

博士論文

**Molecular pathogenesis of bone marrow disorders in dogs**

(犬の骨髓疾患における分子病態の解析)

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## **General Introduction**

Bone marrow disorder is one of the major causes of cytopenia including non-regenerative anemia in dogs and is categorized into neoplastic, hypoplastic, and dysplastic disorders (Weiss, 2006). Neoplastic bone marrow disorders include acute leukemia, chronic leukemia, lymphocytic neoplasms, and myeloproliferative neoplasms. In a retrospective study of canine bone marrow disorders, non-neoplastic bone marrow disorders were reported to account for more than 60% of all bone marrow disorders in a single center of America (Weiss, 2006). Among non-neoplastic bone marrow disorders, hypoplastic disorders include aplastic anemia (AA), non-regenerative immune-mediated anemia (NRIMA), and pure red cell aplasia (PRCA) (Brazzell & Weiss, 2006; Stokol et al., 2000; Weiss, 2002; Weiss & Smith, 2002) (Table 0). Dysplastic bone marrow disorder is designated as myelodysplastic syndrome (MDS) (Weiss & Smith, 2000). Despite the high incidence of non-neoplastic bone marrow disorders in dogs, only a limited number of studies on their clinical features have been conducted.

AA is diagnosed when pancytopenia, which simultaneously shows anemia, leukopenia, and thrombocytopenia, occurs and a replacement of hematopoietic area with adipose tissue is shown on bone marrow examination (Brazzell & Weiss, 2006). AA is poorly reported in veterinary medicine, but possible causes include infectious diseases, medications, toxins, and exposure to radiation (Weiss et al., 1999; Weiss & Klausner, 1990). PRCA is characterized by nonregenerative anemia with severe depletion or absence of erythroid precursor cells (Stokol et al., 2000; Weiss, 2002). Severe depletion of erythroid precursor cells is defined by myeloid/erythroid ratio  $> 75$  (Watson et al., 1975). NRIMA is diagnosed when non-regenerative anemia is continuously present

without any other diseases to cause non-regenerative anemia such as PRCA, neoplastic diseases, dysplastic diseases, and infectious diseases (Stokol et al., 2000). MDS is diagnosed when continuous cytopenia and dysplastic features were found and the number of blast cells was less than 20-30% of bone marrow cells (Bennett et al., 1982). Dysplastic features include megaloblastic appearances, fragmented nuclei and binucleate cells in erythroid cells; ring neutrophils, giant metamyelocytes, giant band neutrophils, and pseudo-Pelger-Huet cell morphology in myeloid cells; dysmorphic megakaryocytes with multiple nuclei, mononucleate megakaryocytes, and micro-megakaryocytes in megakaryocytes (Jain et al., 1991; Weiss, 2006). Since dysplastic features are often found even in normal bone marrow cells, the frequency of dysplastic features among each lineage is used to recognize “true” dysplastic features (Blue, 2003; Jain et al., 1991; Weiss & Smith, 2000). However, the threshold of the frequency of dysplastic features has been various among reports in veterinary medicine (10-25%) (Blue, 2003; Weiss & Smith, 2000).

In human medicine, the threshold of the frequency of dysplastic features to identify “true” dysplastic features is defined as  $> 10\%$  (Arber et al., 2016; Senent et al., 2013). Meanwhile, interobserver differences to identify dysplastic features were reported, and it was suggested that cytogenetic analysis or clonality analysis were important for the objective diagnosis of MDS (Font et al., 2013; Valent et al., 2007). Recently, MDS-unclassifiable (MDS-U) was defined as a subtype of MDS where MDS-specific cytogenetic aberration was found although the frequency of dysplastic features was  $< 10\%$  (Margolskee et al., 2017; Valent et al., 2007). Therefore, the frequency of the dogs

diagnosed with MDS might be changed if detailed cytogenetic aberrations are revealed and the diagnostic classifications are reestablished.

In dogs, clinicopathological features of various non-neoplastic bone marrow disorders, including PRCA, NRIMA, AA, and MDS have been rarely investigated. In addition, the molecular pathophysiology of non-neoplastic bone marrow disorders has been still to be uncovered. Hence, molecular biological investigations are needed to elucidate the pathogenesis of canine non-neoplastic bone marrow disorders.

Based on these backgrounds, a series of studies in this thesis was carried out to investigate the pathogenesis of non-neoplastic bone marrow disorders in dogs. A retrospective study on clinicopathological features and clinical outcome of canine non-neoplastic bone marrow disorders was conducted in Chapter 1. The results showed that Miniature Dachshund (MD) was a predisposed breed to be diagnosed with non-neoplastic bone marrow disorders. Moreover, immunosuppressive treatment-resistant MDs showed distinctive features compared with treatment-responsive MDs. Based on these findings, it was speculated that genetic background to develop non-neoplastic bone marrow disorder exists in MDs and bone marrow disorders of treatment-resistant MDs were different from those of treatment-responsive MDs. Therefore, whole exome sequencing analysis was conducted to explore genetic mutations in MDs with non-neoplastic bone marrow disorders in Chapter 2. Based on the results of Chapter 2, epigenetic aberration in MDs with non-neoplastic bone marrow disorders was investigated in Chapter 3. Since distinct gene expression profiles (GEPs) were suggested in treatment-resistant MDs compared with treatment-responsive MDs in Chapter 3, the analysis of GEPs was

conducted in treatment-resistant MDs in Chapter 4.

Table 0. Classification of non-neoplastic bone marrow disorders.

Disease	Cause	Characteristics	References
Aplastic anemia (AA)	Various factors (i.e infectious diseases, medications, toxins, radiation)	<ul style="list-style-type: none"> <li>• Pancytopenia in blood</li> <li>• Hypocellular bone marrow with the hematopoietic spaces replaced by adipose tissue</li> </ul>	Brazzell & Weiss, 2006
Non-regenerative immune-mediated anemia (NRIMA)	Immune-mediated destruction of erythroid precursor cells	<ul style="list-style-type: none"> <li>• Non-regenerative anemia</li> <li>• Normal to increased erythroid precursor cells in bone marrow</li> </ul>	Stokol et al., 2000; Weiss, 2002
Pure red cell aplasia (PRCA)		<ul style="list-style-type: none"> <li>• Non-regenerative anemia</li> <li>• Erythroid aplasia (Myeloid-to-Erythroid ratio &gt; 75:1)</li> </ul>	
Myelodysplastic syndrome (MDS)	Genetic and epigenetic aberrations in hematopoietic stem cells	<ul style="list-style-type: none"> <li>• Cytopenia of at least 1 lineage (Non-regenerative anemia, leukopenia, or thrombocytopenia)</li> <li>• Dysplastic features of at least 1 lineage</li> <li>• Clonal proliferation of hematopoietic stem cells</li> <li>• Blast cell ratio &lt; 20-30%</li> </ul>	Weiss & Smith, 2002



## **Chapter 1**

Clinicopathological features and clinical outcome of  
non-neoplastic bone marrow disorders in dogs

## **ABSTRACT**

Non-neoplastic bone marrow disorders such as non-regenerative immune-mediated anemia, pure red cell aplasia, and myelodysplastic syndrome are major causes of non-regenerative anemia in dogs. However, the clinical and clinicopathological features of canine non-neoplastic bone marrow disorders have been rarely reported. Hence, I first investigated the breed disposition of non-neoplastic bone marrow disorders that induce anemia and found that Miniature Dachshund (MD) was a predisposed breed. Based on this finding, I compared the clinical and clinicopathological features of non-neoplastic bone marrow disorders between MDs and other breeds (non-MDs) and found that MDs showed an increased platelet count and increased frequencies of dysplastic features in the peripheral blood compared with non-MDs. Furthermore, I found that treatment-resistant MDs showed an increased platelet count and increased frequencies of dysplastic features in the peripheral blood when compared to treatment-responsive MDs. These results indicated that bone marrow disorders in treatment-resistant MDs might manifest distinct features compared with those in other dogs, and the responsiveness to treatments could be predicted based on the platelet count and dysplastic features in the peripheral blood.

## **Introduction**

Bone marrow disorder is one of the major causes of non-regenerative anemia in dogs and is categorized into neoplastic, hypoplastic, and dysplastic diseases (Weiss, 2006). Besides neoplastic diseases, non-neoplastic bone marrow disorders that could induce anemia in dogs include aplastic anemia, myelofibrosis, bone marrow necrosis, myelodysplastic syndrome (MDS), non-regenerative immune-mediated anemia (NRIMA), and pure red cell aplasia (PRCA) (Brazzell & Weiss, 2006; Stokol et al., 2000; Weiss, 2002, 2005; Weiss & Smith, 2000, 2002). It has been previously reported in the United States that Labrador Retrievers and American Eskimos are predisposed to NRIMA and bone marrow necrosis, respectively (Stokol et al., 2000; Weiss, 2005). Meanwhile, the breed predisposition of non-neoplastic bone marrow disorders has not been conducted in Japan. Hence, the primary objective of this study was to investigate the canine breed disposition of non-neoplastic bone marrow disorders that can induce anemia. In this retrospective study, Miniature Dachshund (MD) was significantly overrepresented to be diagnosed with non-neoplastic bone marrow disorders that induce anemia. Thus, my secondary objective was set to investigate the clinical and clinicopathological features of non-neoplastic bone marrow disorders observed in this breed and compare them with those in other breeds (non-MDs).

## Materials and Methods

### *Retrospective investigation of predisposed breeds of bone marrow disorders that induce anemia*

Medical records of dogs referred to the Veterinary Medical Center of the University of Tokyo (VMC-UT) and diagnosed with non-neoplastic bone marrow disorders between April 2017 and March 2018 were retrospectively reviewed. Non-regenerative anemia was defined as moderate to severe anemia (Hct < 30%) of minimum 5-day history with absolute reticulocyte count <  $60 \times 10^3 /\mu\text{l}$  according to a previous study (Stokol et al., 2000). All dogs underwent a physical examination, complete blood count (CBC), blood biochemistry, radiographic and ultrasonographic examinations, and bone marrow aspiration. Dogs were excluded if they had apparent causes other than bone marrow disorders that could induce anemia based on the results of the blood examinations and diagnostic images described above. Dogs diagnosed with any neoplastic diseases were also excluded. Information on age, sex, and breed of the dogs were extracted from the medical records, and the predisposed breed was investigated in comparison with other breeds referred to VMC-UT in the same time period.

### *Clinical and clinicopathological features of bone marrow disorders in MDs and non-MDs*

In the first retrospective study, I found that MD was the significantly overrepresented breed diagnosed with non-neoplastic bone marrow disorders that induce anemia. Therefore, I decided to investigate the clinical and clinicopathological features of non-neoplastic bone marrow disorders observed in MDs and compare them with those in non-

MDs. For this purpose, the medical records of dogs that were referred to VMC-UT and diagnosed with non-regenerative anemia due to non-neoplastic bone marrow disorders between April 2015 and March 2018 were retrospectively reviewed according to the same inclusion criteria as those in the first retrospective study.

The information on clinical history, signalments, the results of physical examination, CBC, blood biochemistry, autoagglutination test, direct Coombs test, radiographic and ultrasonographic examinations, and bone marrow aspiration, treatments, and outcomes were extracted from the medical records.

In the examinations of bone marrow smears, 1000 cells were evaluated on each slide, and the cellularity, myeloid-to-erythroid (M-E) ratios, and blast cell to all nucleated cell (ANC) ratios were evaluated as previously described (Turinelli et al., 2015). Granulocyte maturation ratio and erythroid maturation ratio were also investigated as previously reported (Weiss & Aird, 2001). Granulocyte maturation ratio was calculated using the following formula:  $(\text{myeloblasts} + \text{promyelocytes} + \text{myelocytes}) / (\text{metamyelocytes} + \text{band neutrophils} + \text{segmented neutrophils})$ . Erythroid maturation ratio was calculated using the following formula:  $(\text{rubriblasts} + \text{prorubricytes}) / (\text{rubricytes} + \text{metarubricytes})$ . The reference ranges of granulocyte maturation and erythroid maturation ratios were set as 0.04–0.15 and 0.02–0.12, respectively, according to previous studies (Weiss & Aird, 2001). The following dysplastic features in each cell lineage were investigated: megaloblastic appearances, fragmented nuclei and binucleate cells in erythroid cells; ring neutrophils, giant metamyelocytes, giant band neutrophils, and pseudo-Pelger-Huet cell morphology in myeloid cells; dysmorphic megakaryocytes with multiple nuclei,

mononucleate megakaryocytes, and micro-megakaryocytes in megakaryocytes (Jain et al., 1991; Weiss & Smith, 2000), and the frequency of each dysplastic feature was calculated by dividing the number of cells with dysplastic features by a total number of cells of the same cell lineage in each case. Regarding megakaryocytes, 25 cells of this lineage were examined in each slide to calculate the frequency of dysplastic features, according to a previous study (Bowen et al., 2003). In addition, the bone marrow core biopsy specimens were evaluated using hematoxylin-eosin staining to examine fibrosis in the bone marrow.

X-chromosome inactivated pattern analysis (XCIP) is a method to assess the clonality of cells derived from female tissues (Mochizuki et al., 2015; Tomita et al., 2019). Briefly, heterozygous repeat sequences of genes in X-chromosomes are used to distinguish the paternal or maternal X-chromosome, and the methylation status was distinguished by a methylation sensitive endonuclease. Since the inactivation ratio between paternal and maternal X-chromosome should be equal in normal tissues, skewed inactivation allele ratio indicates clonal cell expansion. As a previous study, clonality of cells was indicated when the corrected inactivation allele ratio (CIAR) was  $> 3.8$  (Tomita et al., 2019). To assess the clonality of peripheral blood, total DNA was extracted from peripheral blood, and XCIP was conducted with the same threshold.

Drugs used for the treatment and responsiveness to the treatments were also investigated. Since dogs with NRIMA or PRCA have been shown to respond to treatment within a maximum of 10 weeks after treatment initiation (Stokol et al., 2000), dogs that could be followed for more than 2 months after treatment initiation were included for the

evaluation of treatment responsiveness. The hematological improvement due to treatments was defined as the absence of blood transfusion for more than 2 months after the last blood transfusion according to the response criteria in human medicine (Cheson et al., 2006).

#### *Comparison of clinical and clinicopathological features*

The distributions of age and sex, the results of CBC at diagnosis, and findings of peripheral blood and bone marrow smears were compared between MDs and non-MDs. The same comparisons were also conducted among MDs based on treatment responsiveness. The MDs were divided into treatment-responsive and -resistant groups based on the existence of hematological improvement by treatments, and the clinical and clinicopathological features described above were compared between the two groups. Because the number of platelets was significantly different between the two groups, receiver operator characteristic (ROC) analysis was performed to calculate sensitivity, specificity, and an optimal cut-off value of the number of platelets to predict the resistance to treatment.

#### *Immunohistochemical (IHC) examinations for immune cells in bone marrow specimens*

I conducted IHC examinations for lymphocytes and macrophages in bone marrow specimens of MDs and healthy beagle dogs. This animal experiment was approved by the Animal Care Committee of The University of Tokyo (approval no. P19-119). Paraffin-embedded bone marrow biopsies were retrospectively evaluated. Two  $\mu\text{m}$  paraffin

sections were generated from paraffin-embedded bone marrow core biopsies. Heat-induced antigen retrieval was conducted using Tris-EDTA (pH 9.0) or citric acid (pH 6.0) in the autoclave at 120°C for 15 min. Endogenous peroxidase in each section was inactivated, and the sections were incubated in 8% skimmed milk for 40min at 37°C followed by incubations with primary antibodies against CD3, CD20, CD204 and Iba-1. The details of primary antibodies and antigen retrieval procedures were as shown in Table 1-1. As secondary antibody, a horseradish peroxidase (HRP)-conjugated anti-mouse antibody (Envision+ System-HRP Labelled polymer, K4001, DAKO, Glostrup, Denmark) or a HRP-conjugated anti-rabbit antibody (Envision+ System-HRP Labelled polymer, K4002, DAKO) were used. Then, the sections were incubated with 3,3'-diaminobenzidine (DAB; Dojindo Laboratories, Rockville, MD, USA) solution, and counterstained with Mayer's hematoxylin. The numbers of CD3-positive lymphocytes, CD20-positive lymphocytes, CD204 positive macrophages and Iba-1 positive histiocytes were counted, and their ratios to 300 mononuclear bone marrow cells were calculated.

#### *Detection of apoptotic cells in bone marrow specimens*

Two µm paraffin sections were generated from paraffin-embedded bone marrow core biopsies. Apoptotic cells were investigated in the deparaffinized and rehydrated slides using in situ terminal deoxynucleotidyl transferase-catalyzed DNA nick end labeling (TUNEL) staining (ApopTag™ plus peroxidase kit, ONCOR Inc., MD, U.S.A.) following manufacture's instruction. The rates of reaction-positive cells to 500 mononuclear bone marrow cells were calculated.



### *Statistical analysis*

The Fisher's exact test was used to investigate the predisposed breed and sex and to compare the frequencies of morphological findings in peripheral blood and bone marrow smears. Overall survival time was defined as the time from the initial presentation to VMC-UT until the death. The Kaplan-Meier method and log-rank test were used to compare overall survival times. ROC curves were used to evaluate the accuracy of the number of platelets in predicting the prognosis. The Mann-Whitney U test was used to compare age and results of CBC. The Steel-Dwass test was used to compare the rates of immune cells in bone marrow smears. A  $P$ -value  $< 0.05$  was considered statistically significant. All statistical analyses were performed using the statistical program R 3.5.0 (<http://cran.r-project.org>).

## **Results**

### *Investigation of the predisposed breed of bone marrow disorders that induce non-regenerative anemia*

Thirty-three dogs were included in the first retrospective study. The median age was 10.9 years (range 1–15.8 years). Sixteen dogs were male (10 were castrated) and 17 were female (13 were neutered). There were 22 MDs, 3 Toy Poodles, 2 mix-breed dogs, and 1 each of Cavalier King Charles Spaniel, French Bulldog, Shetland Sheepdog, Shiba Inu, Pug, and Yorkshire Terrier. The odds ratios (ORs) of these dogs against all dogs admitted to VMC-UT for the same period are shown in Table 1-2. Among these breeds, the OR in

MD was 12.2 (95% confidence interval: 5.61–28.12), and this breed was significantly overrepresented ( $P < 0.001$ ).

#### *Clinical and clinicopathological features of bone marrow disorders in MDs*

Based on the results of the first retrospective study, I decided to investigate the clinical and clinicopathological features of bone marrow disorders that induce non-regenerative anemia in MDs and compare them with those in non-MDs. Twenty-one MDs were included in the second retrospective study. The median age was 12.5 years (range 4.3–15.8 years). Seven dogs were male (5 were castrated) and 14 were female (12 were spayed). During the same period, 311 male, 510 castrated male, 201 female, and 535 spayed female MDs were referred to VMC-UT, and it was revealed that spayed female MDs were significantly overrepresented ( $P = 0.035$ ). MDs were treated at the primary hospital using corticosteroids (17 dogs), antibiotics (14 dogs), blood transfusion (7 dogs), cyclosporine (3 dogs), mycophenolate mofetil (2 dogs), and leflunomide (1 dog). On the first admission to VMC-UT, physical examination showed mild to severe pale mucous membranes in 21 dogs, cardiac murmur in 5 dogs, tachycardia in 1 dog, and tachypnea in 1 dog.

Regarding laboratory tests, all dogs showed moderate to severe non-regenerative anemia (Ht, range 7.0–24.7%) (Table 1-3). The white blood cell (WBC) count was within the reference range in 16 dogs. Five dogs showed leukocytosis (range  $23\text{--}50 \times 10^3/\mu\text{L}$ ). The platelet count was within the reference range in 9 dogs. Eleven dogs showed thrombocytosis (range  $520\text{--}2260 \times 10^3/\mu\text{L}$ ), and 1 dog showed thrombocytopenia ( $130 \times$

$10^3 /\mu\text{L}$ ). Regarding blood biochemistry, the elevation of C-reactive protein (CRP) was commonly observed (14 dogs, range 0.9–6.9 mg/dL). Thirteen dogs showed elevated alkaline phosphatase (ALP, range 259–8186 U/L), of which 9 were treated with corticosteroids before their admission to VMC-UT. Nine dogs showed elevated alanine transaminase (ALT, range 93–907 U/L), of which 6 were treated with corticosteroids before their admission to VMC-UT. All the 19 examined dogs were negative for autoagglutination test, and one of the 19 dogs was positive for direct Coombs test.

In the examinations of peripheral blood smears, hypersegmented neutrophils were found in 12 dogs (Fig. 1-1a). Ten of these 12 dogs were treated with corticosteroids before admission to VMC-UT. In addition, spindle-shaped platelets were found in 8 dogs (Fig. 1-1b).

Radiographic examinations for the thorax and ultrasonographic examinations for the abdomen were performed in all 21 dogs. While 12 dogs showed no abnormalities, 5 showed mild splenomegaly, 2 showed mild hepatomegaly, and 2 showed both mild splenomegaly and hepatomegaly.

In the examinations of bone marrow smears, the cellularity could not be evaluated in 10 dogs due to the dilution by peripheral blood. For the remaining 11 dogs, the cellularity was normocellular in 5 and hypercellular in 6. Next, the ratios of each progenitor cell were calculated by counting 1000 nucleated cells in 17 dogs. The median M-E ratio was 1.1 (range 0.2–14), and the median blast cell ratio was 1% (range 0.2–2.6%). Erythroid cells were hypercellular in 6 dogs, normocellular in 5 dogs, and hypocellular in 6 dogs. The median erythroid maturation ratio was 0.7 (range 0.08–3.84), and it was above the

reference range in 16 dogs. Myeloid cells were hypercellular in 6 dogs, normocellular in 5 dogs, and hypocellular in 6 dogs. The median granulocyte maturation ratio was 0.1 (range 0.05–0.27), and it was above the reference range in 5 dogs. Megakaryocytes were hypercellular in 2 dogs, normocellular in 6 dogs, and hypocellular in 3 dogs.

