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Neutrophilic leucocytosis induced by granulocyte colony-stimulating factor and interleukin-6 in canine primary lung adenocarcinoma

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Abstract

Background: Neutrophilic leucocytosis as a paraneoplastic syndrome may occur in dogs with lymphoma, renal carcinoma, rectal polyps and metastatic fibrosarcoma. However, the information on canine lung adenocarcinoma with neutrophilic leucocytosis is lacking.

Objective: This study aimed to describe the clinical features and cytokine profiles of canine patients with primary lung adenocarcinoma and neutrophilic leucocytosis.

Methods: Two dogs (cases #1 and #2), each with a solitary lung adenocarcinoma, were included. Both cases had leucocytosis and underwent lung lobectomy. The resected tumours were analysed for the expression of granulocyte colony-stimulating factor (G-CSF), granulocyte macrophage colony-stimulating factor (GM-CSF) and interleukin-6 (IL6) by quantitative real-time PCR compared with normal lung tissues.

Results: At the initial examination, neither patient had any clinical signs or fever. White blood cell count (WBC) was 58,300/μl and 32,900/μl in cases #1 and #2, respectively. The gene expression of *G-CSF* increased 6.7-and 19.7-fold in cases #1 and #2, respectively. The gene expression of *IL6* markedly increased (30-fold) in case #1, whereas it increased slightly (1.9-fold) in case #2. On the other hand, that of *GM-CSF* was slightly changed in both cases. The WBC count postoperatively decreased to within the normal range in both cases. The postoperative survival times were 347 and 118 days in cases #1 and #2, respectively.

Conclusions: This study describes G-CSF and IL6 producing lung adenocarcinoma associated with neutrophilic leucocytosis in dogs. Canine patients with pulmonary adenocarcinomas that have elevated *G-CSF* and *IL6* levels may have a guarded prognosis. Further investigations are needed to clarify the prognosis of canine cytokine-producing lung adenocarcinoma.

KEYWORDS
Dog, Granulocyte colony-stimulating factor, Interleukin-6, Leukocytosis, Lung adenocarcinoma

1 | INTRODUCTION

Paraneoplastic syndrome (PNS) is a structural or functional abnormality of the body that occurs in association with a neoplasm and in a location remote from the tumour (Bergman, 2013). Various PNSs have been reported, including anaemia, hypoglycaemia, hypercalcaemia and leucocytosis. In veterinary medicine, neutrophilic leucocytosis has been reported to occur in dogs with lymphoma, renal carcinoma, rectal polyps and metastatic fibrosarcoma (Chinn et al., 1985; Lappin & Lattimer, 1988; Madewell et al., 1990; Thompson et al., 1992). A previous report described leucocytosis caused by expression of granulocyte colony-stimulating factor (G-CSF) and granulocyte macrophage colony-stimulating factor (GM-CSF) in a dog with primary lung adenocarcinoma (Sharkey et al., 1996). However, the detailed mechanism underlying neutrophilic leucocytosis as a PNS in canine lung cancer remains unclear.

In human medicine, lung cancer is a cancers with high mortality (Siegel et al., 2014). Lung primary cancer is divided into two main subtypes: small cell lung carcinoma and non-small cell lung carcinoma (NSCLC), including squamous-cell carcinoma, adenocarcinoma and large-cell carcinoma (Goldstraw et al., 2016). In NSCLC, several gene mutations and biological characteristics have been described (Jamal-Hanjani et al., 2017; Tfayli et al., 2017; Zappa & Mousa, 2016). Lung adenocarcinoma may induce synthesis of haematopoietic cytokines (Kasuga et al., 2001); for instance, production of G-CSF and interleukin-6 (IL6) has been reported in human lung cancers (Notsuda et al., 2010; Kawakami et al., 2020). G-CSF-producing human lung cancers are characterised by rapid proliferation and are refractory to treatment, resulting in a poor prognosis (Kasuga et al., 2001).

In dogs, adenocarcinoma is the most common primary lung tumour, as in human medicine (Rebhum & Culp, 2013). However, information on the genetic characteristics of canine adenocarcinomas is limited. In a previous study, reverse transcription-polymerase chain reaction (RT-PCR) showed that expression of G-CSF and GM-CSF was increased in paraneoplastic leucocytosis (Sharkey et al., 1996). However, other factors associated with neutrophilic leucocytosis have not yet been investigated. In addition, information on clinical features and prognosis in canine lung adenocarcinoma with neutrophilic leucocytosis is lacking.

Therefore, the purposes of this study were to report the clinical features of canine cytokine-producing lung adenocarcinoma and to demonstrate the association between the cytokine and leucocyte profiles.

