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Original Article

Angiogenesis-related gene expression profile in clinical cases of canine cancer

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Abstract

The balance between pro- and anti-angiogenic signalling is tightly regulated in normal tissues to maintain the functions of the vasculature. In contrast, the overproduction of angiogenic factors and enhanced angiogenesis are frequently observed in several types of tumours. Although there have been many reports on the correlation between tumour progression and angiogenesis in humans, little is known about tumour angiogenesis in canines. Hence, we attempted to clarify whether angiogenesis contributes to tumour progression in canines as well as humans. In this study, we investigated the expression of several angiogenesis-related genes, including *CD34*, *VEGF-A*, *VEGFR-1*, *VEGFR-2*, *Ang-1*, *Ang-2*, *Tie1,* and *Tie2*, in 66 canine tumour tissues and in the normal tissues surrounding the tumours by quantitative real-time PCR analysis. Our comparative analysis between canine tumour tissues and normal tissues revealed that several angiogenesis-related genes, such as vascular endothelial growth factor (*VEGF*) and VEGF-receptor genes, were significantly upregulated in canine tumour tissues when compared to the normal tissues. We also found that the angiopoietin (*Ang*)*-1/Ang-2* gene expression ratio was lower in canine tumour tissues than in the normal tissues, suggesting less association between vascular endothelial cells and perivascular cells in the canine tumour tissues. Taken together, our results suggest that several angiogenesis-related genes may contribute to the malignant progression of canine tumours via tumour angiogenesis.

Keywords: angiogenesis, angiopoietin, canine, tumour, VEGF-A, VEGFR.

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Introduction

Angiogenesis, which enables the adequate supply of oxygen and nutrients throughout the body, is critical for normal embryonic development; however, it is also involved in the development of pathological conditions, such as cancer (Folkman 1990; Carmeliet 2005). Angiogenesis differs between normal tissues and rapidly growing solid tumour tissues (Bergers & Benjamin 2003; Carmeliet 2005). A variety of angiogenic factors that regulate angiogenesis, including vascular endothelial growth factor (VEGF) and angiopoietin (Ang), are secreted from vascular endothelial cells and tumour cells (Shibuya & Claesson-Welsh 2006; Augustin *et al.* 2009; Goel & Mercurio 2013). A recent trend for cancer therapy is the suppression of tumour angiogenesis by inhibiting the function of angiogenic factors (Wedam *et al.* 2006; Ellis & Hicklin 2008).

VEGF-A, which belongs to the VEGF family, has important roles in the growth and survival of vascular endothelial cells (Holash *et al.* 1999; Meeson *et al.* 1999; Lobov *et al.* 2002). The production of VEGF-A also results as a response to tumour growth under hypoxic conditions, when it is upregulated by hypoxia-inducible factor (Pichiule *et al.* 2004; Goel & Mercurio 2013). VEGF-A signalling is mediated by the receptor tyrosine kinases VEGF receptor-1 (VEGFR-1, also known as Flt-1) and VEGF receptor-2 (VEGFR-2, also known as KDR or Flk-1) (Takahashi *et al.* 2001; Ferrara *et al.* 2003; Goel & Mercurio 2013). VEGFRs are expressed not only in

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vascular endothelial cells, but also in many tumour types, and they mediate VEGF signalling in tumour cells (Goel & Mercurio 2013; Dang *et al.* 2017). Signal transduction of VEGFR-1 and VEGFR-2 promotes survival and growth of vascular endothelial cells (Ferrara *et al.* 2003; Shibuya & Claesson-Welsh 2006). Interestingly, it has been reported that although VEGF-A associates more strongly with VEGFR-1 than with VEGFR-2, the signal intensity of VEGFR-1 is weaker than that of VEGFR-2 (Waltenberger *et al.* 1994; Ferrara *et al.* 2003; Goel & Mercurio 2013). Therefore, VEGFR-1 is considered as negative regulator of angiogenesis, especially in embryogenesis (Shibuya & Claesson-Welsh 2006).

