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# Evaluation of circulating IGF-I and IGFBP-3 as biomarkers for tumors in dogs

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## ABSTRACT

**Background:** Serum-based parameters are considered non-invasive biomarkers for cancer detection. In human studies, insulin-like growth factor-I and II (IGF-I and IGF-II) and insulin-like growth factor binding protein-3 (IGFBP-3) are useful as diagnostic or prognostic markers and potential therapeutic targets.
**Objectives:** This study examined the diagnostic utility of circulating IGF-I, IGF-II, and IGFBP-3 levels in healthy dogs and dogs with tumors.
**Methods:** The serum concentrations of these biomarkers in 86 dogs with tumors were compared with those in 30 healthy dogs using an enzyme-linked immunosorbent assay (ELISA).
**Results:** The ELISA results showed no difference between healthy dogs and dogs with tumors in the serum IGF-II concentrations. On the other hand, there was a significant difference in the circulating IGF-I and IGFBP-3 levels between healthy dogs and dogs with tumors. The concentrations of serum IGF-I (median [interquartile range], 103.4 [59.5–175] ng/mL) in dogs with epithelial tumors were higher than those (58.4 ng/mL [43.5–79.9]) in healthy dogs. Thus, the concentrations of serum IGFBP-3 (43.4 ng/mL [33.2–57.2]) in dogs with malignant mesenchymal tumors were lower than those (60.8 ng/mL [47.6–70.5]) in healthy dogs.
**Conclusions:** The serum IGF-I and IGFBP-3 levels can be used as diagnostic biomarkers in dogs with tumors.

**Keywords:** Tumors; biomarkers; insulin-like growth factor; insulin-like growth factor binding proteins; dogs

## INTRODUCTION

Tumors grow faster than normal tissues. Various factors related to abnormal cell proliferation have been identified and studied. In humans, insulin-like growth factors (IGF-I and IGF-II) and insulin-like growth factor binding protein-3 (IGFBP-3) are useful for the diagnosis and treatment of tumors.

IGF produced by the liver is a polypeptide hormone that regulates cellular proliferation and apoptosis [1]. IGF-I and IGF-II play similar roles, but the time at which they are secreted

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**Conflict of Interest**

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

**Author Contributions**

Conceptualization: Song DW, Park SH, Park HM; Data curation: Song DW, Ro WB; Formal analysis: Ro WB; Funding acquisition: Sur JH; Investigation: Song DW, Sur JH, Seung BJ, Kang HM, Kim JW; Resources: Sur JH, Seung BJ; Supervision: Park HM; Writing - original draft: Song DW. Writing - review & editing: Song DW, Park SH, Park HM.

is different. IGF-II is expressed during the fetal period to facilitate development, whereas IGF-I plays a role in adults [2]. Both are involved in cell proliferation through autocrine and paracrine mechanisms. On the other hand, excessive IGF-I and IGF-II expression causes the cells to over-proliferate and interfere with apoptosis, inducing tumors [3]. In veterinary medicine, studies on IGF-I have also been reported in relation to tumors, but there is a significant difference in the methodology for measuring the concentration, which may be unreliable [4-9]. In addition, in the case of IGF-II, there are only a few reports on veterinary research [10,11]. Therefore, it is necessary to analyze the concentration of total IGFs in serum accurately and compare it in healthy dogs and dogs with tumors.

Approximately 90% of circulating IGFs are bound to IGFBP-3. Circulating IGFBP-3, which is freely bioavailable and inhibits cell proliferation, modulates the amount of IGF [12,13]. In humans, the relationship between IGFBP-3 and certain cancers has been reported. The circulating IGFBP-3 levels in patients with lung, pancreatic, and colorectal cancers are lower than those in healthy individuals [14-16]. The activity of IGFs increases when circulating IGFBP-3 decreases, which prevents the inhibition of excessive cell proliferation, resulting in tumors.

Until recently, various serological markers have been studied in healthy dogs and those with cancers [4-11]. The present study evaluated the circulating IGFs and IGFBP-3 in dogs to identify the diagnostic utility between dogs with tumors and dogs without tumors.

