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# Serum big endothelin-1 as a clinical marker for cardiopulmonary and neoplastic diseases in dogs

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ABSTRACT

*Aims:* Many studies of human subjects have demonstrated the utility of assessing serum levels of endothelin-1 (ET-1) and big ET-1 as clinical biomarkers in cardiopulmonary and neoplastic diseases. In this study we explored the feasibility of using serum big ET-1 as a reliable veterinary marker in dogs with various cardiopulmonary and neoplastic diseases.

*Main methods:* Serum big ET-1 levels were measured by ELISA in dogs with cardiopulmonary ($n = 21$) and neoplastic diseases ($n = 57$). Dogs exhibiting cardiopulmonary disease were divided into two groups based on the velocity of tricuspid valve regurgitation ($3.0 > m/s$) measured by ultrasound: without and with pulmonary hypertension. Big ET-1 levels for the dogs with the diseases were compared with levels in normal healthy dogs ($n = 17$).

*Key findings:* Dogs with cardiopulmonary disease ($4.6 \pm 4.6$ pmol/l) showed a significantly ($P < 0.01$) higher level of big ET-1 than healthy control dogs ($1.1 \pm 0.53$ pmol/l). Serum levels in the dogs with pulmonary hypertension ($6.2 \pm 5.3$ pmol/l) were significantly ($P < 0.01$) higher than those without pulmonary hypertension ($2.0 \pm 0.6$ pmol/l). Dogs with hemangiosarcoma ($5.6 \pm 2.2$ pmol/l), adenocarcinoma ($2.0 \pm 1.8$ pmol/l), histiocytic sarcoma ($3.3 \pm 1.9$ pmol/l), chondrosarcoma or osteosarcoma ($3.0 \pm 1.6$ pmol/l) and hepatocellular carcinoma ($2.7 \pm 1.8$ pmol/l) showed significantly ($P < 0.05$) higher levels than healthy control dogs.

*Significance:* These findings point to the potential of serum big ET-1 as a clinical marker for cardiopulmonary and neoplastic diseases in dogs.



## Introduction

There have been many studies reporting the importance of evaluating endothelin-1 (ET-1) or big ET-1 in blood as a clinical marker for various diseases, especially cardiopulmonary and neoplastic diseases in human patients (Simpson et al., 2000; Yildirim et al., 2008; Ishikawa et al., 1995a, 1995b; Miyauchi et al., 1989). In cardiopulmonary diseases including cardiac valvular insufficiency, cardiomyopathy and primary pulmonary hypertension, ET-1 and big ET-1 blood levels have been reported to correlate with disease severity (Ishikawa et al., 1995a, 1995b; Miyauchi et al., 1989). In patients with tumors including breast, hepatocellular, gastric and prostate cancer, blood levels have been shown to elevate at an advanced stage with distant metastasis to the lymph nodes or lungs (Yildirim et al., 2008; Nakamuta et al., 1993; Ferrari-Bravo et al., 2000; Nelson et al., 1995). Prompted by these findings, clinicians have investigated the feasibility of using blood ET-1 and big ET-1 levels as clinical markers for predicting prognosis, evaluating therapeutic benefit and diagnosing these diseases (Yildirim et al., 2008; Nakamuta et al., 1993; Ferrari-Bravo et al., 2000; Nelson et al., 1995). In veterinary medicine, however, there is less information on the clinical significance of blood ET-1 and big ET-1 levels.

In this study we investigated the potential of big ET-1 as a clinical marker for canine cardiopulmonary and neoplastic diseases by comparing serum levels in canine patients diagnosed with cardiopulmonary or neoplastic disease through clinical and pathological examinations. Serum levels of N-terminal pro-brain natriuretic peptide (NT-proBNP), which is a reliable clinical marker for cardiac disease (Hori et al., 2012), was also measured and compared with big ET-1 levels in dogs with cardiopulmonary disease.