Ang and its receptor, Tie, are reported to have important roles in vascular remodelling and maturation (Thurston *et al.* 1999; Augustin *et al.* 2009; Thomas & Augustin 2009). Ang-1 is mainly secreted by perivascular cells, and it binds to Tie2 receptors on vascular endothelial cells (Suri *et al.* 1996, 1998; Augustin *et al.* 2009). After binding, the ligand induces the auto-phosphorylation of Tie2, stimulates intracellular signalling, and then promotes the survival of endothelial cells and suppresses apoptosis (Maisonpierre *et al.* 1997; Bogdanovic *et al.* 2006; Augustin *et al.* 2009; Thomas & Augustin 2009). Although Tie1 is also expressed on vascular endothelial cells, the function of Tie1 in angiogenesis remains mostly unknown (Augustin *et al.* 2009; Thomas & Augustin 2009). In contrast, Ang-2 is secreted by endothelial cells, and it binds to Tie2 in an autocrine manner Augustin *et al.* 2009. However, since the association between Ang-2 and Tie2 does not significantly induce the auto-phosphorylation of Tie2 (Maisonpierre *et al.* 1997; Bogdanovic *et al.* 2006; Reiss *et al.* 2007), Ang-2 likely interrupts Tie2-mediated signalling via an antagonistic action and destabilises endothelial cells (Falcón *et al.* 2009). Thus, the balance between Ang-1 and Ang-2 is tightly regulated in normal adult tissues to maintain vascular stability.

In the field of veterinary oncology, the importance of angiogenesis in tumour progression has been recognised (Restucci *et al.* 2003; Millanta *et al.* 2006; Yonemaru *et al.* 2006; Camacho *et al.* 2014), and several therapies targeting tumour angiogenesis are being used for some tumour types (Scharf *et al.* 2013; Li *et al.* 2016). However, although several studies have reported on the expression of angiogenesis-related genes in canine tumours, few studies have compared the expression profiles of angiogenesis-related genes in normal and tumour tissues. It was reported that the expression of VEGF-A was correlated with malignant progression of canine squamous cell carcinomas (SCC) and seminomas (Al-Dissi *et al.* 2007; Sleeckx *et al.* 2014). In addition, the upregulation of VEGFR-2 was also observed in canine SCC Al-Dissi *et al.* 2007. Kool *et al.* (2014) reported that the relative expression of Ang-2 was higher in adrenocortical tumours compared with normal adrenal tissues and the change in balance between Ang-1 and Ang-2 could be enhanced tumour progression.

In this study, we investigated the expression of several angiogenesis-related genes, including *CD34*, *VEGF-A*, *VEGFR-1*, *VEGFR-2*, *Ang-1*, *Ang-2*, *Tie1,* and *Tie2*, in canine tumours and the normal tissues surrounding the tumours. The gene expression of *CD34* gene was evaluated for amounts of vascular endothelial precursor cells, *VEGF-A*, *VEGFR-1* and *VEGFR-2* gene were evaluated for proliferation character of vascular endothelial cells, and *Ang-1*, *Ang-2*, *Tie1* and *Tie2* gene were evaluated for the structural stability of vascular endothelial cells. We found that several angiogenesis-related genes were upregulated in tumour tissues as compared to the normal tissues. Furthermore, the *Ang-1/Ang-2* gene expression ratio was significantly lower in benign and malignant tumours than in the normal tissues. Taken together, canine tumours have a high angiogenic potency, and the characteristic features of canine angiogenesis may provide an effective target for canine cancer therapy.

Materials and Methods

Canine tumour and normal tissue samples

Canine tumour tissues and the normal tissues surrounding the tumours were collected from 66 canine cancer patients undergoing surgery at Taguchi Animal Hospital (Saitama, Japan) between 2010 and

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2011. Written informed consent was obtained from all dog owners. The sampling of all tumour tissues and normal tissues surrounding each tumour was performed according to standard surgical resection procedures. Consequently, we always excised normal tissues close to the tumour, and both the tumour and this adjacent normal tissue were used for the study. The final diagnosis of that tumour or normal tissue was determined by histopathological examination, and the classification of the tumour was also based on the pathological diagnosis. Consequently, 28 of 66 cases were benign tumours and 38 were malignant tumours. The types of each tumour are shown in Tables 1 and 2.

RNA extraction and quantitative real-time PCR

Resected tissues were immediately immersed in RNA later (QIAGEN Inc., Hilden, Germany) and stored at 4°C. Total RNA was prepared using an RNeasy Mini Kit (QIAGEN) according to the manufacturer's instructions and the total RNA samples were treated with DNase I to degradation and contamination of genomic DNA. cDNA was reverse-transcribed from 1 μg of total RNA using Transcriptor First Strand cDNA Synthesis Kit (Roche Applied Science, Mnnheim, Germany). The measurement of gene expression by quantitative analysis was performed using the LightCycler system (Roche Applied Science). Quantitative real-time PCR analysis of the *GAPDH*, *CD34, VEGF-A, VEGFR-1, VEGFR-2 Ang-1, Ang-2, Tie1,* and *Tie2 genes* was performed using the LightCycler FastStart DNA MasterPLUS SYBR Green I system (Roche Applied Science). Primers used for PCR were listed in Table 3. PCR amplification of the housekeeping gene, *GAPDH*, was performed for each sample as control for sample loading and to allow normalisation among samples. To determine the absolute copy number of the target transcripts, the fragments of target gene amplified by PCR using the primer set were constructed with pGEM-T-easy cloning vector (Promega, Madison, WI). The concentrations of these purified plasmids were measured by absorbance at 260 nm and copy numbers were calculated from concentration of samples. A standard curve was created by plotting the threshold cycle (Ct) versus the known copy number for each plasmid template in the dilutions. The copy numbers for all unknown samples were determined according to the standard curve using LightCycler version 3.5.3 (Roche Applied Science). To correct for differences in both RNA quality and quantity between samples, each target gene was first normalised by dividing the copy number of the target by the copy number of *GAPDH*.

