FULL PAPER
Internal Medicine

Genome-wide DNA methylation analysis in canine gastrointestinal lymphoma

Hiroshi OHTA[1], Jumpei YAMAZAKI[2]*, Jaroslav JELINEK[3], Teita ISHIZAKI[4,5],
Yumiko KAGAWA[5], Nozomu YOKOYAMA[2], Noriyuki NAGATA[1],
Noboru SASAKI[1] and Mitsuyoshi TAKIGUCHI[1]*

[1]Laboratory of Veterinary Internal Medicine, Graduate School of Veterinary Medicine, Hokkaido University, N18 W9, Kita-ku, Sapporo, Hokkaido 060-0818, Japan
[2]Veterinary Teaching Hospital, Graduate School of Veterinary Medicine, Hokkaido University, N18 W9, Kita-ku, Sapporo, Hokkaido 060-0818, Japan
[3]Coriell Institute for Medical Research, 403 Haddon Ave, Camden, NJ 08103, USA
[4]Laboratory of Comparative Pathology, Graduate School of Veterinary Medicine, Hokkaido University, N18 W9, Kita-ku, Sapporo, Hokkaido 060-0818, Japan
[5]North Lab, Hokkaido, Hondori 2-chome, Kita 8-35, Shiroishi-ku, Sapporo, Hokkaido 003-0027, Japan

ABSTRACT. DNA methylation is the covalent modification of methyl groups to DNA mostly at CpG dinucleotides and one of the most studied epigenetic mechanisms that leads to gene expression variability without affecting the DNA sequence. Genome-wide analysis of DNA methylation identified the signatures that could define subtypes of human lymphoma patients. The objective of this study was to conduct the genome-wide analysis of DNA methylation in dogs with gastrointestinal lymphoma (GIL). Genomic DNA was extracted from endoscopic biopsies from 10 dogs with GIL. We performed Digital Restriction Enzyme Assay of DNA Methylation (DREAM) for genome-wide DNA methylation analysis that could provide highly quantitative information on DNA methylation levels of CpG sites across the dog genome. We successfully obtained data of quantitative DNA methylation level for 148,601–162,364 CpG sites per GIL sample. Next, we analyzed 83,132 CpG sites to dissect the differences in DNA methylation between GIL and normal peripheral blood mononuclear cells (PBMCs). We found 383–3,054 CpG sites that were hypermethylated in GIL cases compared to PBMCs. Interestingly, 773 CpG sites including promoter regions of 61 genes were identified to be commonly hypermethylated in more than half of the cases, suggesting conserved DNA methylation patterns that are abnormal in GIL. This study revealed that there was a large number of hypermethylated sites that are common in most of canine GIL. These abnormal DNA methylation could be involved in tumorigenesis of the canine GIL.

KEY WORDS: DNA methylation, dog, epigenetics, gastrointestinal lymphoma

DNA methylation is the covalent modification of methyl groups to DNA mostly at CpG dinucleotides to convert cytosine to 5-methylcytosine (5mC), causing structural change in the interactions between DNA and protein (s). DNA methylation is catalyzed by DNA methyltransferases (DNMTs) in mammalian cells and CpG-rich regions called 'CpG islands' (CGIs) are found to be at approximately half of the human gene promoters. CGI methylation is associated with gene expression silencing of the involved promoter exemplified by the inactive X chromosome in women [13] and in imprinting [1, 5].

Recently, genome-wide studies of DNA methylation in human medicine have revealed that hundreds of genes are aberrantly methylated in a variety of diseases such as tumors [8, 23]. Although global changes in DNA methylation or hypermethylation at single locus were found in several studies in dogs [7, 21, 30, 36], these studies are not capable of clarifying changes in genomic location modified by DNA methylation. To identify specific regions with DNA methylation changes with certain types of conditions such as cancer and inflammatory diseases in dogs, we previously established digital restriction enzyme analysis of methylation for dogs (Canine DREAM), which is genome-wide analysis of DNA methylation in dogs based on next-generation sequencing of methylation-specific signatures created by sequential digestion of genomic DNA with *Sma*I and *Xma*I restriction enzymes [37], which could provide highly quantitative information on methylation levels in dogs, and showed more than one hundred thousand of CpG sites could be quantitatively analyzed in lymphoma cell lines from dogs.

Gastrointestinal lymphoma (GIL) is one of common intestinal tumors in dogs and shows poor clinical prognosis in spite of

multidrug chemotherapy, causing short median survival time [24]. However, the etiology of this disease is largely unknown except for the fact that there is T-cell phenotype dominance [14, 24, 28]. In human medicine, gastrointestinal lymphoma occurs mostly in stomach followed by small bowel and there are two most frequent subtypes, mucosa-associated lymphoid tissue (MALT) and diffuse large B cell lymphoma [2]. Furthermore, aberrant DNA methylation has been reported in gastric MALT lymphoma in human [19] suggesting that epigenetic abnormality could be one of the cause of this disease. Since most of the reports of DNA methylation studies in canine lymphoma comes from multicentric-type lymphoma [6, 30], the purpose of this study is to observe genome-wide DNA methylation change in canine gastrointestinal lymphoma.

