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Big endothelin-1 as a tumour marker for canine haemangiosarcoma

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ABSTRACT

Haemangiosarcoma (HSA) is an important malignant neoplasm of dogs that originates from vascular endothelial cells. This study explored the suitability of using serum big endothelin-1 (ET-1) as a tumour marker for canine spontaneous HSA. Serum big ET-1 was measured in dogs with splenic HSA ($n = 14$), splenic malignant tumours other than HSA ($n = 10$), benign splenic lesions ($n = 11$) and normal healthy dogs ($n = 17$) by ELISA. Serum big ET-1 levels in dogs with HSA were significantly ($P < 0.01$) higher than in other dogs. High sensitivity (100%, 95% confidence interval 86–100%) and specificity (95%, 95% confidence interval 86–95%) for HSA diagnosis were obtained using a cut-off of 17 pg/mL according to receiver operating characteristic (ROC) curves (area under ROC curve 0.93). *PPET1*, *ETA*, *VEGF* and *Hif1-α* mRNA expression, measured by real-time PCR, were elevated in HSA compared with normal tissues. These findings suggest that elevated serum big ET-1 could be used as a diagnostic marker for canine HSA.

Introduction

Haemangiosarcoma (HSA) is a relatively common malignant neoplasm in dogs, accounting for approximately 20% of all soft tissue sarcomas (Smith, 2003) and 4% of all malignant canine neoplasms (Göritz et al., 2013). Although canine HSA can occur at any site, the spleen, right atrium of the heart, liver and skin are the most common primary sites (Smith, 2003). In view of the aggressive behaviour of HSAs, the prognosis is poor, with reported 1 year survival rates of <10% (Hammer et al., 1991; Vail et al., 1995; Sorenmo et al., 2000). Early diagnosis and treatment of canine HSA is correlated with improved prognosis (Spangler and Culbertson, 1992; Sorenmo et al., 2000) and therefore a tumour marker for early diagnosis of HSA would be useful.

Endothelin-1 (ET-1) is a bioactive peptide originally isolated from vascular endothelial cells (Yanagisawa et al., 1988). ET-1 is expressed in a range of human tumours and is involved in tumourigenesis (Smollich and Wülfing, 2007; Bagnato and Rosanò, 2008). Blood concentrations of ET-1 are increased in human patients with prostatic, ovarian, breast, intestinal, pulmonary or bone tumours (Nakamuta et al., 1993; Nelson et al., 1995; Ferrari-Bravo et al., 2000; Simpson et al., 2000), suggesting the potential of serum ET-1 as a tumour marker. We recently demonstrated that serum concentrations of big ET-1, a precursor of ET-1, are higher in canine HSA than in other types of tumours (Fukumoto et al., 2014).

In this study, we investigated the potential of big ET-1 as a tumour marker for canine HSA because it is stable in blood and has a longer half-life than ET-1 (Hemsén et al., 1995). We also studied transcriptional correlation of the preproendothelin-1 (*PPET-1*) gene with the endothelin-related genes for endothelin type A receptor (*ETA*), endothelin type B receptor (*ETB*), vascular endothelial growth factor (*VEGF*) and hypoxia inducible factor 1-α (*Hif1-α*) in canine HSA.

Materials and methods

Dogs

Thirty-five dogs that were found by ultrasonographic examination to have splenic lesions and could be given a definitive diagnosis based on pathology of tissues obtained by splenectomy or biopsy at Rakunou Gakuen University, Japan, from April 2010 to October 2013 were enrolled in this study. The lesions were diagnosed by ultrasound with convex (6–8 MHz) or linear (10–13 MHz) probes in conjunction with a scanner (EUB-6500, Hitachi Medical Corporation) by three veterinarians. Dogs were excluded if they had pulmonary hypertension (tricuspid regurgitation speeds >3.0 m/s by echocardiography with 3–7 MHz sector probes) or renal disease (serum creatinine levels >2.0 mg/dL) because blood levels of big ET-1 increase in these states

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Table 1
Clinical features of dogs enrolled in this study.