Table 1. Sample list (benign tumour)

Histopathological diagnosis	Number of cases
Breast adenoma	5
Benign mixed adenoma of the breast	4
Skin adnexal neoplasms	4
Leydig cell adenoma	3
Trichoepithelioma	3
Perianal gland adenoma	2
Trichoblastoma	2
Apocrine adenoma	1
Apocrine cystadenoma	1
Hemangioma	1
Inflammatory colorectal polyp	1
Mixed apocrine adenoma	1
Total	28

Table 2. Sample list (malignant tumour)

Histopathological diagnosis	Number of cases
Mastocytoma	9
Breast carcinoma	6
Hemangiosarcoma	3
Malignant mixed tumour of the breast	2
Sertoli cell tumour	2
Soft tissue sarcoma	2
Adrenal carcinoma	1
Colorectal carcinoma	1
Hemangiopericytoma	1
Hepatocellular carcinoma	1
Leiomyoma	1
Lymphoma	1
Mixed apocrine carcinoma	1
Nictitating membrane gland tumour	1
Osteosarcoma	1
Peripheral nerve tumour	1
Squamous cell carcinoma	1
Thyroid tumour	1
Transitional cell carcinoma	1
Undifferentiated sarcoma	1
Total	38

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Table 3. Primers used for quantitative real-time PCR

Gene	Sequence (5′ → 3′)
GAPDH	F: AACGGGAAGCTCACTGGCAT R: CTTGACAAAGTGGTCATTGAGGG
CD34	F: AGTCTGAGGTGAGGCCTCACT R: TGCGGCGGTTCATCAGGAAGT
VEGF-A	F: CCCACTGAGGAGTTCCAACATCAC R: GGGTTTATACCGGGATTTCTTG
VEGFR-1	F: GATGCACAGTGAAATACCCGAAA R: CAGGTTATTCGCTTCCCATCA
VEGFR-2	F: TAGTAGGCACGGCAGTGATTG R: GTCGATTCCAAAGGCATCTGC
Ang-1	F: AAAGTGTCACACTGGGACAG R: CAGCAGTGTAGAACATTCCA
Ang-2	F: TAAAGGACTTACAGGGACGG R: GATCATCATGGTTGTGCCCT
Tie1	F: CTTGTGAGAACCGAGGTTAC R: GTCTCTGTGGATGAACTGCT
Tie2	F: AGCTTCTCCATTTCGCAGCGG R: ACTCGATTGCCATCCAGCGCAC

F, Forward primer. R, Reverse primer.

Statistical analyses

The Statcel 3 add-in (OMS Publishing, Saitama, Japan) for Microsoft Excel was used for the statistical analysis. The relative expression of each of angiogenesis-related genes was compared between tumour tissues and the normal tissues surrounding the tumours using the Wilcoxon rank-sum test. The *Ang-1* to *Ang-2* gene expression ratio was also compared between tumour tissues and the normal tissues surrounding the tumours using the Wilcoxon rank-sum test. A correlation between the gene expression of *VEGF-A* and *VEGFR-2* calculated by Spearman rank correlation coefficient (Rs). Values of $P < 0.05$ were considered statistically significant.

Results

Several angiogenesis-related genes are upregulated in canine tumour tissues

To investigate whether angiogenesis-related genes are upregulated in canine tumours, we measured the mRNA expression levels of the genes in 66 canine tumour tissues (28 benign, 38 malignant) as well as the normal tissues surrounding the tumours by quantitative real-time PCR. As shown in Fig. 1, the gene expression of each of the *VEGF-A*, *VEGFR-1*, *VEGFR-2*, *Ang-1*, *Tie1,* and *Tie2* genes was significantly upregulated in the benign tumour tissues when compared to the normal tissues surrounding the tumours (Fig. 1b–e,g,h). In contrast, in malignant tumour tissues, significant upregulation was only observed for the expression of the *VEGFR-2* gene (Fig. 2d). Because VEGFR-2 mediates the angiogenic signalling by VEGF-A, we investigated a correlation between the gene expression of *VEGFR-2* and *VEGF-A*. Consequently, a positive correlation was observed in both benign (Rs = 0.57, $P = 0.003$) and malignant (Rs = 0.52, $P = 0.002$) tumour tissues. These results suggested that several angiogenesis-related gene expressions may be upregulated in tumour tissues. Especially, the upregulation of *VEGFR-2* gene was observed in common with benign and malignant tumours, but it remains unknown whether the *VEGFR-2* gene is required for tumour progression.