2 | MATERIALS AND METHODS

2.1 | Case histories

Two dogs (cases #1 and #2) were referred to our hospital for closer inspection of a lung tumour and demonstrated leucocytosis of unknown cause. Case #1 was an 11-year-old female American cocker spaniel. Body weight was 8.3 kg. She had a history of a resected subcutaneous mast cell tumour near the 2nd left mammary gland. On physical examination, she was in good condition without fever (38.6 °C). The complete blood count (CBC) revealed marked leucocytosis (58,300/μl), among which segmented neutrophils comprised 53,300/μl, and few stab neutrophils were observed. The numbers of lymphocytes (875/μl) were decreased, and monocytes (2624/μl) and eosinophils (1458/μl) were increased (Table 1). Platelet count was slightly increased (411 $\times$ 10^3/μl). Abnormalities in the serum chemistry profile included high C-reactive protein (CRP; 2.35 mg/dl) and low albumin (2.1 g/dl) levels (Table 1). Urinalysis showed no evidence of bacterial infection. Thoracic radiography showed a large pulmonary mass (Figure 1), and computed tomography (CT) revealed the mass involving the right middle lung lobe (Figure 2). On CT, the length, width and height of the mass size were 52, 46 and 53 mm, respectively (126.7 cm^3), and no enlargement of the pulmonary lymph nodes was observed. Abdominal radiography, ultrasound and CT showed no evidence of other primary or metastatic neoplasia. In this case, the TNM classification (Martano et al., 2012) was defined as T1N0M0.

Case #2 was a 10-year-old male mixed-breed dog. Body weight was 7.1 kg. He had a history of a left adrenal tumour (its complete resection

TABLE 1 Hematology and serum chemistry examinations in cases #1 and #2

Parameter		Unit	Case #1	Case #2	Normal range
RBC		10^6/μl	5.8	6.2	5.5–8.5
PCV		%	38.6	41.6	37–55
WBC		/μl	58,300	32,900	6000–17,000
	Stab	/μl	0	0	0–300
	Seg	/μl	53,300	28,300	3000–11,500
	Lym	/μl	875	2400	1000–4800
	Mono	/μl	2624	1700	150–1350
	Eos	/μl	1458	532	100–750
Plt		10^3/μl	411	532	200–500
TP		g/dl	5.9	7.9	5.2–8.2
Alb		g/dl	2.1	3.0	2.7–3.8
Glu		mg/dl	102	103	77–125
AST		U/L	33	17	0–50
ALT		U/L	24	29	10–100
ALP		U/L	156	34	23–212
GGT		U/L	4	8	0–7
BUN		mg/dl	9	29	7–27
Cr		mg/dl	0.5	0.7	0.5–1.8
Na		mEq/L	146	146	134–153
K		mEq/L	4.3	4.2	3.4–4.6
Cl		mEq/L	114	111	105–118
CRP		mg/dl	2.2	0.55	0–1.0

RBC, red blood cell count; PCV, packed cell volume; WBC, white blood cell count; Stab, stab neutrophil; Seg, segmented neutrophil; Lym, lymphocyte; Mono, monophil; Eos, eosinophil; Plt, platelet count; TP, total protein; Alb, albumin; AST, aspartate aminotransferase; ALT, alanine aminotransferase; ALP, alkaline phosphatase; GGT, gamma-glutamyltransferase; BUN, blood urea nitrogen; Cr, creatinine; CRP, c-reactive protein.