## Materials and methods

### Dogs

Twenty-one dogs (eight female and thirteen male, average age $7.8 \pm 3.6$ years old, age range 5–13 years old) with cardiopulmonary

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disease and 57 dogs with neoplastic disease (twenty five female and thirty two male, average age 8.9 ± 4.1 years old, age range 6–14 years old), diagnosed at Rakuno Gakuen University Veterinary Teaching Hospital during a period from April 2010 to July 2013, were enrolled in this study. Seventeen healthy dogs (eleven female and six male, average age 8.3 ± 3.4 years old, age range 4–10 years old) kept at Kitasato University or Rakuno Gakuen University were used as controls. There was no significant difference in the results based on age or sex among cardiopulmonary, neoplastic and control groups.

All experimental procedures in this study progressed under approval by ethics committees in Kitasato and Rakuno Gakuen Universities (ethics committee approval numbers 10-028 and VH24A13, respectively).

*Diagnosis of cardiopulmonary and neoplastic diseases*

Cardiopulmonary diseases were diagnosed by ultrasound with sector probes (3 to 7.5 MHz) in conjunction with a scanner (EUB-6500, HITACHI Medical Corporation, Tokyo, Japan) by three experienced veterinarians in accordance with commonly recognized ultrasound criteria. The cardiopulmonary disease studied was valvular (mitral and tricuspid valves) insufficiency (twenty-one cases). The presence of pulmonary hypertension was confirmed based on tricuspid valve regurgitation (TR) velocity (3.0 > m/s) in accordance with previous reports (Hori et al., 2012). Neoplastic diseases were diagnosed by histopathological examination using excised tumor tissues after surgery. The neoplastic diseases enrolled in this study were hemangiosarcomas (eight cases), malignant melanomas (eight cases), adenocarcinomas (nine cases), histiocytic sarcoma (six cases), lymphomas (seven cases), chondrosarcoma or osteosarcoma (four cases), mast cell tumors (four cases), transitional cell carcinomas (three cases), hepatocellular carcinomas (four cases), gastrointestinal stromal tumor (one case), granulosa cell tumor (one case), multiple myeloma (one case) and schwannoma (one case). Because a decrease in renal excretion lengthens the half-life of big ET-1 in blood (Rossi et al., 2013), dogs with renal disease were excluded from this study based on blood tests and urinalysis.

*Serum big ET-1 levels*

We prepared serum samples according to methods described in previous studies (Yildirim et al., 2008; Rossi et al., 2013; Gruber et al., 2000; Glaus et al., 2004). Briefly, venous blood was collected in a serum separating plastic tube. Immediately after centrifugation at 1200 *g* for 10 min at 4 °C, serum samples were stored at −80 °C until measurement.

Serum big ET-1 concentrations were assessed with a commercial ELISA kit for human big ET-1 (Big ET-1 EIA Kit, IBL, Japan) after solid phase extraction using Sep-Pak C18 cartridges (Waters, USA) according to the manufacturer's protocol. This assay kit is demonstrated by the manufacturer to have no significant cross-reactivity (<0.1%) with ET-1, ET-2, ET-3, big ET-2 or big ET-3. To show the feasibility of using a human ELISA kit for estimation of canine big ET-1, we performed a dilution experiment ($2^n$ dilution) using canine serum samples. The resulting curves obtained from the dilution experiment were parallel to the standard curve. Linearity of the curves was observed between 0.18 and 23 pmol/l. To evaluate the intra-assay and inter-assay coefficients of variance, we measured three samples five times for three alternating days. The intra-assay and inter-assay coefficients of variance were 3.9% and 2.5% respectively. Serum NT-proBNP levels were measured by a commercial laboratory (IDEXX Laboratories, Japan).

*Statistical analysis*

The Mann–Whitney *U* test was used to analyze differences in serum big ET-1 or NT-pro BNP levels with $P < 0.05$ considered significant. Spearman's rank correlation coefficient was used to analyze the correlation between serum big ET-1 levels and TR speeds. Data analyses were carried out with Excel Toukei 2010 (SSRI, Tokyo, Japan). All data in this study are shown as mean ± standard deviation.