The expression of angiogenesis-related receptor genes on vascular endothelial precursor cells

The *CD34* gene is broadly accepted as a marker of vascular endothelial precursor cells (Fina *et al.* 1990; Asahara *et al.* 1997; Sideny *et al.* 2008). As shown in Figs 1a and 2a, the expression of the *CD34* gene did not differ significantly between the benign or malignant tumour tissues and the normal tissues surrounding the tumours; this suggested that the number of vascular endothelial precursor cells did not differ between the tumour tissues and the normal tissues. We further investigated whether the expression of angiogenesis-related receptor genes per vascular endothelial precursor cell differed between the tumour tissues and the normal tissues surrounding the tumours. To quantify the expression of angiogenesis-related receptor genes per vascular endothelial precursor cell, the mRNA copy numbers were normalised to that of the *CD34* gene [(mRNA copy number of target gene/mRNA copy number of the *GAPDH* gene)/(mRNA copy number of *CD34* gene/mRNA copy number of the *GAPDH* gene)] (Mori *et al.* 2008). As shown in Figs 3a,c,d and 4a,c,d, the

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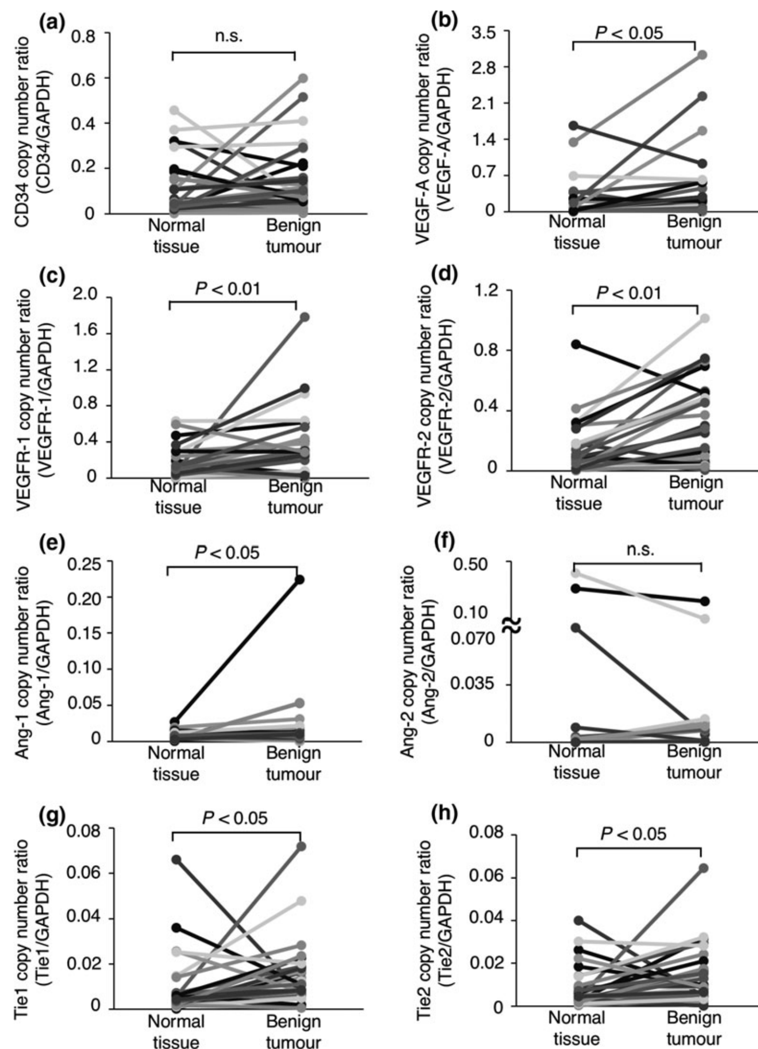

