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Canine oral malignant melanoma:

evaluation of radiotherapy and profiling of plasma metabolites

using gas chromatography mass spectrometry

犬の口腔内悪性メラノーマ：放射線治療の評価とガスクロマトグラフィー

マススペクトロメトリーによる血漿中代謝物プロファイリング解析

川部 美史

2015年
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Introduction

Oral malignant melanoma is one of the most common and aggressive tumors in dogs (Bradley et al., 1984; Smith et al., 2002; Todoroff and Brodey, 1979). Canine oral malignant melanoma carries a poor prognosis due to its rapid invasion of the surrounding normal tissue and the high likelihood of regional and distant metastasis early in the course of the disease (Bolon et al., 1990; Harvey et al., 1981).

Effective chemotherapy or immunotherapy protocols are in demand for the adjunctive management of dogs with oral malignant melanoma because of the high metastatic risk. Unfortunately, most oral melanoma in dogs is relatively resistant to standard chemotherapy (Withrow et al., 2012). The vaccine which safety and efficacy as adjunctive treatment was reported got approval of the United States FDA, and was commercialized for the first time in the world (Grosenbaugh et al., 2011). However, in a recent report, dogs that received the vaccine did not achieve a greater progression-free survival, disease-free interval or median survival time than dogs that did not receive the vaccine (Ottnod et al., 2013). Further consideration will be desired about its effect.

Surgery and radiotherapy are the most common treatments used to locally control oral tumors. The reported median survival time (MST) for untreated dogs with oral malignant melanoma is 65 days (Harvey et al., 1981). In comparison, the MST for dogs treated by surgery ranges from 9.1 to 9.9 months (Kosovsky et al., 1991; MacEwen et al., 1986; Wallace et al., 1992),
and in dogs treated by radiation therapy alone or in combination with surgery, the MST ranges from 7.0 to 7.9 months (Bateman et al., 1994; Proulx et al., 2003). Due to these reports of extended survival times, aggressive surgery is the first choice for local treatment of oral malignant melanoma (Bergman, 2007). However, a variety of complications such as difficulty eating or esthetic changes may occur after surgery (Fox et al., 1997).

Oral melanoma responds to hypofractionated radiotherapy in both humans and dogs (Bateman et al., 1994; Freeman et al., 2003; Overgaard et al., 1986; Theon et al., 1997). In the report which compared palliative radiation therapy (coarse fractions; 9 to 10 Gy weekly fractions to a total dose of 30 to 36 Gy) with conventional radiation therapy (2 to 4 Gy fractions to as high as 45 Gy or more, with or without surgery and/or chemotherapy) for canine oral malignant melanoma, differences could not be discerned (Proulx et al., 2003). Additionally, several prognostic factors that affect radiotherapy outcomes have been identified. Proulx et al. reported that the overall survival time was longer in dogs without radiologic evidence of bone destruction than in dogs with radiographically evident bone changes (Proulx et al., 2003). Blackwood et al. also found that dogs with tumors measuring less than 5 cm³ in volume were more likely to achieve a complete response, and they found that tumor volume affected MST; the MST was 86 weeks in dogs with tumors measuring less than 5 cm³, 16 weeks in dogs with tumors between 5 and 15 cm³, and 20.5 weeks in dogs with tumors greater than 15 cm³ in size (Blackwood and Dobson, 1996). Although Bateman et al. did not find any association between the clinical stage of cancer and the response to radiotherapy or survival time (Bateman et al., 1994), the study lacked the number of cases required to
make a definitive determination.

In surgery, some reports showed that MST in canine oral malignant melanoma changes according to stage and becomes shorter in higher stages (Hahn et al., 1994; MacEwen et al., 1986). Early detection of oral malignant melanoma is desirable but difficult, since there are few opportunities to check the inside of the mouth. In addition, although oral lesion can be checked with the naked eye, it is still more difficult to detect a metastatic focus. Systemic imaging method (ex. radiograph, ultrasound or computed tomography) may be required for the detection of metastasis as well as an ocular inspection or palpation, since melanoma may metastasize to the abdominal lymph nodes, liver, adrenal glands, and other sites as well as lung (Withrow et al., 2012). Furthermore, Williams and Packer reported that approximately 70% of dogs with oral malignant melanoma had local lymph node metastasis when lymphadenomegaly was present, and approximately 40% had local lymph node metastasis when no lymphadenomegaly was present (Williams and Packer, 2003). Development of the less-invasive biomarker which can evaluate a condition of dogs is expected, since more aggressive examination (ex. CT or biopsy under anesthesia) may not be desired for dogs with advanced age or advanced disease.

A biomarker is a measurable indicator of the severity or presence of some disease state, and can be used as an indicator of a particular disease state. Serum metabolomics analysis has been shown to be useful to identify potential biomarkers of colorectal cancer, pancreatic cancer, and gastric cancer (Kobayashi et al., 2013; Nishiumi et al., 2012; Song et al., 2012). The concept of metabolomics was recently introduced as the global analysis of all
metabolites in a sample (Weckwerth and Morgenthal, 2005). Metabolomic investigations attempt to detect and profile changes in metabolites, which reflect changes in metabolic pathways and may provide information concerning a disease state or the biological stress of an organism (Bando et al., 2010).

In the past few years metabolomics has combined data-rich advanced analytical techniques such as mass spectrometry with multivariate statistical analysis. Gas chromatography with mass spectrometry (GC-MS) provides high separation efficiency for resolving complex biological mixtures. It has been indicated that metabolites profile with GC-MS may be useful tool for detection of potential biomarker and diagnosis of canine lymphoma and of canine epilepsy (Hasegawa et al., 2014; Tamai et al., 2014).

In chapter 1, we evaluated the clinical outcomes of dogs with oral malignant melanoma treated by radiotherapy with or without cytoreductive surgery according to World Health Organization (WHO) staging guidelines, and examined the utility of radiotherapy in these cases. And in chapter 2, we used GC-MS to examine metabolites profiles in serum from dogs with oral malignant melanoma with the goal of identifying biomarkers for diagnosis of canine oral malignant melanoma and for prediction of its prognosis.
**List of abbreviations**