## MATERIALS AND METHODS

### Serum samples

The archived serum samples stored at −70°C until analysis were retrieved from 30 healthy dogs and 86 dogs with tumors. All serum samples were obtained from 15 animal hospitals between 2016 and 2018 for diagnostic or routine health examinations. The serum samples from dogs with tumors were obtained at diagnosis. One hundred and sixteen dogs with ≥ 0.3 mL serum remaining were classified retrospectively as healthy dogs or dogs with tumors based on a physical examination and blood work, including the complete blood counts and serum chemistry profiles. The remaining serum was stored after the blood work. The stored serum was collected from the jugular vein into 5-mL serum separating tubes (BD Vacutainer SST Tube; Becton Dickinson, USA). The tube was inverted gently approximately five times and allowed to stand for 20–30 min at −4°C before centrifugation at 3,000 rpm for 15 min. The serum was then aliquoted into cryovials and stored at −70°C until needed. The protocol for serum sampling was approved by the Institutional Animal Care and Use Committee of Konkuk University (KU16106, KU17162, and KU18168).

### Inclusion and exclusion criteria

Healthy dogs had no remarkable findings on the physical examination or blood work. There was no history of abnormalities or evidence of tumors (**Table 1**). Dogs with various tumors were included in the tumor group. The diagnosis was based on a histological examination. The tumors were further classified into three general types based on the histological classification (**Table 2**). A certain terminology was used depending on the origin of the tumor, such as epithelial, mesenchymal, and hematopoietic and lymphoreticular. In addition, they were classified as benign or malignant. Patients with diseases other than tumors in both healthy dogs and those with tumors were excluded.

**Table 1.** Characteristics of 30 healthy dogs and 86 dogs with epithelial, mesenchymal, and hematopoietic and lymphoreticular tumors in this study

| Signalment information | Healthy (n = 30) | Tumor types | | | | | |
|---|---|---|---|---|---|---|---|
| | | Epithelial (n = 35) | | Mesenchymal (n = 28) | | H & L (n = 23) | |
| | | Benign | Malignant | Benign | Malignant | Benign | Malignant |
| No. | 30 | 12 | 23 | 5 | 23 | 6 | 17 |
| Age (median [IQR]) | 4.0 [3.3–6.8] | 5.3 [3.0–5.8] | 5.2 [3.9–5.7] | 7.0 [4.0–7.2] | 5.1 [3.4–8.0] | 5.2 [4.6–12.3] | 5.8 [4.2–10.2] |
| Sex | F (7), SF (7)<br>M (6), CM (10) | F (0), SF (2)<br>M (3), CM (7) | F (10), SF (8)<br>M (2), CM (3) | F (3), SF (0)<br>M (2), CM (0) | F (8), SF (5)<br>M (3), CM (7) | F (0), SF (0)<br>M (4), CM (2) | F (3), SF (3)<br>M (1), CM (10) |
| Breed | Maltese (10)<br>Beagle (4)<br>Yorkshire Terrier (3)<br>Chihuahua (2)<br>Italian Greyhound (2)<br>Poodle (2)<br>Shih Tzu (2)<br>Others* (5) | Poodle (3)<br>Italian Greyhound (2)<br>Maltese (2)<br>Mongrel (2)<br>Pomeranian (2)<br>Shetland Sheepdog (1) | Pug (6)<br>Pomeranian (5)<br>Schnauzer (3)<br>Old English Sheepdog (2)<br>Others† (7) | Cocker Spaniel (1)<br>Golden Retriever (1)<br>Miniature Pinscher (1)<br>Pomeranian (1)<br>Pug (1) | Mongrel (5)<br>Pinscher (3)<br>Pug (3)<br>Schnauzer (3)<br>Maltese (2)<br>Miniature Pomeranian (2)<br>Old English Sheepdog (2)<br>Others‡ (3) | Beagle (2)<br>Boston Terrier (1)<br>Chihuahua (1)<br>Golden Retriever (1)<br>Italian Greyhound (1) | Poodle (5)<br>Miniature Pinscher (2)<br>Schnauzer (2)<br>Others§ (8) |

F, female; SF, spayed female; M, male; CM, castrated male; IQR, interquartile range; H&L, hematopoietic and lymphoreticular.
*Bichon Frise, Cocker Spaniel, Mixed, Pomeranian, Schnauzer; †Boxer, Chihuahua, Italian Greyhound, Maltese, Miniature Pinscher, Mixed, Poodle
‡Cocker Spaniel, Golden Retriever, Italian Greyhound; §Boxer, Chihuahua, Italian Greyhound, Maltese, Mixed, Pomeranian, Pug, Old English Sheepdog.