Fig. 1 The expression of angiogenesis-related genes in canine benign tumour tissues and the normal tissues surrounding each tumours. (a) The expression of the *CD34* gene. (b) The expression of the *VEGF-A* gene. (c) The expression of the *VEGFR-1* gene. (d) The expression of the *VEGFR-2* gene. (e) The expression of the *Ang-1* gene. (f) The expression of the *Ang-2* gene. (g) The expression of the *Tie1* gene. (h) The expression of the *Tie2* gene. The expression of each gene was compared between tumour tissues and the normal tissues surrounding the tumours using the Wilcoxon rank-sum test. n. s. = not significant.

expression of the *VEGFR-1*, *Tie1* or *Tie2* gene per vascular endothelial precursor cell did not differ significantly between the benign or malignant tumour tissues and the normal tissues surrounding the tumours. In contrast, the expression of the *VEGFR-2* gene per vascular endothelial precursor cell was significantly upregulated in the benign and malignant tumour tissues when compared to that in the normal tissues surrounding the tumours (Figs 3b,4b). These results suggested that the upregulation of the *VEGFR-2* gene on tumour-associated vascular endothelial precursor cells may play an important role in tumour angiogenesis regardless of the benign or malignant status of the tumour.

The Ang-1/Ang-2 gene expression ratio in canine tumour tissues

The Ang-1/Tie2 receptor signalling pathway plays important roles for the structural stability of the

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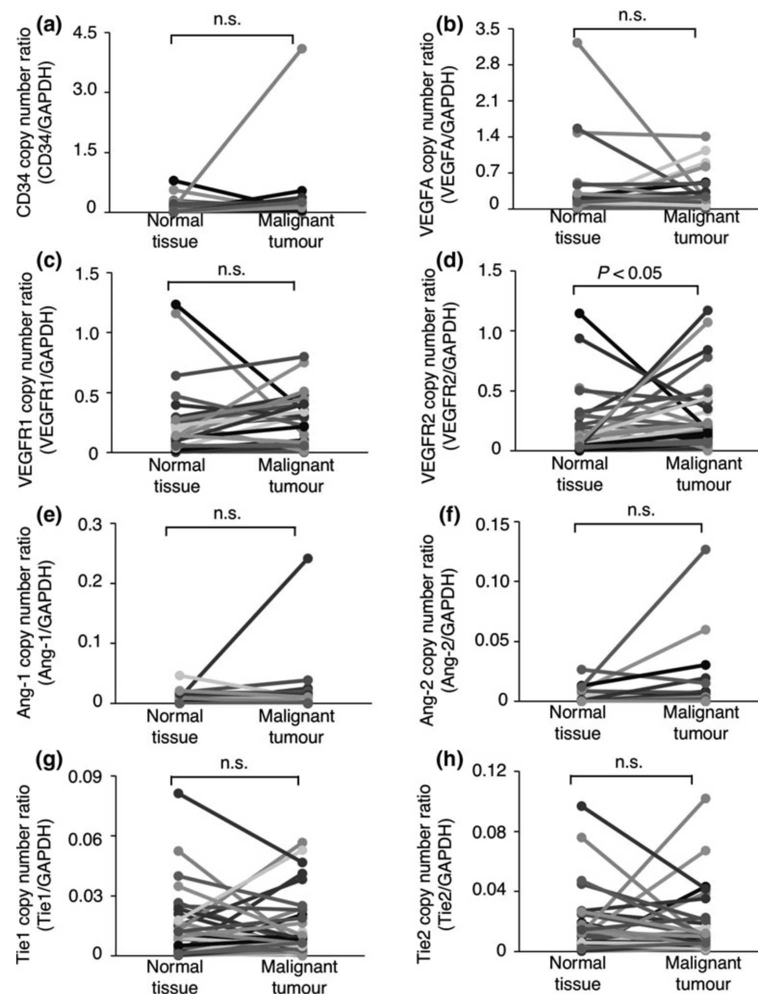

Fig. 2 The expression of angiogenesis-related genes in canine malignant tumour tissues and the normal tissues surrounding each tumours. (a) The mRNA expression of the *CD34* gene. (b) The expression of the *VEGF-A* gene. (c) The expression of the *VEGFR-1* gene. (d) The expression of the *VEGFR-2* gene. (e) The expression of the *Ang-1* gene. (f) The expression of the *Ang-2* gene. (g) The expression of the *Tie1* gene. (h) The expression of the *Tie2* gene. The expression of each gene was compared between tumour tissues and the normal tissues surrounding the tumours using the Wilcoxon rank-sum test. n. s. = not significant.