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<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>COX-2</td>
<td>cyclooxygenase-2</td>
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<tr>
<td>CR</td>
<td>complete response</td>
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<td>CT</td>
<td>computed tomography</td>
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<td>CTV</td>
<td>clinical target volume</td>
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<td>GC-MS</td>
<td>gas chromatography with mass spectrometry</td>
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<td>GTV</td>
<td>gross tumor volume</td>
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<td>MRI</td>
<td>magnetic resonance imaging</td>
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<tr>
<td>MST</td>
<td>median survival time</td>
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<tr>
<td>MVX</td>
<td>megavoltage X-ray</td>
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<tr>
<td>OPLS-DA</td>
<td>orthogonal projection to latent structure with discriminant analysis</td>
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<tr>
<td>OVX</td>
<td>orthovoltage X-ray</td>
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<tr>
<td>PD</td>
<td>progressive disease</td>
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<td>PR</td>
<td>partial response</td>
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<td>QOL</td>
<td>quality of life</td>
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<td>RECIST</td>
<td>response evaluation criteria in solid tumors</td>
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<td>SD</td>
<td>stable disease</td>
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<td>WHO</td>
<td>World Health Organization</td>
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Chapter 1: Evaluation of radiotherapy for canine oral malignant melanoma

It was shown that oral malignant melanoma responds to hypofractionated radiotherapy in dogs (Bateman et al., 1994; Freeman et al., 2003; Theon et al., 1997). However, the information is not enough, and the survival time of every stage is not compared, either. Although aggressive surgery is the first choice for local treatment of oral malignant melanoma (Bergman, 2007), a variety of complications such as difficulty eating or esthetic changes may occur after surgery (Fox et al., 1997). If the MSTs for dogs treated by surgery and by radiation therapy are equal or the latter is longer than the former, there is a possibility that the range of choices for treatment is extended.

In the present chapter, I evaluated the clinical outcomes of dogs with oral malignant melanoma treated by hypofractionated radiotherapy with or without cytoreductive surgery according to World Health Organization staging guidelines, and examined the utility of radiotherapy in these cases.
Section 1: Materials and Methods

Case selection

The study included dogs treated by radiotherapy for spontaneous oral malignant melanoma at the Animal Medical Center of Gifu University between July 2006 and December 2012. Case records that did not include the date of death were excluded.

All tumor specimens were evaluated by board-certified veterinary pathologists who had 10 or more years of experience. All lesions were diagnosed as malignant melanoma, although the actual biopsy specimens and slides were not examined for this study. Dogs that underwent radiotherapy with or without cytoreductive surgery and/or chemotherapy were included. In order to appropriately evaluate the efficacy and tolerability of radiotherapy and to compare outcomes from past surgeries, the records of dogs that underwent extensive surgery to obtain a radical cure (e.g., mandibulectomy or maxillectomy) were excluded from the analysis.

Medical records review

We reviewed the medical records of dogs that met the inclusion criteria. Long-term post-treatment outcomes were obtained from the medical records or by telephone interviews with the owners.

Procedures

All dogs underwent CT (Asteion™ Super4 Edition, Toshiba Medical Systems, Tokyo, Japan) just prior to the first radiotherapy treatment in order to stage the tumor and plan treatment. The mandibular lymph nodes, retropharyngeal lymph nodes, and entire thorax were imaged. Biopsy specimens were collected after the CT, and the tumors were classified as
stage I, II, III, or IV according to WHO staging guidelines (Table 1). Any enlarged local lymph nodes were also biopsied; normal sized lymph nodes were not evaluated.

All dogs underwent radiotherapy delivered with Radioflex 300EMG (Rigaku Corporation, Tokyo, Japan), which had an X-ray energy output of 300 kV, or PRIMUS Mid-Energy (Toshiba Medical Systems, Tokyo, Japan), which had an X-ray energy output of 4 MV. The radiation beam provided orthovoltage X-ray (OVX), megavoltage X-ray (MVX), or electron radiotherapy. Each radiotherapy treatment was performed as follows. OVX: The treatment position varied according to the location of the tumor. The exposure field was positioned such that the entire gross tumor volume (GTV) was contained within the irradiation range. Planning target volume margins were contoured greater than 0.5 cm around the GTV to include regions at risk for microscopic disease extension. Treatment beams were delivered from one or two directions while avoiding organs at risk when feasible, such as the eyes and brain. The rated output was 300 kV, the dose prescription was 6.3 to 10.0 Gy per fraction in 4–6 fractions for a total dose of 37.8 to 40 Gy, and irradiations were carried out at 7–10 day intervals. MVX: Planning CT scans were performed for each patient in the treatment position (sternal recumbency) with a bite block system. Individualized treatment plans were constructed using a 3-dimensional CT-based computer generated treatment planning system, XiO version 4.6 (Elekta AB, Stockholm, Sweden). Planning target volume margins were contoured to include regions at risk for microscopic disease extension and ranged from 0.3 to 1.0 cm around the GTV. In dogs that underwent debulking surgery before radiotherapy, the clinical
target volume (CTV) was contoured based on the location and extent of the tumor. The planning target volume margins were contoured from 0.3 to 1.0 cm around the CTV. Adjacent critical normal tissues or organs at risk were also contoured on the CT images. A multileaf collimator was used on the linear accelerator to shape the fields exposing the target volume and block the surrounding normal tissues. The isocenter and beam arrangements for each plan were determined by the locations of the tumors and adjacent critical normal structures. Treatments were delivered in five to nine unequally weighted fields in multiple oblique beam arrangements. The energy output of the X-ray beam was 4 MV. The dose prescription was 6.0–10.0 Gy per fraction in 4–8 fractions for a total dose of 40–50 Gy, and irradiations were carried out at 7–10 day intervals. Portal imaging was performed during the initial setup to ensure an accurate in patient position. Port films and orthogonal port films were acquired before the first treatment to assist in the setup. Electron radiotherapy: The treatment position varied with the location of the tumor. The exposure field was positioned such that the entire GTV was contained within the radiation treatment field. The planning target volume margins were contoured greater than 0.5 cm around the GTV. Treatment beams were delivered from one direction, and the electron energy was 3 MeV. The treatment dose was 6.0 Gy for a total dose of 36 Gy, and radiation treatments were carried out at 7-day intervals.

The therapeutic response was evaluated within 1 month after final radiotherapy treatment. Primary tumor measurements were performed using appropriate methods (i.e., CT, palpation, and visual examination). The therapeutic response was classified according to the canine RECIST
guidelines described by the Veterinary Cooperative Oncology Group (Nguyen et al., 2013). A complete response was defined as the disappearance of all target lesions. A partial response was defined as an at least 30% reduction in the sum of diameters of the target lesions compared to the baseline sum. Stable disease was defined as a less than 30% reduction or as a 20% increase in the sum of diameters of the target lesions, with the smallest sum of the diameters during the study designated as the reference. Progressive disease was defined as either the appearance of one or more new lesions or an at least 20% increase in the sum of diameters of the target lesions, with the smallest sum during the study designated as the reference; the sum must also have increased by at least 5 mm.