	Haemangiosarcoma	Other malignant splenic tumours	Benign splenic lesions	Normal healthy dogs
Number of dogs	$n = 14$	$n = 10$	$n = 11$	$n = 17$
Breed	Miniature Schnauzer ($n = 4$) Golden Retriever ($n = 2$) Beagle ($n = 2$) Other purebreds ($n = 6$)	Mixed ($n = 2$) Other purebreds ($n = 8$)	Miniature Dachshund ($n = 4$) Shih Tzu ($n = 2$) Other purebreds ($n = 5$)	Beagle ($n = 17$)
Weight (kg) mean ± SD (range)	15.1 ± 7.1 (3–32)	7.8 ± 10.5 (3–28)	6.7 ± 5.4 (4–12)	11.9 ± 1.4 (10–14)
Age (years) mean ± SD (range)	11.8 ± 1.7 (10–15)	11.6 ± 3.3 (8–16)	9.7 ± 2.6 (6–14)	8.3 ± 3.4 (4–10)
Sex	Female ($n = 6$) Male ($n = 8$)	Female ($n = 6$) Male ($n = 4$)	Female ($n = 5$) Male ($n = 6$)	Female ($n = 11$) Male ($n = 6$)
Histopathological diagnosis	HSA ($n = 14$)	Lymphoma ($n = 7$) Histiocytic sarcoma ($n = 3$)	Haematoma ($n = 6$) Infarction ($n = 2$) Necrosis ($n = 1$) Myelolipoma ($n = 1$) Haemangioma ($n = 1$)	
Major axis of splenic mass (cm) mean ± SD (range)	3.4 ± 2.5 (0.5–12)	2.8 ± 2.0 (0.4–10)	2.4 ± 2.1 (0.8–7)	No mass
Metastasis	Absent ($n = 8$) Present ($n = 6$, Liver)	Absent ($n = 3$) Not applicable ($n = 7$, lymphoma)	Absent ($n = 11$)	Absent ($n = 17$)
Coagulation abnormalities	$n = 13$	$n = 3$	$n = 0$	$n = 0$
Anaemia (haematocrit <25%)	$n = 12$	$n = 4$	$n = 1$	$n = 0$
Weight loss (>5%)	$n = 10$	$n = 7$	$n = 1$	$n = 0$
Anorexia	$n = 13$	$n = 7$	$n = 1$	$n = 0$
Collapse	$n = 9$	$n = 0$	$n = 0$	$n = 0$

SD, standard deviation; HSA, dogs with haemangiosarcoma; OMT, dogs with other malignant tumours (splenic malignant tumours other than HSA); BL, dogs with benign splenic lesions.

Table 2
Oligonucleotide primers and cycling conditions for amplification of mRNA by reverse transcriptase PCR.

Gene	Nucleotide sequence (5′–3′)	Amplicons (bp)	Amplification conditions	GenBank
PPET-1	F: GGACAGCAGGGGGGAGAA R: GTTGACCCAGATGATGTCAAGGT	130	95 °C for 30 s, 63 °C for 30 s, 72 °C for 30 s	AB115087 (dog)
ETA	F: TTCATCGTGGGAATGGTGGG R: ACCTGTCAACACTGAGAGGG	280	95 °C for 30 s, 60 °C for 30 s, 72 °C for 30 s	NM_001031632 (dog)
ETB	F: CCTGCGAATCTGCTTGCTTC R: AGCAATCTGCATGCCACTC	177	95 °C for 30 s, 60 °C for 30 s, 72 °C for 30 s	NM_001010943 (dog)
VEGF	F: CACTGAGGAGTTCAACATCAC R: TGCTATGCTGCAGGAAACTC	92	95 °C for 30 s, 63 °C for 30 s, 72 °C for 30 s	NM_001003175 (dog)
HIF-1α	F: GGACAGCCTCACCAAACAG R: AGTTGCACGATCATCTGGC	244	95 °C for 30 s, 63 °C for 30 s, 72 °C for 30 s	XM_003639201 (dog)
RP19	F: CCTTCCTCAAAAAGTCTGCG R: GTTCTCATCGTAGGGAGC	95	95 °C for 30 s, 60 °C for 30 s, 72 °C for 30 s	XM_538673 (dog)

PPET-1, preproendothelin-1 gene; *ETA*, endothelin type A receptor gene; *ETB*, endothelin type B receptor gene; *VEGF*, vascular endothelial growth factor gene; *HIF-1α*, hypoxia inducible factor 1α gene; *RP19*, ribosomal protein S19 gene; F, forward primer; R, reverse primer; bp, base pairs.