vasculature, such as interaction between vascular endothelial cell and perivascular cell (Augustin *et al.* 2009; Thomas & Augustin 2009). In contrast, Ang-2 inhibits the Tie2 signalling pathway and decreases the interaction between vascular endothelial cell and perivascular cell (Maisonpierre *et al.* 1997; Falcón *et al.* 2009). To evaluate whether the balance of *Ang-1 and Ang-2* gene expression is changed between the tumour tissues and the normal tissues surrounding the tumours, the *Ang-1*/*Ang-2* gene expression ratio was calculated [(mRNA copy number of *Ang-1* gene/mRNA copy number of the *GAPDH* gene) / (mRNA copy number of *Ang-2* gene/mRNA copy number of the *GAPDH* gene)]. The *Ang-1/Ang-2* gene expression ratio was significantly lower in both the benign and malignant tumour tissues than in the normal tissues surrounding the tumours (Fig. 5a,b). These results suggested that Ang-2 may be dominant over Ang-1 in canine tumour tissues, resulting that may induce the decrease in structure stability in vascular formations.

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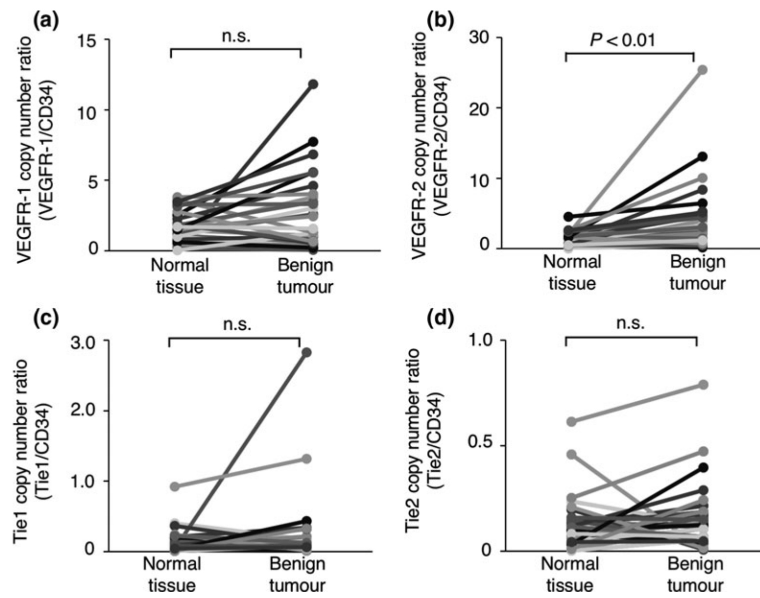

Fig. 3 The expression of angiogenesis-related receptor genes on vascular endothelial precursor cells in canine benign tumour tissues and the normal tissues surrounding each tumours. (a) The expression of the *VEGFR-1* gene. (b) The expression of the *VEGFR-2* gene. (c) The expression of the *Tie1* gene. (d) The expression of the *Tie2* gene. The expression of each gene was compared between tumour tissues and the normal tissues surrounding the tumours using the Wilcoxon rank-sum test. n. s. = not significant.

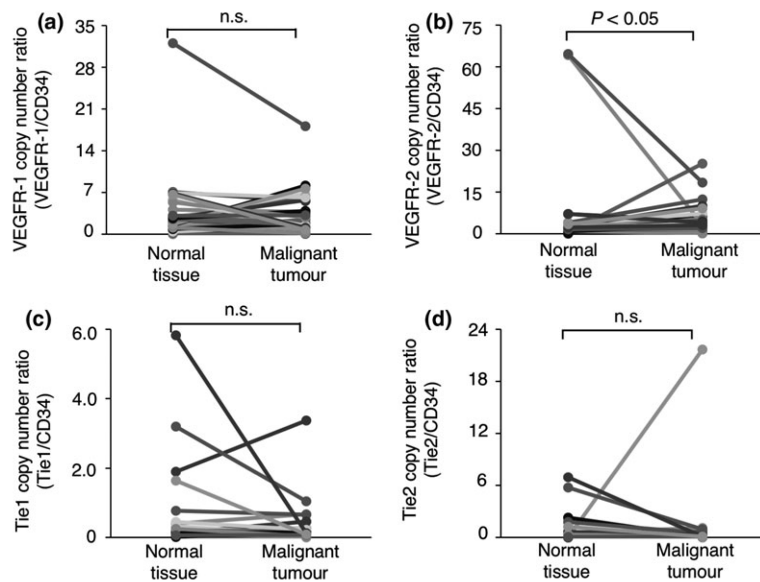

Fig. 4 The expression of angiogenesis-related receptor genes on vascular endothelial precursor cells in canine malignant tumour tissues and the normal tissues surrounding each tumours. (a) The expression of the *VEGFR-1* gene. (b) The expression of the *VEGFR-2* gene. (c) The expression of the *Tie1* gene. (d) The expression of the *Tie2* gene. The expression of each gene was compared between tumour tissues and the normal tissues surrounding the tumours using the Wilcoxon rank-sum test. n. s. = not significant.