Radiation-induced tissue damage was assessed and characterized using the radiation morbidity scoring scheme described by the Veterinary Radiation Therapy Oncology Group (Ladue et al., 2001). Serial physical examinations were performed for all dogs during weekly treatments and at follow-up visits in order to assess radiation damage, and the scores were assessed retrospectively based on findings in the medical records.

**Statistical analyses**

Survival time was defined as the time between the first radiation treatment and the date of death. Survival curves were generated for all the dogs using the Kaplan–Meier method. Surviving cases at the end of the investigation period were treated as censored data. The effects of clinical stage were examined using the log-rank tests to detect any significant differences between the curves. The difference between each stage was evaluated by Tukey’s multiple comparisons test. The difference in effect
between OVX and MVX treatments was evaluated using the log-rank test. In addition, the difference between OVX and MVX in the median total dose was examined using the Mann Whitney test. Chi-squared tests were used to examine differences in sex, the therapeutic response, and side effects. These analyses were performed with GraphPad Prism 6 for Windows version 6.01 (GraphPad Prism Software Inc., La Jolla, CA, USA); P < 0.05 was considered significant.

The interaction of treatment and survival was evaluated using the Cox proportional hazards model. The breed, weight, sex, age, clinical stage, radiation technique (MVX and OVX), and radiation dose were included in the multivariate analysis. These analyses were performed using JMP 10.0.0 (SAS Institute Inc., Cary, NC, USA); P < 0.05 was considered significant.
Section 2: Results

A total 157 dogs were treated by radiotherapy. Of these, 111 dogs were included in the study; 45 dogs with an unknown date of death and one dog that received extensive surgery were excluded from the analysis. Twenty-four breeds were represented. Mixed breeds (22 dogs; 19.8%), Miniature Dachshunds (17 dogs; 15.3%), and Labrador Retrievers (10 dogs; 9.0%) were the most commonly affected. Other represented breeds were as follows: Shih Tzu (8 dogs), Shiba Inu (8), Beagle (7), Golden Retriever (6), Yorkshire Terrier (6), Toy Poodle (4), Pomeranian (3), German Shepherd (2), Chihuahua (2), Pug (2), Flat-Coated Retriever (2), Miniature Schnauzer (2), American Cocker Spaniel (2), Shetland Sheepdog (1), Scottish Terrier (1), Dalmatian (1), Bernese Mountain Dog (1), Basset Hound (1), Papillon (1), Miniature Pinscher (1), and Akita (1). The median weight of the dogs was 9.8 kg (range, 2.5–52 kg), and the median age of the dogs at first visit was 12.2 years (range, 3–17 years). There were 36 sexually intact females, 16 spayed females, 45 sexually intact males, and 14 castrated males in the study population. There was no significant difference observed between female and male dogs (P = 0.64), but a significant difference was observed between intact and fixed (spayed and castrated) dogs (P < 0.001). There were no significant differences in the clinical stage of disease according to weight, age, or sex (Table 2). Metastasis to the mandibular lymph node was confirmed by biopsy in 39 dogs, and pulmonary metastasis was confirmed by imaging in 31 dogs.

OVX was used in 68 dogs, MVX in 39 dogs, and electron radiotherapy in four dogs. Five of 39 dogs that underwent MVX treatment first received OVX,
but only for the first treatment. These five dogs were switched from OVX to MVX for the following reasons: insufficient time to plan the first treatment (3 dogs); mechanical problems during the first treatment (1 dog); and owner’s request (1 dog). Irradiation treatment was discontinued mid-course in 31 of 111 dogs because they either died (8 dogs), their clinical condition deteriorated (8 dogs), or at the request of the owner (15 dogs). In all seven cases of stage I and II melanoma, treatment was discontinued at the owner's request (7/7, 100%). The therapeutic response in most of these cases was CR or PR (4/7, 57%) but was unknown in the remaining three dogs. In contrast, in stage III and IV cases, the most common reason for discontinuation was deterioration of the dog’s condition or death (16/24, 67%). The median total radiation dose was 37.8 Gy (range, 6.3–74.3 Gy). In one geriatric dog (age 15 years), lymph node metastasis was confirmed at the initial physical examination; the patient was not expected to survive long-term was thus administered over 70 Gy of radiation. A significant difference was observed between the OVX and MVX median total doses (31.5 Gy versus 49.0 Gy, respectively; \( P < 0.0001 \)).

Debulking surgery (45 dogs), chemotherapy (local only, 26 dogs; systemic only, 26 dogs; local and systemic, 14 dogs; total, 66 dogs), COX-2 inhibitor drugs (firocoxib, 5 mg/kg p.o. SID; 29 dogs), corticosteroids (prednisolone, 0.5–1.0 mg/kg p.o. SID; 29 dogs), mebendazole (5 mg/kg p.o. SID; 5 dogs), and hyperthermia (1 dog) were used as adjunct treatments. In 27 dogs, both debulking surgery and chemotherapy were used. In seven dogs that underwent debulking surgery, there were no macroscopically measurable lesions at the time of radiation therapy. All seven dogs were
classified as stage I prior to resection. Cisplatin (0.5 mg/head/treatment; 0.5 mg/mL cisplatin solution injected directly into the tumor every 1 or 2 weeks) was used for local chemotherapy, and carboplatin (180–250 mg/m² i.v. every 3 weeks) was used for systemic chemotherapy. The number of dogs administered chemotherapy according to clinical stage was shown in Table 3. Local chemotherapy was administered in combination with radiotherapy in two dogs, and was administered after radiotherapy was complete in 38 dogs. Systemic chemotherapy was administered concurrently with radiotherapy in 32 dogs, and administered after radiotherapy was complete in eight dogs.

The anatomic locations included 40 maxillary, 38 mandibular, 11 lip, 11 buccal mucosa, 4 hard palate, 4 soft palate, and 3 tongue tumors. The survival curves did not vary significantly between the various tumor sites (P = 0.16, Figure 1). The survival curves did not vary significantly between 29 rostral and 9 caudal maxillary cases (P = 0.20), and between 29 rostral and 5 caudal mandibular cases (P = 0.51). Six dogs were excluded from the analysis because their tumors were too large to classify as rostral or caudal.