MATERIALS AND METHODS

Patients

Dogs diagnosed with GIL that presented to Hokkaido University Veterinary Teaching Hospital between September 2013 and November 2017 with upper gastrointestinal endoscopy (10 dogs) and additional lower gastrointestinal endoscopy (5 out of 10 dogs) were included. The median age of these dogs was 9.5 years (range, 7–13 years), and they included 7 males (2 intact and 5 neutered) and 3 neutered females. The median body weight was 6.4 kg (range, 4.0–23.2 kg) and they comprised three Miniature Dachshunds, two Shiba, and one each of Labrador Retriever, Jack Russel Terrier, French Bulldog, Pug, and American Coker Spaniel. Prior to the endoscopic biopsy procedure, the dogs were sedated with midazolam (0.1 mg/kg, intravenously [IV]) and butorphanol tartrate (0.2 mg/kg, IV). Then, anesthesia was induced with propofol (4–6 mg/kg, IV) and maintained with isoflurane in oxygen. Gastroduodenoscopy and lower gastrointestinal endoscopy was performed under anesthesia using a flexible video endoscope (VQ-8143A, AVS, Tokyo, Japan), and multiple (6–8) mucosal biopsies were obtained from the stomach, duodenum, jejunum, ileum and colon using serrated biopsy forceps (FB-53Q-1, AVS). During the endoscopic procedures, electrocardiogram, respiratory rate, rectal temperature, arterial blood pressure, pulse oximetry values, and capnography values were monitored and recorded. All endoscopic procedures were completed within 2 hr. Biopsy samples for histologic examination were fixed in neutral buffered 10% formalin, embedded in paraffin wax, and hematoxylin and eosin-stained sections were prepared. Paraffin embedded biopsy samples were subjected to PCR for antigen receptor gene rearrangement (PARR). One of the mucosal biopsy specimens was stored at −80°C for Canine DREAM.

Histopathologic diagnosis was carried out by an American College of Veterinary Pathologists board-certified pathologist (Y. K.). Informed consent was obtained from all owners of dogs involved in this study.

Normal PBMCs

Peripheral blood mononuclear cells (PBMCs) from three healthy beagle dogs (one intact male and two intact females, aged 1 to 4 years) were used as normal controls as described in previous study [37]. All dogs were considered to be healthy based on physical examination and complete blood count and serum biochemistry. All procedures were approved by the Hokkaido University Animal Care and Use Committee (approval number 16-0067). PBMCs were separated by Ficoll/Hypaque gradient centrifugation (Lymphoprep, Nycomed Pharma, Zurich, Switzerland) for genomic DNA extraction.

Digital restriction enzyme analysis of methylation (DREAM)

Genome-wide DNA methylation analysis using next-generation sequencing was performed as previously [37] for the above samples. Briefly, genomic DNA (2 μg) extracted from the samples was mixed with 2 pg of a set of artificial methylation standards. These mixes were digested with *Sma*I and *Xma*I endonuclease (New England Biolabs, Tokyo, Japan) followed by filling in and 3′-dA tails by Klenow DNA polymerase lacking 3′-to-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 to 450 bp in size. Purified DNA was amplified using KAPA Hifi HotStart ReadyMix (Kapa Biosystems, Woburn, MA, USA) and 11 cycles of amplification followed by sequencing on an Illumina HiSeq 2000 (Illumina, San Diego, CA, USA). Sequencing reads were mapped to *Sma*I/*Xma*I sites in the dog genome (canFam3.1), and signatures corresponding to methylated and unmethylated CpGs were enumerated for each *Sma*I/*Xma*I site to calculate methylation frequencies for individual *Sma*I/*Xma*I sites. Finally, we corrected methylation levels measured by DREAM based on the values obtained from the spiked in standards. We used at least 20 sequencing reads to analyse methylation levels at individual *Sma*I/*Xma*I sites. It has been confirmed that DREAM could distinguish differences in methylation of >10% with a False Discovery Rate (FDR) of 2.4% based on technical replicate experiments [17]. We used the University of California, Santa Cruz (UCSC) definition of CpG islands: GC content of 50% or greater, length >200 bp, ratio greater than 0.6 of observed number of CG dinucleotides to the expected number on the basis of the number of Gs and Cs in the segment [10]. Sites at promoter regions are defined as being located within 1 kb from transcription start sites (TSS) of given genes.

Gene ontology analysis

Gene ontology analysis was done using DAVID [15, 16]. DAVID analyses were performed online using parameters of count of >10, and Benjamini-Hochberg-corrected P values <0.05.

RESULTS

Seven dogs were diagnosed as large cell GIL (GIL 1, GIL 3, GIL 5–8, and GIL 10), and three dogs were diagnosed as small cell GIL (GIL 2, GIL 4, and GIL 9). Histopathological diagnosis of stomach, duodenum, jejunum, ileum and colon in each dog were summarized in Table 1. Duodenal or ileal mucosal biopsy samples obtained from 9 dogs (GIL 1–2, GIL 4–10) or 1 dog (GIL 3) with GIL were used for Canine DREAM. PARR was performed in 9 dogs with GIL. Eight out of 9 GIL cases showed clonal rearrangements of T cell receptor gene (GIL 1–2, GIL 3, GIL 5–6, GIL 8–10). One dog with GIL did not show clonal rearrangements of T cell receptor and immunoglobulin genes (GIL 4).