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Discussion

The vasculature is generally quiescent and stable in normal adult tissues, with the exception of injured or developing tissues (Bergers & Benjamin 2003; Carmeliet 2005). To maintain normal vasculature, the production of angiogenic factors is tightly regulated in normal tissues (Shibuya & Claesson-Welsh 2006; Augustin *et al.* 2009; Thomas & Augustin 2009; Goel & Mercurio 2013). In contrast, angiogenic factors are frequently upregulated in tumour tissues, and the tumour vasculature is often architecturally distorted (Ellis & Hicklin 2008; Falcón *et al.* 2009). Physiological stimuli resulting from the rapid growth of a tumour, including hypoxia and nutrient starvation, activate the expression of angiogenesis-related genes in tumour and vascular endothelial cells (Zhang *et al.* 2003; Pichiule *et al.* 2004).

In this study, we investigated mRNA expression levels of the angiogenesis-related genes in 66 canine tumour tissues as well as the normal tissues surrounding the tumours. Those canine tissues contained various tumour types and lacked homogeneity. However, since the main purpose of this study was to see the common tendency of angiogenesis-related gene expression in various kinds of canine tumours, we did not focus on the heterogeneity of tumour types in this study. Also, we used *GAPDH* gene as an internal control for the quantitative real-time PCR. *GAPDH* gene has been commonly considered as a housekeeping gene that be expressed stably regardless of experimental conditions. However, the expression of *GAPDH* gene may change greatly under certain conditions, such as hypoxia (Zhong & Simons 1999). Therefore, we cannot rule out the possibility that parts of our quantitative real-time PCR data might be influenced by the change in the expression of *GAPDH* gene. For more precisely experiment, it would be necessary to measure several housekeeping genes and choose adequate internal controls in each cell and tissue type.

As a result of analysis, we observed that several angiogenesis-related genes were upregulated in canine tumour tissues and tumour-associated vascular endothelial precursor cells. The expression of the *VEGFR-2* gene was significantly upregulated in both canine tumour tissues and tumour-associated vascular endothelial precursor cells as compared to normal

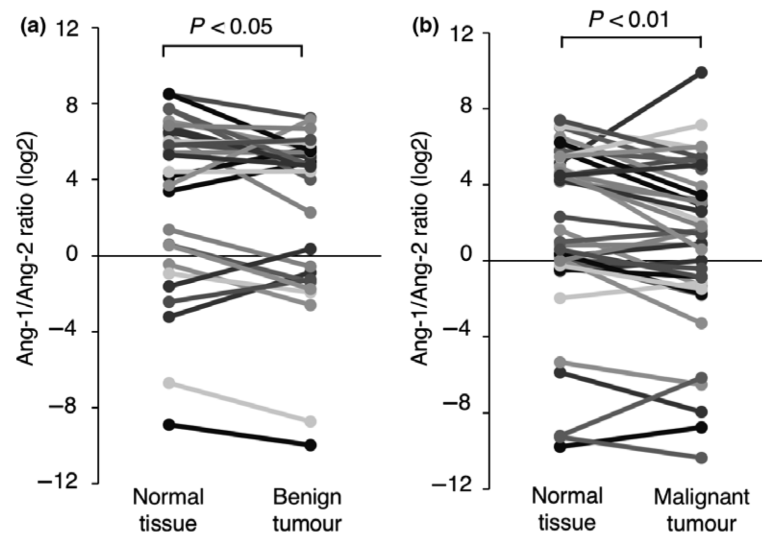

Fig. 5 The expression ratio of *Ang-1* to *Ang-2* in canine tumour tissues and the normal tissues surrounding each tumours. (a) The *Ang-1* to *Ang-2* gene expression ratio in benign tumour tissues and the normal tissues surrounding each tumours. (b)The *Ang-1* to *Ang-2* gene expression ratio in malignant tumour tissues and the normal tissues surrounding each tumours. The gene expression ratio in each tissue is represented by log2. The gene expression ratio was compared between tumour tissues and the normal tissues surrounding each tumours using the Wilcoxon rank-sum test.