Of the 111 dogs treated with radiotherapy, 19 were classified as stage I, 24 as stage II, 37 as stage III, and 31 as stage IV. The MST for stages I, II, III, and IV were 758 days, 278 days, 163 days, and 80 days, respectively. The survival times were significantly different between the stages (P < 0.0001, Figure 2). The MST for all 111 dogs was 171 days (5.7 months; range, 3–1620 days). Significant differences were observed between stages I and II (P < 0.05), stages I and III (P < 0.001), and stages I and IV (P < 0.01). There was no significant difference observed between stages II and III (P = 0.83), stages II and IV (P = 0.90), and stages III and IV (P = 1.0).
Fifteen dogs were still alive at study completion (stage I, 8 dogs [1620, 1159, 675, 598, 577, 468, 402, and 339 days]; stage II, 3 dogs [966, 769, and 479 days]; stage III, 2 dogs [424 and 392 days]; and stage IV, 2 dogs [343 and 312 days]). Of the 96 dogs that died, three were euthanized, 66 dogs died from melanoma, and 11 dogs died from other conditions (i.e., other tumors or unrelated disease); the cause of death was unknown in 16 dogs. The cause of death was confirmed as melanoma in 69 dogs, including the three euthanized dogs, and the mechanism of death included pulmonary metastasis, brain metastasis, anemia resulting from tumor hemorrhage, and respiratory failure from physical obstruction. Unfortunately, exact numbers could not be determined because many case records did not include detailed information on the cause of death.

The MSTs of the 107 dogs that received OVX (68 dogs) and MVX (39 dogs) were 121.5 days (range, 11–1620 days) and 233 days (range, 3–966 days), respectively, and a significant difference was observed between the two treatment groups (P < 0.05, Figure 3). When OVX and MVX were compared according to stage, there was no significant difference observed between stage I (997 days [12 dogs] versus 934 days [6 dogs]), stage II (246 days [12 dogs] versus 258.5 days [10 dogs]), or stage IV (78.5 days [24 dogs] versus 97 days [7 dogs]). However, the MST values differed significantly different in cases of stage III disease (OVX, 98.5 days [20 dogs] versus MVX, 209.5 days [16 dogs], P < 0.01). When the influence on MST of clinical stage, radiation energy, and radiation dose was evaluated, a significant difference was observed according to clinical stage (P < 0.0001), but no significant difference was observed according to breed, weight, sex, age, radiation
energy, and radiation dose (P = 0.08, P = 0.60, P = 0.45, P = 0.07, P = 0.37 and P = 0.09, respectively). In stage III cases, a significant difference was observed between OVX and MVX (risk ratio = 2.91; P < 0.05), but there was no significant difference according to the total radiation dose (P = 0.79). The results were essentially unchanged when the five dogs that underwent OVX initially followed by MVX were excluded from the analysis. When these five dogs were excluded, the MSTs for OVX (68 dogs) and MVX (34 dogs) were 121.5 days and 214.5 days, respectively, and a significant difference was observed between the groups (P < 0.05). The MSTs according to stage were as follows: stage I (OVX, 997 days [12 dogs] versus MVX, 934 days [5 dogs]); stage II (246 days [12 dogs] versus 233 days [9 dogs]); stage III (98.5 days [20 dogs] versus 187 days [13 dogs], P < 0.01); and stage IV (78.5 days [24 dogs] versus 97 days [7 dogs]).

The therapeutic response was able to be evaluated in 87 dogs. A complete response (CR) was observed in 38 dogs (43.7%), partial response (PR) in 36 (41.4%), stable disease (SD) in 7 (8.0%), and progressive disease (PD) in 6 (6.9%). A significant difference was observed according to therapeutic response (P < 0.0001). Unfortunately, the response rate could not be evaluated in all 111 dogs because some patients did not visit the hospital after radiotherapy (15 dogs) or underwent partial resection during radiotherapy (9 dogs).

Acute radiation damage was indicated in the medical records and scored as follows: score 1 in 50 dogs (hair loss, 21; dermatitis, 11; skin pigmentation, 10; and conjunctivitis, 8); score 2 in 36 dogs (stomatitis, 23; keratoconjunctivitis, 9; corneal ulcer, 2; epidermal hypertrophy, 1; and
keratoconjunctivitis sicca, 1); and score 3 in 9 dogs (cutaneous necrosis, 4 [OVX, 2; MVX, 2]; cutaneous edema, 2 [OVX, 1; MVX, 1]; necrosis of the oral mucosa, 2 [OVX, 1; MVX, 1]; and cutaneous ulcer, 1 [MVX]). When the acute damage was classified according to the radiation type, scores of 1, 2, and 3 were observed in 18, 14, and 4 dogs that underwent OVX, respectively. Acute damage was scored as 1, 2, and 3 in 31, 21, and 5 dogs that underwent MVX, respectively. The acute radiation damage was scored as 1 and 2 in two dogs that underwent electron radiotherapy. When classified according to the affected site, the skin, oral cavity, and eye were involved in 19, 12, and 5 dogs that underwent OVX, respectively, and in 30, 12, and 15 dogs that underwent MVX, respectively. In the two dogs that underwent electron radiotherapy, acute damage occurred in the skin and oral cavity. Late radiation-induced damage, which is scored as 2 or 3, was not observed. The following serious side effects resulted from tumor reduction in dogs with large tumors: oronasal fistula (6 dogs, 5.4%), jaw fracture (2 dogs, 1.8%), and trismus (1 dog, 0.9%). In all six dogs with oronasal fistula, the lesion occurred in the hard or soft palate region surrounding the tumor. In the two dogs with jaw fracture, the fracture site displayed osteolysis before radiotherapy. In the dog with trismus, the original tumor encompassed a large area of the left cheek, and the volume of muscle in the area was markedly decreased after radiotherapy. Globe rupture occurred in a dog with a tumor that invaded the orbital cavity (0.9%).

Thirty-four of 66 dogs that received chemotherapy as an adjunct treatment experienced side effects (local only, 10/26 dogs; systemic only, 14/26 dogs; local and systemic, 10/14 dogs; total, 34/66 dogs). Side effects
were observed in 15 of 45 dogs that did not receive chemotherapy. There was no significant difference according to chemotherapy use (P = 0.73).
Section 3: Discussion

In our study, oral malignant melanomas was nearly equally distributed according to sex, although slightly fewer females (n = 52; 47%) than males (n = 59; 53%) were represented. This result is consistent with findings in some reports, but contradicts most reports of oral melanoma, which find that males are significantly overrepresented (Borthwick et al., 1982; Brodey, 1960; Hoyt and Withrow, 1984; Prier and Brodey, 1963; Ramos-Vara et al., 2000; Todoroff and Brodey, 1979). The present study also included a large number of intact dogs (female, 36/52 dogs, 69%; male, 45/59, 76%), which may reflect the low rate of spaying and neutering of dogs in Japan.

This study shows that the MST in canine oral malignant melanoma differs significantly according to the stage and becomes shorter in higher stages. The results are similar to those reported after surgical treatment (Hahn et al., 1994; MacEwen et al., 1986). This particular staging system may be effective for predicting the radiotherapy prognosis in cases of oral malignant melanomas. Bergman stated that established therapeutic approaches such as surgery, chemotherapy, and fractionated radiation therapy are not curative after metastasis in canine malignant melanoma (Bergman, 2007). The reduction in survival time with increasing stage may be related to the existence of metastasis; however, because information on the cause of death and the number of dogs dying from metastasis was insufficient, we were unable to clarify this further in the present report.