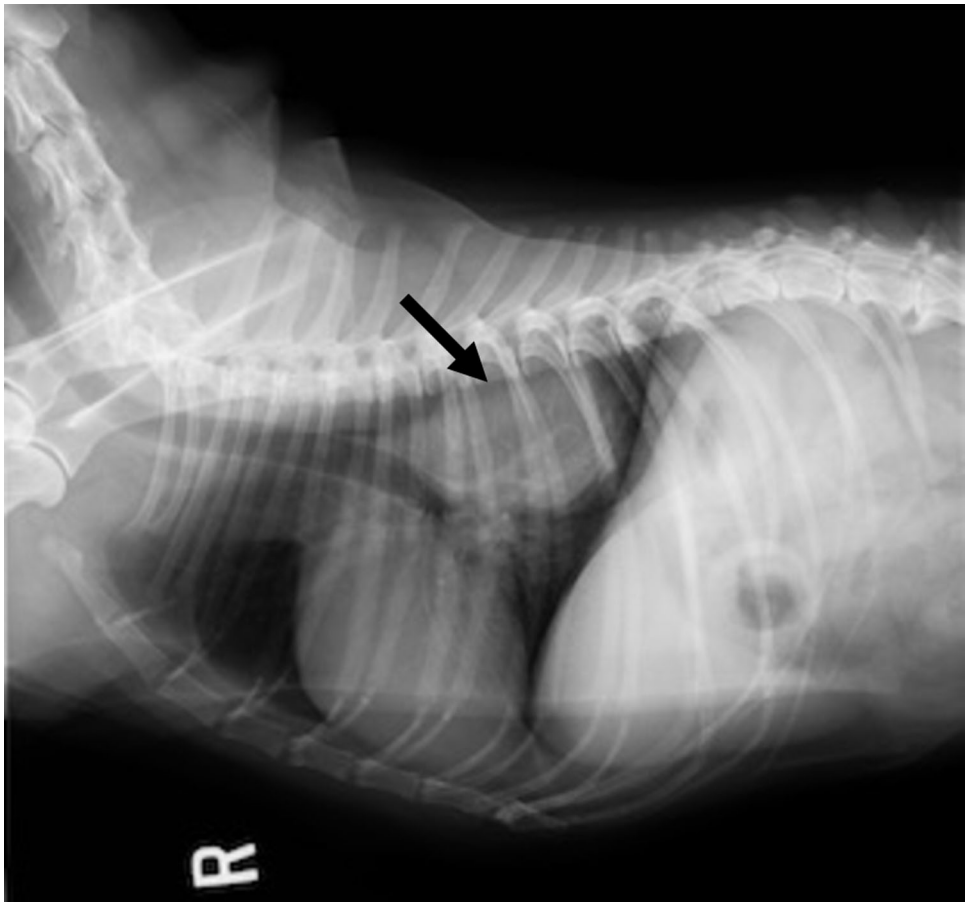

FIGURE 1 Radiographic lateral image in case #1. Radiographic image of the thorax in case #1 (right lateral view), showing a nodule with soft tissue density in the middle lung lesion (black arrow)

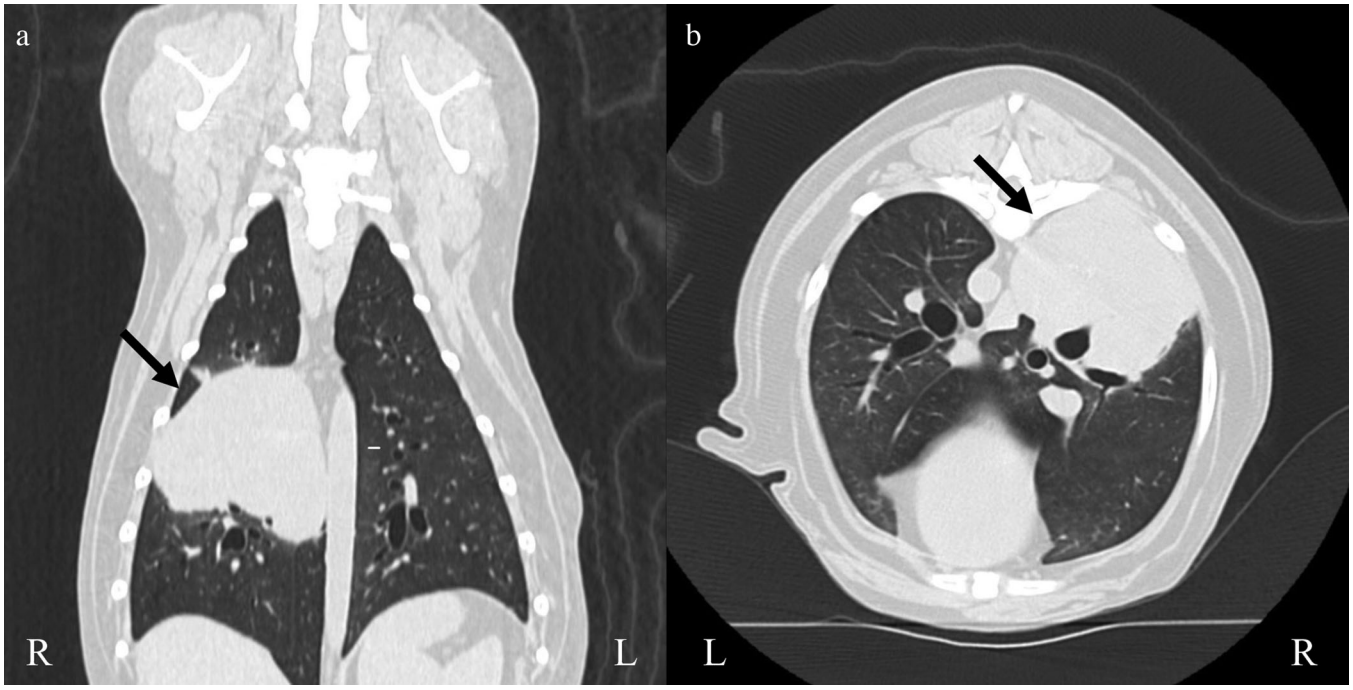

FIGURE 2 Computed tomography (CT) images in case #1. Pre-contrast CT images of coronal (a) and axial (b) views in case #1. A large nodule was found in the right middle lobe (black arrow)