## Results

*Serum NT-pro BNP and big ET-1 levels in cardiopulmonary disease*

Dogs with cardiopulmonary disease were divided into two groups: without and with pulmonary hypertension based on TR velocity measured by ultrasound. No significant difference was observed in serum NT-pro BNP levels between the two cardiopulmonary disease groups: those without (848 ± 1018 pmol/l) and with (1398 ± 1746 pmol/l) pulmonary hypertension. In contrast, serum big ET-1 levels were significantly ($P < 0.01$) elevated in dogs with pulmonary hypertension (6.2 ± 5.3 pmol/l) compared to those without pulmonary hypertension (2.0 ± 0.6 pmol/l) (Fig. 1). Regardless of the presence or absence of pulmonary hypertension, dogs with cardiopulmonary disease (4.6 ± 4.6 pmol/l) showed a significantly ($P < 0.01$) higher level of big ET-1 than healthy controls (1.1 ± 0.49 pmol/l). Through correlation analysis between the serum levels of NT-pro BNP or big ET-1 and TR velocity values obtained by ultrasound, which represent systolic pulmonary artery pressure, big ET-1 levels ($R^2 = 0.74$, $P < 0.01$) were shown to correlate better with the severity of pulmonary hypertension than NT-pro BNP levels ($R^2 = 0.32$, $P = 0.24$). These results suggest that serum big ET-1 could be a more reliable clinical marker than serum NT-pro BNP for detecting cardiopulmonary disease with pulmonary hypertension in dogs.

*Serum big ET-1 levels in neoplastic disease*

To investigate the potential of big ET-1 as a tumor maker, we assessed serum levels in dogs exhibiting various tumors. Serum big ET-1 levels in dogs with hemangiosarcoma (5.6 ± 2.2 pmol/l), adenocarcinoma (2.0 ± 1.8 pmol/l), histiocytic sarcoma (3.3 ± 1.9 pmol/l), chondrosarcoma or osteosarcoma (3.0 ± 1.6 pmol/l) and hepatocellular carcinoma (2.7 ± 1.8 pmol/l) were significantly ($P < 0.05$) higher than those in healthy control dogs (1.1 ± 0.53 pmol/l) (Fig. 2). Interestingly, hemangiosarcoma, a tumor derived from vascular endothelial cells, produced the highest level of all tumors examined.

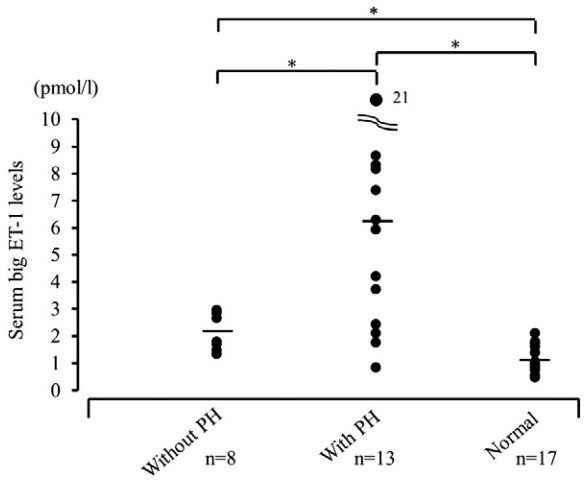


**Fig. 1.** Serum big ET-1 levels in dogs with cardiopulmonary diseases. Dogs with cardiopulmonary diseases were divided into two groups, dogs without and with pulmonary hypertension (PH), based on TR velocity measured by ultrasound. Serum big ET-1 levels were assessed in the groups without and with PH (groups with cardiopulmonary disease), compared to those of healthy control group. Serum big ET-1 levels in the dogs with the disease were significantly ($P < 0.01$) higher than those of control dogs. Significant ($P < 0.01$) and remarkable increase was observed in dogs with PH compared to the group without PH and healthy group. Each point represents an individual serum level. The horizontal lines represent the means for each group.