In this study, irradiation treatment was discontinued mid-course in 31 of 111 dogs. In stage I and II cases, the owner may have believed that the dog was cured and wished to discontinue the therapy as most of these cases
achieved CR or PR. In stage III and IV cases, radiotherapy may have been performed in order to improve the quality of life (QOL) despite the serious illness as euthanasia tends to be avoided in Japan.

In one report, stage I dogs treated with surgery had a MST of 559 days (n = 21 dogs), whereas stage II and III dogs had an MST of 121 days (n = 26) (MacEwen et al., 1986). In another report, the MSTs of dogs treated with surgery for stage I, II, and III disease were 943 (n = 10), 193 (n = 5), and 161 days (n = 5), respectively (Hahn et al., 1994). In this study, the MSTs following radiotherapy for stages II and III were similar to or longer than these previously reported values. That is, radiotherapy was as effective as surgery for stage II and III disease.

Bateman et al. reported the MST in dogs with oral malignant melanoma treated by radiotherapy was 7.0 months (Bateman et al., 1994), and Proulx et al. reported 7.9 months (Proulx et al., 2003). In our study, the MST for all 111 dogs was 5.7 months, which was shorter than in previous reports. However, the MST changed significantly with the stage of disease. The former study included six dogs each in stages I, II, and III, and in the latter study, the staging was unknown. Because the stage distribution within the populations differed, the MSTs may have also differed from our observations.

In a surgical study, Hahn et al. reported that rostral mandible and caudal maxilla locations had significantly longer survival times than other locations (Hahn et al., 1994). In this study, a difference was not detected in MST according to the primary region. Complete surgical excision may be difficult or may create problematic changes in facial configuration, depending on the primary region. In addition, cases judged unsuitable for surgical treatment
were not included in the present study. Because radiotherapy can be delivered to all locations, MST was not expected to be influenced by the primary region in patients that received radiotherapy. Radiotherapy can be performed on large tumors in the caudal maxilla that are discovered during more advanced disease stages when surgery may not be possible for the tumor. In this scenario, the MST may vary according to stage rather than to the primary region.

The therapeutic responses of the 87 dogs that were evaluated were CR in 43.7% (n = 38), PR in 41.4% (n = 36), stable disease in 8.0% (n = 7), and progressive disease in 6.9% (n = 6). In past reports, CR rates of 51%–69% and PR rates of 25%–31% were achieved, yielding overall response rates of 82%–94% (Bateman et al., 1994; Blackwood and Dobson, 1996; Murphy et al., 2005; Proulx et al., 2003). The overall response rate in this chapter was similar to those observed in earlier reports. Because RECIST was not used to evaluate the response rates in earlier reports, the CR and PR rates may have differed.

A significant difference was observed between the MSTs of dogs receiving OVX versus MVX. When compared according to stage, the data suggest that this difference originated in the stage III case group and likely resulted from differences in radiation energy. In stage I and II cases, because the tumor is small, a sufficient dose can be delivered by an OVX unit. By contrast, in stage III cases, the tumor is large; therefore, a uniform dose distribution cannot be delivered by an OVX unit. In addition, it is difficult to increase the total dose due to potential radiation injury in the surrounding normal tissue, as well as the increased radiation deposition in bone compared
to soft tissue when using OVX radiation therapy. In contrast, MVX can deliver a sufficient dose to a large tumor because the dose distribution is more easily adjusted by computer planning, which may have resulted in the different clinical outcome. In stage IV cases, we hypothesize that the difference in local radiation did not affect the therapeutic outcome as the MST was influenced by metastatic lesions rather than the primary tumor, but this hypothesis could not be confirmed in this study because the cause of death was unclear in many cases.

Radiation toxicity may impact the patient’s QOL. A standardized radiation scoring system for acute and late effects on oral mucosa and bone has been published by the Veterinary Radiation Therapy Oncology Group (Ladue et al., 2001). The hair loss, dermatitis, skin pigmentation, and conjunctivitis observed in this study were given a score of 1. Cutaneous necrosis, cutaneous edema, necrosis of the oral mucosa, and cutaneous ulcers were given scores of 3; these side effects are the most critical and may decrease QOL. Although a score of 1 was recorded in the medical records of 50 cases (45% of the total), I found evidence of this level of damage in all cases. Nine cases were given scores of 3 (8% of the total), and this severity of damage was clearly indicated in the medical records, as it was critical in nature.

When acute radiation damage was classified according to the radiotherapy type, I found that MVX generally resulted in greater damage than did OVX, especially to the eyes. The ocular damage was attributed to the following mechanisms. During OVX, the eye at risk of radiation exposure is protected by a lead shield, and the radiation is directed away from the eyes,
which is relatively simple as the radiation beam is only delivered from one or two directions. By contrast, the exposure to the eyes may be far greater during MVX compared with OVX because a lead shield is not used and the larger or deeper tumors that are typically targeted in MVX require irradiation from many directions, although effort was made to reduce the radiation exposure to the eyes during therapy planning.

In addition to direct damage, secondary radiation damage was also observed. Oral malignant melanomas are locally aggressive and may invade adjacent tissue and bone (Veena et al., 2012). In the two cases of jaw fracture, the fractures were not considered related to the radiotherapy but were instead attributed to bone lysis caused by the tumor beforehand; the fractures occurred in areas of bone lysis that was identified on CT before therapy. Similarly, I observed an oro nasal fistula in a region of normal tissue that was invaded by the tumor before radiotherapy. In one case in which the tumor developed near the eye, the globe ruptured when the dog rubbed the eye forcefully; notably, the eye was initially affected by radiation-induced conjunctivitis. This particular dog was a Shih Tzu and had a history of a descemetocele in the eye. The risk of secondary damage as observed in these cases can be predicted by assessing the position and size of the tumor before beginning radiotherapy. Before beginning treatment, the owner should be thoroughly educated on these potential complications.

The possibility that both the fractures and the globe rupture resulted from direct radiation damage cannot be denied. The fractures occurred 233 and 463 days after the start of radiotherapy, which are times when late radiation morbidity may occur. Osteonecrosis is considered a type of late radiation
morbidity (score 3) and may have occurred at the fracture site. The globe rupture was considered a secondary injury because it occurred after the dog rubbed its eye, but the eye also had pre-existing conjunctivitis that had not resolved despite treatment (i.e., the eye was protected physically and treated with ointment). However, the breed and clinical history of the patient may have also contributed to the rupture; therefore, evaluation of the radiation injury was difficult in this case. One weakness of this study is its retrospective nature, specifically the retrospective application of the radiation scoring system. The findings would have been more reliable had the radiation side effects had been scored in real time. A prospective study evaluating acute and late radiation morbidity is necessary.