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tissues surrounding the tumours. In particular, when the gene expression of *VEGFR-2* was examined in nine breast adenomas (five adenomas and four mixed adenomas of the breast) and eight breast carcinomas (six carcinomas and two mixed carcinomas of the breast), these tumours were also observed the upregulation as compared to normal tissues as well as other types of tumour, but there was no difference in adenoma and carcinoma in its gene expression (data not shown). VEGFR-2 is a key receptor that mediates VEGF-A signalling; it is expressed not only in vascular endothelial cells but also in many tumour types, and it promotes the proliferation of vascular endothelial cells and tumour cells (Waltenberger *et al.* 1994; Shibuya & Claesson-Welsh 2006; Goel & Mercurio 2013). Several studies have reported that VEGFR-2 is associated with the progression of some canine tumour types (Yonemaru *et al.* 2006; Al-Dissi *et al.* 2007). A positive correlation between the gene expression of *VEGFR-2* and *VEGF-A* was observed in benign and malignant tumours, suggesting that these gene expressions might be regulated by a similar control mechanism and work in cooperation with each other on tumour angiogenesis.

Even if the vascular endothelial precursor cells in tumour tissue are derived from these cells in normal tissues, the gene expressions would be influenced by various tumour-derived factors, such as angiogenic factors and hypoxic condition (Bergers & Benjamin 2003). Therefore, we speculated that there was difference in gene expression profile in vascular endothelial precursor cells between the niches of normal and tumour tissue. The gene expression of receptor genes, such as *VEGFR*-1, *VEGFR* -2, *Tie1 and Tie 2*, was normalised to that of *CD34* gene that is well known as a marker of vascular endothelial precursor cells, and examined amount of these gene expression (Fina *et al.* 1990; Asahara *et al.* 1997; Sideny *et al.* 2008). Consequently, *VEGFR-2* gene expression per vascular endothelial precursor cell in the tumour niche was upregulated as compared to normal. These data raise the possibility that gene expression profiles in vascular endothelial precursor cells might be different in depending on the niche.

In addition, we showed that the expression of the *VEGFR-1*, *Tie1,* and *Tie2* genes was also upregulated in benign tumour tissues when compared to the normal tissues surrounding the tumours. However, because the upregulation of these receptor genes of vascular endothelial precursor cells that normalised to *CD34* gene was not observed in a tumour niche, the upregulation of these receptor genes might occur mainly in tumour cells. Although these genes are mainly expressed in vascular endothelial cells and perivascular cells, they were occasionally observed to be upregulated in several types of tumours (Plate *et al.* 1992; Brown *et al.* 2000; Dang *et al.* 2017). Taken together, these results indicated that the upregulation of angiogenesis-related genes may be important for canine tumourigenesis, and *VEGFR-2* may be a candidate target gene for cancer therapy regardless of the benign or malignant status of the tumour.

We also demonstrated that the *Ang-1/Ang-2* gene expression ratio was lower in benign and malignant tumour tissues than in the normal tissues surrounding the tumours. These data suggested that Ang-2 gene expression could be dominant as compared to Ang-1 in tumour tissues. It is widely accepted that the Ang/Tie system plays important roles in the maturation and structural stability of blood vessels and the maintenance of the association between endothelial cells and perivascular cells (Augustin *et al.* 2009; Thomas & Augustin 2009). The binding of Ang-1 to the Tie2 receptor enhances the stability of endothelial cells by inducing auto-phosphorylation of the receptor and activating downstream signalling (Maisonpierre *et al.* 1997; Bogdanovic *et al.* 2006; Reiss *et al.* 2007). In contrast, Ang-2 would decrease the stability of endothelial cells by antagonising Ang-1 signalling, and would promote the invasion of endothelial cells and the extravasation of endothelial precursor cells from blood vessels (Ahmad *et al.* 2001; Lobov *et al.* 2002; Ellis & Hicklin 2008). It has been reported that the expression of Ang-2 in tumour cells is upregulated by various growth factors and physiological stimuli, such as VEGF-A and hypoxia (Zhang *et al.* 2003; Pichiule *et al.* 2004). Taken together, our analysis that is the *Ang-1/Ang-2* gene expression ratio was lower in tumour than normal tissues would be in agreement with these reports, suggesting that vascular instability in tumour is responsible for the balance of Ang-1/Ang-2.

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In conclusion, our results provide useful information on the expression profiles of angiogenesis-related genes in canine tumours, suggesting that tumour angiogenesis is potentially an attractive target for canine cancer therapy.

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Conflicts of interest

The authors declare that they have no conflicts of interest.

Ethics statement

The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to and the appropriate ethical review committee of Azabu University Veterinary Teaching Hospital approval has been received.

Contributions

Hiroeki Sahara designed the research; Daisuke Kobayashi and Koki Maeda carried out the research; Atsushi Tanabe performed the data analysis; Masayuki Taguchi collected canine tissue samples; Atsushi Tanabe and Hiroeki Sahara drafted the manuscript.

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