We employed digital restriction enzyme analysis of methylation using next-generation sequencing in dogs, which we had termed Canine DREAM, for ten gastrointestinal lymphoma samples. From all ten samples, 8.6–11.5 million unique usable reads after conservative filtering (quality filtered and aligned to the dog genome) were successfully generated for DNA methylation analyses (Table 2). We used CpG sites that have more than 20 reads (148–162 thousands CpG sites per sample) to assure quantitative ability and also the ones common among the samples for inter-sample comparisons.

We used 83,132 CpG sites that had >20 reads in all GIL samples to compare across the samples. We also compared with the previously analyzed DNA methylation data from healthy normal control PBMCs [37] to analyze DNA methylation patterns in GIL compared to PBMCs. Of those, 30,006 sites are in CGIs and 53,126 sites in non-CGIs (NCGIs). First, we conducted hierarchical clustering analysis with all 13 samples consisted of the ten GIL and three normal PBMCs to observe clusters according to genome-wide DNA methylation patterns and found that PBMCs and GIL were clustered separately (Fig. 1). Interestingly, GIL samples seems to have more variable DNA methylation patterns than PBMC samples that showed uniform DNA methylation patterns shown by clusters formed in this analysis.

Since DNA methylation status is well known to behave differently by regions of CpGs, we analyzed CpG sites in CGIs and NCGIs separately to examine DNA methylation change in GIL. Figure 2 showed density plots of DNA methylation status for CpG sites in CGI or NCGI in comparisons of each GIL with normal PBMCs. Interestingly, GIL showed a notable hypermethylated CpG sites in CGI; increase in DNA methylation for the sites that are unmethylated in normal PBMCs. On the other hand, we found profound hypomethylation at CpG sites in NCGI; decrease in DNA methylation for the sites that are fully methylated in normal PBMCs.

Table 1. Histopathological diagnosis of stomach, duodenum, jejunum, ileum, and colon in 10 dogs with gastrointestinal lymphoma (GIL)

Cases	Stomach	Duodenum	Jejunum	Ileum	Colon
GIL1	Chronic gastritis	Large cell lymphoma	NE	NE	NE
GIL2	NE	Small cell lymphoma	Small cell lymphoma	Small cell lymphoma	Small cell lymphoma
GIL3	NE	Not significant	Not significant	Large cell lymphoma	Large cell lymphoma
GIL4	Not significant	Small cell lymphoma	Small cell lymphoma	Small cell lymphoma	Not significant
GIL5	Large cell lymphoma	Large cell lymphoma	Large cell lymphoma	Large cell lymphoma	NE
GIL6	Not significant	Large cell lymphoma	Large cell lymphoma	NE	NE
GIL7	NE	Large cell lymphoma	Large cell lymphoma	NE	NE
GIL8	Not significant	Large cell lymphoma	NE	Not significant	Large cell lymphoma
GIL9	Not significant	Small cell lymphoma	Small cell lymphoma	NE	NE
GIL10	NE	Large cell lymphoma	Large cell lymphoma	NE	NE

NE, not examined.

Table 2. The number of unique usable reads and CpG sites covered by more than 20 reads of each sample for DNA methylation analysis with digital restriction enzyme analysis of methylation

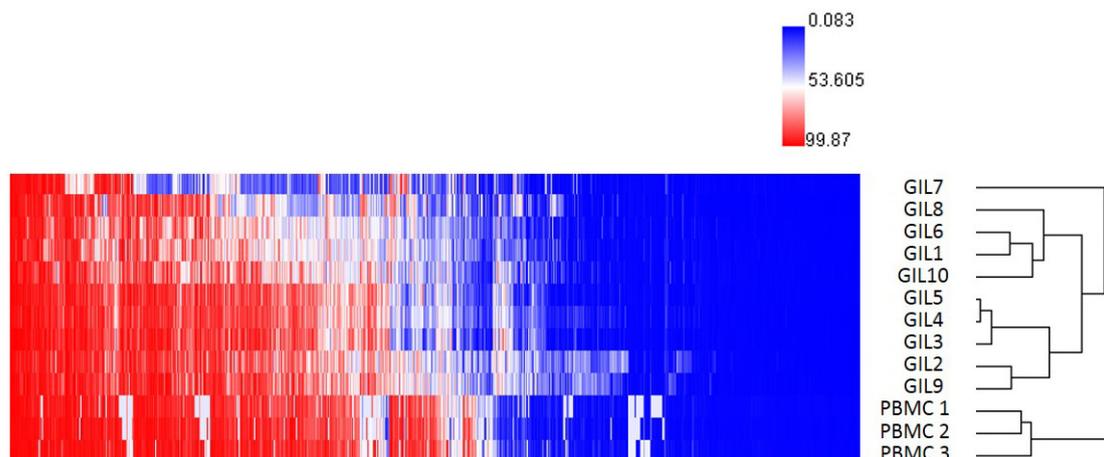| Samples	Number of reads	Number of CpG sites covered
GIL1	9,325,645	155,698
GIL2	8,607,456	152,574
GIL3	9,682,936	153,446
GIL4	8,832,885	148,601
GIL5	9,069,684	151,452
GIL6	10,188,750	160,971
GIL7	10,842,362	162,364
GIL8	11,528,871	160,815
GIL9	9,328,796	154,739
GIL10	9,347,409	157,296

GIL, gastrointestinal lymphoma.