(Hori et al., 2012; Rossi et al., 2013; Fukumoto et al., 2014). Seventeen healthy dogs kept at Kitasato University or Rakunou Gakuen University were used as controls. All experimental procedures in this study were approved by the ethics committees at Kitasato University for collection of blood and tissue samples from normal healthy dogs (approval number 10–028; date of approval 15 March 2010) and at Rakuno Gakuen University for collection of blood and tissue samples from dogs with tumours and from normal healthy dogs (approval number VH24A13; date of approval 14 February 2010).

Serum and tissue samples

Serum samples for analysis of big ET-1 levels were obtained from dogs with splenic lesions ($n = 35$) at initial presentation. On the basis of the postoperative histopathology ($n = 25$) or cytology ($n = 10$) of 35 dogs with splenic lesions (Table 1), 24 had malignant splenic tumours (14 HSA, 3 histiocytic sarcoma and 7 lymphosarcoma/lymphoma) and 11 had benign splenic lesions (6 haematoma, 2 infarction, 1 necrosis, 1 myelolipoma and 1 haemangioma). Dogs enrolled in the study were divided into three groups according to their pathological diagnosis: (1) splenic HSA (HSA group, $n = 14$); (2) splenic malignant tumours other than HSA (other malignant tumours group, $n = 10$); and (3) benign splenic lesions (benign lesions group, $n = 11$). Control serum samples were obtained from all healthy dogs ($n = 17$). Postoperative serum samples, which were available from 5/25 dogs subjected to splenectomy (serum from the remaining dogs was not suitably collected or stored), were collected 10–14 days after surgery. To investigate the relationship between serum big ET-1 levels and tumour progression, we followed a splenic HSA case for 140 days after splenectomy; the dog was confirmed by histopathological examination to have several small metastatic lesions in the liver and the largest of these was monitored for changes in size by ultrasound. Splenic tissue samples for analysis of mRNA expression levels were collected by biopsy from 11/14 dogs with HSA and 6/17 healthy dogs. Serum and tissue samples were stored at –80 °C until used for protein and molecular analyses.

RNA isolation and real-time PCR

Total RNA was extracted from neoplastic and normal tissues with the RNeasy Mini kit (Qiagen) and treated with the RNase-Free DNase kit (Qiagen). cDNA was synthesised from 1 μg total RNA using the ReverTra Ace reverse transcriptase kit (Toyobo) and oligo dT primers (Toyobo). Specific primers used to amplify canine *PPET-1*, *ETA*, *ETB*, *VEGF* and *Hif1-α* cDNA were designed based on sequence data obtained from GenBank[1] (Table 2). The gene for ribosomal protein S19 (*RP19*) was used as an internal ('housekeeping') control. Quantitative analysis of mRNA expression levels by real-time PCR was performed according to Fukumoto et al. (2013). Relative expression levels (RELs) of *PPET-1*, *ETA*, *ETB*, *VEGF* and *Hif1-α* mRNAs were obtained by normalising the cDNA values of *PPET-1*, *ETA*, *ETB*, *VEGF* or *Hif1-α* to those of *RP19* (Brinkhof et al., 2006).

Serum big endothelin-1 levels

Venous blood was collected into a serum-separating plastic tube (Serum Collection Tube, Nipro). Immediately after centrifugation at 1200 *g* for 10 min at 4 °C, serum samples were stored at –80 °C until analysed. Serum big ET-1 concentrations

[1] See: www.ncbi.nlm.nih.gov/genbank/.