences in the CA or SCC samples (Table 2). The PI ranged from 0.8 to 77%. The median of the PI was 21.3%. There was no significant difference between the partial response and stable-disease samples (the mean of the PI: 25.1% versus 23.9%; P=0.336), and there were no differences in the different nasal tumor types (Table 2).

The expression of survivin was localized in the nucleus and/or cytoplasm of tumor cells (Fig. 1). Eighteen dogs (54.6%) were survivin positive in the nucleus, and 22 dogs (66.7%) were positive in the cytoplasm. Twenty-eight dogs (84.9%) 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 3. 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 4.5. Eighteen dogs showed high survivin expression (survivin IHC score >3). High survivin expression was significantly associated with an advanced clinical stage (T3+T4) of

| Response to RT | Number of tumors | Indices (mean ± SD) |
|---------------|-----------------|---------------------|
|               |                 | AI                  | PI                  |
| Partial response | 19              | 0.32 ± 0.19         | 25.1 ± 18.9        |
| ADC            | 10              | 0.34 ± 0.20         | 26 ± 20             |
| CA             | 8               | 0.29 ± 0.20         | 23.5 ± 19.9        |
| SCC            | 1               | 0.3                | 28.7                |
| Stable disease | 14              | 0.22 ± 0.20         | 23.9 ± 20.9        |
| ADC            | 6               | 0.11 ± 0.12         | 13.6 ± 8.4         |
| CA             | 2               | 0.3 ± 0.28          | 29 ± 1.1           |
| SCC            | 6               | 0.32 ± 0.24         | 32.4 ± 29          |

RT: Radiation therapy. ADC: Adenocarcinoma. CA: Undifferentiated carcinoma. SCC: Squamous cell carcinoma.
Moreover, high survivin expression was also significantly associated with an SD in response to treatment in canine nasal carcinomas \((P=0.026, \text{Table 4})\). The distribution of survivin in the nucleus or cytoplasm was not associated with the AI or PI (Table 5). The IHC score for survivin had a significant negative correlation with the AI in canine nasal carcinomas \((r=-0.388; P=0.026)\).

**Analysis of survival time:** The median survival time of all 43 dogs with nasal carcinomas was 235 days (Table 6). The dogs with SCCs had the shortest MST (179 days), compared with the MSTs for CAs (203 days) and ADCs (242 days), although a significant difference was not observed between dogs with SCCs and ADCs \((P=0.102)\). Dogs with a PR and SD to radiation therapy had MSTs of 249 and 173 days. Dogs with a PR were significantly correlated with longer survival times than those with an SD \((P=0.016)\). The respective MSTs in the high-AI and low-AI dogs were 275 days and 192 days, but the difference was not significant \((P=0.056)\). The PI was not significantly related to survival time in this study \((P=0.211)\). However, combining the two factors, the dogs with the favorable factors of a high AI and low PI had a significantly longer MST than those with the two unfavorable factors of a low AI and high PI \((P=0.012)\). The dogs with a low IHC score for survivin had a better survival time than the dogs with a high IHC score \((P=0.031; \text{Fig. 2})\). The dogs positive for survivin in the nucleus had a significantly shorter MST \((P=0.034)\), but there was no significant difference between cytoplasmic survivin-positive and survivin-negative dogs \((P=0.356)\).

**DISCUSSION**

Several studies have reported the prognostic factors in canine nasal tumors, including age [13], epistaxis [21],
A longer survival time for dogs with ADCs than those with SCCs and CAs has been reported in previous studies [1, 31]. In our study, the dogs with SCCs had the worst response rate and had the shortest MSTs. SCCs tend to be the most radioresistant among these three types of carcinoma.

One aim of this study was to assess the outcome of radiation therapy using IHC. IHC has not been widely used to predict the response to radiation therapy in veterinary medicine. One study used COX-2 expression in relation to outcome in dogs with nasal carcinomas treated with hypofractionated radiotherapy. There was no significant association between survival and COX-2 expression in that study [3]. Ki-67 is a proliferation marker generally used to assess malignancy and prognosis in several tumors in humans and dogs [4, 18, 26]. Low levels of Ki-67 have been associated with 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. Several studies have concluded that high levels of Ki-67 yielded a better prognosis in radiation [6, 12, 16]. However, despite the tendency for a high PI to be correlated with poor survival in dogs with nasal carcinomas, there were no significant differences in the final results in our study. In addition, no difference was observed in PI between good and poor responses to treatment in canine nasal carcinomas. Additional studies of Ki-67 in a larger cohort of dogs with nasal carcinomas are therefore necessary.

The relationship between apoptosis and the outcome of radiation has been studied in several cancers in human medicine. High apoptosis rates have a strong relation to good outcomes in patients treated with radiation therapy [2, 27, 33]. In our study, although there were no statistically significant correlations, a higher AI had a correlation with a good response to radiation therapy and a longer survival time in canine nasal carcinomas. Our study also found that the combination of the AI and PI was useful for predicting prognosis in canine nasal carcinomas. The combination of the two favorable markers of a high AI and low PI was significantly correlated with survival, as evidenced by comparison with the combination of the two unfavorable markers.

A few studies on human cervical cancer have reported that different tumor types have different relationships with the levels of apoptosis and prognosis. In squamous cell carcinoma of the cervix, highly apoptotic regions are correlated with hypoxic areas; apoptosis reflects the level of hypoxia. Therefore, a high AI indicates high radiosensitivity and thus a good prognosis in adenocarcinomas of the cervix [27]. In our study of canine nasal carcinoma, we found that the dogs with ADCs with...
a good response (PR group) showed a significantly higher spontaneous apoptosis rate than those with a poor response (SD group), but there was no association for undifferentiated carcinomas or squamous cell carcinomas. Therefore, the pretreatment apoptosis rate should be considered for determining prognosis in canine nasal adenocarcinoma.