The dysplastic morphological features were also examined in bone marrow smears, and the number of dogs with dysplastic features and the frequencies of each feature in the smears were shown in Table 1-4. In erythroid cells, fragmented nucleus was most frequently observed (15 dogs; Fig. 1-2a), and the median frequency of this morphologic feature was 1.8%. In myeloid cells, ring neutrophil was the most frequent and observed in 5 dogs (Fig. 1-2b), and the median frequency was 0.15%. In megakaryocytes, dysmorphic megakaryocytes with multiple nuclei were most commonly observed (13 dogs; Fig. 1-2c), and the median frequency was 12%. One or more dysplastic morphological features were observed in all 21 dogs.

Histopathological examinations of bone marrow core samples were conducted in 19 dogs. Fibrocyte augmentation was observed in 3 dogs, and moderate to severe fibrosis was observed in 6 dogs (Fig. 1-3). XCIP analysis was conducted using peripheral blood in 11 female dogs, and the clonality of cells was indicated in 6 of 11 dogs.

Based on these observations, all dogs were considered to harbor bone marrow disorders with features of both MDS and NRIMA, and a definitive diagnosis could not be reached.

After bone marrow examinations, all dogs were treated with prednisolone (0.2–3.4 mg/kg/day). Immunosuppressive drugs were also used as follows: cyclosporine (4–13

mg/kg/day) in 15 dogs, mycophenolate mofetil (20–40 mg/kg/day) in 6 dogs, leflunomide (3.4–4 mg/kg/day) in 2 dogs, and azathioprine (0.5–2 mg/kg/day) in 2 dogs. In addition, antibiotics were used in 20 dogs, menatetrenone (0.7–2.1 mg/kg/day) in 14 dogs, and hydroxycarbamide (12.5–25 mg/kg/day) in 1 dog.

One dog could not be followed for 2 months and one died from acute enteritis at 7 weeks after diagnosis. Therefore, treatment responsiveness and prognosis could be investigated in 19 dogs. Three dogs responded to corticosteroid alone, whereas 6 responded to corticosteroid and immunosuppressive drugs, and the median duration from treatment initiation to response was 78 days (range; 61–150 days). Ten dogs did not respond until 6 months after treatment initiation. Based on these observations, 9 dogs were assigned to the treatment-responsive group, and 10 to the treatment-resistant group. During the study period, 5 dogs died from variable causes and all these dogs were treatment-resistant dogs. Three dogs died from acute hemolytic transfusion reaction during blood transfusion on day 78, 82, and 90, respectively. One dog died on day 507 and was diagnosed with disseminated histiocytic sarcoma on autopsy. The cause of death was unclear in the remaining 1 dog.

#### *Clinical and clinicopathological features of the bone marrow disorders occurred in non-MDs*

The bone marrow disorders that induced non-regenerative anemia were observed in 15 non-MDs. There were 4 French Bulldogs, 2 mix breeds, 2 Chihuahuas, and 1 each of Bichon Frise, Jack Russell Terrier, Miniature Schnauzer, Pomeranian, Pug, Shiba Inu, and

Yorkshire Terrier.

The median age was 9.4 years (range; 0.8–14.4 years). Five dogs were male (3 were castrated) and 10 were female (8 were spayed), and there was no significant predisposition in sex distribution. The dogs were treated at the primary hospital using corticosteroids (10 dogs), antibiotics (11 dogs), blood transfusions (6 dogs), cyclosporine (4 dogs), mycophenolate mofetil (2 dogs), azathioprine (1 dog), human intravenous immunoglobulin (2 dogs). On the first admission to VMC-UT, physical examinations showed a mild to a severe pale mucous membrane in 13 dogs, cardiac murmur in 3 dogs, tachycardia in 3 dogs, and tachypnea in 1 dog.

With regards to laboratory tests, all dogs showed moderate to severe non-regenerative anemia (range; 8.6–26.7%) (Table 1-5). WBC count was within the reference range in 6 dogs. Three dogs showed leukocytosis (range;  $24\text{--}47 \times 10^3 /\mu\text{L}$ ), and 6 dogs showed leukopenia (range;  $2.0\text{--}4.7 \times 10^3 /\mu\text{L}$ ). Platelet count was within the reference range in 5 dogs. Three dogs showed thrombocytosis (range;  $560\text{--}1290 \times 10^3 /\mu\text{L}$ ), and 7 dogs showed thrombocytopenia (range;  $11\text{--}144 \times 10^3 /\mu\text{L}$ ). In blood biochemistry, elevation of ALP was commonly observed (11 dogs, range; 341–4282 U/l), and 8 of these 11 dogs were treated with corticosteroids before their first admission to VMC-UT. Eight dogs showed elevated ALT (range; 81–734 U/l), and 7 of these 8 dogs were treated with corticosteroids before their first admission to VMC-UT. Nine dogs showed elevated CRP (range; 0.8–4.8 mg/dl). Autoagglutination test was negative in 13 examined dogs, and direct Coombs test was positive in 1 of 13 examined dogs.

In the examinations of peripheral blood smears, hypersegmented neutrophils were

seen in 3 dogs. Two of these 3 dogs were treated with corticosteroids before their first admission to VMC-UT. In addition, platelets with spindle shapes were found in 1 dog.

Radiographic examinations of the thorax and ultrasonographic examinations for the abdomen were performed in all 15 dogs. While 13 dogs showed no abnormalities, 2 showed mild splenomegaly.

In the examinations of bone marrow smears, the cellularity could not be evaluated in 6 dogs due to the dilution by peripheral blood. As for the remaining 9 dogs, the cellularity was normocellular in 8 dogs and hypercellular in 1 dog. The ratios of each progenitor cell could be calculated by counting 1000 nucleated cells in 11 dogs. The median value of M-E ratio was 1.25 (range; 0.12–82). The median value of blast cell ratios was 1.2% (range; 0.4–7.3%). Erythroid cells were hypercellular in 4 dogs, normocellular in 2 dogs, and hypocellular in 5 dogs. The median value of erythroid maturation ratio was 0.4 (range; 0.09–10), and it was above the reference range in 7 dogs. Myeloid cells were hypercellular in 5 dogs, normocellular in 2 dogs, and hypocellular in 4 dogs. The median value of granulocyte maturation ratio was 0.06 (range; 0.01–0.25), and it was above the reference range in 2 dogs. Megakaryocytes were hypercellular in 2 dogs, normocellular in 8 dogs, and hypocellular in 1 dog.

The dysplastic morphological features were also examined in bone marrow smears, and the number of dogs with dysplastic features and the frequency of each feature in smears were shown in Table 1-6. With regards to erythroid cells, fragmented nucleus was the most frequent and was observed in 9 dogs, with a median frequency of 2.9%. For myeloid cells, ring neutrophil was observed in 2 dogs, with a median frequency of 0.4%.

With regards to megakaryocytes, dysmorphic megakaryocytes with multiple nuclei were the most frequent (5 dogs), with a median frequency of 12%. One or more dysplastic morphological features were observed in all 15 dogs.

Histopathological examinations of bone marrow samples were conducted in 11 dogs, and mild to severe fibrosis was observed in 3 dogs. XCIP analysis was conducted using peripheral blood in 2 of 11 dogs, and clonality of cells was negative in these 2 dogs.

One dog was diagnosed with PRCA since the M-E ratio was 82 (Weiss, 2002). Meanwhile, the other 14 dogs were considered to harbor bone marrow disorders with features of both MDS and NRIMA, and a definitive diagnosis could not be reached.

After bone marrow examinations, all dogs were treated with prednisolone (0.13–2.4 mg/kg/day). Immunosuppressive drugs were used as follows: cyclosporine (2.5–15 mg/kg/day) in 13 dogs, mycophenolate mofetil (17–23 mg/kg/day) in 2 dogs, azathioprine (4.2 mg/kg/day) in 1 dog, and cytosine arabinoside (2.7 mg/kg/day) in 1 dog. Antibiotics were administered in 12 dogs, and menatetrenone (0.9–1.8 mg/kg/day) in 9 dogs.

Three dogs could not be followed for 2 months after diagnosis and two dogs suddenly died from unknown causes at 4 and 7 weeks after the initial admission. Therefore, treatment responsiveness could be investigated in only 10 dogs. One dog responded to corticosteroid alone, 6 responded to corticosteroid and immunosuppressive drugs, and the median duration from treatment initiation to response was 64 days (range 60–181 days). Three dogs did not respond to treatments by 6 months after treatment initiation. During the study period, 3 dogs died from variable causes and these were treatment-resistant dogs.



One dog died from meningitis on day 853, and the other 2 dogs died from unknown causes on days 80 and 676.

#### *Comparison of clinical and clinicopathological features between MDs and non-MDs*

In the comparisons of clinical features between MDs and non-MDs, a dog diagnosed with PRCA in the non-MD group was excluded and only dogs that were considered to have features of both NRIMA and MDS were included.

When comparing signalments, I found that MDs tended to be older than non-MDs ( $P = 0.06$ , Table 1-7). Besides, the number of platelets was significantly higher in MDs than in non-MDs ( $P = 0.01$ , Fig. 1-4). At initial presentations, 20 MDs and 7 non-MDs were suffering from anemia, 1 MD and 1 non-MDs were suffering from anemia and thrombocytopenia, and 6 non-MDs were suffering from pancytopenia. The ratio of cases that showed anemia but not leukocytopenia or thrombocytopenia was significantly higher in MDs than non-MDs ( $P = 0.003$ ).

When evaluating peripheral blood smears, hypersegmented neutrophils were more frequently observed in MDs than in non-MDs ( $P = 0.046$ ). There was no significant difference in the proportions of dogs treated by corticosteroid before diagnosis between the two groups ( $P = 0.43$ ). Spindle-shaped platelets tended to be more frequently observed in MDs than in non-MDs ( $P = 0.056$ ). When evaluating bone marrow samples, no significant difference was observed in the M-E ratio, erythroid maturation ratio, granulocyte maturation ratio, the frequencies of erythroid cells with fragmented nuclei, ring neutrophils, dysmorphic megakaryocytes with multiple nuclei, or the rates of dogs

with mild to severe myelofibrosis between MDs and non-MDs.

The treatment response rates to immunosuppressive treatments were not different between MDs and non-MDs ( $P = 0.43$ ).

*Comparison of clinical and clinicopathological features between treatment-responsive and -resistant MDs*

Based on the response to treatment, 9 MDs were assigned to the treatment-responsive MD group and 10 were assigned to the treatment-resistant MD group. No significant difference was observed in age and sex distribution between the two groups. The number of platelets of MDs in the treatment-resistant group was significantly higher than that of MDs in the treatment-responsive group ( $P = 0.004$ , Fig. 1-5). When evaluating peripheral blood smears, I found that hypersegmented neutrophils tended to be more frequently observed in the treatment-resistant MD group than in the treatment-responsive MD group ( $P = 0.07$ , Table 1-8). No significant difference was observed in the proportions of dogs treated with corticosteroid before diagnosis between the two groups. Spindle-shaped platelets were significantly more frequent in the treatment-resistant MD group than in the treatment-responsive MD group ( $P = 0.02$ ). On XCIP analysis, the clonality of peripheral blood cells was suggested only in the treatment-resistant MD group ( $P = 0.015$ ).

I could examine bone marrow samples of 8 treatment-responsive MDs and 8 treatment-resistant MDs. The granulocyte maturation ratio was significantly higher in the treatment-responsive MD group than in the treatment-resistant MD group ( $P = 0.01$ ), and all dogs with granulocyte maturation ratio above the reference range were treatment-

responsive dogs. On the other hand, there was no significant difference in the M-E ratio, erythroid maturation ratio, and the frequencies of erythroid cells with fragmented nuclei, ring neutrophils, and dysmorphic megakaryocytes with multiple nuclei between the two groups. Mild to severe myelofibrosis was observed in 4 treatment-resistant MDs, although there was no significant difference in the frequency of myelofibrosis between the two groups ( $P = 0.087$ ). Treatment-responsive dogs showed significantly longer survival times compared with -resistant dogs (Fig 1-6,  $P = 0.03$ ).

ROC curve analysis was also conducted to calculate sensitivity, specificity, and an optimal cutoff value of the number of platelets to predict the resistance to treatment. The area under the ROC curve was 0.89, and a cut-off number of platelets of  $1115 \times 10^3 /\mu\text{l}$  was determined as a point where the sum of sensitivity and specificity values were the highest. When this cut-off value was used, the sensitivity and specificity to predict the resistance to treatments were 0.60 (6/10) and 1.00 (0/9), respectively. The median survival time of dogs with number of platelets of  $> 1115 \times 10^3 /\mu\text{l}$  was 354 days (range: 82–766 days) and one with number of platelets of  $< 1115 \times 10^3 /\mu\text{l}$  was 878 days (range: 78–2986 days). Although the difference in overall survival was not statistically significant, the overall survival of dogs with platelets of  $> 1115 \times 10^3 /\mu\text{l}$  tended to be shorter than that of dogs with platelets of  $< 1115 \times 10^3 /\mu\text{l}$  ( $P = 0.052$ , Fig. 1-7).

#### *IHC examinations of immune cells in bone marrow specimens*

IHC analysis was conducted using bone marrow specimens obtained from 4 healthy beagles and 17 MDs diagnosed with non-neoplastic bone marrow disorders (6 treatment-

responsive MDs and 11 treatment-resistant MDs). The number of Iba-1 positive histiocytes ( $P = 0.004$ ) and CD204 positive macrophages ( $P = 0.014$ ) were significantly increased in MDs diagnosed with non-neoplastic bone marrow disorders compared with healthy beagles. No significant difference was observed in the number of CD3 and CD20 positive cells between MDs diagnosed with non-neoplastic bone marrow disorders and healthy beagles. In comparisons among treatment-responsive MDs, -resistant MDs, and healthy dogs, the number of Iba-1 positive histiocytes was significantly increased in treatment-responsive MDs ( $P = 0.028$ ) and -resistant MDs ( $P = 0.049$ ) MDs compared with healthy beagles (Table 1-9). The number of CD204 positive macrophages was significantly increased in treatment-resistant MDs compared with healthy beagles ( $P = 0.024$ ). Meanwhile, significant difference was not observed in the number of any immune cells between treatment-responsive and -resistant MDs.

#### *Apoptotic cells detection in bone marrow specimens*

The proportion of TUNEL-positive apoptotic mononuclear cells was compared among 4 healthy dogs, 2 treatment-responsive MDs, and 6 treatment-resistant MDs. However, no significant difference was observed among the 3 groups (Fig 1-8).

### **Discussion**

In this study, the breed predisposition of dogs diagnosed with non-neoplastic bone marrow disorders that induce anemia was investigated. As a result, MD was identified as a predisposed breed of non-neoplastic bone marrow disorders in Japan. In the United

States, Labrador Retrievers and American Eskimos were reported as the predisposed breeds for NRIMA and bone marrow necrosis, respectively (Stokol et al., 2000; Weiss, 2005). The difference in the predisposed breeds for bone marrow disorders might be due to the difference in the popular breeds among countries. It was reported that MD was a popular breed in Japan (Inoue et al., 2015), and it was also reported that MD was a breed commonly predisposed to inflammatory colorectal polyps and sterile panniculitis, which were rare in other breeds (Ohmi et al., 2012; Yamagishi et al., 2007). Therefore, breed-specific genetic aberration might be an underlying cause of non-neoplastic bone marrow disorders observed in MDs in Japan.

Next, I retrospectively investigated clinical and clinicopathological features of MDs diagnosed with non-neoplastic bone marrow disorders and compared them with those in non-MDs. As for signalment, spayed female was significantly overrepresented in MDs. In previous studies, spayed female was also significantly predisposed to be diagnosed with NRIMA and PRCA (Stokol et al., 2000; Weiss, 2002), but the underlying causes have been unknown.

With regard to laboratory tests, autoagglutination tests were negative in all MDs and non-MDs, and the direct Coombs test was positive only in 1 MD and 1 non-MD dog. Since it was previously reported that 57% of dogs diagnosed with NRIMA or PRCA showed a positive direct Coombs test (Stokol et al., 2000), the proportions of dogs that were positive for direct Coombs test seemed to be low in the present study. Meanwhile, one or more dysplastic morphological features were found in bone marrow smears of all dogs in the present study. However, the frequencies of these features in each smear were

considered to be low because previous reports showed that dysplastic features were observed in more than 10% or 25% of each lineage cells in canine MDS (Blue, 2003; Weiss & Smith, 2000). In addition, 16 of 17 MDs and 7 of 11 non-MDs showed an elevation in the erythroid maturation ratio. In previous studies, an increase in the erythroid maturation ratio or left-shifted erythroid maturation was reported in both MDS and NRIMA (Stokol et al., 2000; Weiss & Smith, 2000). Therefore, most dogs except for a non-MD dog with PRCA were thought to harbor non-neoplastic bone marrow disorders with features of both NRIMA and MDS, and the diagnosis was not confirmed in the present study.

Thrombocytosis was observed in 11 of 21 MDs and 3 of 15 non-MDs at initial admission, and the number of platelets was significantly increased in MDs compared with non-MDs. Additionally, the number of platelets was significantly increased in treatment-resistant MDs compared with treatment-responsive MDs. Hypersegmented neutrophils were also significantly more frequently observed in MDs compared with non-MDs, and they tended to be more frequently observed in treatment-resistant MDs compared with treatment-responsive MDs. Furthermore, spindle-shaped platelets tended to be more frequently observed in MDs compared with non-MDs, and they were significantly more frequently observed in treatment-resistant MDs compared with treatment-responsive MDs. These observations indicated that bone marrow disorders in treatment-resistant MDs might harbor distinct features compared with other dogs, and sensitivity to immunosuppressive treatments could be predicted based on the number of platelets and the morphological features in peripheral blood.

Hypersegmented neutrophils are regarded as a dysplastic feature in human MDS patients (Yoshida, 1996). Although hypersegmented neutrophils were reported to be observed after administration of corticosteroids (Liles et al., 1995), no significant difference was observed in the frequency of corticosteroid administration between treatment-responsive MDs and -resistant MDs. Thus, it is reasonable to regard hypersegmented neutrophils as dysplastic features in the present study. Spindle-shaped platelets have been poorly reported in veterinary medicine, but platelets with similar morphological features were reported in human hematopoietic neoplasms (Hattori et al., 1981) Therefore, this morphological features might be also one of the dysplastic features.

Clonality of cells indicated by XCIP analysis was positive only in treatment-resistant MDs, and the proportion of cases with the clonality was significantly higher in treatment-resistant MDs compared with -responsive ones. It has been shown that the clonality of blood cells are observed in human MDS patients by DNA-methylation based human androgen receptor (HUMARA) assay (Mossner et al., 2013), which is a counterpart of canine XCIP analysis. The results of the present study might indicate that treatment-resistant MDs harbored non-neoplastic bone marrow disorder that was similar to human MDS.

The granulocyte maturation ratio was significantly higher in treatment-responsive MDs compared with -resistant MDs. An increase of this ratio has been observed in various conditions including infection, corticosteroid treatment, hemorrhage, metabolic acidosis, and postoperative states (Honda et al., 2016), but the causes of this increase in treatment-responsive MDs were unclear in the present study.

IHC examinations using bone marrow specimens revealed that Iba-1-positive histiocytes and CD204-positive macrophages were increased in MDs diagnosed with non-neoplastic bone marrow disorders compared with healthy controls. In human MDS, excessive apoptosis of hematopoietic cells and increased reactive phagocytosis of macrophages were observed (Shetty et al., 2002). Based on these findings, the number of apoptotic cells was investigated using TUNEL assay, but no significant difference in the number of apoptotic cells was observed between MDs diagnosed with non-neoplastic bone marrow disorders and healthy dogs. Although the TUNEL assay has been frequently used for apoptosis detection, it was reported that detection of activated caspase-3 was more sensitive to detect apoptotic cells than the TUNEL method (Duan et al., 2003). Therefore, It might be needed to detect the activated caspase-3 to investigate the proportions of apoptotic cells. It is also possible that dysregulation of immune systems might be the pathogenesis of non-neoplastic bone marrow disorders in MDs, because it was shown that macrophages increase in immune-mediated bone marrow disorders, such as precursor-targeted immune-mediated anemia (Lucidi et al., 2017).

Interestingly, it was reported that non-regenerative anemia, thrombocytosis, and dysplastic features of blood cells with low frequencies, which were observed in treatment-resistant MDs, were also observed in a mouse model of human MDS where the *Additional sex combs like 1 (ASXL1)* gene was mutated (Nagase et al., 2018). In addition, the mutations in *ASXL1* gene were shown to be related to epigenetic dysregulation (Asada et al., 2019). Taken together, it was reasonable to think that genetic and epigenetic aberrations might be related to the pathogenesis of the bone marrow disorder in treatment-



resistant MDs.

One of the limitations of the present study is that a standardized treatment protocol could not be conducted for all dogs because this study was retrospective in nature. An additional limitation is that the present study was conducted at a single facility. Furthermore, the number of dogs included in the present study was relatively low, and some comparisons might not have enough statistical power to show significant differences.

In conclusion, it was showed that MD was a predisposed breed to be diagnosed with bone marrow disorder that induced anemia in Japan. The investigations of the clinical and clinicopathological features of non-neoplastic bone marrow disorders in MDs and non-MDs revealed that bone marrow disorders of treatment-resistant MDs might harbor distinct features compared with other dogs.

Table1-1. Primary antibodies and protocols used for immunohistochemistry.

Antibody	Clone no.	Isotype	Manufacturer	Antigen retrieval	Dilution	Incubation
CD3	-	rabbit IgG	DAKO, Glostrup, Denmark	Tris-EDTA (pH 9.0), autoclave	1:100	4°C, overnight
CD20	-	rabbit IgG	Thermo Fisher Scientific, Waltham, MA, U.S.A.	Citric buffer (pH 6.0), autoclave	1:100	4°C, overnight
Iba-1	-	rabbit IgG	Wako, Osaka, Japan	Tris-EDTA (pH 9.0), autoclave	1:250	4°C, overnight
CD204	SRA-E5	mouse IgG1	Transgenic, Kobe, Japan	Tris-EDTA (pH 9.0), autoclave	1:50	37°C, 90min

Table 1-2. Prevalence of each breed diagnosed with non-neoplastic bone marrow disorders with non-regenerative anemia.