**Table 2.** Classification and number of 86 tumors according to the cytologic and/or histologic diagnosis in this study

| Tumors | n |
|---|---|
| Epithelial | 35 |
| Benign | 12 |
| Sebaceous gland adenoma | 6 |
| Hepatoid gland adenoma | 4 |
| Trichoblastoma | 2 |
| Malignant | 23 |
| Sebaceous gland carcinoma | 1 |
| Hepatoid gland carcinoma | 1 |
| Squamous cell carcinoma | 8 |
| Hepatocellular carcinoma | 6 |
| Mammary gland carcinoma | 7 |
| Mesenchymal | 28 |
| Benign | 5 |
| Fibroma | 4 |
| Hemangioma | 1 |
| Malignant | 23 |
| Hemangiosarcoma | 4 |
| Fibrosarcoma | 7 |
| Osteosarcoma | 4 |
| Hemangiopericytoma | 4 |
| Melanoma | 4 |
| Hematopoietic and Lymphoreticular | 23 |
| Benign | 6 |
| Histiocytoma | 6 |
| Malignant | 17 |
| Lymphoma | 7 |
| Mast cell tumor | 8 |
| Histiocytic sarcoma | 2 |
| Total number of tumors | 86 |

Specific terminology is used depending on the origin of the tumor (epithelial, mesenchymal, and hematopoietic and lymphoreticular). It is also divided into benign or malignant.


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### Enzyme-linked immunosorbent assay (ELISA)

The IGF-I (Mediagnost, Germany), IGF-II (Mediagnost), and IGFBP-3 (MyBioSource Inc., USA) concentrations were measured using commercially available ELISA kits according to the manufacturer's instructions. IGFBP-blocked double-antibody sandwich ELISA was used for IGF-I and IGF-II. The IGF-I and IGF-II ELISA kits have been validated previously for use in dogs [17-19]. Briefly, the standards at different concentrations and serum samples were reconstituted with an acid buffer to dissociate and block IGFBP. Therefore, the standards and samples were added to 96-well plates, which were then incubated with the antibody conjugate for 1 h at 25°C. After several washes, horseradish peroxidase (HRP)-conjugate reagent was added to each well and incubated for 30 min at 25°C. After several washes, a substrate solution was added to each well and incubated in the dark for 15 min at 25°C. The reaction was quenched by adding a stop solution, and the optical density was measured at 450 nm. The protein levels were calculated using the standard curves. A double-antibody sandwich ELISA was used for IGFBP-3. Briefly, the standards at different concentrations and serum samples were added to 96-well plates, which were then incubated for 90 min at 37°C. After several washes, a biotinylated canine IGFBP-3 antibody was added to each well and incubated for 60 min at 37°C. The plates were washed several times, and the enzyme-conjugate reagent was added to each well and incubated for 30 min at 37°C. After several washes, a mixture of color reagents A and B was added to each well and incubated in the dark for 15 min at 37°C. The reaction was quenched by adding a color reagent C solution, and the optical density was measured at 450 nm. The protein levels were calculated using standard curves.

### Statistical analysis

SPSS software (version 25.0; SPSS Inc., USA) was used for statistical analysis. The Mann-Whitney U test and Kruskal-Wallis test were used to compare the IGF-I, IGF-II, and IGFBP-3 concentrations between the healthy dogs and dogs with tumors. The Spearman correlation was used to relate the serum IGF-I, IGF-II, and IGFBP-3 concentrations. The values are presented as the median and interquartile range (IQR). Statistical significance was set to $p < 0.05$.