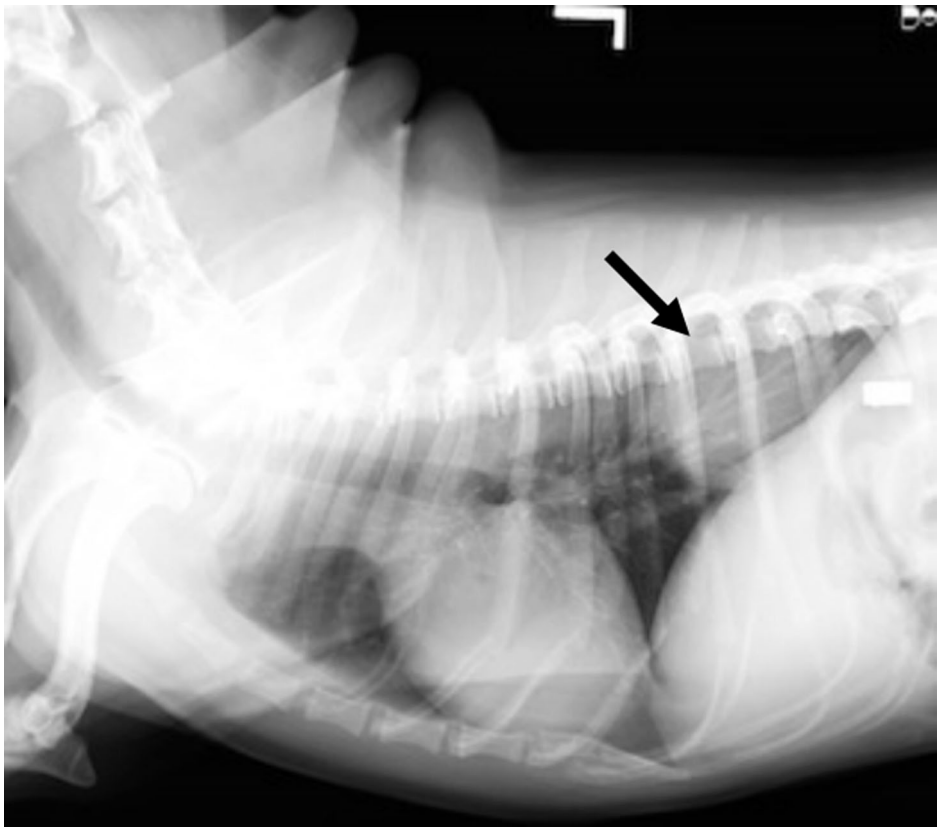

FIGURE 3 Radiographic lateral image in case #2. Radiographic image of the thorax in case #2 (left lateral view), showing a nodule with soft tissue density in the caudal lung lesion (black arrow)

had been performed 2 years ago, and the histopathological diagnosis was made as an adenocarcinoma) and an extrahepatic portosystemic shunt (complete surgical ligation was concurrently performed). On physical examination, he was in good condition, without fever (38.1°C). The CBC revealed marked leucocytosis (32,900/μl), among which segmented neutrophils comprised 28,300/μl, and few stab neutrophils were observed. The numbers of lymphocytes (2400/μl) and eosinophils (500/μl) were in the normal range. However, monocytes (1700/μl) were increased (Table 1). Platelet count was slightly increased (532 $\times$ 10^3/μl). The serum chemistry profile was normal. CRP level was 0.5 mg/dl (Table 1). Urinalysis showed no evidence of bacterial infection. Thoracic radiography showed a large pulmonary mass (Figure 3), and CT revealed the mass involving the left caudal lung lobe (Figure 4). On CT, the length, width and height of the mass size were 94.5, 54 and 25 mm, respectively (127.5 cm^3), and pulmonary lymphadenopathy was not observed. Abdominal radiography, ultrasound and CT showed no other neoplastic diseases and metastases. In this case, the TNM classification (Martano et al., 2012) was defined as T1N0M0.

2.2 | Surgery

Surgical treatment was performed 13 and 14 days after the first examination in cases #1 and #2, respectively. In both cases, general anaesthesia was induced by subcutaneous injection of 0.04 mg/kg atropine sulphate (Mitsubishi Tanabe Pharma Co., Osaka, Japan), followed by intravenous injection of 0.1 mg/kg midazolam (Dormicum; Astellas Pharma Inc., Tokyo, Japan) and 0.1 ml/kg fentanyl citrate–droperidol (Thalamonal; Daiichi-Sankyo Propharma Co., Ltd., Tokyo, Japan). General anaesthesia was induced with propofol (Mylan; Mylan Seiyaku Ltd., Tokyo, Japan). Subsequently, endotracheal intubation was performed. Each dog was mechanically ventilated with a mixture of isoflurane (IsoFlo; Zoetis Japan, Tokyo, Japan) and oxygen. For analgesia, intra- and post-operative continuous drip infusions of remifentanil (5–40 μg/kg/h) (Ultiva; Janssen Pharmaceutical K.K. Tokyo, Japan) and pre- and post-operative intramuscular injections of morphine hydrochloride (0.3 mg/kg each dose) (Takeda Pharmaceutical Co. Ltd. Osaka, Japan) were used.

Case #1 was positioned in left lateral recumbency. The thoracotomy was performed at the right 5th intercostal space, followed by the right middle lung lobectomy. Case #2 was positioned in right lateral recumbency. The left caudal lung lobectomy was done after the 5th intercostal incision. In both cases, the thoracic wall, subcutaneous tissues and skin were routinely closed.