Breed	Number of cases	Number of dogs admitted to UT-VMC	Odd's ratio	<i>P</i> -value
Miniature Dachshund	22	402	12.2	< 0.001
Toy Poodle	3	377	0.622	0.612
Mix	2	222	0.728	1
Cavalier King Charles Spaniel	1	34	2.52	0.34
French Bulldog	1	94	0.877	1
Shetland Sheepdog	1	52	1.624	0.471
Shiba Inu	1	104	0.789	1
Pug	1	41	2.08	0.394
Yorkshire Terrier	1	109	0.75	1

UT-VMC; Veterinary Medical Center of the University of Tokyo

Table 1-3. Results of CBC in Miniature Dachshunds at the first admission to Veterinary Medical Center of the University of Tokyo.

	Median (Range)	Reference range
Hematocrit (%)	19.1 (7–24.7)	37.3–61.7
RBC ( $\times 10^6/\mu l$ )	2.7 (0.9–4.3)	5.85–8.67
Hemoglobin (g/dl)	6.2 (1.9–8.4)	13.1–20.5
Reticulocyte ( $\times 10^3/\mu l$ )	15.8 (5.3–55.9)	<60.0
WBC ( $\times 10^3/\mu l$ )	11.3 (5.9–50.4)	5.05–16.76
Platelet ( $\times 10^3/\mu l$ )	524 (127–2268)	148–484

Table 1-4. Number of Miniature Dachshunds with dysplastic features and the frequencies of these features in bone marrow smears.

	Number of cases	Median frequency (Range) (%)
Erythroid lineage		
Fragmented nuclei	15	1.8 (0.2–5.1)
Megaloblastic cells	8	0.6 (0.2–6.2)
Binucleate cells	3	0.5 (0.2–2.0)
Myeloid lineage		
Ring neutrophils	5	0.2 (0.1–0.3)
Giant metamyelocytes and neutrophils	3	8 (5.8–8.3)
Pseudo-Pelger-Huet cell morphology	1	0.6
Megakaryocytic lineage		
Dysmorphic megakaryocytes with multiple nuclei	13	12 (4–52)
Mononucleate megakaryocytes	5	4 (4–12)
Micro-megakaryocytes	4	4 (4–8)

Table 1-5. Results of CBC in dogs of breeds other than Miniature Dachshund at the first admission to Veterinary Medical Center of the University of Tokyo.

	Median (Range)	Reference range
Hematocrit (%)	16.2 (8.6–26.7)	37.3–61.7
RBC ( $\times 10^6/\mu\text{l}$ )	2.5 (1.3–4.1)	5.85–8.67
Hemoglobin (g/dl)	5.8 (2.9–9.1)	13.1–20.5
Reticulocyte ( $\times 10^3/\mu\text{l}$ )	26.7 (6.9–58)	<60.0
WBC ( $\times 10^3/\mu\text{l}$ )	6.5 (2.0–46.7)	5.05–16.76
Platelet ( $\times 10^3/\mu\text{l}$ )	243 (11–1291)	148–484

Table 1-6. Number of dogs of other breeds other than Miniature Dachshund with dysplastic features and the frequencies of these features in bone marrow smears.

	Number of cases	Median frequency (Range) (%)
Erythroid lineage		
Fragmented nuclei	9	2.9 (0.6–26.4)
Megaloblastic cells	5	1.1 (1.0–2.6)
Binucleate cells	3	0.6 (0.1–8.1)
Myeloid lineage		
Ring neutrophils	2	0.4 (0.2–0.5)
Giant metamyelocytes and neutrophils	0	NE
Pseudo-Pelger-Huet cell morphology	0	NE
Megakaryocytic lineage		
Dysmorphic megakaryocytes with multiple nuclei	5	12 (8–80)
Mononucleate megakaryocytes	5	6 (4–8)
Micro-megakaryocytes	2	12 (4–20)

Table 1-7. Comparisons of the clinical and clinicopathological features between Miniature Dachshunds (MDs) and other breed dogs (non-MDs).

	MDs (n=21)	non-MDs (n=14)	<i>P</i> -value
Age (years)	12.5 (4.3–15.8)	9.5 (4.4–14.4)	0.06
Platelet ( $\times 10^3/\mu l$ )	524 (127–2268)	194 (11–1291)	0.013
Peripheral blood smear			
Hypersegmented neutrophil	12	3	0.046
Spindle shape platelet	8	1	0.056

Table 1-8. Comparisons of the clinical and clinicopathological features between treatment-responsive and -resistant Miniature Dachshunds (MDs).

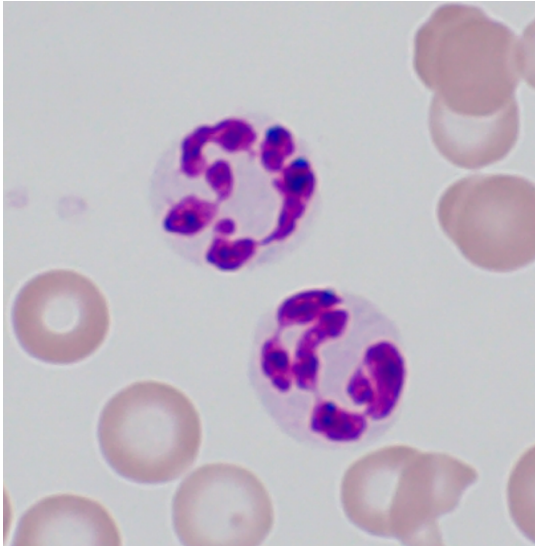
	Responsive-MDs (n=9)	Resistant-MDs (n=10)	<i>P</i> -value
CBC			
Platelet ( $\times 10^3/\mu l$ )	200 (127–951)	1116 (178–2268)	0.004
Peripheral blood smear			
Hyper-segmented neutrophil	3	8	0.07
Spindle shape platelet	1	7	0.02
XCIP (positive/negative)	0/4	6/1	0.015
Bone marrow smear			
Granulocyte maturation ratio	0.19 (0.06–0.27)	0.07 (0.05–0.12)	0.01

Table 1-9. Results of immunohistochemical examinations of immune cells in healthy dogs and Miniature Dachshunds (MDs) diagnosed with non-neoplastic bone marrow disorders.

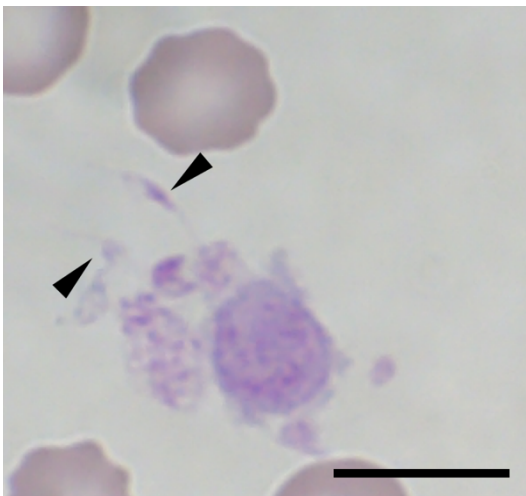
	Healthy dogs (%)	Treatment-responsive MDs(%)	Treatment-resistant MDs (%)
	median (range)	median (range)	median (range)
CD3	5.3 (4.4 - 7.1)	7.5 (2.3 - 22.5)	5.1 (3.7 - 19.3)
CD20	12.7 (9.6 - 14.8)	13.2 (9.0 - 17.3)	14.7 (6.0 - 17.9)
Iba1	11.1 (8.8 - 13.7)	19.7* (16.8 - 22)	17.3* (8.6 - 23.2)
CD204	14.3 (11-15.3)	19.1 (13.9 - 24.2)	17.7* (14.8 - 30.2)

\* indicates  $P < 0.05$  compared with healthy beagles

**Fig 1-1a**

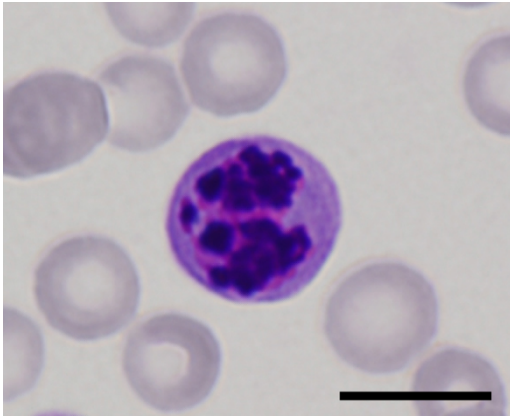


**Fig 1-1b**

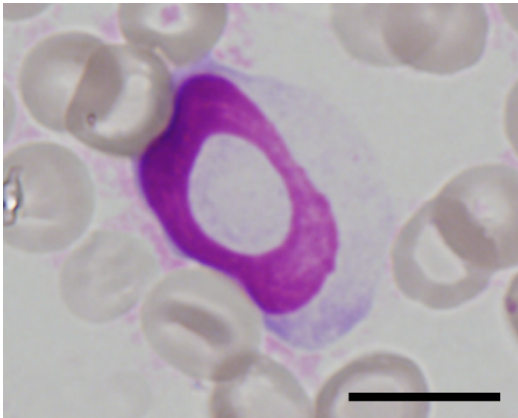


Hypersegmented neutrophils (a) and platelets with spindle shape (b) observed in peripheral blood smears stained with Wright-Giemsa stain. Arrowheads indicate spindle-shaped platelets. Bar = 10  $\mu\text{m}$

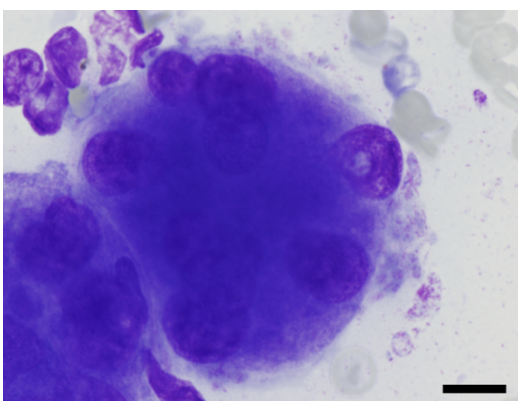
**Fig 1-2a**



**Fig 1-2b**

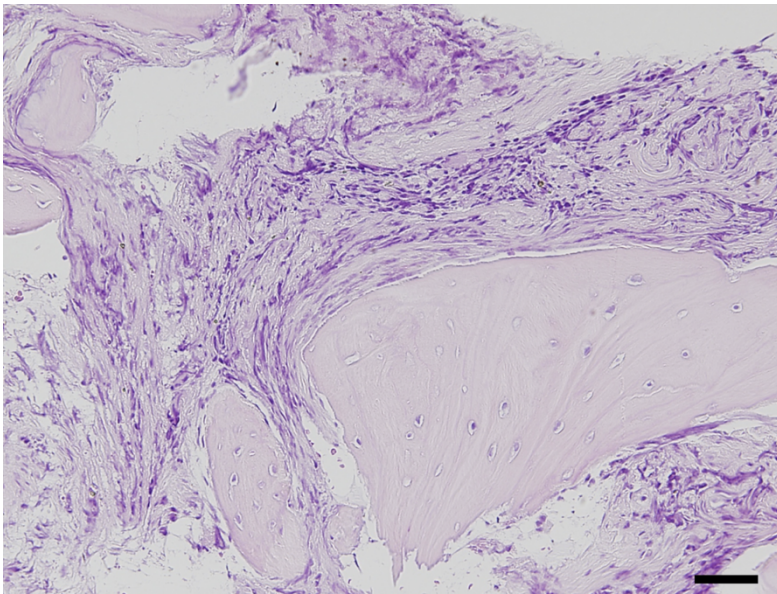


**Fig 1-2c**



Fragmented nucleus in the erythroid lineage (a), ringed neutrophil in the myeloid lineage (b), and dysmorphic megakaryocyte with multiple nuclei (c) observed in bone marrow smears stained with Wright-Giemsa stain. Bar = 10  $\mu\text{m}$

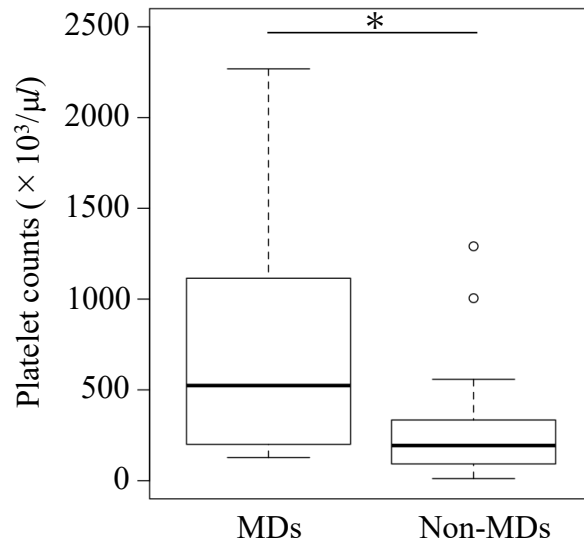
**Fig 1-3**



Severe fibrosis and bone marrow hypoplasia was observed in bone marrow core biopsy stained by hematoxylin-eosin stain. Bar = 50  $\mu$ m

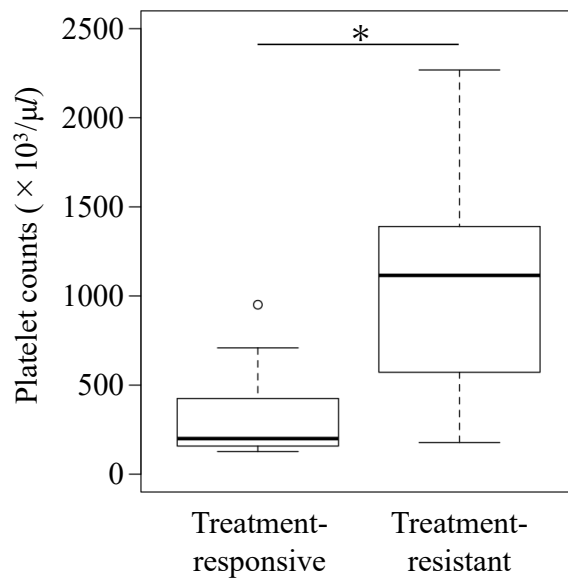


**Fig 1-4**



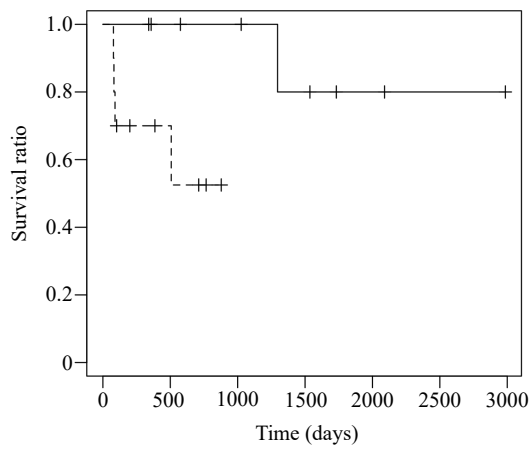
Comparison of platelet counts between Miniature Dachshunds (MDs) and other breeds (non-MDs). \* =  $P < 0.05$

**Fig 1-5**



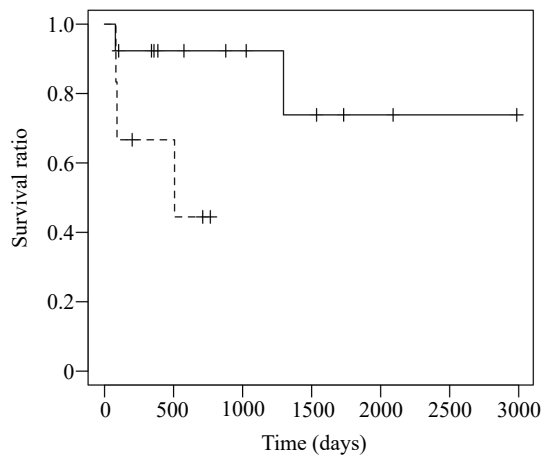
Comparison of platelet counts between treatment-responsive and treatment-resistant Miniature Dachshunds. \* =  $P < 0.05$

**Fig 1-6**



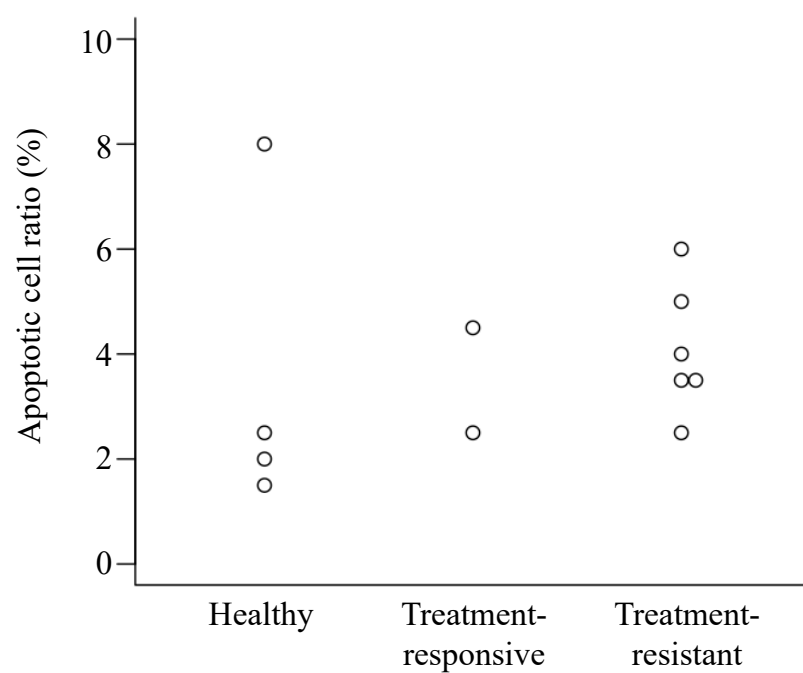
Comparison of overall survival between treatment-responsive (solid line) and treatment-resistant (dotted line) Miniature Dachshunds.

**Fig 1-7**



Comparison of overall survival between Miniature Dachshunds with platelets of < 1115 × 10<sup>3</sup> /μl (solid line) and those with platelets of > 1115 × 10<sup>3</sup> /μl (dotted line).

**Fig 1-8**



Comparison of the percentages of apoptotic cells among healthy control dogs, treatment-responsive Miniature Dachshunds (MDs), and -resistant MDs.

## **Chapter 2**

Comprehensive analysis of gene mutations in  
Miniature Dachshunds affected with non-neoplastic  
bone marrow disorders

## ABSTRACT

In Chapter 1, it was showed that Miniature Dachshund (MD) was a predisposed breed to be diagnosed with non-neoplastic bone marrow disorder, and treatment-resistant MDs showed distinctive clinicopathological features compared with treatment-responsive MDs. Since molecular abnormalities were suspected in non-neoplastic bone marrow disorders in MDs, I comprehensively investigated genetic mutations in MD cases using whole exome sequencing analysis (WES), and the gene mutations extracted in WES were validated by Sanger sequencing analysis. In addition, enrichment analysis using the genes with mutations extracted by WES was performed. As a result, a missense germline mutation in *Uromodulin like 1 (UMODL1)* gene, which encodes protein relating to T cell maturation, was found in MDs diagnosed with non-neoplastic bone marrow disorders but not in MDs without bone marrow disorders. However, gene enrichment analysis did not reveal any specific gene groups with mutations related to the pathogenesis of bone marrow disorders. The results of the present study suggested that the mutation of *UMODL1* gene might be related to the susceptibility of non-neoplastic bone marrow disorders in MDs.

## Introduction

Non-neoplastic bone marrow disorders are main causes of non-regenerative anemia in dogs (Weiss, 2006). In Chapter 1, it was revealed that Miniature Dachshund (MD) was the predisposed breed to be diagnosed with non-neoplastic bone marrow disorders in Japan. MD was also reported as a predisposed breed of inflammatory colorectal polyps and sterile panniculitis, which were rare in other breeds (Ohmi et al., 2012; Yamagishi et al., 2007). These breed-specific diseases implied that genetic aberration might be the underlying cause of non-neoplastic bone marrow disorders in MDs.

In Chapter 1, I also compared clinicopathological features between treatment-responsive and -resistant MDs. As a result, treatment-resistant MDs showed thrombocytosis, frequent dysplastic features in peripheral blood, and frequent clonality of peripheral blood cells. In a myelodysplastic syndrome (MDS) model mouse, which has *Additional sex combs like 1 (ASXL1)* mutation, non-regenerative anemia, thrombocytosis, and dysplastic features, which were similar to the clinicopathological features of treatment-resistant MDs, were reported (Nagase et al., 2018). These observations also indicated the molecular abnormalities especially in treatment-resistant MDs.

Based on these backgrounds, it was hypothesized that genetic abnormalities were related to the pathogenesis of non-neoplastic bone marrow disorders in MDs. Moreover, I also speculated that the molecular abnormalities in treatment-resistant MDs might be different from those in treatment-responsive MDs. Therefore, the purpose of this study was set to explore gene mutations in genome-wide scale by whole exome sequencing (WES) using samples obtained from MDs diagnosed with non-neoplastic bone marrow

disorders.

## **Materials and Methods**

### *Samples*

In the present study, 25 MDs diagnosed with non-neoplastic bone marrow disorders and 18 MDs without hematological abnormalities (control MDs) were included, and all dogs were referred to the Veterinary Medical Center of the University of Tokyo (VMC-UT) between April 2013 and March 2020. The signalment of each dog is shown in Table 2-1. MDs were diagnosed with bone marrow disorders when non-regenerative anemia was present and any apparent causes other than bone marrow disorders that could induce anemia were excluded based on the results of the blood examinations, diagnostic images, and bone marrow examinations. Dogs diagnosed with any neoplastic diseases were also excluded. The responsiveness to immunosuppressive treatments, which includes corticosteroid drugs and immunosuppressive drugs, were evaluated in MDs diagnosed with non-neoplastic bone marrow disorders, and the treatment responsiveness was defined as the absence of blood transfusion for more than 2 months after the last blood transfusion according to the response criteria in human medicine (Cheson et al., 2006). Based on these criteria, MDs diagnosed with non-neoplastic bone marrow disorders were divided into two groups; treatment-responsive MDs (n=13) and treatment-resistant MDs (n=12). MDs were included as control MDs if they showed no abnormality on complete blood count tests and were not diagnosed with any neoplastic disorders.