Fig. 1.   Hierarchical clustering analysis of DNA methylation patterns in all samples. Note that gastrointestinal lymphoma (GIL) are clearly separated from normal peripheral blood mononuclear cells (PBMCs). DNA methylation levels of high and low are shown in red and blue, respectively, according to the color scale where 100 is fully methylated and 0 is unmethylated.

Hypermethylation in CGI is a well-known phenomenon that correlates with gene expression silencing [18]. Therefore, we focused on the CpG sites that showed obvious increase in DNA methylation in CGI for the following analyses. By using the temporary criteria for CGI hypermethylation (>20% increase in CGI DNA methylation in the lymphoma from basal DNA methylation level ranging 0–15% in normal PBMCs [12, 32, 37]), we found 383–3,054 CpG sites (1.3–10% of the sites analyzed in CGI) which were hypermethylated in the ten GIL (Fig. 3). Of these, we found 773 CpG sites hypermethylated in more than half of GIL cases (>5 cases). To gain insight into the characteristics of these hypermethylated CGI sites, we looked into their genomic coordinates to address the biological relevance of gaining DNA methylation in GIL. Importantly, we found a considerable number of hypermethylated CGI sites that are close to TSS of dog genes. We used genes that have hypermethylated CGI sites within-1,000 bp of their TSS and found 61 genes with their promoters hypermethylated in GIL compared to normal PBMCs (Table 3). Functional annotations of 'Homeobox' and 'DNA-binding' are significantly found to be involved with the genes with promoter methylation.

DISCUSSION

We previously reported a novel method measuring DNA methylation in the dog genome based on methylation-specific signatures generated by methylation sensitive/insensitive restriction enzymes followed by next-generation sequencing (Canine DREAM) [37]. We utilized Canine DREAM in this study for clinical samples for the first time and obtained descent amount of unique usable reads for quantitative DNA methylation which allowed us to analyze more than 100,000 common CpG sites in all samples. These numbers are comparable to those in canine lymphoma cell lines [37], suggesting that Canine DREAM is a powerful tool that is applicable to clinical samples to provide a more in-depth view of DNA methylation statue than single-locus studies [3, 7].

Hierarchical clustering analysis with 10 GIL samples as well as healthy normal control PBMCs showed clear difference in global DNA methylation patterns between GIL and normal samples. This is consistent with the reports of canine lymphoma cell lines and human lymphoma samples [20, 37], indicating distribution of cytosine methylation is profoundly perturbed in GIL in dogs.

Interestingly, GIL showed a notable increase in DNA methylation for the sites that are unmethylated in normal PBMCs as well as decrease in DNA methylation for the sites that are fully methylated in normal PBMCs. These results indicate that GIL have similar characteristics in DNA methylation as shown in tumors in human [29] and canine lymphoma cell lines [37] characterized by gain of methylation at normally unmethylated CGI sites and loss of methylation at normally methylated NCGI sites.

DNA methylation in CGI promoter region of genes is well known to repress gene transcription. Therefore, we focused on DNA methylation status of CpG sites in promoter CGIs in GIL samples. As a result, 383–3,054 de novo hypermethylated CpG sites were found in GIL compared with normal PBMCs. This variable numbers of hypermethylated CpG sites in CGIs could partly be attributed to the patient characteristics of GIL in this study since wide-spread CpG island promoter methylation, referred to as CpG island methylator phenotype (CIMP) was indicated in a variety of tumors in human [23, 31]. Interestingly, patients with CIMP are also associated with aggressive clinicopathological features of human mantle cell lymphoma [4]. Given these facts, CIMP-like patients could exist in canine GIL cases with a poor prognosis. In fact, GIL2 and GIL9 showed higher number of hypermethylated CpG sites in CGIs (3,054 and 2,881 sites, respectively) as well as a clearly separated cluster in the clustering analysis. However, further study with larger numbers of cases is necessary to elucidate the association between CIMP and prognosis or cell phenotype of canine GIL.