2.3 | Histopathological examination

For histopathological examination, the resected lung masses were sliced and fixed in 10% neutral-buffered formalin, and subsequently

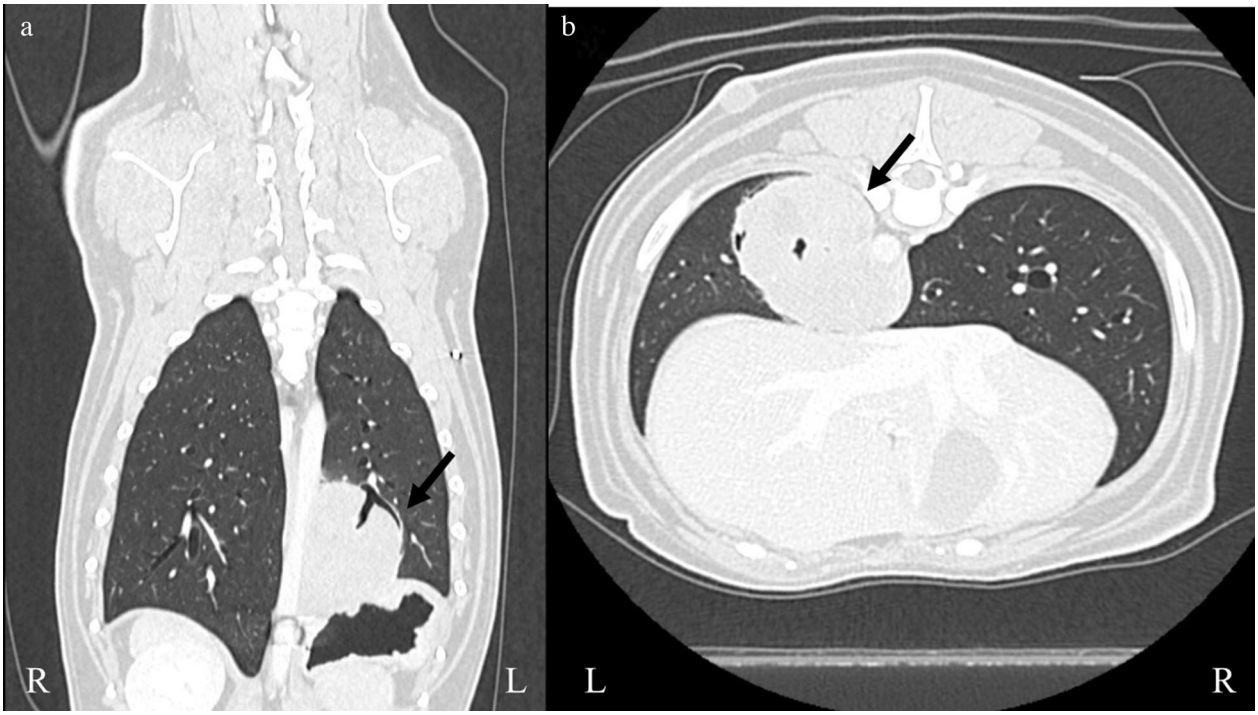

FIGURE 4 Computed tomography (CT) images in case #2. Pre-contrast CT images of coronal (a) and axial (b) views in case #2. A large nodule was found in the left caudal lobe (black arrow)

embedded in paraffin for histopathological diagnosis. Sections (4 μm thick) were deparaffinised in xylene and rehydrated through ethanol to water. The slides were then stained with haematoxylin and eosin, and histopathologically evaluated.

2.4 | Gene expression analysis

The resected lung masses were also subjected to gene expression analysis. Using RNA nucleospin columns (TAKARA Bio Inc, Shiga, Japan), total RNA was extracted from the masses of both cases, and normal lung tissues (control), which had been obtained from five healthy beagles and stored. Tissue samples were homogenised using a motorised tissue grinder (Fisher brand®, Thermo Fisher Scientific, Waltham, MA, USA), according to the manufacturer's instructions. The quality and quantity of the RNA were determined spectroscopically using a NanoDrop 1000 spectrophotometer (Invitrogen, Carlsbad, CA, USA). A High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, CA, USA) was used for cDNA synthesis, according to the manufacturer's instructions.