Survivin is expressed in two different locations, the nucleus and the cytoplasm, in tumor cells. Additionally, the different locations of survivin are related to different functions [9, 36]. Expression of survivin in the nucleus is associated with cell proliferation, but expression in the cytoplasm is correlated with apoptosis inhibition and the control of cellular survival. In the nucleus, survivin binds to the microtubules of the mitotic spindle during the G2/M phase of the cell cycle. In the cytoplasm, 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 [9]. In our study, 54.6% of the dogs with nasal carcinomas were found to be positive for the expression of nuclear survivin, and 66.7% of the dogs were positive for cytoplasmic survivin. A relationship between the expression of survivin in the nucleus and poor survival in canine nasal carcinoma.

### Table 6. Survival time in dogs with nasal carcinoma

|                        | Number of cases | Survival time | P value  |
|------------------------|-----------------|---------------|----------|
| **Tumor type**         |                 | Median (range), days |          |
| ADC                    | 22              | 242 (75–1979) | *P* = 0.102 |
| CA                     | 12              | 203 (40–1558) |          |
| SCC                    | 9               | 179 (124–302) |          |
| **Tumor stage**        |                 |               |          |
| T1+T2                  | 13              | 443 (102–1979) | *P* = 0.004 |
| T3                     | 12              | 262 (173–1641) |          |
| T4                     | 18              | 111 (40–639)  |          |
| **Response to RT**     |                 |               |          |
| PR                     | 27              | 249 (40–1979) | *P* = 0.016 |
| SD                     | 16              | 173 (83–678)  |          |
| **AI**                 |                 |               |          |
| <Median                | 17              | 192 (40–871)  | *P* = 0.056 |
| >Median                | 16              | 275 (104–1979) |          |
| **PI**                 |                 |               |          |
| <Median                | 16              | 254 (74–1979) | *P* = 0.211 |
| ≥Median                | 17              | 192 (40–871)  |          |
| **AI and PI**          |                 |               |          |
| Low AI and high PI     | 6               | 113 (40–639)  | *P* = 0.012 |
| All others             | 18              | 223 (74–720)  |          |
| High AI and low PI     | 9               | 625 (147–1979) |          |
| **Survivin (IHC score)**|               |               |          |
| Low                    | 15              | 261 (111–1979) | *P* = 0.031 |
| High                   | 18              | 182 (40–871)  |          |
| nSurvivin              |                 |               |          |
| Positive               | 18              | 182 (40–871)  | *P* = 0.034 |
| Negative               | 15              | 262 (111–1979) |          |
| cSurvivin              |                 |               |          |
| Positive               | 22              | 223 (102–871) | *P* = 0.356 |
| Negative               | 11              | 249 (40–1979)  |          |

Fig. 2. Kaplan-Meier curves for canine nasal carcinomas with high survivin expression (IHC score >3) and low survivin expression (IHC score ≤3). Dogs with low survivin expression had a longer survival time than those with high survivin expression (*P* = 0.031).
was observed. However, cytoplasmic expression of survivin had no correlation with survival time. In our study, although expression of survivin in the nucleus or cytoplasm was not associated with the AI or PI, there was a weak but significant negative correlation between the total survivin IHC score and AI (r = −0.388; P=0.026). This result indicates that survivin may be related to apoptosis inhibition in canine nasal carcinoma. Overexpression of survivin was observed in the advanced clinical stage and poor response to radiation in dogs with nasal carcinoma. Therefore, survivin overexpression is also suggested to be an unfavorable prognostic factor for predicting the outcome of treatment with radiation.

Currently, survivin is considered to be a new target for cancer therapy. Some studies have demonstrated that YM155, a small molecule suppressant of survivin, has antitumor activity in vitro and in vivo [8, 17]. A single agent of survivin inhibition has been found to induce apoptosis in tumor cells, and in combination with a chemotherapeutic agent, it can enhance the chemotherapeutic effect in human malignant melanoma [34]. One other study showed that YM155 also increased the sensitivity of tumor cells to radiation in vitro and in vivo [8]. YM155 has currently been studied in phase II clinical trials in human cancers [14].

Regarding the limitations of our study, due to it being a retrospective study, all the treatment schemes were heterogeneous. The total radiation dose varied, and in some cases, treatment had to be discontinued, because of severe acute toxicity. However, the total radiation dose was not significantly related with treatment response (P=0.397). Also, the different treatment schemes did not affect survival in our study (P=0.284). Although survivin expression was associated with prognosis, the apoptosis and proliferation indices were not found to be predictable values in our study. Small biopsy specimens taken from a large tumor might not be representative of the whole tumor, stated in previous studies [3]. An additional study with a larger cohort of canine nasal carcinomas may be necessary.

In conclusion, a number of studies have reported that the expression of survivin is related to malignancy and poor prognosis in canine tumors [20, 22, 26, 28]. Similarly, survivin expression has the ability to reflect malignancy and predict prognosis in these tumors. One study of the enhanced effect of chemotherapeutic drugs in combination with survivin inhibition in a canine osteosarcoma cell line has been reported [28]. Although our study was targeted at a limited population, overexpression of survivin was shown to be an unfavorable prognostic factor in dogs with nasal carcinomas. A study of the efficacy of radiation therapy combined with an inhibitor of survivin in veterinary medicine is needed, especially in advanced-stage tumors and those that often have a poor response to radiation therapy.

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