Of these de novo hypermethylation, we found 61 gene promoters to be overlapped in more than half cases (>5 cases). These include mostly genes annotated for 'Homeobox' and 'DNA-binding' by their putative functions. Interestingly, Homeobox genes are polycomb targets, frequently methylated in cancer [26, 34]. In addition, CpG islands in Homeobox gene promoters
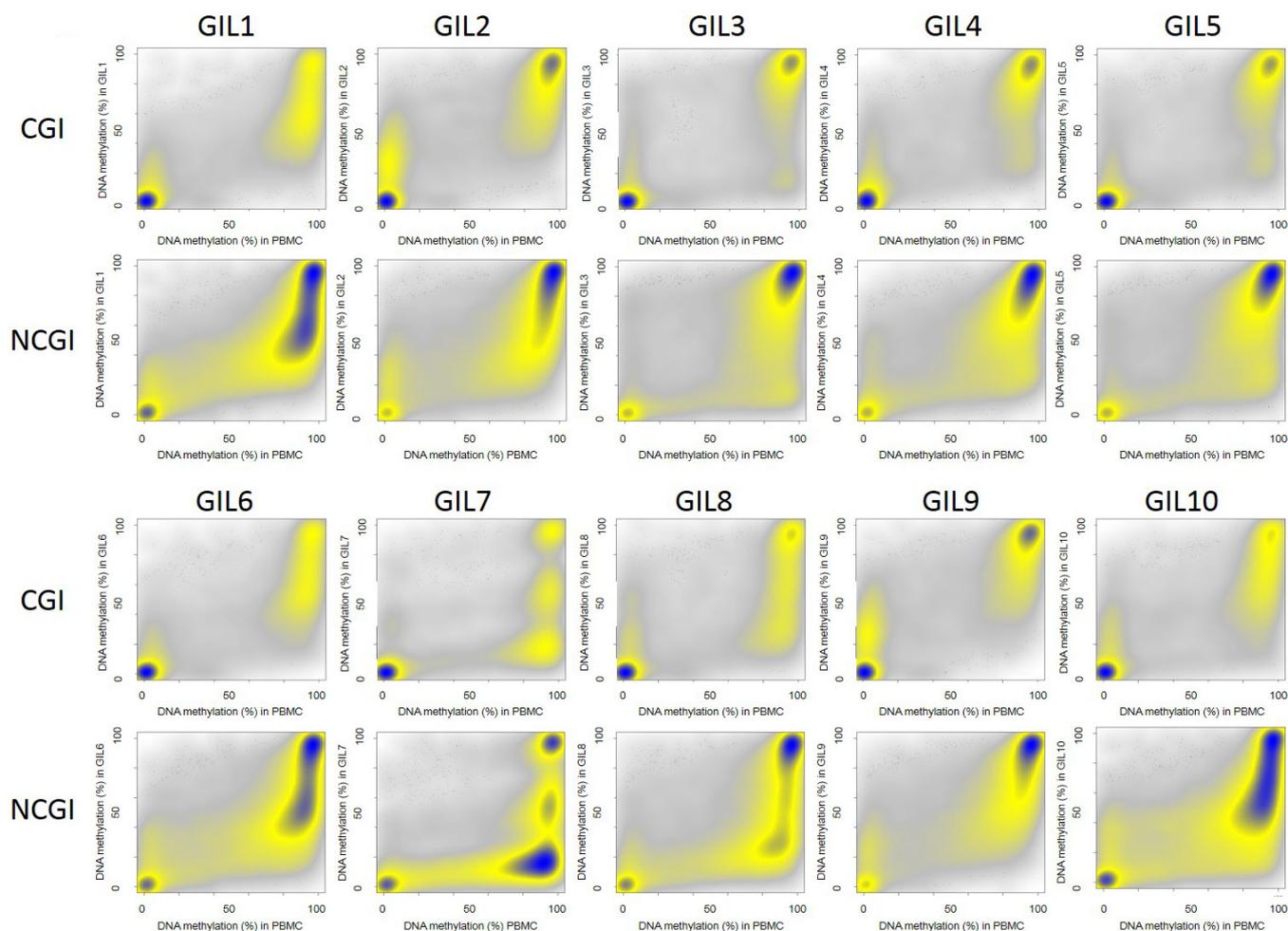
Fig. 2. Representative scatter plots for DNA methylation levels of 10 gastrointestinal lymphoma (GIL) compared to normal peripheral blood mononuclear cells (PBMCs) analyzed separately in CpG islands (CGI) (upper; 30,006 CpG sites) and non-CpG islands (NCGI) (lower; 53,126 CpG sites). DNA methylation levels in PBMCs from healthy dog is plotted on the x-axis, DNA methylation level in GILs are plotted on the y-axis. The density in number of CpG sites increases from white to grey to yellow to blue.
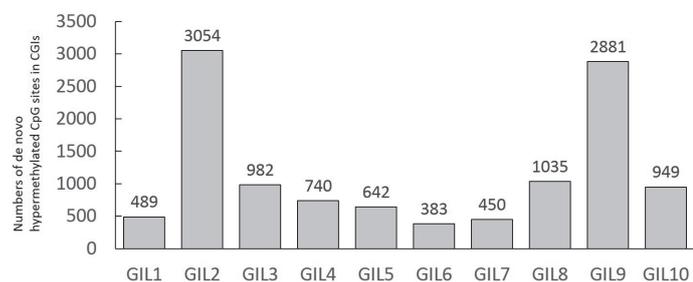

Fig. 3. Numbers of *de novo* hypermethylated sites in CpG islands (CGI) in gastrointestinal lymphoma (GIL) samples. 383–3,054 CpG sites are hypermethylated by using the temporary criteria for *de novo* CGI hypermethylation (>20% increase in CGI DNA methylation in the lymphoma from basal DNA methylation level ranging 0–15% in normal peripheral blood mononuclear cells). Note that GIL2 and GIL9 showed more *de novo* hypermethylated sites than the others.