Using the cDNA generated, the expression levels of *CSF3* (encoding G-CSF), *CSF2* (encoding GM-CSF), *colony-stimulating factor 3 receptor* (*CSF3R*) and *IL6*, as well as that of a housekeeping gene, *GAPDH*, were analysed by real-time PCR based on the use of TB Green™ Premix EX Taq™ II (TAKARA Bio Inc). The following primers were used: dog *CSF3* (5'-AGA GCT TCC TGC TCA AGT GC-3' and 5'-AAC TCC TCA GGA TGG CAC AG-3'), dog *CSF2* (5'-TCT CTG AAG TGT TTG ACC CTG A-3' and 5'-AAG GGA TTC TTG AGG CTG GT-3'), dog *CSF3R* (5'-TAC CAG AAC ATG GGC ATC TG-3' and 5'-TGT ACA GGC TTG GCT TCC AT-3'), dog *IL6* (5'-CCA CTC ACC TCT GCA AAC AA-3' and 5'-TTT TCT GCC AGT GCC TCT TT-3'), dog *GAPDH* (5'-ATG ATT CTA CCC ACG GCA AA-3' and 5'-TCT CCA TGG TGG TGA AGA CC-3'). Primers were mostly designed using Primer3plus (http://www.bioinformatics.nl/cgibin/primer3plus/primer3plus.cgi) and BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The ΔCt values (target gene Ct value – housekeeping gene Ct value) for each gene were calculated in the control and tumour samples. The ΔCt values of the controls were then averaged, and the ΔΔCt values (tumour sample target gene ΔCt – average control sample gene ΔCt value) were calculated from these values and the ΔCt values of each tumour sample. The relative quantification of each gene in the tumour sample, relative to the normal tissue, was determined using the comparative method ($2^{-\Delta\Delta Ct}$) (Livak & Schmittgen, 2001).

2.5 | Postoperative evaluation

The patients were postoperatively evaluated by physical examination, CBC and serum chemistry profile on every consultation day. Postoperative outcome was determined from medical records and telephonic contact with the referring veterinarians and/or owners. Survival time of the patients was calculated from the time of surgery to death.

3 | RESULTS

3.1 | Histopathological evaluation

In both cases, histopathological diagnosis was adenocarcinoma derived from the bronchial epithelium or alveolar epithelium, and the tumours

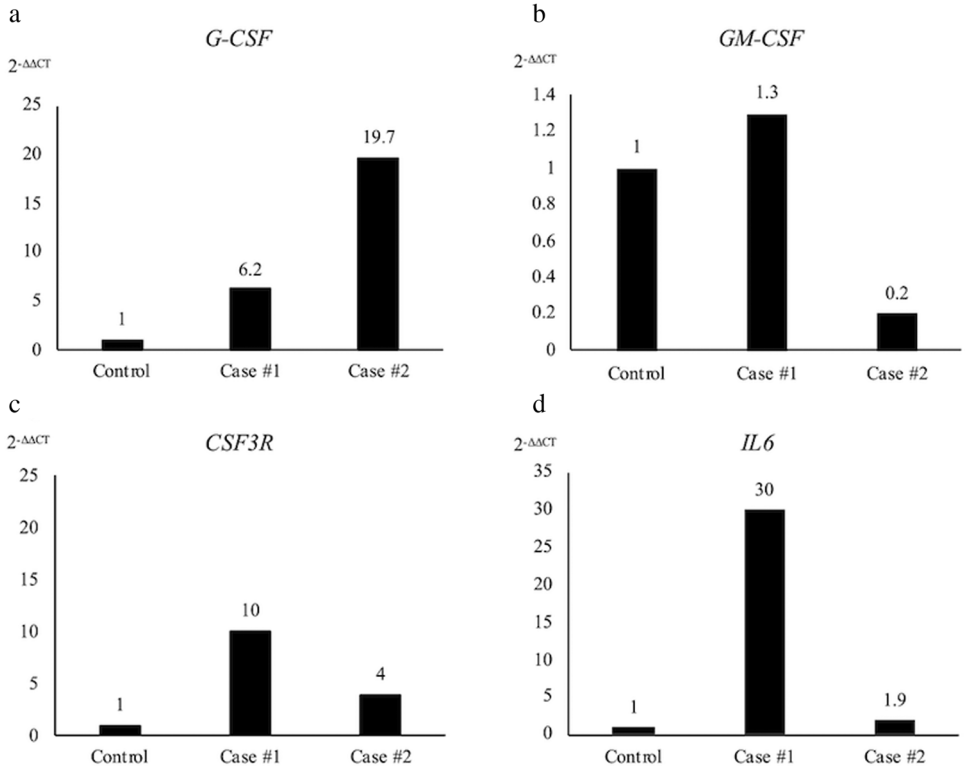

FIGURE 5 Gene expression of *G-CSF* (*CSF3*), *GM-CSF* (*CSF2*), *CSF3R* and *IL6* in canine primary lung cancer with leucocytosis. Real-time PCR analysis of *G-CSF* (a), *GM-CSF* (b), *CSF3R* (c) and *IL6* (d) expression in both cases. Data were normalised to *GAPDH*, compared to normal lung tissues as 1 (control)

consisted of neoplastic growth of papillary to tubular epithelial cells. The tumour grew into the lung parenchyma in both cases, whereas no infiltration of inflammatory cells was observed in the lung tumours. In addition, no bacterial or fungal infections were observed, and any necrotic tissues were not seen. The surgical margin was clean in both cases.