## RESULTS

### Serum IGF-I, IGF-II, and IGFBP-3 concentrations in healthy dogs and those with tumors

The median serum IGF-I, IGF-II, and IGFBP-3 concentrations in healthy dogs were 58.4 [43.5–79.9] ng/mL, 127.5 [104.2–169.9] ng/mL, and 60.8 [47.6–70.5] ng/mL, respectively. The serum concentrations of IGF-I in dogs with epithelial, mesenchymal, and hematopoietic and lymphoreticular tumors were 103.4 [59.5–175] ng/mL, 62.7 [41.8–93.6] ng/mL, and 79.2 [42.5–160.6] ng/mL, respectively (**Fig. 1A**). The serum IGF-I concentration in dogs with epithelial tumors was higher than in healthy dogs ($p = 0.002$). The serum IGF-II concentrations in dogs with epithelial, mesenchymal, and hematopoietic and lymphoreticular tumors were 159.4 [105.3–194.8] ng/mL, 131.7 [109.8–170.7] ng/mL, and 145.4 [87.4–183.3] ng/mL, respectively (**Fig. 1B**). There was no significant difference in the serum IGF-II concentrations between the healthy dogs and those with tumors. The serum concentrations of IGFBP-3 in dogs with epithelial, mesenchymal, and hematopoietic and lymphoreticular tumors were 51.9 [38.8–74.1] ng/mL, 45.8 [34.6–57.2] ng/mL, and 49.2 [40.2–64] ng/mL, respectively (**Fig. 1C**). The serum IGFBP-3 concentrations in dogs with mesenchymal tumors were significantly lower than in healthy dogs ($p = 0.007$).

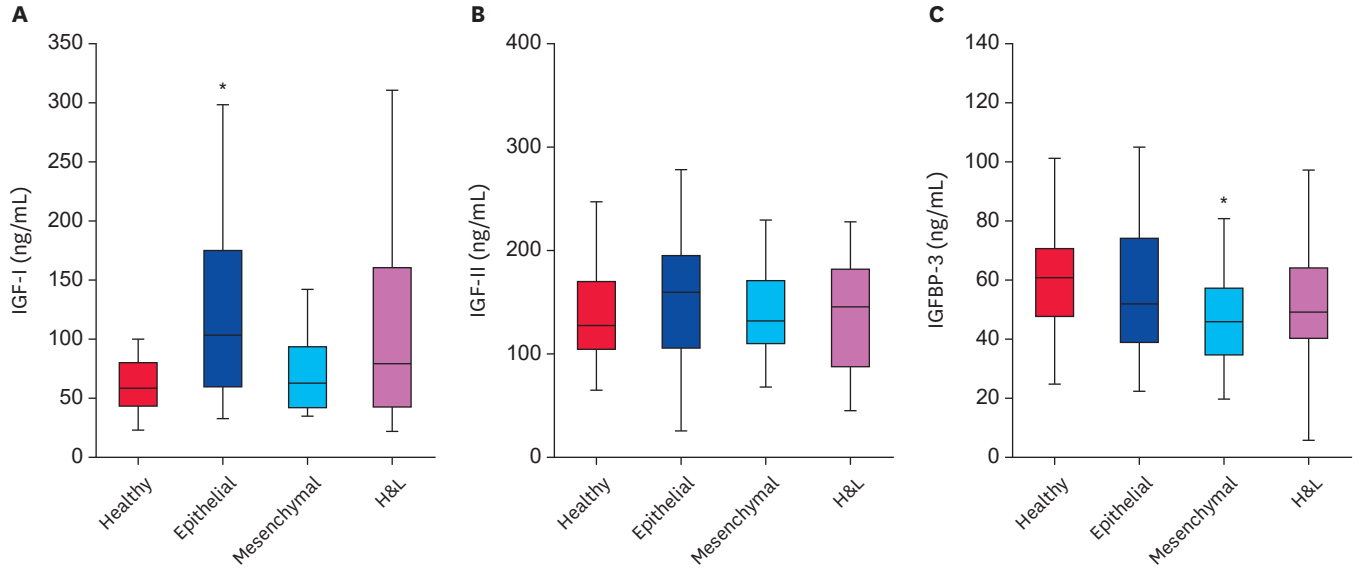


**Fig. 1.** Boxplots of IGF-I, IGF-II, and IGFBP-3 concentration for healthy dogs and dogs with tumors (epithelial, mesenchymal, and hematopoietic and lymphoreticular). (A) In the IGF-I concentrations in serum, dogs with epithelial tumors showed significantly higher IGF-I concentrations than healthy dogs. (B) In IGF-II concentrations in serum, there was no difference between healthy dogs and dogs with tumors. (C) In IGFBP-3 concentrations in serum, dogs with a mesenchymal tumor showed significantly lower IGFBP-3 levels than healthy dogs.
IGF, insulin-like growth factor; IGFBP-3, insulin-like growth factor binding protein-3; H&L, hematopoietic and lymphoreticular.
*Asterisk indicates a significant difference at $p < 0.05$.