Table 3. A list of the genes with *de novo* promoter hypermethylation in more than half of the cases (>5 cases) analyzed in this study

GRP	SFMBT2	HLX	NCF2	LBX2
OSR2	EDNRB	BSX	ADAM23	TCF24
FBN2	OXTR	MN1	DUOX1	PDE1B
TMEM215	GRM7	CBLN4	SCG3	JAKMIP3
KDR	MYF5	TRIM67	CCDC105	HECW1
GRIN3A	MICU3	AGAP1	TPM1	COL15A1
NPY	STOX2	GBX2	HS3ST3A1	DCHS2
HOXA9	ZNF366	MLPH	NOL4	TDRKH
ELMO1	SFRP2	GAD1	S1PR4	NPBWR1
RSPO1	CA3	KIAA1614	ARHGAP45	
PGR	IRX5	FOXI2	TRAF1	
POMC	ADCY2	ADAMTSL3	ACKR1	
SOX9	DKK2	LMX1A	SP7	

are hypermethylated in several tumors [25] and aberrant Homeobox gene expression is reported in tumors [27]. For example, a transcription factor HLX1 was found to be one of the genes hypermethylated in GILs and classified to be a member of the genes that have function of DNA-binding and homeobox. Recently, Xu *et al.* suggested that the decreased HLX1 expression was closely associated with the downregulation of T-bet and Runx3, two key regulators in immunoregulation, and might contribute to the development of gastric cancer [35]. The exact mechanism has to be addressed in the future experiments where these hypermethylated gene will be more directly focused by gene-specific silencing in gene expression.

In conclusion, we identified aberrant DNA methylation pattern in canine GIL by genome-wide analysis using DREAM. There are limitations about this study. Since we used PBMCs as control, we cannot exclude the possibility of the effects of the cells other than lymphocytes which should be compared directly to lymphoma cells in GIL. Unfortunately, we do not have data about proportion of lymphocytes and monocytes in the control samples used in our previous study and current study. However previous studies indicated that proportion of monocytes is usually less than 20% [11, 33]. Thus, it is more likely that DNA methylation levels of PBMCs were mainly derived from lymphocytes, and that overall DNA methylation levels should not dramatically changed compared to the samples with only lymphocyte. Collectively, we concluded that difference derived from the heterogeneity in PBMCs is much smaller than dynamic change between normal and tumor cells. However, genome-wide DNA methylation data for each subset of white blood cells will still be important and should be addressed in the future with strictly sorted cells with specific antibodies and flow cytometry analysis. Another possibility of the effect of contamination within the GIL samples is normal intestinal epithelial cells which were hard to be appropriately removed. Quantitative results of DNA methylation levels obtained from 10 GIL samples included the effect of overall population within the samples. Therefore, we consider that there would be even more genes and CpG sites identified to be hypermethlyated if contaminated normal epithelial cells could be removed and the hypermethylated CpG sites and genes identified in this study will still stand even after removing. Future investigation is warranted with appropriately isolated cell populations of lymphocytes, normal epithelial cells, and lymphoma cells in this context. One of intriguing discussion of canine GIL has been raised by the reports that suggested a possibly equivocal mechanisms between chronic enteropathy and GIL, which could not easily be clarified by using the diagnostic technique of their clonality with antigen receptor gene rearrangement as well as histopathological evaluation to differentiate intestinal diseases in dogs [9, 14, 22]. It is widely accepted that *H. pylori* infection plays a crucial role in the development of human gastric MALT lymphoma through inflammation [2]. Further studies should be conducted to address canine GIL as well as chronic enteropathy cases in dogs to clarify difference of DNA methylation features.

CONFLICT OF INTEREST. Authors declare no conflicts of interest.