3.2 | Gene expression levels

The gene expression levels of *G-CSF (CSF3)*, *GM-CSF (CSF2)*, *CSF3R* and *IL6* are shown in Figure 5. The expression level of *G-CSF* was increased 6.2- and 19.7-fold in cases #1 and #2 compared to the control, respectively. Furthermore, *CSF3R* levels were increased by 10- and 4-fold in cases #1 and #2, respectively. *IL6* levels were increased 30-fold in case #1, whereas they were slightly increased, by 1.9-fold, in case #2. There was no difference in *GM-CSF* expression between case #1 and the control. On the other hand, *GM-CSF* expression in case #2 was decreased by 0.2-fold compared to that in the control.

3.3 | Postoperative progress

In case #1, no postoperative complications, including bleeding or pneumothorax, were observed, and the patient was discharged 6 days after surgery. No postoperative chemotherapy has been performed. The preoperatively increased leucocyte count decreased to 45,600/μl, 12,800/μl and 7300/μl at 1, 5 and 17 days after the surgery, respectively (Figure 6a). Similarly, CRP decreased to 1.3 and 0.3 mg/dl by 5 and 17 days after the surgery, respectively (Figure 6b). Platelet count did not change. Case #1 died on 347 days after the operation due to unknown causes.

In case #2, no postoperative complications, including bleeding or pneumothorax, were observed, and the patient was discharged 7 days after surgery. The preoperatively increased leucocyte count decreased to 27,900/μl, 19,700/μl and 6900/μl at 1, 7 and 40 days after the surgery, respectively (Figure 6a). Platelet count did not change. CRP had increased to 1.15 mg/dl and decreased to 0.1 mg/dl by 7 and 40 days after surgery, respectively (Figure 6b). On 95 days after surgery, pulmonary metastasis was revealed by thoracic radiography, so 2.1 mg/kg of toceranib (Palladia®; Zoetis, Florham Park, NJ, USA) was administered every other day. Case #2 died 118 days after the operation; thoracic radiography revealed many pulmonary nodules suspected to be metastasized. The cause of death was not identified because the necropsy was not performed.

4 | DISCUSSION

In this study, leucocytosis was found in the presence of increased expression of genes such as *G-CSF* in two dogs with naturally

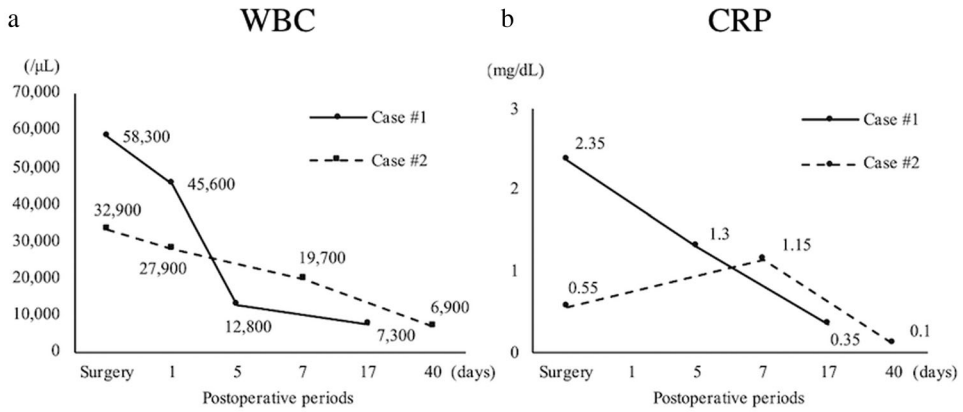

FIGURE 6 Postoperative changes in white blood cell count (WBC) and serum C-reactive protein (CRP). The graphs show WBC (a) and serum CRP (b) from the time of surgery to the postoperative period for both cases

occurring lung adenocarcinoma. In addition, the expression of cytokines such as *CSF3R* was also upregulated, a finding that has not been previously described in this population.

There are many factors involved in leucocytosis without PNS, including infection (bacterial, mycotic, viral), inflammation, metabolism (stress, endogenous or exogenous corticosteroids) (Tvedten & Raskin, 2011). In both cases, there was a marked peripheral blood neutrophilia without a significant left shift. While a stress or steroid response in case #1 may have contributed somewhat to the neutrophilia given the lymphopenia, the degree of neutrophilia in these cases is quite notable. The expression of G-CSF and GM-CSF has been previously reported to be increased in paraneoplastic leucocytosis. (Sharkey et al., 1996). However, our cases demonstrated an increase in gene expression of *G-CSF* in contrast to *GM-CSF*. The causes of this phenomenon are still unclear, so further investigations on the GM-CSF in canine cytokine-producing lung adenocarcinoma are required.

G-CSF and GM-CSF are cytokines that promote production of neutrophils, monocytes, eosinophils and basophils, (Mehta et al., 2015; Shochat et al., 2007) and may have been related to leucocytosis in both our cases. Asano et al. (1997) demonstrated that G-CSF was produced autonomously in the plasma of nude mice transplanted with human lung cancer cells; this was the first report of G-CSF-producing lung cancer. Diagnostic criteria for G-CSF-producing tumours in human medicine are as follows: (1) increased leucocytes due to unknown causes; (2) increased serum G-CSF level; (3) decreased white blood cell count after surgery and (4) evidence of G-CSF expression in the tumour tissue (Hanaoka et al., 2015). Definitions (1), (3) and (4) were fulfilled in both cases, whereas the increase in the serum G-CSF concentration could not be investigated because anti-canine G-CSF antibodies were not available. These results suggested that the lung masses in both cases were G-CSF producing lung adenocarcinomas.