### *DNA extraction*

Peripheral bloods were collected from all 43 dogs and buccal swabs were collected from 2 of the 43 dogs. DNA was extracted from these samples using DNeasy blood & tissue kit (Qiagen, Milan, Italy) following the manufacture's instruction. Extracted DNA was examined through gel electrophoresis to check the absence of DNA laddering when it was used for WES.

### *Whole exome sequencing (WES)*

Genomic DNAs (500 ng) that were obtained from 4 treatment-resistant MDs and 3 control MDs were mechanically sheared to fragments of approximately 150 bp (Covaris S220, Woburn, MA, USA). Fragment sizes were verified for quality control using Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA, USA). SureSelect XT Canine All Exon V2 kit (Agilent) and AMPure XP beads (Beckman Coulter, Brea, CA, USA) were used to prepare libraries for sequence analysis. The library was sequenced using a paired-end 2x100 bp read protocol on NextSeq500 instrument (Illumina, San Diego, CA, USA). Signal captures were converted into FASTQ files using bcl2fastq (v2.18.0.12) and trimmed with trimgalore (v0.6.5) and Cutadapt (v1.15). The alignment of processed reads to a canine reference genome (CanFam 3.1, GenBank assembly accession: GCA\_000002285.2), local realignment, and variants calling in each sample were performed using DRAGEN software (v 07.021.572.3.6.3) (Illumina) with the standard protocol, and mutations were annotated using SnpEff (v5.0). The information on canine genetic variation, single nucleotide polymorphisms (SNPs) and insertions and deletions



(Indels), was available from the Ensembl variation database (CanFam3.1, dog release 101) and used as a variant database.

Extracted mutations in WES were filtered by the following criteria; 1) mutations found in treatment-resistant MDs but not in control MDs, 2) mutations predicted to affect amino acid sequences, 3) heterozygous mutations, and 4) mutations with total read depths of more than 10. Then, functional impacts of the filtered mutations on coded proteins were predicted by online software including Polymorphism Phenotyping v2 (PolyPhen 2, <http://genetics.bwh.harvard.edu/pph2/>), Sorting Intolerant from Tolerant (SIFT, <http://sift.jcvi.org/>), and Protein Variation Effect Analyzer (PROVEAN, <http://provean.jcvi.org/index.php>), and the mutations were included in the following examinations if they were predicted as deleterious or damaging to protein function by at least one software.

Enrichment analysis was conducted to investigate the biological functions associated with the genes with mutations. In this analysis, the genes with mutations that were common in more than 3 treatment-resistant MDs was extracted, and they were subjected to enrichment analysis using Database for Annotation, Visualization, and Integrated Discovery (DAVID, v6.8, <https://david.ncifcrf.gov>) to figure out significantly enriched Gene Ontology (GO) terms and pathways.

#### *Validations of the gene mutations extracted in WES*

The mutations that were common in 4 treatment-resistant MDs in WES were examined by Sanger sequence analysis in dogs used for WES and additional MD cases;

15 control MDs, 13 treatment-responsive MDs and 8 treatment-resistant MDs (Table 2-1). DNAs were extracted from the samples of these cases as previously described, and polymerase chain reaction (PCR) was conducted using these DNAs and PrimeSTAR HS (Takara Bio, Shiga, Japan) following the manufacture's instruction. Primers used for PCR were shown in Table 2-2. PCR products were purified by QIAquick PCR Purification Kit (Qiagen), and the purified PCR products were sequenced using BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystem, Foster City, CA, USA). The investigation for the mutation in each gene was terminated at the point the mutation was found in control MDs.

The genes commonly mutated in human MDS was also focused, because Chapter 1 indicated that treatment-resistant MDs harbored clinicopathological features that were similar to those in a MDS model mouse (Nagase et al., 2018). According to a previous study in human medicine (Ogawa 2019), 65 genes were selected as commonly mutated genes in human MDS (Table 2-3). I examined the mutations in these 65 genes in 4 treatment-resistant MDs using the data obtained from WES, and the identified gene mutations were also investigated in dogs used for WES and additional MD cases using Sanger sequencing analysis as described above. The investigation for the mutation in each gene was terminated at the point the mutation was found in control MDs.

## **Results**

### *WES*

WES analysis was performed for 3 control MDs and 4 treatment-resistant MDs (Table

2-1). Total raw reads were more than 99 million reads and mapping rates were more than 99% in each sample (Table 2-4). Approximately 600 thousand mutations were called in each sample by comparisons with canine reference genome (CanFam 3.1), and 1,047,648 mutations were called only in treatment-resistant MDs. SnpEff predicts 6,503 mutations as high or moderate to affect protein sequences, and 4,058 mutations were heterozygous mutations. Finally, 3,377 mutations were extracted as those with a total read depth>10.

#### *GO and pathway analysis using extracted genes with mutations*

Of 3,377 mutations extracted as above, 302 mutations in 243 genes were commonly found in more than 3 of 4 treatment-resistant MDs. Then, enrichment analysis was conducted using these genes to find common GOs and pathways related to the pathogenesis in treatment-resistant MDs. As a result, 3 GO-biological process terms, 3 GO-cellular component, and 3 GO-molecular function terms were shown to be significantly enriched, and pathway analysis showed 2 terms were significantly enriched as shown in Table 2-5.

#### *Validation of gene mutations extracted in WES*

Of 3,377 mutations extracted as above, 70 mutations were extracted as those common in all 4 treatment-resistant MDs. Then, I extracted 7 gene mutations after excluding the mutations that were registered as SNPs in Ensembl variation database or that were not predicted as functionally damaging ones. Extracted genes were as follows; *Pleckstrin homology domain-containing family G member 1 (PLEKHG1)*, *Dishevelled binding*

*antagonist of beta-catenin 1 (DACT1)*, *5'-nucleotidase domain containing 1 (NT5DC1)*, *Methyltransferase like 25 (METTL25)*, *Thyroid hormone receptor interactor 10 (TRIP10)*, *ZW10 interacting kinetochore protein (ZWINT)* and *Uromodulin like 1 (UMODL1)* genes. Because of the difficulty to amplify by PCR, Sanger sequencing analysis for validation could not be conducted for *DACT1* gene, and mutations in other 6 genes were examined in 18 control MDs, 13 treatment-responsive MDs and 12 treatment-resistant MDs (Table 2-6). As a result, it was shown that the mutation of *UMODL1* gene was not found in any control MDs but detected in 4 treatment-responsive and 6 -resistant MDs. The c.3724C>G mutation of *UMODL1* gene was predicted to cause an amino acid substitution of L1242V, and Polyphen 2 and Provean predicted the mutation as a damaging variant. Then, this mutation in *UMODL1* gene was investigated using gDNA derived from buccal swabs obtained from 2 of 10 MDs with the mutation, and the mutation was also found in gDNA derived from buccal swabs of both two dogs.

Then, I investigated the mutations in 65 genes that were commonly mutated in human MDS (Ogawa, 2019), and 5 mutations in 4 genes, *Stromal Antigen 2 (STAG2)*, *BCL6 Corepressor Like 1 (BCORL1)*, *Nuclear Receptor Corepressor 2 (NCOR2)* and *Fms Related Receptor Tyrosine Kinase 3 (FLT3)*, were found in 4 treatment-resistant MDs used in WES. Thus, the mutations in these 4 genes were examined by Sanger sequence analysis in 18 control MDs, 13 treatment-responsive MDs and 8 treatment-resistant MDs. As a result, a mutation in *STAG2* gene was revealed not to be found in any control dogs (Table 2-7). However, the mutation was found only in a treatment-resistant MD, where the mutation was detected in WES. When the *STAG2* gene mutation was examined using

DNA extracted from buccal swabs obtained from the case with the mutation, it was revealed that this mutation was also found in DNA derived from buccal swabs.

## Discussion

In this study, I investigated genetic mutations related to the pathogenesis of non-neoplastic bone marrow disorders in MDs. WES identified 3,377 mutations that were observed in treatment-resistant MDs but not in control MDs, and further examinations using Sanger sequencing revealed that a missense mutation of *UMODL1* gene was found in treatment-responsive MDs and -resistant MDs but not in control MDs.

*UMODL1* encodes  $\text{Ca}^{2+}$ -dependent EGF-like membrane-bound protein. Although the function of *UMODL1* has not been fully understood, it is known to be related to T cell immunodeficiency (Wang et al., 2012). Mutations of *UMODL1* gene were reported in human lung adenocarcinoma, MDS, and acute myeloid leukemia (AML) (Imielinski et al., 2012; Walter et al., 2012). In the study on human MDS, 7 patients were conducted whole-genome sequencing using the samples of 7 patients, and 2 of 7 patients were shown to harbor missense mutations of *UMODL1* gene. In the present study, the mutation of *UMODL1* gene seemed to be a germline one, because the mutation was also found in gDNA derived from buccal swabs. Therefore, the germline mutation of *UMODL1* might be related to the susceptibility of non-neoplastic bone marrow disorders in MDs. However, the mutation was found in both treatment-responsive MDs and -resistant MDs, and molecular mechanisms other than gene mutations were suggested to be underlying causes of the differences in clinical features between these two groups.

In enrichment analysis using extracted genes with mutations, GO terms or pathways that were apparently related to ineffective anemia or non-neoplastic bone marrow disorders were not identified. In addition, the investigation of the mutations in genes that were reported to be commonly mutated in human MDS could not extract any common somatic mutations in MDs diagnosed with non-neoplastic bone marrow disorders. These findings implied two possibilities. One is that non-neoplastic bone marrow disorders in MDs include quite heterogeneous diseases, and it was difficult to identify common somatic gene mutations using small number of cases in the present study. The other is that genetic aberrations are not direct triggers of the pathogenesis of non-neoplastic bone marrow disorders in MDs.

As a limitation of this study, the number of cases was small and it might be difficult to detect important mutations that were not common but were observed in a part of cases. It is also limitation that control MDs in the present study might develop bone marrow disorders in the future and control MDs might have had mutations related to the pathogenesis of non-neoplastic bone marrow disorders. As another limitation, DNA samples were extracted from peripheral blood cells rather than bone marrow cells. Although high concordance rates in detection of genetic abnormalities were reported between peripheral blood and bone marrow cells in human MDS (Mohamedali et al., 2013), DNA extracted from bone marrow might be needed to examine genetic aberrations.

In conclusion, WES revealed that a missense germline mutation of *UMODL1* gene was found in MDs diagnosed with non-neoplastic bone marrow disorders but not in control MDs. Since the mutation was found in both treatment-responsive MDs and -

resistant MDs, the mutation might be related to the susceptibility of non-neoplastic bone marrow disorders in MDs. However, molecular mechanisms other than gene mutations were suggested to be underlying causes of the differences in clinical features between treatment-responsive MDs and -resistant MDs.

Table 2-1. Signalment of dogs included in Chapter 2.

Number	Breed	Age	Sex	Treatment responsiveness	Exome	Sanger
Dog1	Miniature Dachshund	14.8	NF	control	○	○
Dog2	Miniature Dachshund	12.5	NF	control	○	○
Dog3	Miniature Dachshund	12.8	NF	control	○	○
Dog4	Miniature Dachshund	12	F	control	-	○
Dog5	Miniature Dachshund	13.4	NF	control	-	○
Dog6	Miniature Dachshund	10.4	NF	control	-	○
Dog7	Miniature Dachshund	10.6	F	control	-	○
Dog8	Miniature Dachshund	12.5	NF	control	-	○
Dog9	Miniature Dachshund	13.1	NF	control	-	○
Dog10	Miniature Dachshund	11.9	NF	control	-	○
Dog11	Miniature Dachshund	14.2	NF	control	-	○
Dog12	Miniature Dachshund	14.4	F	control	-	○
Dog13	Miniature Dachshund	11.7	F	control	-	○
Dog14	Miniature Dachshund	9.2	F	control	-	○
Dog15	Miniature Dachshund	13	NF	control	-	○
Dog16	Miniature Dachshund	12.1	NF	control	-	○
Dog17	Miniature Dachshund	11.5	F	control	-	○
Dog18	Miniature Dachshund	11.2	NF	control	-	○
Dog19	Miniature Dachshund	12.5	NF	resistant	○	◎
Dog20	Miniature Dachshund	12.1	F	resistant	○	○
Dog21	Miniature Dachshund	10.8	NF	resistant	○	◎
Dog22	Miniature Dachshund	17.4	F	resistant	○	○
Dog23	Miniature Dachshund	12.2	F	resistant	-	○
Dog24	Miniature Dachshund	11.9	M	resistant	-	○
Dog25	Miniature Dachshund	11.7	NF	resistant	-	○
Dog26	Miniature Dachshund	14.4	F	resistant	-	○
Dog27	Miniature Dachshund	12.6	F	resistant	-	○
Dog28	Miniature Dachshund	12.8	F	resistant	-	○
Dog29	Miniature Dachshund	14.2	F	resistant	-	○
Dog30	Miniature Dachshund	5.7	CM	resistant	-	○
Dog31	Miniature Dachshund	14.5	CM	responsive	-	○
Dog32	Miniature Dachshund	15.2	NF	responsive	-	○
Dog33	Miniature Dachshund	14.1	CM	responsive	-	○
Dog34	Miniature Dachshund	11.2	NF	responsive	-	○
Dog35	Miniature Dachshund	10.2	CM	responsive	-	○
Dog36	Miniature Dachshund	9.7	F	responsive	-	○
Dog37	Miniature Dachshund	11.7	F	responsive	-	○
Dog38	Miniature Dachshund	15	NF	responsive	-	○
Dog39	Miniature Dachshund	8.6	NF	responsive	-	○
Dog40	Miniature Dachshund	10.7	NF	responsive	-	○
Dog41	Miniature Dachshund	9.1	NF	responsive	-	○
Dog42	Miniature Dachshund	12.1	NF	responsive	-	○
Dog43	Miniature Dachshund	13.1	NF	responsive	-	○

○ indicates DNA sample of peripheral blood, ◎ indicates DNA samples of peripheral blood and buccal swab,

- indicates no DNA sample



Table 2-2. Primer sequences used for PCR amplification.

Gene	Forward	Reverse	Product size (bp)
<i>ANKRD31</i>	TCCAGAGCACCAACTCCTTT	TGGTTTGTCTCTTGGAAGAT	208
<i>ARHGEF</i>	CCATTCTCCCATCCACTCAA	GGTCCTTAGGCCCCAGTAAG	224
<i>BCORL1</i>	CATTGGAGACCTTCGAACC	ATCCCTGGATAACCCTCACC	494
<i>DAAM1</i>	GGGGGATTTCGTTTCATCTTTTG	ACCAAACCAAGCACATCCTC	229
<i>FLT3</i>	ACAGAAACCCAAGCTGGAG	CATTCTGAGAGCCCACAGG	276
<i>FZD3</i>	TCAGCTTGCTATGGTGGATG	TTCAGCTTCTTTTCAGAATTGG	194
<i>KRT1</i>	TAGTTCAGGTCTGGGTTC	GCATCTCCATAAGTGTGGCC	235
<i>LEF1</i>	TTAGCACGGAAGGAAAAGACAG	TGGTTTCAGGAATCCTCTGG	220
<i>METTL25</i>	GAAGCCTTGTCATTTCCTAA	CTGAGGGACTCTCGGCTTC	187
<i>NCOR2</i> ①	TTCTCGATCTCTGTGCCTCA	CACACACTCACCTCCTTGTC	230
<i>NCOR2</i> ②	CCCAAGTGCCACATCTACC	CTGCCAACCAAAAGTCCTG	271
<i>NFATC4</i>	CTAGGGAGCACCTTGGACAG	AATGTCTGTCTCCCCCTTCC	237
<i>NKD1</i>	TGAATGAAGGGTCCAGAAGG	GCCCCAACTGGGATGTGTAG	230
<i>NKD2</i>	GCACCTCTCCTCACCTG	CTGGGGAGGATCTTTTCTGG	249
<i>NT5DC1</i>	GGATTTCCTGTTTGTCTCTC	AGACAGGACAGTGCTCGTG	258
<i>PLEKHG</i>	CCTCAGGAGAACGAGGATGA	GAGAGCCACCACTGTCCCTA	247
<i>PPP2R5D</i>	GGTAGGAGACAGCTGCTTGG	AAACCTCCAGTCTGCCAATG	420
<i>STAG2</i>	GGTTTGTAAAATGAGAGTTTGAAG	CCGACTGGTAAAAAGCTCCA	232
<i>TRIP10</i>	GTGAGCTCAGGAAACCTCCA	CTCTCTGGCATCCACACTCA	248
<i>WNT1</i>	CAAGTGCCACGGGATGTC	AATTGGGCGATTCTCAAAG	239
<i>WNT16</i> ①	GAGGAACAAGGAGGGGAAAG	AAGAATCGAAAAGTCGCCAG	331
<i>WNT16</i> ②	ACGACCTGCTGTACGTCAAC	CGTAGCAGCACCAGATGAAC	205
<i>ZWINT</i>	GACCTTCCTCCGGCTGCT	GGATGGCACACTCCTGTCTT	151

Table 2-3. Sixty-five genes commonly mutated in human MDS

<i>DNMT3A</i>	<i>TET2</i>	<i>IDH1</i>	<i>IDH2</i>	<i>WT1</i>
<i>EZH2</i>	<i>SUZ12</i>	<i>EED</i>	<i>JARID2</i>	<i>ASXL1</i>
<i>KMT2</i>	<i>KDM6A</i>	<i>ARID2</i>	<i>PHF6</i>	<i>ATRX</i>
<i>SF3B1</i>	<i>SRSF2</i>	<i>U2AF1</i>	<i>U2AF2</i>	<i>ZRSR2</i>
<i>SF1</i>	<i>PRPF8</i>	<i>LUC7L2</i>	<i>STAG2</i>	<i>RAD21</i>
<i>SMC3</i>	<i>SMC1A</i>	<i>CTCF</i>	<i>NIPBL</i>	<i>ESCO2</i>
<i>PDS5B</i>	<i>RUNX1</i>	<i>ETV6</i>	<i>GATA2</i>	<i>IRF1</i>
<i>CEBPA</i>	<i>BCOR</i>	<i>BCORL1</i>	<i>NCOR2</i>	<i>CUX1</i>
<i>FLT3</i>	<i>KIT</i>	<i>JAK2</i>	<i>MPL</i>	<i>CALR</i>
<i>CSF3R</i>	<i>PTPN11</i>	<i>NF1</i>	<i>NRAS</i>	<i>KRAS</i>
<i>CBL</i>	<i>RIT1</i>	<i>BRAF</i>	<i>GNAS</i>	<i>GNB1</i>
<i>FBWX7</i>	<i>PTEN</i>	<i>BRCC3</i>	<i>FANCL</i>	<i>SETBP1</i>
<i>DDX41</i>	<i>TP53</i>	<i>ATM</i>	<i>NPM1</i>	<i>CDKN2A</i>

Table 2-4. Total read depth, mapping rate, and coverage obtained in each sample in whole exome sequencing.

Number	Raw reads (M)	Mapping rate (%)	Coverage
Dog1	120.9	99.7	173.1
Dog2	102.5	99.7	144.8
Dog3	111.4	99.7	165.9
Dog19	99.7	99.7	137.7
Dog20	112.8	99.7	159.5
Dog21	102.5	99.7	143.2
Dog22	105	99.7	154.1

Table 2-5. Gene ontology (GO) terms and pathways that were shown to be enriched by enrichment analysis using extracted genes with mutations.

Analysis	Term	<i>P</i> -Value	Fold Enrichment
GO-biological process	Sensory perception of sound	0.029	4.292
GO-biological process	Endodermal cell differentiation	0.039	9.478
GO-biological process	Notch signaling pathway	0.048	4.860
GO-cellular component	Z disc	0.001	7.814
GO-cellular component	Calcium channel complex	0.013	16.669
GO-cellular component	Sarcolemma	0.015	7.577
GO-molecular function	Ryanodine-sensitive calcium-release channel activity	0.033	59.125
GO-molecular function	Voltage-gated calcium channel activity	0.034	10.233
GO-molecular function	Calcium-induced calcium release activity	0.044	44.344
Pathway	Circadian entrainment	0.003	5.859
Pathway	Dorso-ventral axis formation	0.032	10.417

Table 2-6. Frequencies of gene mutations extracted as common ones in cases diagnosed with non-neoplastic bone marrow disorders.

Gene name	Reference	Altered	Control MDs			Treatment-responsive MDs			Treatment-resistant MDs		
			WT	hetero	homo	WT	hetero	homo	WT	hetero	homo
<i>PLEKHG1</i>	G	A	8/12	3/12	1/12	-	-	-	-	-	-
<i>ANKRD31</i>	C	T	3/6	3/6	0/6	-	-	-	-	-	-
<i>NT5DC1</i>	A	G	4/7	3/7	0/7	-	-	-	-	-	-
<i>METTL25</i>	GAGGCC	G	8/12	3/12	1/12	-	-	-	-	-	-
<i>TRIP10</i>	G	A	9/12	2/12	1/12	-	-	-	-	-	-
<i>ZWINT</i>	C	T	4/6	2/6	0/6	-	-	-	-	-	-
<i>UMODL1</i>	C	G	18/18	0/18	0/18	9/13	4/13	0/13	6/12	6/12	0/12

MD; Miniature Dachshund, WT; Wild Type

Table 2-7. Frequencies of the mutations in genes that were reported to be commonly mutated in human MDS.