REFERENCES

1. Barlow, D. P. 1995. Gametic imprinting in mammals. *Science* **270**: 1610–1613. [Medline] [CrossRef]
2. Bautista-Quach, M. A., Ake, C. D., Chen, M. and Wang, J. 2012. Gastrointestinal lymphomas: Morphology, immunophenotype and molecular features. *J. Gastrointest. Oncol.* **3**: 209–225. [Medline]
3. Bryan, J. N., Jabbes, M., Berent, L. M., Arthur, G. L., Taylor, K. H., Rissetto, K. C., Henry, C. J., Rahmatpanah, F., Rankin, W. V., Villamil, J. A., Lewis, M. R. and Caldwell, C. W. 2009. Hypermethylation of the DLC1 CpG island does not alter gene expression in canine lymphoma. *BMC Genet.* **10**: 73. [Medline] [CrossRef]
4. Enjuanes, A., Fernàndez, V., Hernández, L., Navarro, A., Beà, S., Pinyol, M., López-Guillermo, A., Rosenwald, A., Ott, G., Campo, E. and Jares, P. 2011. Identification of methylated genes associated with aggressive clinicopathological features in mantle cell lymphoma. *PLoS One* **6**: e19736. [Medline] [CrossRef]
5. Ferguson-Smith, A. C. 2011. Genomic imprinting: the emergence of an epigenetic paradigm. *Nat. Rev. Genet.* **12**: 565–575. [Medline] [CrossRef]
6. Ferraresso, S., Aricò, A., Sanavia, T., Da Ros, S., Milan, M., Cascione, L., Comazzi, S., Martini, V., Giantin, M., Di Camillo, B., Mazzariol, S., Giannuzzi, D., Marconato, L. and Aresu, L. 2017. DNA methylation profiling reveals common signatures of tumorigenesis and defines epigenetic prognostic subtypes of canine diffuse large B-cell Lymphoma. *Sci. Rep.* **7**: 11591. [Medline] [CrossRef]
7. Ferraresso, S., Bresolin, S., Aricò, A., Comazzi, S., Gelain, M. E., Riondato, F., Bargelloni, L., Marconato, L., Kronnie, G. and Aresu, L. 2014. Epigenetic silencing of TFPI-2 in canine diffuse large B-cell lymphoma. *PLoS One* **9**: e92707. [Medline] [CrossRef]
8. Figueroa, M. E., Skrabanek, L., Li, Y., Jiemjit, A., Fandy, T. E., Paietta, E., Fernandez, H., Tallman, M. S., Greally, J. M., Carraway, H., Licht, J. D., Gore, S. D. and Melnick, A. 2009. MDS and secondary AML display unique patterns and abundance of aberrant DNA methylation. *Blood* **114**: 3448–3458. [Medline] [CrossRef]
9. Fukushima, K., Ohno, K., Koshino-Goto, Y., Uchida, K., Nomura, K., Takahashi, M., Nakashima, K., Fujino, Y. and Tsujimoto, H. 2009. Sensitivity for the detection of a clonally rearranged antigen receptor gene in endoscopically obtained biopsy specimens from canine alimentary lymphoma. *J. Vet. Med. Sci.* **71**: 1673–1676. [Medline] [CrossRef]
10. Gardiner-Garden, M. and Frommer, M. 1987. CpG islands in vertebrate genomes. *J. Mol. Biol.* **196**: 261–282. [Medline] [CrossRef]
11. Goto-Koshino, Y., Ohno, K., Nakajima, M., Mochizuki, H., Kanemoto, H. and Tsujimoto, H. 2011. A rapid and simple method to obtain canine peripheral blood-derived macrophages. *J. Vet. Med. Sci.* **73**: 773–778. [Medline] [CrossRef]
12. Guo, H., Zhu, P., Yan, L., Li, R., Hu, B., Lian, Y., Yan, J., Ren, X., Lin, S., Li, J., Jin, X., Shi, X., Liu, P., Wang, X., Wang, W., Wei, Y., Li, X., Guo, F., Wu, X., Fan, X., Yong, J., Wen, L., Xie, S. X., Tang, F. and Qiao, J. 2014. The DNA methylation landscape of human early embryos. *Nature* **511**: 606–610. [Medline] [CrossRef]
13. Heard, E., Clerc, P. and Avner, P. 1997. X-chromosome inactivation in mammals. *Annu. Rev. Genet.* **31**: 571–610. [Medline] [CrossRef]
14. Hiyoshi, S., Ohno, K., Uchida, K., Goto-Koshino, Y., Nakashima, K., Fukushima, K., Kanemoto, H., Maeda, S. and Tsujimoto, H. 2015. Association between lymphocyte antigen receptor gene rearrangements and histopathological evaluation in canine chronic enteropathy. *Vet. Immunol. Immunopathol.* **165**: 138–144. [Medline] [CrossRef]
15. Huang, W., Sherman, B. T. and Lempicki, R. A. 2009. Bioinformatics enrichment tools: paths toward the comprehensive functional analysis of large

gene lists. *Nucleic Acids Res.* **37**: 1–13. [Medline] [CrossRef]

16. Huang, W., Sherman, B. T. and Lempicki, R. A. 2009. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nat. Protoc.* **4**: 44–57. [Medline] [CrossRef]

17. Jelinek, J., Liang, S., Lu, Y., He, R., Ramagli, L. S., Shpall, E. J., Estecio, M. R. and Issa, J. P. 2012. Conserved DNA methylation patterns in healthy blood cells and extensive changes in leukemia measured by a new quantitative technique. *Epigenetics* **7**: 1368–1378. [Medline] [CrossRef]

18. Jones, P. A. and Baylin, S. B. 2007. The epigenomics of cancer. *Cell* **128**: 683–692. [Medline] [CrossRef]

19. Kondo, T., Oka, T., Sato, H., Shinnou, Y., Washio, K., Takano, M., Morito, T., Takata, K., Ohara, N., Ouchida, M., Shimizu, K. and Yoshino, T. 2009. Accumulation of aberrant CpG hypermethylation by Helicobacter pylori infection promotes development and progression of gastric MALT lymphoma. *Int. J. Oncol.* **35**: 547–557. [Medline]

20. Leshchenko, V. V., Kuo, P. Y., Shaknovich, R., Yang, D. T., Gellen, T., Petrich, A., Yu, Y., Remache, Y., Weniger, M. A., Rafiq, S., Suh, K. S., Goy, A., Wilson, W., Verma, A., Braunschweig, I., Muthusamy, N., Kahl, B. S., Byrd, J. C., Wiestner, A., Melnick, A. and Parekh, S. 2010. Genomewide DNA methylation analysis reveals novel targets for drug development in mantle cell lymphoma. *Blood* **116**: 1025–1034. [Medline] [CrossRef]

21. Morimoto, C. Y., Tedardi, M. V. da F., II, Kimura, K. C., Sanches, D. S., Epiphanio, T. F., de Francisco Strefezzi, R. and Dagli M. L. 2016. Evaluation of the global DNA methylation in canine mast cell tumour samples by immunostaining of 5-methyl cytosine. *Vet. Comp. Oncol.* 10.1111/vco.12241.