### Serum IGF-I concentration in dogs with benign/malignant epithelial tumors

The median serum concentrations of IGF-I in healthy dogs were 58.4 [43.5–79.9] ng/mL, whereas it was 133.8 [50.5–187.4] ng/mL and 98.5 [59.5–158.1] ng/mL in dogs with benign epithelial tumors and malignant epithelial tumors, respectively. Compared to healthy dogs, there were significant differences between the serum IGF-I levels in dogs with benign and malignant epithelial tumors ($p = 0.007$ and $p = 0.01$, respectively). The serum IGF-I levels in dogs with epithelial tumors were higher than those found in healthy dogs, regardless of the malignancy (**Fig. 2A**). Furthermore, the median serum concentrations of IGF-I in dogs with a sebaceous gland adenoma, hepatoid gland adenoma, squamous cell carcinoma, hepatocellular carcinoma, and mammary gland carcinoma were 160.7 [110.9–210.0], 82.6 [36.7–234.0], 127.8 [75.0–175.9], 53.7 [38.1–120.4], and 101.4 [59.5–145.0] ng/mL, respectively (**Fig. 3A**). Compared to healthy dogs, there were significant differences between the serum IGF-I levels in dogs with sebaceous gland adenoma, squamous cell carcinoma, and mammary gland carcinoma ($p = 0.000233$, $p = 0.000362$, and $p = 0.026$, respectively).

### Serum IGFBP-3 concentration in dogs with benign/malignant mesenchymal tumors

The median serum concentration of IGFBP-3 in healthy dogs was 60.8 [47.6–70.5] ng/mL, whereas it was 49.8 [33.6–90.9] ng/mL and 43.4 [33.2–57.2] ng/mL in dogs with a benign mesenchymal tumor and malignant mesenchymal tumors, respectively. A significant difference in the serum IGFBP-3 levels was observed between healthy dogs and those with malignant mesenchymal tumors ($p = 0.002$). The serum IGFBP-3 levels in dogs with malignant mesenchymal tumors were lower than those in healthy dogs (**Fig. 2B**). Furthermore, the median serum concentrations of IGFBP-3 in dogs with hemangiosarcoma, fibrosarcoma, osteosarcoma, hemangiopericytoma, and melanoma were 40.7 [23.0–51.2], 39.0 [28.5–42.9], 42.6 [35.3–53.7], 53.4 [43.2–61.2], and 62.8 [52.4–73.2] ng/mL, respectively (**Fig. 3B**). Compared to healthy dogs, there were significant differences between the serum

IGFBP-3 levels in dogs with hemangiosarcoma, fibrosarcoma, and osteosarcoma ($p = 0.036$, $p = 0.001$, and $p = 0.031$, respectively).