Gene name	Reference	Altered	Control MDs			Treatment-responsive MDs			Treatment-resistant MDs		
			WT	hetero	homo	WT	hetero	homo	WT	hetero	homo
<i>STAG2</i>	C	CT	18/18	0/18	0/18	13/13	0/13	0/13	11/12	1/12	0/12
<i>BCORL1</i>	A	G	6/7	1/7	0/7	-	-	-	-	-	-
<i>NCOR2</i> ①	A	T	3/8	3/8	2/8	-	-	-	-	-	-
<i>NCOR2</i> ②	G	A	9/11	1/11	1/11	-	-	-	-	-	-
<i>FLT3</i>	C	T	5/7	2/7	0/7	-	-	-	-	-	-

MD; Miniature Dachshund, WT; Wild Type

## **Chapter 3**

Genome-wide DNA methylation analysis in  
Miniature Dachshunds with  
non-neoplastic bone marrow disorders

## ABSTRACT

In Chapter 1, Miniature Dachshund (MD) was shown to be a predisposed breed diagnosed with non-neoplastic bone marrow disorders in Japan, and immunosuppressive treatment-resistant MDs harbored different clinicopathological features from those of treatment-responsive MDs. Thus, whole-exome sequencing analysis was conducted to investigate gene mutations related to the pathogenesis of MDs diagnosed with non-neoplastic bone marrow disorders in Chapter 2. However, no difference in genetic aberration was observed between treatment-responsive MDs and -resistant MDs. Therefore, epigenetic aberrations in MDs diagnosed with non-neoplastic bone marrow disorders were investigated using genome-wide DNA methylation analysis in this chapter. Differentially methylated genes (DMGs) were examined by comparisons of MDs diagnosed with non-neoplastic bone marrow disorders and healthy dogs, and 28 genes were extracted as DMGs. Then, I focused on 6 DMGs, which were differentially methylated in a treatment-resistant MD but not in treatment-responsive MDs, and the mRNA expression levels of these genes were relatively quantified using RT-qPCR. As a result, *secreted frizzled-related protein 2 (SFRP2)* gene, which was hypermethylated in a treatment-resistant MD, was shown to be significantly down-regulated in treatment-resistant MDs compared with control dogs and treatment-responsive MDs. Based on this finding, pyrosequencing analysis was conducted to investigate the DNA methylation status in promoter of *SFRP2* gene. However, no significant difference in methylated DNA ratio was observed between treatment-responsive MDs and -resistant MDs. The results of this study indicated that gene expression profiles might be different between treatment-

responsive MDs and -resistant MDs and these differences might be derived from epigenetic aberration other than DNA methylation in promoter regions.

## Introduction

Non-neoplastic bone marrow disorders are the main causes of non-regenerative anemia in dogs (Weiss, 2006). In Chapter 1, I found that Miniature Dachshund (MD) was the predisposed breed to be diagnosed with non-neoplastic bone marrow disorders in Japan. The clinicopathological features of immunosuppressive treatment-resistant MDs were distinct from treatment-responsive MDs and these features were similar to those of *Additional sex combs like 1 (ASXL1)*-mutated mouse model (Nagase et al., 2018). Thus, genetic mutations were suspected to be the molecular pathogenesis in MDs diagnosed with non-neoplastic disorders. However, no difference in genetic mutations were observed between treatment-responsive MDs and -resistant MDs. Since mutations of *ASXL1* were closely related to the aberration of epigenetic mechanisms (Asada et al., 2019), epigenetic alterations, which induce changes in gene expressions without genetic mutations, were suspected to be underlying causes of the differences in the pathophysiology between treatment-responsive MDs and -resistant MDs.

Among various epigenetic mechanisms, the aberration of DNA methylation is the most frequently reported and investigated mechanism (Bernstein et al., 2007). DNA methylation involves the addition of a methyl group at the 5' carbon of a cytosine of a 5'-CpG-3' dinucleotide by DNA methyltransferases (DNMTs) (Bernstein et al., 2007). Regions enriched with CpG, which is called 'CpG islands (CGI)', are found in the promoters of approximately half of the genes. While most CpG dinucleotides in genome are methylated, the majority of those in CGI are unmethylated. DNA methylation in promoter CGI is associated with stable repression of gene expressions via inhibition of



gene transcriptions (Herman & Baylin, 2003). Therefore, aberrant DNA methylation in promoter results in abnormal gene expression patterns.

Based on these backgrounds, I set the purpose of the study in this chapter to explore aberrations in DNA promoter methylation related to the pathogenesis of non-neoplastic bone marrow disorders in MDs using genome-wide DNA methylation analysis.

## **Materials and Methods**

### *Samples*

Bone marrow specimens were collected from 18 MDs diagnosed with non-neoplastic bone marrow disorders (Table 3-1). All MDs were referred to the Veterinary Medical Center of the University of Tokyo (VMC-UT) between April 2013 and March 2020, and bone marrow disorders were diagnosed when non-regenerative anemia was present without any other apparent causes other than bone marrow disorders that could induce anemia based on the results of the blood examinations, diagnostic images, and bone marrow examinations. Dogs diagnosed with any neoplastic diseases were excluded. MDs diagnosed with non-neoplastic bone marrow disorders were evaluated for their responsiveness to immunosuppressive treatments, and the treatment responsiveness was defined as the absence of blood transfusion for more than 2 months after the last blood transfusion according to the response criteria in human medicine (Cheson et al., 2006). Based on the criteria, 10 MDs were considered to be treatment-responsive MDs and 8 MDs were treatment-resistant.

As healthy control dogs, 7 Beagles, 1 mix breed, and 1 MD were included and

conducted bone marrow aspirations. This animal experiment was approved by the Animal Care Committee of The University of Tokyo (approval no. P17-064 and P19-119). All bone marrow specimens were immediately frozen at -80°C until DNA or RNA extraction.

#### *DNA extraction*

DNA was extracted from bone marrow specimens using DNeasy blood & tissue kit (Qiagen, Milan, Italy) following the manufacture's instruction. Extracted DNA was examined through gel electrophoresis to check the absence of DNA laddering when it was used for genome-wide DNA methylation analysis.

#### *RNA extraction and reverse transcription*

Total RNA was extracted from bone marrow specimens using RNeasy Mini Kit (Qiagen, Hilden, Germany). The RNA quantity and quality were assessed using Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). The synthesis of complementary DNA (cDNA) was conducted using ReverTra Ace (TOYOBO, Osaka, Japan) following the manufacture's instruction.

#### *Digital restriction enzyme analysis of methylation (DREAM)*

DREAM analysis, a genome-wide DNA methylation analysis which could distinguish differences in methylation of >10% with a False Discovery Rate (FDR) of 2.4% (Jelinek et al., 2012), was performed as previously described (Yamazaki et al., 2018). Briefly, DNAs (2 µg) extracted from bone marrow specimens were mixed with 2 pg of a set of

artificial methylation standards. They were digested with SmaI and XmaI endonuclease (New England Biolabs, Tokyo, Japan) followed by filling in with a dCTP, dGTP, and dATP mix, and 3'-dA tails were added by Klenow DNA polymerase lacking 3'-5' exonuclease activity (New England Biolabs). Illumina paired-end sequencing adaptors were ligated using T4 DNA ligase (New England Biolabs). The ligation mix was size-selected by Agencourt AMPure XP to obtain DNA fragments ranging from 250 bp to 450 bp. Purified DNA was amplified using KAPA Hifi HotStart ReadyMix (Kapa Biosystems, Woburn, MA, USA) and 11 cycles of amplification reaction followed by sequencing on an Illumina HiSeq 2000 (Illumina, San Diego, CA, USA). Reads were mapped to SmaI/XmaI sites in the canine reference genome (canFam3.1), and signatures corresponding to methylated and unmethylated CpGs were counted for each SmaI/XmaI site to calculate methylation frequencies for individual SmaI/XmaI sites. Finally, I corrected methylation levels measured by DREAM based on the values obtained from the spikes in standards. SmaI/XmaI sites with at least 20 sequencing reads to analyze methylation levels were used. The University of California, Santa Cruz (UCSC) definition of CpG islands: GC content of 50% or greater, length > 200 bp, a ratio greater than 0.6 of the observed number of CG dinucleotides to the expected number on the basis of the number of Gs and Cs in the segment was employed (Gardiner-Garden & Frommer, 1987). SmaI/XmaI sites at promoter regions are defined as being located within 1 kb from transcription start sites (TSS) of given genes.

Differentially methylated sites (DMSs) were identified based on the following temporary criteria for hypermethylation; >20% increase in CGI DNA methylation in each

case from basal DNA methylation level (0-15%) in healthy dogs, or hypomethylation; >20% increase in CGI DNA methylation in healthy dogs from basal DNA methylation level (0-15%) in each case. When DMSs exist within 1kb from TSS of given genes, the genes were defined as differentially methylated genes (DMGs). Genes on X chromosomes were excluded from analyses of DMGs to prevent the influence of female X-chromosome inactivation (Bird, 2002).

### *RT-qPCR*

RT-qPCR to quantify mRNA of DMGs was performed using a Thermal Cycler Dice Real Time System TP800 (Takara Bio, Shiga, Japan). Twenty-five microliters of the PCR mixture containing 12.5 µl of Master Mix (TB Green Premix Ex Taq II (Takara Bio)), 400 nM of forward and reverse primers, and 10 ng of cDNA. The cDNA samples were subjected to activation at 95°C for 10 sec, then 40 cycles of denaturation at 95°C for 5 sec, and annealing/extension at 62°C for 30 sec. All reactions were run in duplicate. Sequences of primers and amplification efficacies were written in Table 3-2. As the internal control gene, *Hypoxanthine phosphoribosyl-transferase 1 (HPRT1)*, which was validated as the most stably expressed gene in canine bone marrow (Peters et al., 2015), was used. The relative quantity of each gene was calculated by plotting  $C_T$  in the prepared standard curve. To normalize the amount of cDNA sample, the ratio of the relative quantity of cDNA for each target gene to that of *HPRT1* gene was adopted. Relative expression level of each gene in a healthy control dog was used for normalization of that in other dogs.

### *Bisulfite-pyrosequencing analysis*

I used bisulfite-pyrosequencing to assess DNA methylation status for promoter CGI (Colella et al., 2003) of *secreted frizzled-related protein 2 (SFRP2)* gene, whose expression was significantly down-regulated in treatment-resistant MDs compared with healthy dogs and treatment-responsive MDs. Briefly, genomic DNA (500 ng) extracted from the bone marrow samples was used for bisulfite conversion by using the innuCONVERT Bisulfite Basic Kit (Analytika yena, Prague, Czech Republic) according to the manufacturer's instructions. I searched primers that are capable of amplifying SmaI/XmaI sites of DMSs in silico analysis by UCSC In-Silico PCR. Primer sequences used for the first amplification were as follows; Forward, GGTTGTAGATAGGAGAGGAAATTAGG; Reverse, AACTACATCTACCCCAATTCTTTATACCTC, and those used for the second amplification were as follows; Forward, GGTTGTAGATAGGAGAGGAAATTAGG; Reverse-biotin, (biotin)- GGGACACCGCTGATCGTTTA, and that used as sequence primer was as follows; GTTTTTAGAGGATAGGTT. Forty cycles were confirmed to completely exhaust the biotinylated primer. I measured DNA methylation levels as the percentage of bisulfite-resistant cytosines at CpG sites by pyrosequencing using a PSQ24 system with Pyro-Gold reagent Kit (QIAGEN), and the results were analyzed using PyroMark Q24 software (QIAGEN). The pyrosequencing assay interrogated DNA methylation levels of the SmaI/XmaI sites of DMSs.

### *Statistical analysis*

Mann-Whitney *U* test was used to compare the methylation levels among cases. Steel-Dwass test was used to compare mRNA expression levels among healthy dogs, treatment-responsive MDs, and -resistant MDs. A *P*-value < 0.05 was considered statistically significant. All statistical analyses were performed using the statistical program R 3.5.0 (<http://cran.r-project.org>).

## **Results**

### *DREAM analysis*

DREAM analysis was conducted using DNA extracted from bone marrow specimens of 3 healthy dogs, 2 treatment-responsive MDs, and 1 treatment-resistant MD (Table 3-1). More than 10 million unique usable reads were obtained and more than 100,000 CpG sites were covered by these reads in all 6 samples (Table 3-3). I extracted CpG sites that were commonly read more than 20 times in all samples, and 83,548 CpG sites were extracted to compare the methylation levels among the cases. Of these, 33,066 sites were in CGIs and 50,482 sites were in non-CGIs regions (NCGIs). Hierarchical clustering analysis according to methylation levels of CGIs (Fig 3-1a) and NCGIs (Fig 3-1b) showed the separated clusters for healthy dogs, treatment-responsive MDs, and -resistant MD.

Since different methylation levels of CGIs in gene promoter regions were known to be related to gene expression switching (Herman & Baylin, 2003), I identified DMSs of CGIs according to the temporary criteria as described above. As a result, 251, 168, and 134 DMSs were identified and the proportions of DMSs in analyzed CGIs were 0.8, 0.5,

and 0.4 %, in two treatment-responsive MDS and a treatment-resistant MD, respectively. According to the extracted DMSs, 13, 11, and 11 genes were identified as DMGs in two treatment-responsive MDs and a treatment-resistant MD, respectively (Fig 3-2). DNA hypermethylation was observed in 22 DMGs including *SFRP2* and *Piwi Like RNA-Mediated Gene Silencing 4 (PIWIL4)*, and DNA hypomethylation was observed in 6 DMGs including *Thioredoxin Like 4A (TXNL4A)*. DNA hypermethylation of *SFRP2*, *ELAV Like RNA Binding Protein 3 (ELAVL3)*, *Endothelin Converting Enzyme Like 1 (ECE1)*, *Catenin Alpha 2 (CTNNA2)*, and *Kinase Insert Domain Receptor (KDR)* genes and DNA hypomethylation of *Sushi, Nidogen And EGF Like Domains 1 (SNED1)* gene were observed in a treatment-resistant MD but not in two treatment-responsive MDs.

#### *Comparisons of mRNA expression levels of extracted DMGs*

Based on the results obtained from DREAM analysis, I decided to examine the mRNA expression levels of 6 DMGs, *SFRP2*, *ELAVL3*, *ECEL1*, *CTNNA2*, *KDR*, and *SNED1* genes, which were hypermethylation or hypomethylation of DNA was observed in a treatment-resistant MD but not in treatment-responsive MDs.

The samples obtained from 5 healthy dogs, 5 treatment-responsive MDs, and 5 treatment-resistant MDs were used for RT-qPCR (Table 3-1) and mRNA expression levels of 6 genes were compared among healthy dogs, treatment-responsive MDs, and treatment-resistant MDs. The gene expression levels of *SNED1* ( $P = 0.024$ ) and *CTNNA2* ( $P = 0.043$ ) were significantly higher in treatment-responsive MDs compared with healthy dogs (Fig 3-3a and 3-3b). Meanwhile, no significant difference was observed

between healthy dogs and treatment-resistant MDs or between treatment-responsive MDs and -resistant MDs. The gene expression levels of *KDR* were significantly higher in treatment-responsive MDs ( $P = 0.024$ ) or treatment-resistant MDs ( $P = 0.024$ ) compared with healthy dogs (Fig 3-3c). No significant difference was observed between treatment-responsive MDs and treatment-resistant MDs. The gene expression levels of *SFRP2* were significantly lower in treatment-resistant MDs compared with healthy control dogs ( $P = 0.043$ ) or treatment-responsive MDs ( $P = 0.024$ ) (Fig 3-3d). No significant difference was observed between healthy dogs and treatment-responsive MDs. No significant difference in the expression levels of *ELAVL3* and *ECEL1* was observed among the 3 groups (Fig 3-3e and 3-3f).

#### *Pyrosequencing analysis of SFRP2*

Since DNA hypermethylation in *SFRP2* gene was observed and the expression levels of this gene was downregulated in treatment-resistant MDs, the DNA promoter methylation levels of *SFRP2* was validated using larger number of dogs by pyrosequencing analysis.

DNAs obtained from 2 healthy dogs, 7 treatment-responsive MDs, and 5 treatment-resistant MDs were used in pyrosequencing analysis. When the methylation levels of the DMS that were evaluated by DREAM analysis was investigated, the median value of methylation levels was 12% (range, 9.9-14.1%), 17.9% (range, 12.0-22.2%), and 18.3% (range, 15.6-26%) in healthy dogs, treatment-responsive MDs, and treatment-resistant MDs, respectively, and there was not significant difference between treatment-responsive



and -resistant MDs (Fig 3-4,  $P = 1.0$ ).

## **Discussion**

In the present study, I conducted genome-wide DNA methylation analysis to investigate epigenetic aberrations in MDs diagnosed with non-neoplastic bone marrow disorders. Hierarchical clustering analysis according to methylation levels of CGIs and NCGIs showed the separated clusters for healthy dogs, treatment-responsive MDs, and a treatment-resistant MD. However, I could not identify the differences in DNA methylation levels that were associated with suppression of gene expressions, when I examined the DNA methylation levels and the mRNA expression levels of some genes using larger number of cases.

The proportions of DMSs in analyzed CGIs were 0.4-0.8 % in MDs diagnosed with non-neoplastic bone marrow disorders in the present study. These proportions were relatively lower than those that were previously reported in canine lymphoma cells and canine intestinal lymphoma tissue, which were more than 1.3% (Ohta et al., 2020; Yamazaki et al., 2018). The low proportions of DMSs might reflect the various cell populations contained in bone marrow specimens, and it was possible that the aberrations in DNA methylation in abnormal cells could not be found due to the contaminations of normal cells. However, a genome-wide DNA methylation analysis for human myelodysplastic syndrome (MDS), which was caused by clonal cell expansion of hematopoietic stem cells (HSCs), showed that methylation patterns of separated HSCs were highly correlated with whole bone marrow cells (Figueroa et al., 2009). Therefore,

it is reasonable to conclude that genome-wide DNA methylation was not significantly altered in bone marrow cells of MDs diagnosed with non-neoplastic bone marrow disorders. Meanwhile, hierarchical clustering analysis according to methylation levels of CGIs and NCGIs showed the separated clusters for healthy dogs, treatment-responsive MDs, and a treatment-resistant MD. Based on this finding, I decided to investigate the differences in DNA methylation levels of specific genes among these three groups.

Among the DMG extracted from the comparisons between healthy dogs and MDs diagnosed with non-neoplastic bone marrow disorders, I focused on 6 DMGs, where DNA hypermethylation or hypomethylation was observed in a treatment-resistant MD but not in treatment-responsive MDs. RT-qPCR analysis for these 6 DMGs showed the significant upregulation of *SNED1* and *CTNNA2* in treatment-responsive MDs compared with healthy dogs. *SNED1* is one of the extracellular matrix proteins and knockout of *SNED1* cause fatal effect in early neonatal mice (Barqué et al., 2020). *CTNNA2* encodes  $\alpha$ -catenin, which is a cell adhesion protein, and was reported as a tumor suppressive gene (Qiao et al., 2019). However, their roles in the pathophysiology of non-neoplastic bone marrow disorders have not been elucidated, and the changes in expressions of these genes might be due to the mechanisms other than DNA methylation because methylation levels of these genes were not different between healthy dogs and treatment-responsive MDs in DREAM analysis.

It was also revealed that the expression levels of *KDR* were significantly higher in treatment-responsive MDs and treatment-resistant MDs compared with healthy dogs. *KDR* encodes vascular endothelial growth factor receptor 2 (VEGFR2), which are

expressed in neutrophils and macrophages (Larrivée et al., 2005). In Chapter 1, I found that the number of macrophages in bone marrow cells was increased in MDs diagnosed with non-neoplastic bone marrow disorders compared with control dogs. Thus, the upregulation of *KDR* might be related to the increase of the number of macrophages. However, the changes in gene expression levels of *KDR* in treatment-resistant MDs were contradictory to the changes in DNA methylations, because DNA hypermethylation of *KDR* was shown in a treatment-resistant MD in DREAM analysis.

The expression levels of *SFRP2* were significantly lower in treatment-resistant MDs compared with healthy dogs and treatment-responsive MDs. *SFRP2* is one of the extracellular soluble Wnt antagonists (MacDonald et al., 2009). Since hypermethylation of *SFRP2* in a treatment-resistant MD was found in DREAM analysis, the gene expression changes of *SFRP2* were thought to be concordant with the changes in DNA methylation status. However, bisulfite pyrosequencing analysis using larger number of cases showed no difference in DNA methylation status in DMS of this gene among healthy dogs, treatment-responsive MDs, and treatment-resistant MDs. Therefore, epigenetic changes other than promoter DNA methylation might be the causes of downregulation of *SFRP2* expressions in treatment-resistant MDs.

As a limitation of this study, the number of cases analyzed by DREAM analysis was quite small. In addition, bisulfite pyrosequencing analysis could be performed for one DMS in *SFRP2* gene, which was extracted by DREAM analysis, but it was possible that DNA methylation of other CpG sites in TSS could be associated with the downregulation of expression of the gene in treatment-resistant MDs.

In conclusion, I conducted genome-wide DNA methylation analysis in MDs diagnosed with non-neoplastic bone marrow disorders. Hierarchical clustering analysis according to methylation levels of CGIs and NCGIs indicated DNA methylation profiles might be different among healthy dogs, treatment-responsive MDs, and a treatment-resistant MD. Among 6 DMGs that were specifically observed in a treatment-resistant MD, the expression of *SFRP2* was significantly down-regulated in treatment-resistant MDs. Although I could not show the associations of DNA methylation with the downregulation of this gene expression, it was implied that gene expression profiles might be different between treatment-responsive MDs and -resistant MDs.

Table 3-1. Signalment of dogs whose bone marrow specimens were used in Chapter 3.