22. Nakashima, K., Hiyoshi, S., Ohno, K., Uchida, K., Goto-Koshino, Y., Maeda, S., Mizutani, N., Takeuchi, A. and Tsujimoto, H. 2015. Prognostic factors in dogs with protein-losing enteropathy. *Vet. J.* **205**: 28–32. [Medline] [CrossRef]

23. Noushmehr, H., Weisenberger, D. J., Diefes, K., Phillips, H. S., Pujara, K., Berman, B. P., Pan, F., Pelloski, C. E., Sulman, E. P., Bhat, K. P., Verhaak, R. G., Hoadley, K. A., Hayes, D. N., Perou, C. M., Schmidt, H. K., Ding, L., Wilson, R. K., Van Den Berg, D., Shen, H., Bengtsson, H., Neuvial, P., Cope, L. M., Buckley, J., Herman, J. G., Baylin, S. B., Laird, P. W., Aldape K., Cancer Genome Atlas Research Network. 2010. Identification of a CpG island methylator phenotype that defines a distinct subgroup of glioma. *Cancer Cell* **17**: 510–522. [Medline] [CrossRef]

24. Rassnick, K. M., Moore, A. S., Collister, K. E., Northrup, N. C., Kristal, O., Chretin, J. D. and Bailey, D. B. 2009. Efficacy of combination chemotherapy for treatment of gastrointestinal lymphoma in dogs. *J. Vet. Intern. Med.* **23**: 317–322. [Medline] [CrossRef]

25. Rodrigues, M. F., Esteves, C. M., Xavier, F. C. and Nunes, F. D. 2016. Methylation status of homeobox genes in common human cancers. *Genomics* **108**: 185–193. [Medline] [CrossRef]

26. Schlesinger, Y., Straussman, R., Keshet, I., Farkash, S., Hecht, M., Zimmerman, J., Eden, E., Yakhini, Z., Ben-Shushan, E., Reubinoff, B. E., Bergman, Y., Simon, I. and Cedar, H. 2007. Polycomb-mediated methylation on Lys27 of histone H3 pre-marks genes for de novo methylation in cancer. *Nat. Genet.* **39**: 232–236. [Medline] [CrossRef]

27. Shah, N. and Sukumar, S. 2010. The Hox genes and their roles in oncogenesis. *Nat. Rev. Cancer* **10**: 361–371. [Medline] [CrossRef]

28. Sogame, N., Risbon, R. and Burgess, K. E. 2018. Intestinal lymphoma in dogs: 84 cases (1997–2012). *J. Am. Vet. Med. Assoc.* **252**: 440–447. [Medline] [CrossRef]

29. Taby, R. and Issa, J. P. 2010. Cancer epigenetics. *CA Cancer J. Clin.* **60**: 376–392. [Medline] [CrossRef]

30. Tomiyasu, H., Fujiwara-Igarashi, A., Goto-Koshino, Y., Fujino, Y., Ohno, K. and Tsujimoto, H. 2014. Evaluation of DNA methylation profiles of the CpG island of the ABCB1 gene in dogs with lymphoma. *Am. J. Vet. Res.* **75**: 835–841. [Medline] [CrossRef]

31. Toyota, M., Kopecky, K. J., Toyota, M. O., Jair, K. W., Willman, C. L. and Issa, J. P. 2001. Methylation profiling in acute myeloid leukemia. *Blood* **97**: 2823–2829. [Medline] [CrossRef]

32. Vorjohann, S., Pitetti, J. L., Nef, S., Gonelle-Gispert, C., Buhler, L., Fish, R. J. and Neerman-Arbez, M. 2013. DNA methylation profiling of the fibrinogen gene landscape in human cells and during mouse and zebrafish development. *PLoS One* **8**: e73089. [Medline] [CrossRef]

33. Wang, Y. S., Chi, K. H., Liao, K. W., Liu, C. C., Cheng, C. L., Lin, Y. C., Cheng, C. H. and Chu, R. M. 2007. Characterization of canine monocyte-derived dendritic cells with phenotypic and functional differentiation. *Can. J. Vet. Res.* **71**: 165–174. [Medline]

34. Widschwendter, M., Fiegl, H., Egle, D., Mueller-Holzner, E., Spizzo, G., Marth, C., Weisenberger, D. J., Campan, M., Young, J., Jacobs, I. and Laird, P. W. 2007. Epigenetic stem cell signature in cancer. *Nat. Genet.* **39**: 157–158. [Medline] [CrossRef]

35. Xu, Y., Gao, J., Su, Z., Dai, X., Li, Y., Liu, Y., Chen, J., Tong, J., Zhang, Y., Wu, C., Zheng, D., Wang, S. and Xu, H. 2012. Downregulation of Hlx closely related to the decreased expressions of T-bet and Runx3 in patients with gastric cancer may be associated with a pathological event leading to the imbalance of Th1/Th2. *Clin. Dev. Immunol.* **2012**: 949821. [Medline] [CrossRef]

36. Yamaya, Y., Sugiya, H. and Watari, T. 2015. Methylation of free-floating deoxyribonucleic acid fragments in the bronchoalveolar lavage fluid of dogs with chronic bronchitis exposed to environmental tobacco smoke. *Ir. Vet. J.* **68**: 7. [Medline] [CrossRef]

37. Yamazaki, J., Jelinek, J., Hisamoto, S., Tsukamoto, A. and Inaba, M. 2018. Dynamic changes in DNA methylation patterns in canine lymphoma cell lines demonstrated by genome-wide quantitative DNA methylation analysis. *Vet. J.* **231**: 48–54. [Medline] [CrossRef]