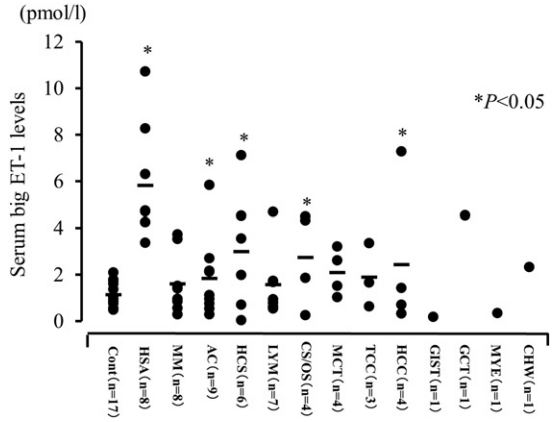


**Fig. 2.** Serum big ET-1 levels in neoplastic diseases. Serum big ET-1 levels were assessed in healthy control dogs (Cont) and patient dogs with hemangiosarcoma (HSA), malignant melanoma (MM), adenocarcinoma (AC), histiocytic sarcoma (HCS), lymphoma (LYM), chondrosarcoma or osteosarcoma (CS/OS), mast cell tumor (MCT), transitional cell carcinoma (TCC), hepatocellular carcinoma (HCC), gastrointestinal stromal tumor (GIST), granulosa cell tumor (GCT), multiple myeloma (MYE) and schwannoma (CHW). Serum big ET-1 levels in dogs with HSA, AC, HCS, CS/OS and HCC were significantly ($P < 0.05$) higher than those in healthy control dogs. Each point represents an individual serum big ET-1 level. The horizontal lines represent the means for each group.

*Comparison of serum big ET-1 levels in cardiopulmonary and neoplastic diseases*

To investigate the specificity of serum big ET-1 as a clinical marker, we compared the levels among cardiopulmonary, neoplastic and control groups (Fig. 3). As a result, we observed no significant difference in the levels between the cardiopulmonary (4.6 ± 4.6 pmol/l) and neoplastic (2.5 ± 2.3 pmol/l) groups, although the groups with the diseases showed a significant ($P < 0.01$) and notable increase compared with the control group (1.1 ± 0.53 pmol/l). These findings show that a primary diagnosis for cardiopulmonary or neoplastic disease based on clinical assessment is essential.

## Discussion

The precursor form proBNP exists as a pro-hormone that is cleaved into the inactive N-terminal fragment NT pro-BNP and active peptide BNP prior to release into blood circulation (Hunt et al., 1997). NT pro-BNP is, at present, used as a surrogate marker for the biologically active

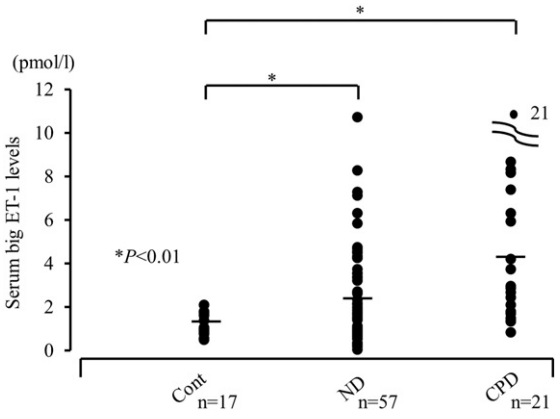


**Fig. 3.** Comparison of serum big ET-1 levels between cardiopulmonary and neoplastic diseases. Serum big ET-1 levels were quantitatively assessed in healthy control dogs (Cont) and patient dogs with neoplastic (ND) and cardiopulmonary (CPD) diseases. Serum big ET-1 levels in dogs with cardiopulmonary and neoplastic diseases were significantly higher than those in healthy control dogs. No significant difference was observed between the cardiopulmonary and neoplastic groups. Each point represents an individual serum big ET-1 level. The horizontal lines represent the means for each group.