IL6 is a cytokine involved in humoral immunity and functions as an inflammatory cytokine, similar to TNF-α and IL1 (Dinarello, 2018; Idriss & Naismith, 2000; Tanaka et al., 2014). Increased expression of IL6 induces CRP expression in the liver. In addition, it may cause fever (Tanaka et al., 2014). IL6-producing tumours were previously reported to increase body temperature and CRP levels in humans (Notsuda et al., 2010). In addition, IL6 has been recognised as an important cytokine for tumour metastasis and is a prognostic marker in human NSCLC (Chang et al., 2013; Shintani et al., 2016). Case #1 showed a marked increase in *IL6* expression, possibly indicating an IL6-producing tumour. In addition, she had no clinical signs related to inflammation and histopathological diagnosis revealed little inflammatory cells infiltration within the tumour. Thus, preoperative elevation of CRP was thought to be associated with IL6-producing lung tumour in case #1; however, the CRP elevation was not related with the fever. Some studies have described that the mechanism of fever is regulated by multiple factors including various inflammatory cytokines, prostaglandins and thermoregulation in the hypothalamus (Bernheim, 1985; Netea et al., 2000). The only increase in *IL6* expression was not thought to be directly related to the fever. On the other hand, IL6 was only slightly expressed in case #2, suggesting that CRP levels and body temperature did not increase.

CSF3R is a known receptor for G-CSF (Beekman & Touw, 2010). It is also involved in tumour growth, and interaction between CSF3R and G-CSF causes tumour growth (Yeo et al., 2018). The expression of *CSF3R* has been reported in a variety of human tumours (Morris et al., 2014; Tsuzuki et al., 1998; Wojtukiewicz et al., 2016); however, to our knowledge, it has not been reported in dogs. Recent studies have shown that G-CSF has a pro-tumourigenic function by promoting migration of breast cancer cells and an anti-inflammatory macrophage phenotype (Hollmén et al., 2016). Furthermore, it has been reported that elevated expression of *CSF3R* promotes an overall pro-tumourigenic immune phenotype (Karagiannidis et al., 2020). In both cases, the lung tumours were >100 cm^3 in size and the expression of *CSF3R* was increased. Thus, although various factors are associated with the timing of development and tumour growth, G-CSF and CSF3R may have been involved in tumour growth in both our cases.

The median survival time (MST) of canine patients with lung cancer has been reported to be 545 days in cases with no clinical signs (Polton et al., 2008) and depends on tumour size, which has been reported as 26, 7 and 3 months in T1, T2 and T3 tumours, respectively (Mcneil et al., 1997). In addition, lymph node metastasis was associated with an MST of 1 month (Polton et al., 2008). However, the association between

prognosis and G-CSF levels has not yet been demonstrated in dogs with lung cancer. In human medicine, G-CSF expression is associated with inferior survival (Nakamura et al., 1997). In our study, both cases were categorised as T1N0M0, and their survival times were shorter than those previously reported. G-CSF-producing lung cancer may have a poor prognosis in dogs as it does in humans.

Limitation of this study included the small number of cases and no necropsies. It is also important to study markers for the early diagnosis of G-CSF-producing lung adenocarcinoma in dogs, mainly the development of anti-G-CSF antibodies in dogs. In addition, effective anticancer and molecular targeted therapies should be explored in the future.

In conclusion, this study describes G-CSF and IL6 producing lung adenocarcinoma associated with neutrophilic leucocytosis in dogs. Canine patients with pulmonary adenocarcinomas that have elevated *G-CSF* and *IL6* levels may have a guarded prognosis. Further investigations are needed to clarify the prognosis of canine cytokine-producing lung adenocarcinoma.

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ANIMAL WELFARE AND ETHICS APPROVAL

Informed owner consent of all dogs was obtained prior to the first evaluation, and all procedures were approved by our Institutional Ethical Committee.

AUTHOR CONTRIBUTIONS

Kei Tamura: conceptualisation (supporting); formal analysis (lead); investigation (lead); methodology (lead); writing – original draft preparation (equal); writing – review and editing (equal). **Kumiko Ishigaki**: conceptualisation (supporting); writing – original draft preparation (equal); writing – review and editing (equal). **Keigo Iizuka**: investigation (supporting); writing – review and editing (equal). **Takahiro Nagumo**: investigation (supporting); writing – review and editing (equal). **Orie Yoshida**: writing – review and editing (equal). **Kazushi Asano**: conceptualisation (lead); formal analysis (supporting); methodology (supporting); writing – original draft preparation (equal); writing – review and editing (equal).

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