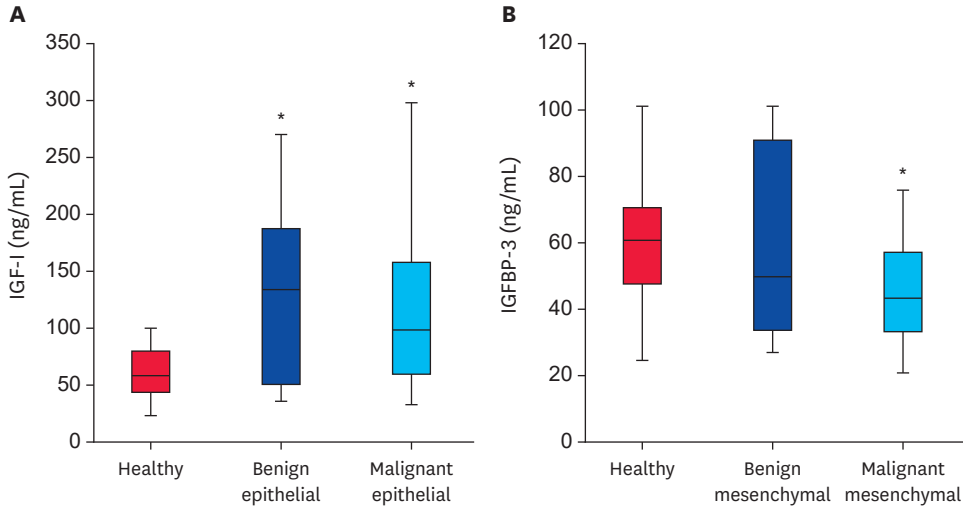


**Fig. 2.** Boxplots of the IGF-I and IGFBP-3 concentration for healthy dogs and dogs with tumors (epithelial, mesenchymal). (A) In IGF-I concentrations in serum, dogs with benign or malignant epithelial tumors showed significantly higher IGF-I concentrations than healthy dogs. (B) In IGFBP-3, dogs with malignant mesenchymal tumors showed significantly lower IGFBP-3 levels than healthy dogs.
IGF, insulin-like growth factor; IGFBP-3, insulin-like growth factor binding protein-3.
*Asterisk indicates a significant difference at $p < 0.05$.

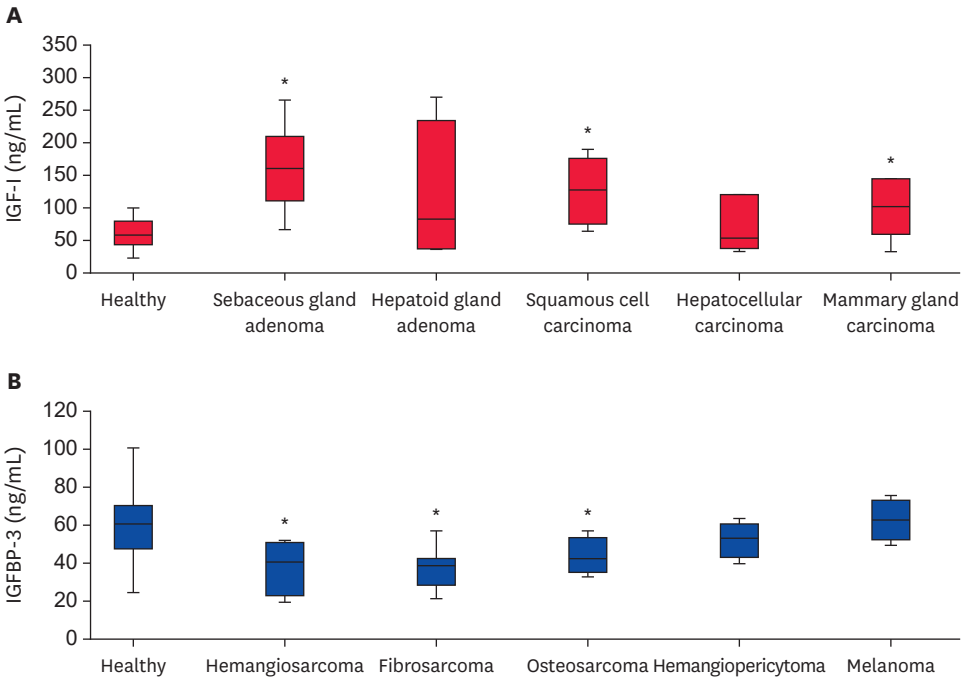


**Fig. 3.** Boxplots of the IGF-I and IGFBP-3 concentration for healthy dogs and dogs with tumors. (A) In IGF-I concentrations in serum, dogs with sebaceous gland adenoma, squamous cell carcinoma, and mammary gland carcinoma showed significantly higher IGF-I concentrations than healthy dogs. (B) In IGFBP-3, dogs with hemangiosarcoma, fibrosarcoma, and osteosarcoma showed significantly lower IGFBP-3 levels than healthy dogs.
IGF, insulin-like growth factor; IGFBP-3, insulin-like growth factor binding protein-3.
*Asterisk indicates a significant difference at $p < 0.05$.


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## DISCUSSION

The early detection of tumors is key to treating and preserving a dog's quality of life. On the other hand, because dogs cannot communicate their condition, many of them have progressed tumors at the time of diagnosis. Tumor treatment includes surgery, chemotherapy, and radiation therapy, and the treatment response is evaluated by the size of the tumor or the survival time. The quantitative parameters for tumor diagnosis, prognosis, and treatment response are scarce. Efforts to find tumor biomarkers are continuing in veterinary medicine, and radioimmunoassay (RIA) methods are used widely. On the other hand, RIA is expensive and complicated. In the current study, an ELISA-based kit that is inexpensive and convenient was used to examine the potential tumor markers.