Number	Breed	Age	Sex	Treatment responsiveness	DREAM	Pyro-sequencing	RT-qPCR
Dog1	Miniature Dachshund	12.5	NF	resistant	○	-	-
Dog2	Miniature Dachshund	11.2	NF	responsive	○	-	-
Dog3	Miniature Dachshund	13.7	NF	responsive	○	○	-
Dog4	Beagle	4.8	NF	healthy	○	-	-
Dog5	Beagle	12.2	NF	healthy	○	-	-
Dog6	Miniature Dachshund	12	NF	healthy	○	-	○
Dog7	Miniature Dachshund	13.4	NF	resistant	-	○	○
Dog8	Miniature Dachshund	12.2	F	resistant	-	○	○
Dog9	Miniature Dachshund	12.6	F	resistant	-	○	-
Dog10	Miniature Dachshund	9.8	NF	resistant	-	○	○
Dog11	Miniature Dachshund	14.4	F	resistant	-	○	-
Dog12	Miniature Dachshund	8.1	F	responsive	-	○	○
Dog13	Miniature Dachshund	11.2	NF	responsive	-	○	○
Dog14	Miniature Dachshund	14.5	CM	responsive	-	○	○
Dog15	Miniature Dachshund	10.2	CM	responsive	-	○	-
Dog16	Miniature Dachshund	9.7	F	responsive	-	○	-
Dog17	Miniature Dachshund	11.7	F	responsive	-	○	-
Dog18	mix breed	9	F	healthy	-	○	-
Dog19	Beagle	3	F	healthy	-	○	-
Dog20	Miniature Dachshund	15.2	NF	responsive	-	-	○
Dog21	Miniature Dachshund	7.8	CM	responsive	-	-	○
Dog22	Beagle	6.5	CM	healthy	-	-	○
Dog23	Beagle	6.2	CM	healthy	-	-	○
Dog24	Beagle	6.4	CM	healthy	-	-	○
Dog25	Beagle	6.3	CM	healthy	-	-	○
Dog26	Miniature Dachshund	11.5	F	resistant	-	-	○
Dog27	Miniature Dachshund	12.8	F	resistant	-	-	○

※DREAM; Digital restriction enzyme analysis of methylation

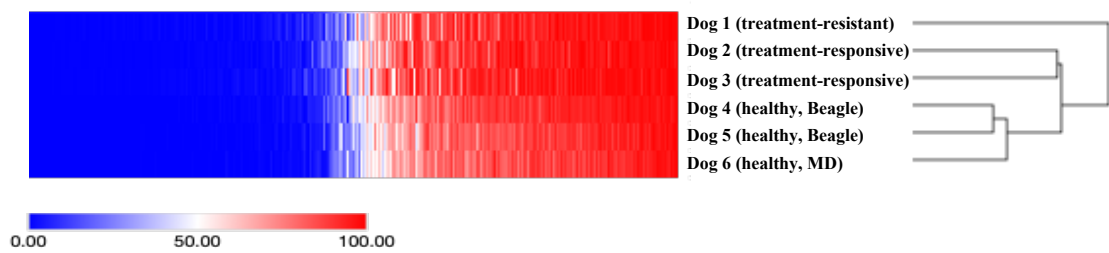
Table 3-2. Sequences of primers used in the present study.

Gene name	Forward	Reverse	Product size (bp)	Ensemble gene number	Amplification efficacy (%)
<i>ELAVL3</i>	AAGACCATCAAGGTGTCCTATGC	GCTGCTCCATTCTTTCTGG	109	ENSCAFG00000017350	98.7
<i>ECEL1</i>	GGCTGGCCAACATCACAG	GGGGTGATCTTCTGCAACTG	100	ENSCAFG00000011211	104
<i>CTNNA2</i>	GAAGACAACAGGAGCTGAAGG	AGGCTGTGTACAGCATCGTG	96	ENSCAFG00000008079	102.4
<i>SNED1</i>	CGCCAAAGAGCTCTTCCC	GATGGAGACCCCACTCTCTTC	67	ENSCAFG00000012816	106.3
<i>KDR</i>	TAGTTGTGGTTGTAGGGTACAAG	GCCTCTCTCCAACAGACAGC	79	ENSCAFG00000002079	108.8
<i>SFRP2</i>	CAACGACCTCTGCATACCCC	GCAGGCTTCGCATACCTTTG	82	ENSCAFG00000008353	101.5
<i>HPRT1</i>	CACTGGGAAAACAATGCAGA	ACAAAGTCAGGTTTATAGCCAACA	123	ENSCAFG00000018870	96.4

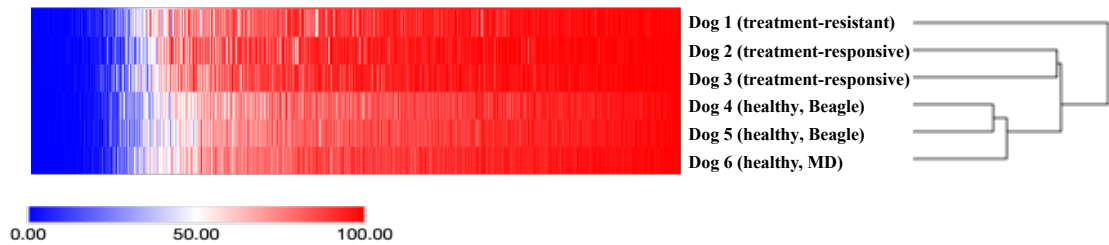
Table 3-3. The number of total reads and CpG sites covered by more than 20 reads in each sample.

Sample	Number of reads	Number of CpG sites covered
Dog1	10,552,081	112,791
Dog2	10,612,929	122,536
Dog3	11,216,639	129,355
Dog4	34,790,675	176,838
Dog5	37,409,787	168,215
Dog6	40,678,544	174,020

**Fig 3-1a.**

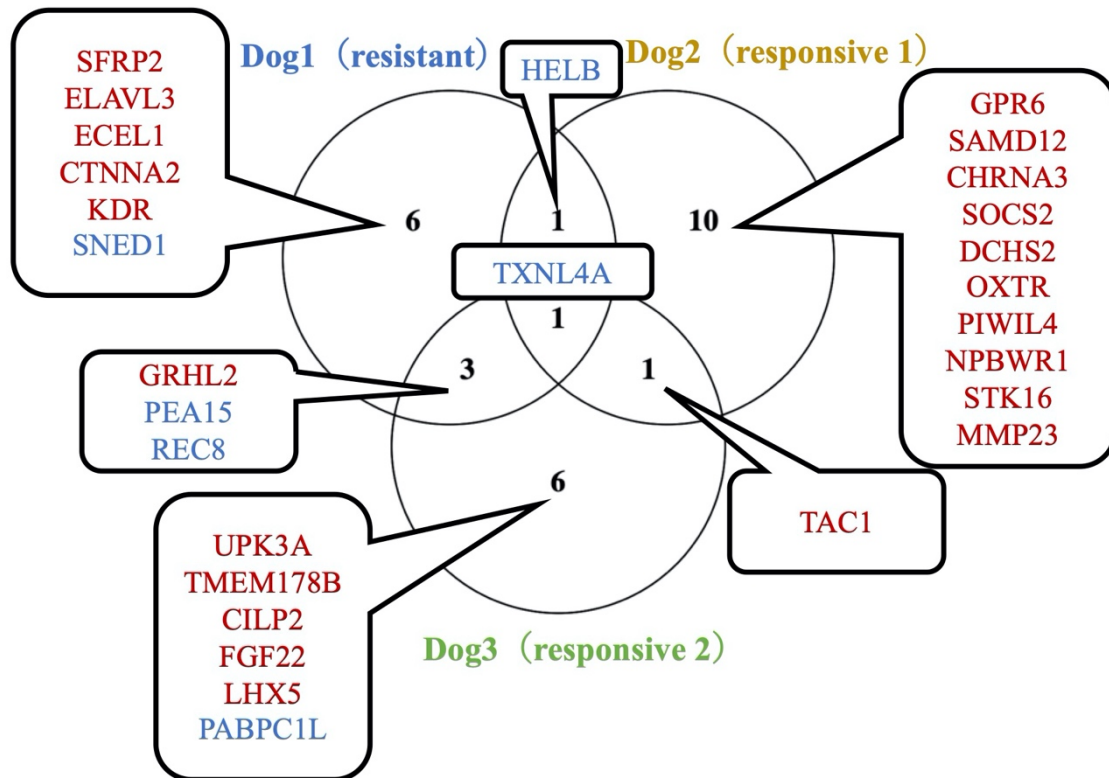


**Fig 3-1b.**



Heatmap of the result of hierarchical clustering using methylation levels of CpG islands (a) and non-CpG islands (b). Healthy dogs (Dog 4-6) and Miniature Dachshunds (MDs) diagnosed with non-neoplastic bone marrow disorders (Dog 1-3) were separately clustered. Healthy dogs included 2 Beagles and 1 MD.

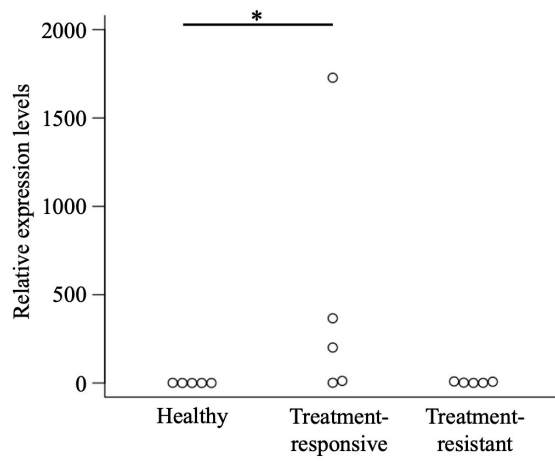
**Fig 3-2**



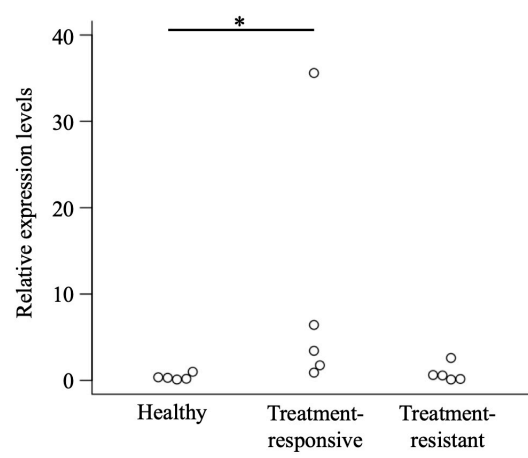
A venn diagram showing differentially methylated genes (DMGs) in 1 treatment-resistant Miniature Dachshund (MD) (Dog1) and 2 treatment-responsive MDs (Dog 2, Dog3). Genes with red-colored names were hypermethylated and genes with blue-colored names were hypomethylated in in each dog compared with healthy dogs.



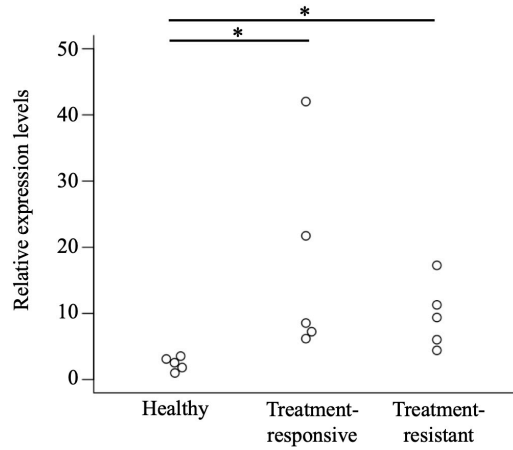
**Fig 3-3a.**



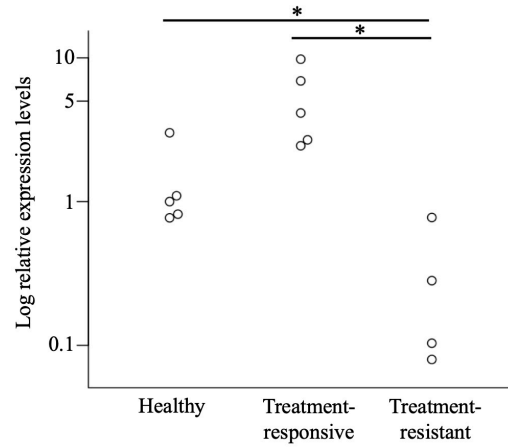
**Fig 3-3b.**



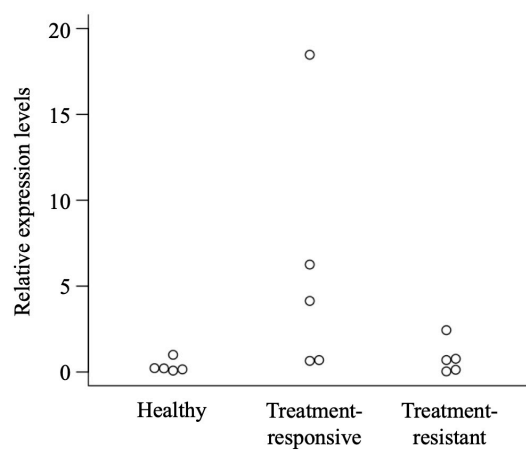
**Fig 3-3c.**



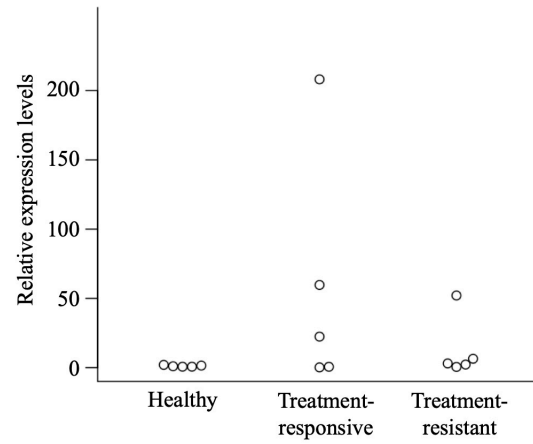
**Fig 3-3d.**



**Fig 3-3e.**

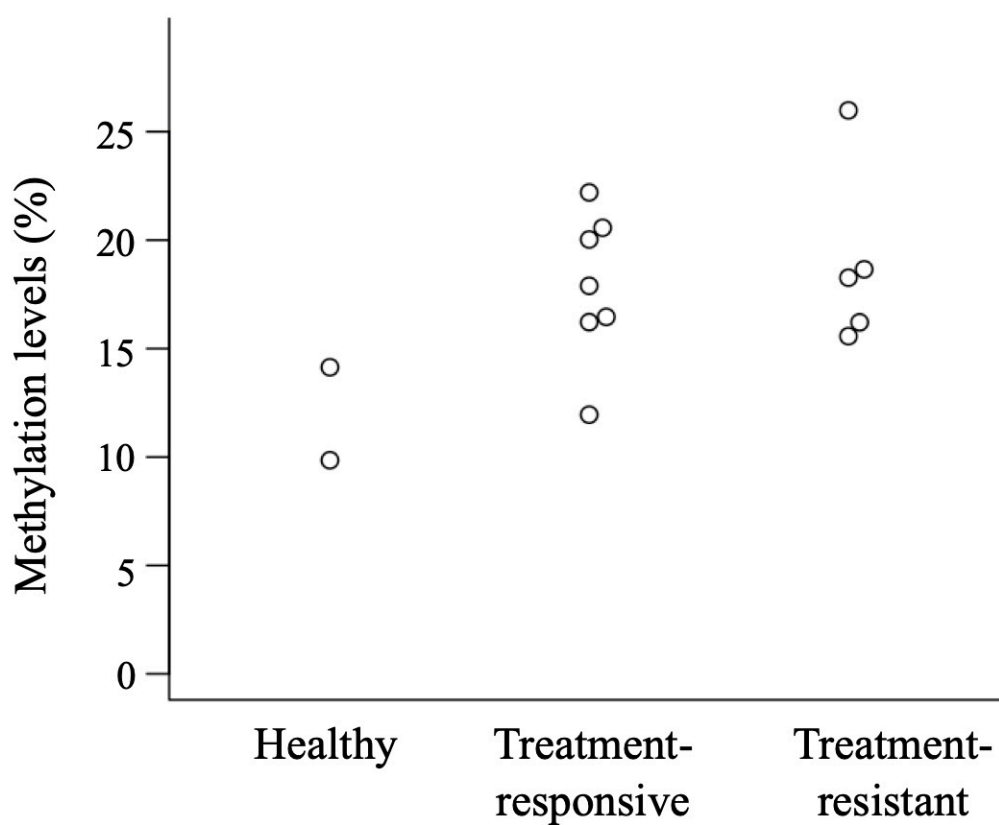


**Fig 3-3f.**



Comparisons of relative mRNA expression levels of *SNED1* (a), *CTNNA2* (b), *KDR* (c), *SFRP2* (d), *ELAVL3* (e), and *ECEL1* (f) among healthy dogs (n=5), treatment-responsive Miniature Dachshunds (MDs) (n=5), and treatment-resistant MDs (n=5). Logarithmic axis was used in *SFRP2*, and the expression of this gene could not be detected in one treatment-resistant Miniature Dachshund. \* indicates  $P < 0.05$

Fig 3-3.



Comparisons of DNA methylation levels in promoter region of *SFRP2* gene among healthy dogs, treatment-responsive Miniature Dachshunds (MDs), and treatment-resistant MDs.

## **Chapter 4**

Transcriptome analysis of bone marrow cells in Miniature  
Dachshunds with non-neoplastic bone marrow disorders

## ABSTRACT

In Chapter 1, Miniature Dachshund (MD) was shown to be a predisposed breed diagnosed with non-neoplastic bone marrow disorder, and treatment-resistant MDs showed distinct features compared with treatment-responsive ones. Thus, I investigated gene mutations and DNA methylation profiles in Chapter 2 and 3, respectively. However, specific changes in genetic mutations or DNA methylation status were not observed in treatment-resistant MDs compared with treatment-responsive MDs. Meanwhile, significant downregulations of *secreted frizzled-related protein 2* (*SFRP2*) gene expression were found in treatment-resistant MDs, and changes in gene expression profiles (GEPs) were suspected to be the molecular pathogenesis of treatment-resistant MDs. Thus, I compared GEPs of 3 treatment-resistant MDs with 3 healthy dogs by RNA-sequencing analysis, and 179 differentially expressed genes (DEGs) were extracted. Next, I performed gene enrichment analysis using these DEGs, and it was indicated that genes associated with Wnt signaling pathway were significantly enriched in these DEGs. Based on these findings, the expression levels of genes associated with Wnt signaling pathway were compared by RT-qPCR among healthy dogs, treatment-sensitive MDs and -resistant MDs. As a result, I found significant upregulation of *cyclin D2* (*CCND2*) expression and significant downregulation of *axin 2* (*AXIN2*) and *SFRP2* expressions in treatment-resistant MDs compared with treatment-responsive MDs and healthy dogs. These alterations implied the activation of canonical Wnt signaling in treatment-resistant MDs and showed the distinct molecular pathogenesis in treatment-resistant MDs compared with treatment-responsive MDs.

## Introduction

In Chapter 1, I found that Miniature Dachshund (MD) was the predisposed breed diagnosed with non-neoplastic bone marrow disorders, and treatment-resistant MDs showed distinct clinicopathological features compared with treatment-responsive MDs. I conducted whole-exome sequencing analysis in Chapter 2, and a missense germline mutation in *Uromodulin like 1 (UMODL1)* gene was detected in MDs diagnosed with non-neoplastic bone marrow disorders but not in control MDs. Meanwhile, no difference in genetic aberration was observed between treatment-responsive MDs and -resistant MDs. Then, genome-wide DNA methylation analysis was conducted in Chapter 3 to find aberrations in DNA methylation in MDs diagnosed with non-neoplastic bone marrow disorders. Although it was indicated DNA methylation profiles might be different among healthy dogs, treatment-responsive MDs, and a treatment-resistant MD, the differences in DNA methylation levels of specific genes among these three groups were not found. Meanwhile, the significant downregulation of *SFRP2* gene expression in treatment-resistant MDs compared with treatment-responsive MDs and healthy dogs was found, and it was implied that gene expression profiles (GEPs) might be different between treatment-responsive MDs and -resistant MDs.

Based on these backgrounds, I set the purpose of the study in this chapter to investigate the changes in GEPs of bone marrow cells derived from MDs diagnosed with non-neoplastic bone marrow disorders.

## Materials and Methods

### *Samples*

RNA samples derived from bone marrow specimens were collected from 11 MDs diagnosed with non-neoplastic bone marrow disorders (Table 4-1). All MDs were referred to the Veterinary Medical Center of the University of Tokyo (VMC-UT) between April 2013 and March 2020. Bone marrow disorders were diagnosed when non-regenerative anemia was present without any other apparent causes than bone marrow disorders that could induce anemia based on the results of the blood examinations, diagnostic images, and bone marrow examinations. Dogs diagnosed with any neoplastic diseases were excluded. MDs diagnosed with non-neoplastic bone marrow disorders were evaluated for their responsiveness to immunosuppressive treatments, and the treatment responsiveness was defined as the absence of blood transfusion for more than 2 months after the last blood transfusion according to the response criteria in human medicine (Cheson et al., 2006). Based on the criteria, 5 MDs were regarded as treatment-responsive and 6 MDs were regarded as treatment-resistant. As normal controls, 4 healthy Beagles and 1 healthy MD were included in this study and bone marrow aspirations were conducted also for these dogs. This animal experiment was approved by the Animal Care Committee of The University of Tokyo (approval no. P19-119). All bone marrow specimens were immediately frozen at -80°C until RNA extraction.

### *RNA extraction and reverse transcription*

Total RNA was extracted from bone marrow specimens using RNeasy Mini Kit

(Qiagen, Hilden, Germany). The RNA quantity and quality were assessed using Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) based on RNA integrity number (RIN). RNAs with RIN value of  $> 7.5$  were used for RNA-sequencing analysis.

#### *RNA-sequencing analysis (RNA-seq)*

The libraries for RNA-seq were constructed with 500 ng of total RNA using NEB-Next Poly(A) mRNA Magnetic Isolation Module (New England Biolabs, Beverly, MA, USA) and NEBNext Ultra Directional RNA Library Prep Kit for Illumina (New England Biolabs). The size distributions and concentrations of libraries were determined using Bioanalyzer (Agilent Technologies). Sequencing was performed using NextSeq500 (Illumina, San Diego, CA, USA) with a paired-end 36-base read method. Sequencing reads in FASTQ format were imported to CLC Genomics Workbench (CLC-GW, ver. 10.1.1, Qiagen) and mapped on the canine reference genome (Canfam 3.1). The number of reads were quantified for 32,220 genes in ENSEMBLE annotation provided in CLC-GW, and total count values were obtained. Total count values were normalized and compared among 3 healthy control dogs and 3 treatment-resistant MDs using “Empirical analysis of DGE”, which was an algorithm to compare GEPs between two groups in CLC-GW. Fold-changes (FC) for each gene was calculated by dividing mean normalized total count values that are derived internally in the Exact Test algorithm in treatment-resistant MDs by those in healthy dogs, and differentially expressed genes (DEGs) were defined by thresholds of  $|FC| > 2$  and false discovery rate (FDR)  $< 0.05$ . Gene ontology (GO) and pathway analyses using extracted DEGs were carried out using Database for Annotation,



Visualization, and Integrated Discovery (DAVID, v6.8).