form BNP because the peptide is more stable in vivo than the active form (Ichiki et al., 2013). Similarly, the biologically active form ET-1 is generated by enzymatic cleavage from the inactive precursor form big ET-1, which is more stable in vivo than the active form (Hemsen et al., 1995). BNP is produced by ventricular myocytes in response to ventricular myocyte stretch (Ichiki et al., 2013), while expression of *preproET-1* mRNA and production of ET-1 increase mainly in the lung of human patients with cardiopulmonary diseases in association with pulmonary vascular remodeling (Gao et al., 2013; Wang et al., 2011). We have previously demonstrated a substantial increase in *preproET-1* mRNA expression in the lungs of dogs with heartworm disease (dirofilariasis), which commonly leads to severe pre-capillary pulmonary hypertension due to the presence of nematodes within the pulmonary artery (Uchide and Saida, 2005). Although elevated *preproET-1* mRNA expression was also observed in canine heart tissues as a result of right heart overload secondary to pulmonary hypertension, vascular structural changes and overproduction of ET-1 in the lung could be closely associated with the pulmonary hypertension. The considerable increase in serum ET-1 levels observed in dogs with the diseases more likely arises from severe and extensive histological changes in the lung rather than from changes in the heart (Munnell et al., 1980; Schaub and Rawlings, 1980). Together with previous reports showing a deeper involvement of ET-1 than BNP in pulmonary disease (Hori et al., 2012; Hemsen et al., 1995) our findings that serum big ET-1 levels correlate with the severity of pulmonary hypertension suggest that serum big ET-1 could be more reliable than NT pro-BNP as a clinical marker for cardiopulmonary disease with pulmonary hypertension in dogs.

In human medicine, many tumor makers, including prostate specific antigen for prostate carcinomas, alpha-fetoprotein for hepatocellular tumors, urine basic fetoprotein for bladder cancer, carcinoembryonic antigen and breast carcinoma-associated antigen 225 for breast cancer and cytokeratin 19 fragment for lung cancer, have been studied and used clinically (Jansen et al., 2010; Morimoto et al., 2012; Imamura et al., 2000; Esteban et al., 1994; Kaneko et al., 1995; Lee et al., 2013). However in veterinary medicine, no reliable tumor marker is currently available for clinical use. Our finding that serum big ET-1 increases in a variety of canine tumors shows its promise as a tumor marker, as has been the case in human medicine (Yildirim et al., 2008; Nakamuta et al., 1993; Ferrari-Bravo et al., 2000; Nelson et al., 1995). In particular, hemangiosarcoma drew our attention because it produced the highest level of all tumors examined. This result could be explained by the fact that HSA is a tumor originating from vascular endothelial cells that have the ability to produce and secrete ET-1 (Smith, 2003; Abraham et al., 2007; Yanagisawa et al., 1988). Another contributing factor may be that disseminated intravascular coagulation occurs with high incidence in canine HSA (Smith, 2003). Reports confirming elevation of serum ET-1 and big ET-1 in human patients with disseminated intravascular coagulation support this hypothesis (Ishibashi et al., 1991, 1994; Asakura et al., 1992). An important next step is to examine serum big ET-1 levels in dogs with disseminated intravascular coagulation in order to better understand the specificity of big ET-1 as a tumor marker for HSA.

Cardiopulmonary and neoplastic diseases are listed as major causes of death in aged dogs (Bronson, 1982; Fleming et al., 2011). Serum big ET-1 levels could aid diagnosis and treatment of these diseases in veterinary medicine. However, because there is no significant difference in serum big ET-1 levels between cardiopulmonary and neoplastic patients, specific diagnostic assessment for each disease in combination with other clinical modalities including X-ray, ultrasound and blood tests would be an important first step when using this peptide as a clinical marker for diverse conditions.

## Conclusion

We have investigated the potential of serum big ET-1 as a clinical marker for canine cardiopulmonary and neoplastic diseases by

comparing the serum levels in dogs diagnosed by clinical and pathological examinations. Although further studies are necessary, our findings point to the potential of serum big ET-1 as an effective clinical marker for cardiopulmonary and neoplastic diseases in dogs.

#### Conflict of interest statement

We all authors have no conflict of interest concerning this work.

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