The roles of IGF-I and IGF-II are similar, and they belong to the same IGF family. In general, IGF-II affects prenatal growth, and IGF-I influences postnatal growth. Both are involved in cellular proliferation and apoptosis, and overexpression can induce cancer or be produced by tumors [3]. In human medicine, the concentration of serum IGF-I is higher in patients with tumors (breast, prostate, colorectal, and lung cancers) than in healthy individuals [1]. IGF-II has been studied in epithelial and mesenchymal tumors [20].

Recently, circulating IGFs have also been studied in veterinary medicine. Several studies have shown that the serum IGF-I concentration in dogs with mammary gland tumors is higher than that in healthy dogs [9,21]. These results are consistent with the results of our study. The serum concentration of IGF-I was higher in dogs with epithelial tumors, excluding liver-related tumors, than in healthy dogs. In particular, it was higher in sebaceous gland adenoma, squamous cell carcinoma, and mammary gland carcinoma tumors. In a previous study, IGF-I was lower in liver-related tumors than healthy [8]. In the present study, however, there was no difference between healthy dogs and dogs with liver-related tumors. The circulating IGF-I levels were similar in dogs with mesenchymal or hematopoietic and lymphoreticular tumors, which is comparable to previous studies [4,7]. The circulating IGF-I concentration varies according to the type of tumor. In healthy dogs, IGF-I showed a significant positive correlation with the body weight and a negative correlation with age, but the differences were not significant. Although the body weight was positively correlated with IGF-I, there was no significant difference between healthy dogs and dogs with tumors, so there was no effect on the study. Furthermore, IGF-I was negatively correlated with age, so IGF-I should decrease with age, but it was higher in dogs with epithelial tumors, so it was considered not to affect the study results. Other than that, there was no significant difference.

The circulating IGF-II levels are increased in dogs with pancreatic islet tumors and mammary gland carcinomas than in healthy dogs [10,11]. On the other hand, the elevation of circulating IGF-II levels varied among the different tumor cases, which appears to be characteristic of each tumor. In humans, IGF-I and IGF-II may show the same or contradictory results for each individual, even with the same type of tumor [22]. Histopathologically, even tumors with the same diagnosis appear to vary in their characteristics.

IGFBPs (IGFBP 1-6) transport IGFs. IGFBP-3 is the main protein secreted and inhibits cell proliferation, migration, and survival by restricting the access of IGFs to their receptors [23,24]. When the levels of IGFBP-3 are decreased, the levels of free IGFs are increased, which can induce tumors by acting as a cell-growth promoter and suppressing apoptosis [25]. In humans, IGFBP-3 has been studied in lung, colorectal, esophageal, and prostate cancers and


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has been associated with tumor development [14,26-28]. Furthermore, IGFBP-3 has an IGF-independent tumor suppressor function [26,28,29]. In this study, dogs with some malignant mesenchymal tumors, specifically hemangiosarcoma, fibrosarcoma, and osteosarcoma, had low IGFBP-3 levels. IGFBP-3 plays a role in regulating and suppressing vimentin expression [29]. Vimentin is a biomarker for mesenchymal tumors and is expressed strongly in mesenchymal tumor tissues. Mesenchymal tumors could be induced because vimentin expression was not regulated because of low IGFBP-3 levels.

As tumor research progresses, various biomarkers have been developed and studied for diagnostic, prognostic, and therapeutic purposes. Studies on the same tumor and biomarkers have shown different results [22]. This is believed to be because the cause and etiology of the tumor are different for each individual, even histopathologically the same. Therefore, it is necessary to identify and evaluate the characteristics of a patient's tumor using various biomarkers. A more tailored and biomarker-driven approach to diagnosis and treatment selection can provide an accurate method for diagnosing and evaluating the prognosis of various tumors.

This study showed that the circulating IGF-II levels does not differ between healthy dogs and dogs with tumors. On the other hand, circulating IGF-I and IGFBP-3 could have useful diagnostic utility in some epithelial tumors and malignant mesenchymal tumors, respectively. Overall, these findings can offer a more straightforward and effective method for tumor diagnosis than a biopsy, but more large-scale studies are needed.

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