#### *RT-qPCR*

The synthesis of cDNA from extracted total RNA was conducted using ReverTra Ace (TOYOBO, Osaka, Japan) following the manufacture's instruction. RT-qPCR for relative quantification of mRNA was performed using a Thermal Cycler Dice Real Time System TP800 (Takara Bio, Shiga, Japan). Twenty-five microliters of the PCR mixture containing 12.5  $\mu$ l of Master Mix (TB Green Premix Ex Taq II (Takara Bio)), 400 nM of forward and reverse primers, and 10 ng of cDNA. The cDNA samples were subjected to activation at 95°C for 10 sec, then 40 cycles of denaturation at 95°C for 5 sec, and annealing/extension at 62°C for 30 sec. All reactions were run in duplicate. Sequences of primers and amplification efficacies were shown in Table 4-2. As the internal control gene, *Hypoxanthine phosphoribosyl-transferase 1 (HPRT1)*, which was validated as the most stably expressed gene in canine bone marrow (Peters et al., 2015), was used. The relative quantity of each gene was calculated by plotting  $C_T$  in the prepared standard curve. To normalize the amount of cDNA sample, the ratio of the relative quantity of cDNA for each target gene to that of *HPRT1* gene was adopted. Relative expression level of each gene in a healthy control dog was used for normalization of that in other dogs.

#### *Statistical analysis*

The Steel-Dwass test was used to compare mRNA expression levels among healthy control dogs, treatment-responsive MDs, and treatment-resistant MDs. Statistical

analyses were performed using the statistical program R 3.5.0 (<http://cran.r-project.org>).

## Results

### *DEGs extracted from comparison between healthy dogs and treatment-resistant MDs*

RNA-seq analysis generated at least 26 million raw reads and > 77% of mapping rates with paired reads for each sample (Table 4-3). Based on the threshold as described above ( $|FC| > 2$  and  $FDR < 0.05$ ), 179 DEGs were extracted from comparison between healthy dogs and treatment-resistant MDs (Table 4-4). Among these genes, 75 genes were upregulated, and 104 genes were downregulated in treatment-resistant MDs compared with healthy dogs. Hierarchical clustering analysis using these DEGs generated separated clusters for the two groups (Fig 4-1).

### *Enrichment analysis of GO terms and KEGG pathway using DEGs*

In enrichment analysis using extracted 179 DEGs, 5 GO biological process terms, 2 GO cellular component terms, and 3 GO molecular function terms were shown to be significantly enriched with DEGs (Table 4-5). Among these terms, cell matrix adhesion, extracellular space, and integrin binding was the most significantly enriched with DEGs among GO biological processes, GO cellular components, and GO molecular functions, respectively. In addition, pathway analysis using DAVID showed 4 significantly pathways enriched with DEGs and Wnt signaling pathway was included in the extracted pathways (Table 4-6).

Based on this finding, I focused on the genes associated with Wnt signaling pathway,

and it was found that *Wnt family member 16 (WNT16)*, *axin 2 (AXIN2)*, *cyclin D2 (CCND2)*, *frizzled class receptor 2 (FZD2)*, and *SFRP2* were included the DEGs extracted from RNA-seq. Among the 5 genes, *SFRP2* was the most dynamically (FC; -83.03) down-regulated in treatment-resistant MDs compared with healthy dogs.

#### *RT-qPCR analysis for genes associated with Wnt signaling pathway*

As genes associated with Wnt signaling pathway, *WNT16*, *AXIN2*, *CCND2*, *FZD2*, and *SFRP2* genes were selected and the expression levels were compared among 5 healthy dogs, 5 treatment-responsive MDs, and 5 treatment-resistant MDs by RT-qPCR. As a result, the expressions of *CCND2* gene were significantly up-regulated in treatment-resistant MDs compared with healthy dogs and treatment-responsive MDs (Fig 4-2a). The expressions of *SFRP2* gene were significantly down-regulated in treatment-resistant MDs compared with healthy dogs and treatment-responsive MDs (Fig 4-2b). The expression levels of *AXIN2* were significantly down-regulated in treatment-resistant MDs compared with treatment-responsive MDs (Fig 4-2c). No significant difference was observed in the expression levels of *WNT16* and *FZD2* among the 3 groups (Fig 4-2d and 4-2e).

## **Discussion**

In the present study, I investigated changes in GEPs of bone marrow specimens derived from treatment-resistant MDs compared with healthy control dogs. Pathway analysis using extracted DEGs revealed that Wnt signaling were significantly enriched with DEGs, and it was suggested that canonical Wnt signaling was activated in treatment-

resistant MDs compared with healthy dogs and treatment-responsive MDs.

In enrichment analysis using DEGs extracted from RNA-seq, GO terms related to extracellular matrix (ECM) such as Cell matrix adhesion, Extracellular space, and Extracellular matrix binding, were extracted as the terms that were enriched with DEGs. ECM plays essential roles in normal hematopoiesis, and dysregulation of ECM was reported to result in myelofibrosis (Hynes, 2009; Leiva et al., 2018). In Chapter 1, mild to severe myelofibrosis was found in treatment-resistant MDs, and the dysregulation of ECM might be associated with the myelofibrosis in treatment-resistant MDs.

Since pathway analysis using extracted DEGs indicated that Wnt signaling pathway was enriched with DEGs, I decided to focus on the expression levels of gene associated with this pathway. Among the genes related to this pathway, *WNT16*, *AXIN2*, *CCND2*, *FZD2*, and *SFRP2* genes were included as DEGs in RNA-seq, and downregulation of *AXIN2* and *SFRP2* expressions and upregulation of *CCND2* expression in treatment-resistant MDs were shown when compared with healthy dogs and treatment-responsive MDs by RT-qPCR. These genes are associated with canonical Wnt signaling, which is regulated by a transcriptional co-activator  $\beta$ -catenin (Clevers, 2006). These results indicated that treatment-resistant MDs harbored different molecular abnormalities from those in treatment-responsive MDs.

SFRPs are a major family protein of Wnt antagonist that inhibit the interaction of Wnt with Frizzled protein (Fz), and SFRP1 and SFRP2 were reported to inhibit interaction of Wnt3a with Fz (Bovolenta et al., 2008; Wawrzak et al., 2007) (Fig. 4-3). In the absence of Wnt interacting with Fz, cytoplasmic  $\beta$ -catenin is continuously degraded by the Axin

complex, which consists of Axin, adenomatous polyposis coli gene product (APC), casein kinase 1 (CK1), and glycogen synthase kinase 3 (GSK3) (MacDonald et al., 2009). When Wnt proteins bind to Fz receptor and its co-receptor, low-density lipoprotein receptor related protein (LRP), Axin complex are inactivated by Fz. Then,  $\beta$ -catenin accumulates in cytoplasm, moves into the nucleus to form a complex with T cell factor/lymphoid enhancer factor (TCF/ LEF) family proteins, and activates expressions of Wnt-target genes such as *CCND1* and *CCND2* (Baek et al., 2003; MacDonald et al., 2009). Therefore, the changes in the expression levels of *AXIN2*, *SFRP2*, and *CCND2* in treatment-resistant MDs indicated the activation of canonical Wnt signaling followed by repression of  $\beta$ -catenin degradation in these cases.

Activation of canonical Wnt-signaling was shown in human myelodysplastic syndrome (MDS) and acute myeloid leukemia (AML) (Valencia et al., 2009; Wang et al., 2013; Xu et al., 2008). DNA hypermethyations in *SFRPs* genes were frequently observed in human MDS (Wang et al., 2013), and existence of non-phosphorylated  $\beta$ -catenin in nucleus was detected and it was associated with unfavorable outcomes in human MDS and AML (Xu et al., 2008). Activation of canonical Wnt signaling pathway leads to induction of self-renewal and inhibition of differentiation in hematopoietic stem cells (HSCs) (Reya et al., 2003). In addition, it was reported that inhibition of Wnt signaling pathway inhibited the development of MDS in a mouse model (Stoddart et al., 2017). When considering these backgrounds, it was possible that treatment-resistant MDs, where the activation of canonical Wnt signaling in bone marrow was suggested, harbored a similar pathophysiology to that of human MDS.

In conclusion, I found that GEPs of bone marrow specimens were different between treatment-resistant MDs and healthy dogs, and it was suggested that canonical Wnt signaling was activated in bone marrow of treatment-resistant MDs when compared with healthy dogs and treatment-responsive MDs. The results of this study indicated that treatment-resistant MDs harbored different molecular abnormalities from those in treatment-responsive MDs. The activation of Wnt signaling might be a novel target to establish effective treatments for MDs diagnosed with non-neoplastic bone marrow disorders that are resistant to immunosuppressive treatments.

Table 4-1. Signalment of dogs whose bone marrow specimens were used in Chapter 4.

Number	Breed	Age	Sex	Treatment responsiveness	RNA-seq	RT-qPCR
Dog1	Miniature Dachshund	13.4	NF	resistant	○	○
Dog2	Miniature Dachshund	12.2	F	resistant	○	○
Dog3	Miniature Dachshund	12.6	F	resistant	○	-
Dog4	Miniature Dachshund	12	NF	healthy	○	○
Dog5	Beagle	6.4	CM	healthy	○	○
Dog6	Beagle	6.3	CM	healthy	○	○
Dog7	Miniature Dachshund	11.5	F	resistant	-	○
Dog8	Miniature Dachshund	12.8	F	resistant	-	○
Dog9	Miniature Dachshund	9.8	NF	resistant	-	○
Dog10	Miniature Dachshund	14.5	CM	responsive	-	○
Dog11	Miniature Dachshund	15.2	NF	responsive	-	○
Dog12	Miniature Dachshund	8.1	F	responsive	-	○
Dog13	Miniature Dachshund	7.8	CM	responsive	-	○
Dog14	Miniature Dachshund	11.2	NF	responsive	-	○
Dog15	Beagle	6.5	CM	healthy	-	○
Dog16	Beagle	6.2	CM	healthy	-	○

Table 4-2 Primer sequences used for RT-qPCR.

Gene name	Forward	Reverse	Product size (bp)	Ensemble gene number	Amplification efficacy (%)
<i>SFRP2</i>	CAACGACCTCTGCATACCCC	GCAGGCTTCGCATACCTTTG	82	ENSCAFG00000008353	101.5
<i>WNT16</i>	ACAACAACGAAGCTGGAAGG	ACATGGTTTTCCAGCAGGTC	108	ENSCAFG00000003496	108.4
<i>AXIN2</i>	GACAGCAGCGTAGATGGAATC	CACACTGCGATGCATTTCTC	78	ENSCAFG00000011236	105.5
<i>CCND2</i>	GATGCTGGAGGTCTGTGAGG	AGCTGCAGATGGGTCTTAGG	114	ENSCAFG00000015381	102.3
<i>FZD2</i>	CCACCTTCTTCACCGTCAC	CATAGTGTAGCAGCCGGACAG	95	ENSCAFG00000014134	106.3
<i>HPRT1</i>	CACTGGGAAAACAATGCAGA	ACAAAGTCAGGTTTATAGCCAACA	123	ENSCAFG00000018870	96.4

Table 4-3. Number of total reads and mapping rates obtained in each case in RNA-sequencing.

Case	Total read (count)	Mapping rate with paired reads (%)
Dog1	39,576,718	81.81
Dog2	33,423,140	79.69
Dog3	37,312,254	82.34
Dog4	36,396,748	86.74
Dog5	26,115,992	77.26
Dog6	33,843,632	78.87



Table 4-4. Differentially expressed genes extracted from comparison of gene expression profiles between treatment-resistant Miniature Dachshunds (MDs) and healthy dogs.

Gene	Healthy dogs (mean)	treatment-resistant		Fold change	False discovery rate
		MDs (mean)	P-value		
<i>ABHD15</i>	3.1666667	56.3888889	0.0000307	11.7496994	0.0113683
<i>ACPP</i>	135.6666667	23.6111111	0.0000292	-7.2591841	0.0112058
<i>ADGRE5</i>	695.9444444	6191.1111111	0.0000161	11.3426675	0.0075097
<i>ADGRG6</i>	95.6111111	16.5000000	0.0000399	-6.2951722	0.0132546
<i>ALDH3A1</i>	49.5555556	0.0000000	0.0000005	-428.6126319	0.0004630
<i>ALPL</i>	354.8333333	35.8888889	0.0000093	-10.0316605	0.0050182
<i>ANKRD6</i>	35.3888889	3.6666667	0.0000090	-10.3222275	0.0050182
<i>APCDD1</i>	362.6666667	58.8888889	0.0001388	-7.2063376	0.0323203
<i>APCDD1L</i>	10.3888889	0.1666667	0.0000642	-26.8106355	0.0193269
<i>ASPA</i>	24.9444444	1.5000000	0.0000837	-14.9785388	0.0232598
<i>AVPRIA</i>	9.0000000	0.0000000	0.0000302	-86.8878911	0.0113285
<i>AXIN2</i>	282.7777778	41.6666667	0.0000030	-7.8157991	0.0023026
<i>B4GALNT1</i>	16.7222222	1.6111111	0.0001684	-10.1052188	0.0359407
<i>BTBD11</i>	24.0555556	281.9444444	0.0000156	11.4800091	0.0073898
<i>C1orf186</i>	23.1111111	0.5000000	0.0000002	-32.0659495	0.0002691
<i>C1QTNF6</i>	15.1666667	426.7222222	0.0000077	24.8579656	0.0045740
<i>CA8</i>	6.6111111	0.0000000	0.0001760	-62.5605207	0.0362683
<i>CAMK1</i>	182.6111111	44.2777778	0.0002505	-4.8359127	0.0472959
<i>CCDC180</i>	14.2777778	0.0000000	0.0001240	-127.3087824	0.0300438
<i>CCND2</i>	72.8333333	535.6666667	0.0000456	6.9538659	0.0146969
<i>CD1E</i>	21.0555556	1.5555556	0.0000790	-14.1529384	0.0225344
<i>CD34</i>	55.1111111	5.2222222	0.0000065	-10.7188695	0.0040689
<i>CD72</i>	64.7777778	7.8333333	0.0000893	-9.5860001	0.0241836
<i>CD83</i>	99.7222222	12.5000000	0.0000026	-8.9112502	0.0020570
<i>CDKN1A</i>	209.5000000	1163.6666667	0.0002619	6.7583434	0.0479514
<i>CLDN1</i>	46.7777778	8.9444444	0.0002355	-5.8763407	0.0457063
<i>CLEC4D</i>	48.2777778	455.1111111	0.0000009	9.5033915	0.0008296
<i>CMA1</i>	431.8888889	34.2777778	0.0000000	-13.7112029	0.0000015
<i>CMBL</i>	163.3333333	20.2222222	0.0001394	-7.9668956	0.0323203
<i>CNTNAP5</i>	50.7222222	619.9444444	0.0000066	13.4376148	0.0040689
<i>COL13A1</i>	1.5555556	235.6111111	0.0000000	111.9027624	0.0000001
<i>CORO2A</i>	791.8888889	101.6666667	0.0000178	-7.4127515	0.0081857
<i>CPA3</i>	140.7222222	1492.7222222	0.0000957	13.2516537	0.0254732
<i>CREG2</i>	0.0000000	38.1111111	0.0000724	246.7205937	0.0211854
<i>CSNK1G3</i>	154.6666667	703.8333333	0.0002371	5.2715492	0.0457533
<i>CYB561</i>	113.1111111	586.1111111	0.0002518	5.5554155	0.0472959
<i>CYP4F3</i>	1215.8888889	8600.3333333	0.0001084	10.9635162	0.0281775
<i>DCN</i>	219.1666667	20.1666667	0.0001067	-10.6197842	0.0279410
<i>DEPDC7</i>	95.7777778	13.1111111	0.0000080	-8.2720766	0.0045741
<i>DHX34</i>	272.1111111	2448.1666667	0.0000323	11.9142940	0.0116813
<i>DIRAS2</i>	6.3888889	0.0000000	0.0001727	-61.7505090	0.0362683
<i>DNTT</i>	233.8333333	7.0555556	0.0000000	-32.0088178	0.0000012
<i>DUSP27</i>	37.7222222	0.8333333	0.0000000	-49.6420810	0.0000269
<i>DUSP9</i>	0.1666667	35.0555556	0.0002596	73.6900898	0.0479514
<i>ENSCAFG00000002230</i>	27.7222222	2.0555556	0.0000255	-13.2426203	0.0101306
<i>ENSCAFG00000003564</i>	206.1111111	0.8333333	0.0000382	-177.2033314	0.0129526
<i>ENSCAFG00000006557</i>	11.3333333	0.3333333	0.0001006	-27.2715480	0.0265672
<i>ENSCAFG00000007004</i>	75.6666667	6.6111111	0.0000079	-14.2845421	0.0045741

ENSCAFG00000008154	77.8333333	9.7777778	0.0001717	-9.1529292	0.0362683
ENSCAFG00000011656	3.1666667	66.4444444	0.0000220	13.8593575	0.0091972
ENSCAFG00000013622	338.8333333	67.8333333	0.0002669	-5.8487344	0.0483178
ENSCAFG00000014139	168.1666667	24.5000000	0.0001570	-7.5950572	0.0344040
ENSCAFG00000014627	256.6111111	1.3888889	0.0001289	-137.8197124	0.0310010
ENSCAFG00000014684	231.0555556	1233.3333333	0.0001682	6.6297693	0.0359407
ENSCAFG00000015584	295.1111111	53.1666667	0.0002103	-5.5345074	0.0418203
ENSCAFG00000015931	42.0555556	441.3333333	0.0002276	10.1493620	0.0447014
ENSCAFG00000015959	2.2222222	27.7222222	0.0002510	7.9995768	0.0472959
ENSCAFG00000016251	78.6666667	5.2222222	0.0000004	-18.4857089	0.0003536
ENSCAFG00000016865	129.7777778	30.8888889	0.0001239	-5.1273792	0.0300438
ENSCAFG00000018187	47.0555556	0.8333333	0.0001195	-49.4776118	0.0295782
ENSCAFG00000023349	106.3888889	7.6111111	0.0000007	-14.5083426	0.0006323
ENSCAFG00000023356	86.1111111	3.2777778	0.0000000	-29.6341590	0.0000465
ENSCAFG00000023772	12.3888889	174.2222222	0.0000327	11.9017368	0.0116981
ENSCAFG00000024748	571.4444444	3043.0000000	0.0000146	7.6885114	0.0073051
ENSCAFG00000025016	0.7777778	91.7777778	0.0000253	71.0270755	0.0101306
ENSCAFG00000028482	20.6666667	2.2777778	0.0001840	-10.0487212	0.0370465
ENSCAFG00000028502	0.2222222	34.3888889	0.0000656	62.7597067	0.0195791
ENSCAFG00000028936	6.0555556	108.5555556	0.0000199	14.4744301	0.0087717
ENSCAFG00000029542	18.1666667	1.1666667	0.0000197	-14.2004707	0.0087717
ENSCAFG00000030089	16.0555556	1615.8333333	0.0000000	119.7602138	0.0000269
ENSCAFG00000030728	0.2222222	12.8888889	0.0000771	26.6804181	0.0221921
ENSCAFG00000030958	21.6666667	413.4444444	0.0000627	17.3104413	0.0190491
ENSCAFG00000031225	32.6111111	5.8333333	0.0001745	-6.7854750	0.0362683
ENSCAFG00000031333	13801.8333333	79.1111111	0.0000000	-175.4388380	0.0000000
ENSCAFG00000031543	109.5000000	726.6111111	0.0001880	7.6848766	0.0376333
ENSCAFG00000031828	9.5000000	0.0000000	0.0000397	-86.0779802	0.0132546
ENSCAFG00000032328	1354.2777778	204.8888889	0.0000884	-6.2645254	0.0241389
ENSCAFG00000033209	1.0000000	50.5555556	0.0001106	31.5171295	0.0285200
ENSCAFG00000034249	0.9444444	28.6666667	0.0000337	18.5846261	0.0117269
ENSCAFG00000034495	1.0555556	39.9444444	0.0000223	24.8720769	0.0092062
ENSCAFG00000035620	0.2777778	13.5555556	0.0001760	25.5337564	0.0362683
ENSCAFG00000035929	0.0000000	40.8333333	0.0000034	268.8638786	0.0024838
ENSCAFG00000036159	1023.6666667	107.0000000	0.0000070	-9.2725170	0.0042731
ENSCAFG00000036275	44.8333333	416.5000000	0.0001203	8.6860555	0.0295782
ENSCAFG00000036642	5.6666667	79.2777778	0.0001349	10.2422337	0.0321900
ENSCAFG00000037677	3973.3333333	25.4444444	0.0000000	-139.9023020	0.0000000
ENSCAFG00000038722	459.5555556	94.4444444	0.0001127	-5.2392362	0.0285849
ENSCAFG00000039524	3.5555556	67.9444444	0.0001428	13.8970285	0.0326383
ENSCAFG00000039606	0.0000000	58.3333333	0.0000046	399.6412600	0.0031742
EPHB6	145.7777778	1701.7777778	0.0001385	10.5508626	0.0323203
FABP2	75.4444444	5.8888889	0.0000205	-13.5612224	0.0089092
FAM213B	71.2777778	8.2777778	0.0000316	-9.8775549	0.0115665
FBN3	22.2222222	2.6111111	0.0002525	-9.8312913	0.0472959
FZD2	13.3888889	0.8333333	0.0000338	-18.6332920	0.0117269
GALNTL6	20.8333333	0.0000000	0.0000002	-184.1019397	0.0002545
GAS2L1	183.7222222	2242.4444444	0.0000150	17.0853807	0.0073051
GCNT1	415.8888889	104.3333333	0.0002549	-4.1960989	0.0474700
GDPD2	106.1111111	3.2777778	0.0000000	-34.1383483	0.0000010
GPNMB	9514.6666667	114.7222222	0.0000000	-80.7357898	0.0000269
HEPH	3.1666667	268.3333333	0.0000000	70.7703722	0.0000575
HHATL	7.0555556	0.0000000	0.0001635	-64.5621498	0.0353597
HIP1	312.6666667	34.3888889	0.0000001	-10.7539207	0.0000989
HK2	1191.0555556	206.5555556	0.0000289	-5.3157245	0.0112058
HRH4	23.6666667	0.0000000	0.0000000	-207.3968547	0.0000509
HSD17B14	5.6666667	94.9444444	0.0001790	12.6989084	0.0362683
HSPB6	14.6666667	590.2222222	0.0000001	38.1869129	0.0001576
IER5L	23.2777778	340.9444444	0.0000230	13.9561995	0.0093828
IGF2BP2	121.8333333	836.1111111	0.0000440	6.8675167	0.0143214