The MST following radiotherapy appeared similar to the MST after surgery reported previously. The results of this study demonstrate that radiotherapy may be useful in the treatment of canine oral malignant melanoma. However, the study also revealed the risk for severe complications depending on the location and size of the tumor. Radiotherapy should be commenced only after performing a thorough examination and after considering the advantages and drawbacks of the treatment.
Table 1. Traditional World Health Organization TNM-based staging scheme for canine oral malignant melanoma

<table>
<thead>
<tr>
<th>T: Primary tumor</th>
<th>N: Regional lymph nodes</th>
<th>M: Distant metastasis</th>
</tr>
</thead>
<tbody>
<tr>
<td>T1 Tumor ≤ 2 cm in diameter</td>
<td>N0 No evidence of regional node involvement</td>
<td>M0 No evidence of distant metastasis</td>
</tr>
<tr>
<td>T2 Tumor 2–4 cm in diameter</td>
<td>N1 Histologic/cytologic evidence of regional node involvement</td>
<td>M1 Evidence of distant metastasis</td>
</tr>
<tr>
<td>T3 Tumor &gt; 4 cm in diameter</td>
<td>N2 Fixed nodes</td>
<td></td>
</tr>
</tbody>
</table>

Stage I = T1 N0 M0
Stage II = T2 N0 M0
Stage III = T2 N1 M0 or T3 N0 M0
Stage IV = Any T, any N, and M1
Table 2. Characteristics and anatomic locations of 111 canine oral malignant melanomas treated with radiotherapy. Weight and age data are presented as the median.

<table>
<thead>
<tr>
<th></th>
<th>Stage I</th>
<th>Stage II</th>
<th>Stage III</th>
<th>Stage IV</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age (y) [mean ± SD]</strong></td>
<td>10.2 [10.6±2.3]</td>
<td>12.3 [12.2±1.4]</td>
<td>12.6 [12.3±2.8]</td>
<td>12.4 [12.2±2.5]</td>
</tr>
<tr>
<td><strong>Sex (n [%])</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>15 [78.9]</td>
<td>14 [58.3]</td>
<td>16 [43.2]</td>
<td>14 [45.2]</td>
</tr>
<tr>
<td>Female</td>
<td>4 [21.1]</td>
<td>10 [41.7]</td>
<td>21 [56.8]</td>
<td>17 [54.8]</td>
</tr>
<tr>
<td><strong>Anatomic location (n [%])</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>maxilla</td>
<td>9 [47.4]</td>
<td>8 [33.3]</td>
<td>9 [24.3]</td>
<td>14 [45.2]</td>
</tr>
<tr>
<td>mandible</td>
<td>5 [26.3]</td>
<td>9 [37.5]</td>
<td>17 [46.0]</td>
<td>7 [22.6]</td>
</tr>
<tr>
<td>tongue</td>
<td>0 [0]</td>
<td>2 [8.3]</td>
<td>1 [2.7]</td>
<td>0 [0]</td>
</tr>
<tr>
<td>buccal mucosa</td>
<td>0 [0]</td>
<td>1 [4.2]</td>
<td>5 [13.5]</td>
<td>5 [16.1]</td>
</tr>
<tr>
<td>hard palate</td>
<td>2 [10.5]</td>
<td>1 [4.2]</td>
<td>1 [2.7]</td>
<td>0 [0]</td>
</tr>
<tr>
<td>soft palate</td>
<td>0 [0]</td>
<td>0 [0]</td>
<td>2 [5.4]</td>
<td>2 [6.5]</td>
</tr>
<tr>
<td><strong>Total (n [%])</strong></td>
<td>19</td>
<td>24</td>
<td>37</td>
<td>31</td>
</tr>
</tbody>
</table>
Table 3. The number of dogs administered chemotherapy.

<table>
<thead>
<tr>
<th>Stage</th>
<th>Total (n)</th>
<th>Local only (n)</th>
<th>Systemic only (n)</th>
<th>Local and Systemic (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stage I</td>
<td>11</td>
<td>3</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Stage II</td>
<td>19</td>
<td>9</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Stage III</td>
<td>20</td>
<td>9</td>
<td>8</td>
<td>3</td>
</tr>
<tr>
<td>Stage IV</td>
<td>16</td>
<td>5</td>
<td>9</td>
<td>2</td>
</tr>
</tbody>
</table>
Fig. 1. The tumor location-related Kaplan–Meier survival curves of 111 dogs with oral malignant melanoma. The survival curves did not vary significantly (P = 0.16) according to the sites of the tumors.
Fig. 2. Clinical stage-related Kaplan–Meier survival curves of 111 dogs with oral malignant melanoma. The survival times were significantly different (P < 0.0001) between the stages.
Fig. 3. Kaplan–Meier survival curves of 107 dogs that received OVX or MVX radiotherapy. The survival times were significantly different (P < 0.05) between the treatments.
Chapter 2: Profiling of plasma metabolites in canine oral melanoma using gas chromatography mass spectrometry

In chapter 1, the author showed that the MST in canine oral malignant melanoma differs significantly according to the stage and becomes shorter in higher stages. The results are similar to those reported after surgical treatment (Hahn et al., 1994; MacEwen et al., 1986). In other words, early detection and early treatment are very important for this tumor.

Most of canine oral malignant melanoma occurs to gingiva (Harvey et al., 1981; MacEwen et al., 1986). Similarly, the author showed that tumors occurred to gingiva in 78 of 111 dogs (70.3 %) in chapter 1. It seems that early detection is easy, since the lesion in the mouth can be confirmed on inspection. Most of owners have few opportunities to check the inside of the mouth, and there are owners who are unable to touch the mouth because their dogs are aggressive. In addition, it is still more difficult to detect a metastatic focus. Although systemic imaging method (ex. radiograph, ultrasound, CT, and MRI) and biopsy may be required for the diagnosis of tumors and detection of metastasis, more aggressive examination under anesthesia may not be desired for dogs with advanced age or advanced disease. It was reported that the mean age at presentation of dogs with oral malignant melanoma was 11.4 years (Ramos-Vara et al., 2000), and that was 12.2 year in my study (see chapter 1). Development of the less-invasive biomarker which can evaluate a condition of dogs is expected.