<i>IGKV2D-26</i>	720.2222222	117.5555556	0.0000299	-6.2231790	0.0113285
<i>ILIR2</i>	323.7222222	4503.1111111	0.0000016	15.5347693	0.0013441
<i>INHBA</i>	271.7222222	18.6666667	0.0000001	-16.7413547	0.0001226
<i>ITGA2B</i>	2147.3888889	19941.5555556	0.0000213	16.6689331	0.0091352
<i>JCHAIN</i>	35430.8888889	6282.0000000	0.0000336	-6.6061070	0.0117269
<i>KIAA1324</i>	61.6666667	9.5000000	0.0000616	-8.2482350	0.0189133
<i>KIF21A</i>	10.0000000	165.0555556	0.0000000	13.9733932	0.0000283
<i>KLHL32</i>	26.3333333	0.9444444	0.0000024	-26.8585266	0.0019688
<i>L3MBTL3</i>	110.6666667	497.8333333	0.0002660	4.8403951	0.0483178
<i>MARCH2</i>	189.6111111	1958.7222222	0.0000292	13.9019512	0.0112058
<i>MEFV</i>	177.1111111	2076.8333333	0.0000012	15.2410224	0.0010387
<i>MET</i>	219.2222222	41.3888889	0.0000148	-6.4289571	0.0073051
<i>MLXIPL</i>	8.8333333	0.0000000	0.0000361	-78.8821163	0.0123643
<i>MMP1</i>	171.4444444	1.0555556	0.0000000	-200.1674554	0.0000000
<i>MMP12</i>	50.9444444	0.0000000	0.0000156	-433.5011688	0.0073898
<i>MMP8</i>	28205.9444444	995.6666667	0.0000000	-25.0005849	0.0000137
<i>MYLK3</i>	6.1111111	91.4444444	0.0000851	11.7432467	0.0234386
<i>MYO1A</i>	123.8333333	12.9444444	0.0001515	-10.5530086	0.0339020
<i>NOV</i>	0.0000000	30.0555556	0.0002619	215.6314300	0.0479514
<i>NPNT</i>	12.1111111	0.9444444	0.0001607	-12.4045133	0.0349881
<i>P2RY2</i>	40.4444444	6.1666667	0.0000218	-7.9587411	0.0091972
<i>PCBP4</i>	74.2222222	423.6111111	0.0001782	5.8529280	0.0362683
<i>PCDC1LG2</i>	59.2222222	8.1666667	0.0000183	-8.9604137	0.0083200
<i>PDGFD</i>	17.2222222	749.5555556	0.0000003	44.2225297	0.0003477
<i>PDLIM1</i>	563.0000000	3381.7222222	0.0000517	9.3157742	0.0163424
<i>PKHD1L1</i>	273.2222222	4.7222222	0.0000000	-59.3768800	0.0000002
<i>PLPP1</i>	105.9444444	17.0555556	0.0000117	-7.3668229	0.0059628
<i>PLPP4</i>	0.6666667	21.2777778	0.0002231	27.9197934	0.0441031
<i>PLXDC1</i>	108.1111111	2.0000000	0.0000031	-60.1121764	0.0023192
<i>PLXNB3</i>	93.7777778	775.9444444	0.0001535	8.7262904	0.0341093
<i>PON3</i>	83.1111111	17.5555556	0.0000534	-5.9373399	0.0167046
<i>PRG3</i>	10.9444444	0.0000000	0.0000113	-97.5491887	0.0058623
<i>PTX3</i>	148.7222222	8.8333333	0.0000000	-18.8666047	0.0000067
<i>RAD9B</i>	18.1111111	0.5555556	0.0001184	-40.7566945	0.0295667
<i>RAG1</i>	29.8888889	0.3888889	0.0000001	-71.2436475	0.0001333
<i>RAVER2</i>	31.5000000	5.6111111	0.0002713	-7.0258522	0.0488273
<i>RD3L</i>	6.6666667	0.0000000	0.0002289	-59.3603859	0.0447014
<i>RXFP1</i>	32.8888889	2.5555556	0.0000042	-14.0610360	0.0030313
<i>SIPRI</i>	42.1111111	409.8333333	0.0000725	10.1554798	0.0211854
<i>SCAMP5</i>	49.4444444	9.7777778	0.0001502	-6.0957605	0.0338439
<i>SERINC2</i>	38.8888889	4.2777778	0.0000606	-12.6204446	0.0187615
<i>SERPINB10</i>	3224.8333333	356.1111111	0.0000022	-7.4400047	0.0017970
<i>SFRP2</i>	59.1111111	0.6111111	0.0000000	-83.0325738	0.0000269
<i>SHE</i>	150.0555556	20.7777778	0.0000093	-7.8758462	0.0050182
<i>SIN3B</i>	1081.1111111	6747.2777778	0.0002399	8.4216829	0.0460004
<i>SLC16A14</i>	66.7777778	8.0555556	0.0000050	-11.1060106	0.0032343
<i>SLC22A16</i>	152.1666667	1.2222222	0.0000000	-134.7448754	0.0000000
<i>SLC30A8</i>	1.1666667	88.1111111	0.0000045	66.1307901	0.0031559
<i>SLC35D3</i>	23.2777778	0.9444444	0.0000050	-24.3732148	0.0032343
<i>SLC38A4</i>	4.0555556	101.0000000	0.0000803	21.4309604	0.0226956
<i>SORL1</i>	2628.1111111	24422.7777778	0.0000834	9.0482014	0.0232598
<i>SPP1</i>	900.4444444	59.3888889	0.0000002	-15.2660924	0.0002518
<i>STXBP5L</i>	12.9444444	0.9444444	0.0000730	-13.3038735	0.0211854
<i>SYT1</i>	0.7222222	34.6666667	0.0000048	43.5537370	0.0031997
<i>TAGLN</i>	21.8888889	1190.5555556	0.0000092	63.2432101	0.0050182
<i>TBC1D16</i>	92.5555556	14.5000000	0.0001125	-7.5756612	0.0285849
<i>TBC1D30</i>	55.0000000	641.6111111	0.0000411	13.0146590	0.0134992
<i>TENM2</i>	11.2222222	280.4444444	0.0000003	21.6692833	0.0003332
<i>TGM5</i>	35.7222222	2.2222222	0.0000096	-22.3996078	0.0050640
<i>TLR8</i>	195.8888889	1097.5000000	0.0001156	7.1921958	0.0290936

<i>TREM2</i>	24.6111111	338.9444444	0.0000000	12.2935064	0.0000640
<i>TRHDE</i>	3.2777778	35.8333333	0.0000945	8.5541966	0.0253854
<i>TRPM1</i>	0.0000000	30.0555556	0.0001476	193.9529686	0.0335009
<i>TSPOAP1</i>	219.5555556	52.1666667	0.0001414	-5.0094675	0.0325332
<i>TTN</i>	0.8333333	37.8333333	0.0000016	30.2269156	0.0013441
<i>USP2</i>	11.2777778	141.5555556	0.0000470	10.5037748	0.0149835
<i>USP35</i>	679.0000000	104.3333333	0.0001558	-6.3444617	0.0343785
<i>VPREB1</i>	39.3333333	0.5000000	0.0000000	-337.7858180	0.0000018
<i>WDR87</i>	1.9444444	34.3888889	0.0001783	14.8025857	0.0362683
<i>WNT16</i>	19.2777778	2.3333333	0.0001392	-13.0274718	0.0323203
<i>ZNF385B</i>	4.2777778	359.6111111	0.0000000	81.4731651	0.0000000

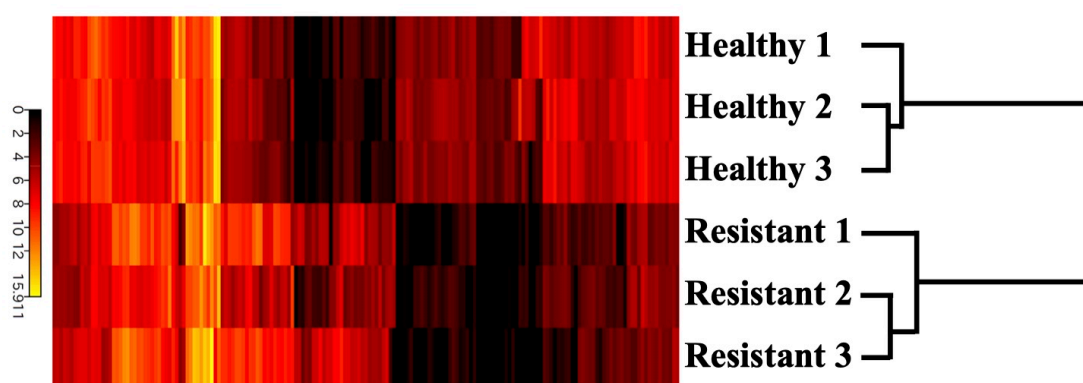
Table 4-5. Gene ontology terms extracted by gene enrichment analysis using differentially expressed genes.

Group	Term	<i>P</i> - Value	Fold Enrichment
Biological process	Cell matrix adhesion	0.0157	7.520
Biological process	Negative regulation of cell growth	0.0363	5.452
Biological process	Cell adhesion	0.0396	3.867
Biological process	Signal transduction	0.0414	2.478
Biological process	Response to vitamin D	0.0446	43.614
Cellular component	Extracellular space	8.778E-04	2.462
Cellular component	Extracellular matrix	1.816E-03	6.817
Molecular function	Integrin binding	0.0115	18.097
Molecular function	Extracellular matrix binding	0.0184	14.163
Molecular function	Immunoglobulin receptor binding	0.0271	72.386

Table 4-6. Pathways extracted by gene enrichment analysis using differentially expressed genes.

Term	<i>P</i> - Value	Fold Enrichment
Hematopoietic cell lineage	8.32E-04	7.937
Wnt signaling pathway	0.031813234	4.089
Signaling pathways regulating pluripotency of stem cells	0.036625693	3.910
Focal adhesion	0.040770695	3.106

**Fig 4-1.**



Results of clustering analysis and heatmap in the comparisons of gene expression profiles between healthy dogs (1 Miniature Dachshund (MD, Healthy1) and 2 Beagles (Healthy 2 and 3) and treatment-resistant Miniature Dachshunds (MDs). Hierarchical clustering analysis showed separated clusters for healthy dogs and treatment-resistant MDs.

Relative expression levels

Healthy Treatment-responsive Treatment-resistant

300  
200  
100  
50  
10  
0

\*

\*

Group	Relative expression levels (approximate values)
Healthy	8, 9, 10, 11
Treatment-responsive	10, 11, 12, 13, 14
Treatment-resistant	12, 35, 40, 135, 285

Scatter plot showing Log relative expression levels for Healthy, Treatment-responsive, and Treatment-resistant groups. The y-axis is logarithmic, ranging from 0.1 to 10. The x-axis shows three groups. Individual data points are shown as open circles. Horizontal lines with asterisks indicate significant differences between the Treatment-responsive group and both the Healthy and Treatment-resistant groups.

Group	Log relative expression levels (approximate values)
Healthy	0.8, 0.9, 1.0, 3.0
Treatment-responsive	2.5, 2.6, 4.0, 7.0, 10.0
Treatment-resistant	0.08, 0.1, 0.3, 0.8

Relative expression levels

Healthy Treatment-responsive Treatment-resistant

\*

Relative expression levels

Healthy Treatment-responsive Treatment-resistant

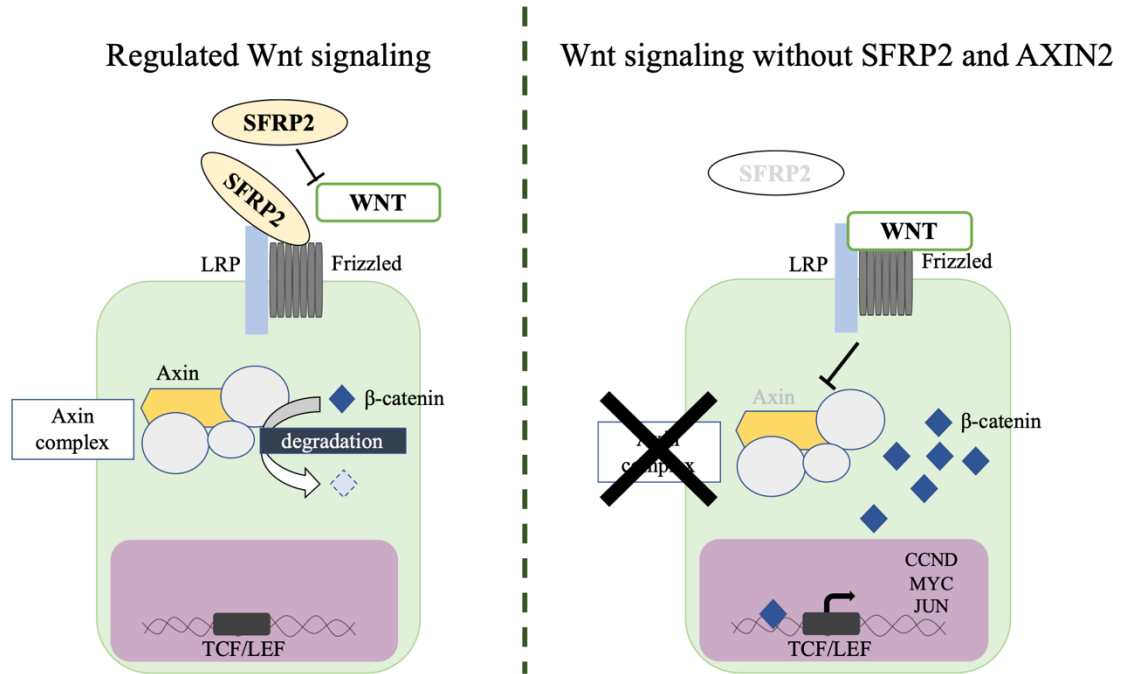
Relative expression levels

Group	Relative expression levels (approximate values)
Healthy	0.5, 1.0, 1.5, 2.0, 2.5
Treatment-responsive	0.5, 1.0, 10.0, 15.0, 68.0
Treatment-resistant	0.5, 1.0, 1.5, 2.0, 2.5

Comparisons of relative mRNA expression levels of *CCND2* (a), *SFRP2* (b), *AXIN2* (c), *WNT16* (d), and *FZD2* (e) among healthy dogs (n=5), treatment-responsive Miniature Dachshunds (MDs) (n=5), and treatment-resistant MDs (n=5). The expression of *SFRP2* gene was not detected in one treatment-resistant MD. \* indicates  $P < 0.05$



**Fig 4-3.**



A Schematic hypothesis for the activation of Wnt signaling pathways that might occur in bone marrows of treatment-resistant Miniature Dachshunds. In normal conditions, interaction of WNT with Frizzled is regulated due to its inhibition by SFRP2, and cytoplasmic  $\beta$ -catenin is continuously degraded by the Axin complex. If the expression of SFRP2 and AXIN2 are suppressed, the interaction of WNT with Frizzled will not be inhibited, and degradation of cytoplasmic  $\beta$ -catenin could be suppressed. Then, the amounts of  $\beta$ -catenin in cytoplasm will increase and they move into the nucleus, leading to the enhancement of the mRNA expressions of Wnt signaling-targeted genes, including *CCND2*.

## **Conclusion**

Bone marrow disorders are the main causes of non-regenerative anemia in dogs. In a retrospective study of canine bone marrow disorders, non-neoplastic bone marrow disorders were reported to account for more than 60% of all bone marrow disorders (Weiss, 2006). Non-neoplastic bone marrow disorders are categorized into aplastic anemia (AA), non-regenerative immune-mediated anemia (NRIMA), pure red cell aplasia (PRCA), and myelodysplastic syndrome (MDS) based on the observations in clinicopathological examinations (Brazzell & Weiss, 2006; Stokol et al., 2000; Weiss, 2002; Weiss & Smith, 2000, 2002). However, the pathophysiology of these canine non-neoplastic bone marrow disorders remains to be uncovered

In Chapter 1, I conducted a retrospective study on the clinical and clinicopathological features of canine non-neoplastic bone marrow disorders, and I revealed that Miniature Dachshund (MD) was a predisposed breed diagnosed with non-neoplastic bone marrow disorders with anemia. Since MD has been also reported as a predisposed breed for other diseases including inflammatory colorectal polyps and sterile panniculitis (Ohmi et al., 2012; Yamagishi et al., 2007), I hypothesized that genetic backgrounds to develop non-neoplastic bone marrow disorders might exist in MDs. In addition, it was also revealed that MD cases that were resistant to immunosuppressive treatments (treatment-resistant MDs) harbored different clinicopathological features from those of MD cases that responded immunosuppressive treatments (treatment-responsive MDs). I found that the clinicopathological features of treatment-resistant MDs were similar to *Additional sex combs like 1 (ASXL1)*-mutated MDS mouse model (Nagase et al., 2018).

Based on these findings, I performed genome-wide analysis of gene mutations in MDs

diagnosed with non-neoplastic bone marrow using whole-exome sequencing in Chapter 2. As a result, a missense germline mutation of *Uromodulin like 1 (UMODL1)* gene was detected in 10 of 25 MDs suffering from non-neoplastic bone marrow disorders but not in 18 MDs without hematological abnormalities. Therefore, this mutation of *UMODL1* might be related to the susceptibility to non-neoplastic bone marrow disorders in MDs. Meanwhile, this mutation was thought not to be a direct trigger of the disorders in MDs, and I could not find any differences in gene mutation status between treatment-responsive MDs and -resistant MDs.

In Chapter 3, I decided to perform genome-wide DNA methylation analysis in MDs diagnosed with non-neoplastic bone marrow, because the epigenetic aberration was suspected to be the underlying causes of the disorders especially in treatment-resistant MDs. Hierarchical clustering analysis according to genome-wide methylation levels indicated DNA methylation profiles might be different among healthy dogs, treatment-responsive MDs, and a treatment-resistant MD. I extracted total 28 differentially methylated genes (DMGs) in treatment-responsive MDs and a treatment-resistant MD from the comparisons of them with healthy dogs. Of these DMGs, 6 DMGs were specifically observed in a treatment-resistant MD, and the expression of *Secreted frizzled-related protein 2 (SFRP2)* gene was significantly down-regulated in treatment-resistant MDs. However, bisulfite pyrosequencing analysis using larger number of MD cases showed no significant difference in DNA methylation status of this gene among healthy dogs, treatment-resistant MDs, and treatment-responsive MDs. Although I could not show the associations of DNA methylation with the downregulation of this gene

expression, it was indicated that gene expression profiles might be different between treatment-responsive MDs and -resistant MDs.

Therefore, I investigated the changes in gene expression profiles (GEPs) of bone marrow specimens of MDs diagnosed with non-neoplastic bone marrow disorders in Chapter 4. RNA sequencing analysis was conducted using the samples obtained from 3 treatment-resistant MDs and 3 healthy dogs, and 179 differentially expressed genes (DEGs) were extracted by the comparison of GEPs between the two groups. Since pathway analysis using these DEGs showed that Wnt signaling pathway was significantly enriched with DEGs, I focused on the changes in expression levels of genes associated with this pathway. Accordingly, it was shown that expressions of *AXIN2* and *SFRP2* were significantly down-regulated and that of *CCND2* was significantly up-regulated in treatment-resistant MDs compared with treatment-responsive MDs and healthy dogs. These results indicated the activation of canonical Wnt signaling pathway in bone marrow of treatment-resistant MDs.

Activation of canonical Wnt-signaling was shown in human myelodysplastic syndrome (MDS) and acute myeloid leukemia (AML) (Valencia et al., 2009; Wang et al., 2013; Xu et al., 2008). DNA hypermethyations in *SFRPs* genes were frequently observed in human MDS (Wang et al., 2013), and existence of non-phosphorylated  $\beta$ -catenin in nucleus was detected and it was associated with unfavorable outcomes in human MDS and AML (Xu et al., 2008). Activation of canonical Wnt signaling pathway leads to induction of self-renewal and inhibition of differentiation in hematopoietic stem cells (HSCs) (Reya et al., 2003). In addition, it was reported that inhibition of Wnt signaling

pathway inhibited the development of MDS in a mouse model (Stoddart et al., 2017).

When considering these backgrounds, it was possible that treatment-resistant MDs, where the activation of canonical Wnt signaling in bone marrow was suggested, harbored a similar pathophysiology to that of human MDS.

In conclusion, the results of a series of the studies indicated that treatment-resistant MDs harbored different clinical features and distinct molecular abnormalities compared with those of treatment-responsive MDs. The activation of Wnt signaling might be a novel target to establish effective treatments for MDs diagnosed with non-neoplastic bone marrow disorders that are resistant to immunosuppressive treatments. I believe that the results obtained in this thesis advanced the research to elucidate the pathophysiology of canine non-neoplastic bone marrow disorders and will contribute to develop effective therapy for canine cases with refractory bone marrow disorders.

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