A biomarker is a measurable indicator of the severity or presence of some disease state, and can be used as an indicator of a particular disease state.
Biomarkers can contain genes, gene products, proteins, peptides, lipid, or any other substance. Recently, a search of biomarkers is performed energetically by progress of mass spectrometric technique. GC-MS is one of these analysis equipments and provides high separation efficiency for resolving complex biological mixtures. It has been indicated that metabolites profile with GC-MS may be useful tool for detection of potential biomarker and diagnosis of canine lymphoma and of canine epilepsy (Hasegawa et al., 2014; Tamai et al., 2014).

In this chapter, we used GC-MS to examine metabolites profiles in plasma from dogs with oral malignant melanoma with the goal of identifying biomarkers for diagnosis of canine oral malignant melanoma and for prediction of its prognosis.
Section 1: Materials and Methods

Case selection

Plasma samples from 9 healthy dogs, which were judged to be normal by physical examination and blood tests, and 32 dogs with oral malignant melanoma were obtained. The melanoma group included dogs treated by radiotherapy for spontaneous oral malignant melanoma at the Animal Medical Center of Gifu University between April 2009 and November 2012. The tumors were classified as stage I, II, III, or IV using WHO staging guidelines (Table 1). Long-term post-treatment outcomes were obtained from the medical records or by telephone interviews with the owners.

Radiation therapy

All melanoma cases had undergone radiotherapy delivered with Radioflex 300EMG (Rigaku Corporation, Tokyo, Japan) or PRIMUS Mid-Energy (Toshiba Medical Systems, Tokyo, Japan), which have X-ray energy outputs of 300 kV and 4 MV, respectively. The radiation beam provided OVX, or MVX. Each radiotherapy treatment was performed as follows. OVX: The treatment position varied according to the location of the tumor. Treatment beams were delivered from one or two directions while avoiding organs at risk when feasible, such as the eyes and brain. The dose prescription was 6.3 to 10.0 Gy per fraction in 4–6 fractions for a total dose of 37.8 to 40 Gy, and irradiations were carried out at 7–10 day intervals. MVX: Planning CT scans were performed for each dog in the treatment position (sternal recumbency) with a bite block system. Individualized treatment plans were constructed using a 3-dimensional CT-based computer generated treatment planning system. Treatments were delivered in five to nine unequally weighted fields.
in multiple oblique beam arrangements. The dose prescription was 6.0–10.0 Gy per fraction in 4–8 fractions for a total dose of 40–50 Gy, and irradiations were carried out at 7–10 day intervals.

Sample for GC-MS analysis

Blood samples from melanoma dogs were collected at the first visit and after radiation treatment. The blood test after radiotherapy was performed at the time of the visit for monitoring the effects of therapy.

Serum preparation

Twenty-five µl of serum were mixed with 5 µl of 1 mg/ml 2-isopropylmalic acid (Sigma-Aldrich, Tokyo, Japan) as an internal standard (IS), and 900 µl of 70 % ethanol was added. The mixture was vortexed and centrifuged at 15,000 rpm for 5 min at 4°C, and then, 900 µl of the supernatant were transferred to a clean vial and dried under a N₂ gas stream. After drying, 40 µl of 20 µg/ml methoxyamine hydrochloride (Sigma-Aldrich, Tokyo, Japan) dissolved in pyridine were mixed with a lyophilized sample for oximation, before being shaken at 1,200 rpm for 90 min at 30°C. Then, 20 µl of N-methyl-N-trimethylsilyl-trifluoroacetamide (MSTFA) (GL Science, Tokyo, Japan) were added for trimethylsilyl derivatization, and the mixture was incubated at 1,200 rpm for 30 min at 37°C. The mixture was then centrifuged at 16,000 x g for 5 min at 4°C, and the resultant supernatant was subjected to GC-MS measurement.

GC-MS analysis

GC-MS analysis was performed using a GCMS-QP2010 Plus (Shimadzu, Kyoto, Japan) with a DB-5 column (Length: 30 m, Film: 1.00 µm, Inside diameter: 0.25 mm, Agilent Technologies, Santa Clara, CA, U.S.A.). The
flow rate was 24.5 ml/min. The GC column temperature was programmed to rise from 100 to 300ºC at a rate of 4ºC per min. The sample was injected in splitless mode. The ion source temperature was 230ºC. Chromatogram acquisition, detection of peaks and data processing were performed using Shimadzu GC-MS solution software ver. 2.71 (Shimadzu). Low molecular weight metabolites were identified using the NIST library (NIST08) with peaks assigned based on a similarity index >75. In addition to the automatic assessment, the identified metabolites were confirmed manually. The peak area of each metabolite was divided by that of the IS to give a relative value. The serum levels of metabolites were shown as fold ratio. The relative value of the dogs with lymphoma was compared with the mean of the healthy dogs.

**Statistical analysis**

Multivariate analysis was performed using orthogonal projection to latent structure with discriminant analysis (OPLS-DA) in SIMCA 13.0 software (Umetrics, Umea, Sweden). Statistical significance was analyzed using a Steel-Dwass test.
Section 2: Results

The characteristics of dogs were shown in Table 4. Of the 32 dogs with oral malignant melanoma, 8 were classified as stage I, 6 as stage II, 10 as stage III, and 8 as stage IV. OVX was used in 10 dogs and MVX in 22 dogs.

GC-MS analysis permits detection of more than 130 metabolites simultaneously, and more than 60 metabolites were identified in canine plasma. Among these metabolites, 46 were present in all samples and were used in statistical analysis. The similarity of metabolic profiles was evaluated using OPLS-DA. Using this method, plasma metabolite profiles from healthy dogs were discriminated from those of melanoma cases at the first visit, which suggests that metabolite profiles in melanoma differ from those of healthy dogs (Fig. 4).

Twelve metabolites showed significant changes in melanoma plasma (p < 0.05) (Fig. 5). Threonine, proline, serine and margaric acid decreased and citric acid, isocitric acid, glycerol, lactic acid, oleic acid, linoleic acid, palmitoleic acid and octadecenoic acid increase in melanoma plasma.

Melanoma plasma samples obtained from each dog after radiotherapy were evaluated using the OPLS-DA model. One of the 32 dogs was predicted to be healthy at 6 months after radiotherapy, despite not being healthy just after radiotherapy (Fig. 6). The dog was in stage I and was found to be in CR after radiotherapy, with no advanced lesions based on inspection and palpation. In a follow-up study, this dog lived for 316 days and died from an unrelated disease with no advanced melanoma lesion.

To evaluate the change of metabolite profile after radiotherapy, an OPLS-DA scores plot was made using the data obtained in this study (Fig. 7).
The plasma metabolite profiles of melanoma dogs after radiotherapy showed a tendency to assemble in another axis compared to those of the healthy and melanoma dogs before the radiation therapy.
Section 3: Discussion

It was possible to detect more than 60 metabolites from the plasma used and 46 metabolites were detected in all of samples. The number of metabolites is similar to our previous and other human plasma experiment (Kobayashi et al., 2013; Tamai et al., 2014), indicating GC-MS measurements were completed satisfactorily. The discrimination was examined using the 46 metabolites with OPLS-DA and the similarity of metabolite profile was shown as OPLS scores plot (Fig. 4). The healthy controls were discriminated from the melanoma patients, indicating metabolite profile of malignant melanoma dogs is different from that of healthy one. The difference of metabolic profile of cancer patients has been reported in many types of human cancer and canine lymphoma (Kobayashi et al., 2013; Nishiumi et al., 2012; Tamai et al., 2014). These suggest that it is possible to search diagnosing biomarker for canine melanoma from plasma metabolites.

Twelve metabolites showed significant changes in melanoma plasma. These included citric acid, an intermediate in energy metabolism; lactic acid, the end product of anaerobic energy metabolism; fatty acids including oleic acid, linoleic acid, palmitoleic acid and octadecenoic acid; and glycerol, a triglyceride component, all of which were elevated in melanoma cases. Elevation of fatty acids in human melanoma has been reported in volatile metabolomics with GC-MS (Abaffy et al., 2013). These results show that energy metabolism is elevated in melanoma cases, which is a typical signature of cancer that is referred to as the Warburg effect (Gatenby and Gillies, 2004). These metabolites could be potential biomarkers for canine
malignant melanoma. It is necessary to examine plasma metabolite specificity for melanoma to provide plasma melanoma diagnosis marker in the future.

Three amino acids, threonine, proline, and serine, were decreased in melanoma plasma. In contrast, the levels of phenylalanine and glutamic acid were increased in serum of dogs with lymphoma (Tamai et al., 2014). Changes of amino acids in tumors may be related to invasiveness and viability (Fu et al., 2003), and further studies are required to determine the mechanisms underlying these changes and their possible use in cancer diagnosis.

Metabolic profile of canine malignant melanoma is different from that of healthy one. It was aimed to apply the model to evaluate the health status of the melanoma patients after radiotherapy by discriminating healthy or melanoma. Only one sample was discriminate as healthy after radiotherapy (Fig. 6). The dog was in stage I and was found to be in CR after radiotherapy, with no advanced lesions based on inspection and palpation. In a follow-up study, this dog lived for 316 days and died from an unrelated disease with no advanced melanoma lesion. In another melanoma case, the dog lived more than 300 days with no advanced lesion found in imaging or by inspection and palpation, the plasma sample collected just after radiotherapy was consistent with melanoma. I was unable to obtain subsequent blood samples. The difference of the both long lived cases was time of blood collection after radiotherapy. The one judged as healthy was taken after 6 months of the therapy and the other was just after the treatment. These findings suggest that the timing of blood collection might be an important factor in prediction
of prognosis, and this requires further evaluation in a controlled clinical trial.

The OPLS-DA scores plot which shows the similarity of plasma metabolites profiles among the healthy and melanoma dogs before and after the radiation therapy indicated that the metabolites profiles of melanoma dogs after radiation therapy tended to assembled along with another axis (Fig. 7). The result indicates that change of metabolites profile of melanoma dog after the radiation therapy seem to be different from those of the healthy and melanoma dogs before the radiation therapy. The changes of metabolites profile of melanoma dogs might be affected by the radiation therapy and the timing of blood collection could be an important factor for diagnosing the disease status of melanoma after therapy using metabolites profiling. It is necessary further evaluation of its availability through a controlled clinical trial.

These results show that the metabolic profile of canine malignant melanoma differs from that in healthy dogs. A further study of the specificity of plasma metabolites for melanoma is required to establish serum diagnostic markers for melanoma.
<table>
<thead>
<tr>
<th>Sample</th>
<th>Number</th>
<th>Age (year)</th>
<th>Gender</th>
<th>Melanoma region</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Male</td>
<td>Female</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(Cast)</td>
<td>(Spay)</td>
</tr>
<tr>
<td>Healthy</td>
<td>9</td>
<td>6.0±2.6</td>
<td>3</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(3)</td>
<td>(2)</td>
</tr>
<tr>
<td>Melanoma</td>
<td>32</td>
<td>11.5±2.4</td>
<td>22</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(9)</td>
<td>(4)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Buccal mucosa</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Hard plate</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Lip</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Mandible</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Maxilla</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Soft plate</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Tongue</td>
<td>1</td>
</tr>
</tbody>
</table>
Fig. 4. OPLS-DA plot for discrimination between healthy and melanoma dogs. Plasma metabolites profiles of melanoma dogs (black circles) were clearly discriminated from those of healthy dogs (white circles).
Fig. 5. Metabolites showing significant changes in melanoma dog plasma. *p < 0.05, **p < 0.01, and ***p < 0.001 by Steel-Dwass test.
Fig. 6 Discrimination of metabolites profiles of melanoma dogs after the radiation therapy. The OPLS-DA discrimination model shown in Fig. 5 was applied to discrimination of metabolites profiles of dogs whether healthy or melanoma. White circles were for healthy dogs, black circles were for melanoma dogs before the radiation therapy and gray circles were for melanoma dogs after the radiation therapy.
Fig. 7 OPLS-DA scores plot for discrimination among healthy (white circles) and melanoma dogs before radiotherapy (black circles) and after radiotherapy (gray circles).
Conclusion

Canine oral malignant melanoma carries a poor prognosis due to its rapid invasion of the surrounding normal tissue and the high likelihood of regional and distant metastasis early in the course of the disease. This study showed that MST in canine oral malignant melanoma treated by radiotherapy changes significantly according to stage and becomes shorter in higher stages. This staging system may be effective in radiotherapy prognosis prediction for oral malignant melanomas.

Early detection is desirable but difficult, since there are few opportunities to check the inside of the mouth. In addition, although oral lesion can be checked with the naked eye, it is still more difficult to detect a metastatic focus. More aggressive examination (ex. CT or biopsy under anesthesia) may be required, but these examinations may not be desired for dogs with advanced age or advanced disease. Here the author examined the metabolite profiling using GC-MS and applied its discriminant model for evaluating its prognosis. The metabolite profiles were discriminated between healthy and melanoma by OPLS-DA. The case predicted as healthy after the radiation therapy by the OPLS model had long survival and no advanced lesions. These indicate that metabolite profiling may have a potential to diagnose canine malignant melanoma and to predict its prognosis.
References


of 140 dogs with oral melanoma treated with external beam radiation.

Vet Radiol Ultrasound, 44, 352-359.


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