Alternative methylation of intron motifs is associated with cancer-related gene expression in both canine mammary tumor and human breast cancer

A-Reum Nam¹, Kang-Hoon Lee¹, Hyeon-Ji Hwang¹, Johannes J. Schabort¹, Jae-Hoon An², Sung-Ho Won² and Je-Yoel Cho¹*

Abstract

Background: Canine mammary tumor (CMT) has long been considered as a good animal model for human breast cancer (HBC) due to their pathological and biological similarities. However, only a few aspects of the epigenome have been explored in both HBC and CMT. Moreover, DNA methylation studies have mainly been limited to the promoter regions of genes.

Results: Genome-wide methylation analysis was performed in CMT and adjacent normal tissues and focused on the intron regions as potential targets for epigenetic regulation. As expected, many tumor suppressors and oncogenes were identified. Of note, most cancer-associated biological processes were enriched in differentially methylated genes (DMGs) that included intron DMRs (differentially methylated regions). Interestingly, two PAX motifs, PAX5 (tumor suppressive) and PAX6 (oncogenic), were frequently found in hyper- and hypomethylated intron DMRs, respectively. Hypermethylation at the PAX5 motifs in the intron regions of CDH5 and LRIG1 genes were found to be anti-correlated with gene expression, while CDH2 and ADAM19 genes harboring hypomethylated PAX6 motifs in their intron region were upregulated. These results were validated from the specimens originally MBD-sequenced as well as additional clinical samples. We also comparatively investigated the intron methylation and downstream gene expression of these genes using human breast invasive carcinoma (BRCA) datasets in TCGA (The Cancer Genome Atlas) public database. Regional alteration of methylation was conserved in the corresponding intron regions and, consequently, gene expression was also altered in HBC.

Conclusions: This study provides good evidence for the conservation of epigenetic regulation in CMT and HBC, and suggests that intronic methylation can be an important factor in better understanding gene regulation in both CMT and HBC.

Keywords: Canine mammary gland tumor, Human breast cancer, Methylome, Transcriptome, Comparative study

* Correspondence: jeycho@snu.ac.kr

¹Department of Biochemistry, BK21 Plus and Research Institute for Veterinary Science, School of Veterinary Medicine, Seoul National University, Gwanak-ro1, Gwanak-gu, Seoul, Korea

Full list of author information is available at the end of the article

© The Author(s). 2020 Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons licence, and indicate if changes were made. The images or other third party material in this article are included in the article’s Creative Commons licence, unless indicated otherwise in a credit line to the material. If material is not included in the article’s Creative Commons licence and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this licence, visit http://creativecommons.org/licenses/by/4.0/. The Creative Commons Public Domain Dedication waiver (http://creativecommons.org/publicdomain/zero/1.0/) applies to the data made available in this article, unless otherwise stated in a credit line to the data.
**Introduction**

Breast cancer (BC) is the most frequently diagnosed and the second leading cause of cancer death in women worldwide [1]. The comparison of 5-year survival rates between cancer stages 4 and 2, 27% vs. 99% in the USA, clearly shows that earlier diagnosis is crucial for increasing patient survival [2]. Many BC risk factors have been reported; some are uncontrollable, such as old age and gene mutations, while some are controllable, such as diet and smoking [3]. Only about 5–10% of BCs are thought to be hereditary [4]. Representatively, inherited mutations in *BRCA1* and *BRCA2*, which have roles in DNA repair, have been known as the most common cause of hereditary BC [5]. In addition to inherited mutations, somatic mutations of dozens of genes, including *CCND1*, *ERBB2*, *PIK3CA*, and *PTEN*, have been revealed as driver mutations that can lead to functional abnormalities and initiate breast tumorigenesis [6, 7]. The fast-growing databases of various human cancers, such as COSMIC and TCGA, now provide researchers with access to genomic data to test their hypothesis in clinical samples (https://cancer.sanger.ac.uk/cosmic; https://www.cancer.gov/tcga) [8, 9]. On the other hand, the molecular biological effects of environmental factors such as smoking, diet, and exercise [3] are not readily accessible in BC and further approaches are needed to investigate epigenomic changes, including DNA methylation [10].

The association of CpG dinucleotide DNA methylation with cancer-related phenotypes [11] is well understood in various types and at all stages of cancer progression [12, 13]. Hypermethylation, which has been known to be associated with repressed gene expression of tumor suppressors, is one of the important paradigms of carcinogenesis [14] and is supported by the activated mutations of DNA methyltransferases (DNMTs) being oncogenic in several tissues [15]. In various human cancers, genome-wide methylation has been profiled [14] and global DNA hypomethylation [16], along with local hyper- (tumor suppressors) and hypo- (oncogenes) methylations concomitant with the respective silencing and activating of gene expression [17, 18] were reported and suggested as potential diagnostic and predictive biomarkers [19]. The use of methylation alteration as a biomarker has several obvious advantages, such as early detection and relative specimen stability, but only a few are currently clinically used (e.g., methylation of *MGMT* in glioblastoma, *SEPT9* in hepatocellular carcinoma, and *PITX2* in breast cancer) [20].

Very similar to BC in human, canine mammary tumor (CMT) is one of the most common cancers in female dogs [21]. Clinical and pathophysiological similarities existing between HBC and CMTs are well-documented, including the spontaneous tumor incidence, comparable onset age, hormonal etiology, and the identical course of the disease [21]. Furthermore, CMT’s molecular characteristics, including several subtype molecular markers such as steroid receptor, epidermal growth factor (EGF), and proliferation markers, are also similar to HBC [22]. Recently, we reported a transcriptome signature in CMT [23] and other high-throughput sequencing studies on the aspects of CMT have been reported [24, 25]. However, no comprehensive genome-wide methylome profiles that are comparable to studies in HBC have been uncovered yet.

In the present study, we profiled the CMT-associated genome-wide methylation signature using methyl CpG binding domain (MBD) sequencing. In particular, altered DNA methylation in the intron region associated with CMT was comparably investigated in both CMT and human breast cancer. Finally, we tried to show the putative function of differentially methylated regions (DMRs) in the intron region on gene expression using motif analysis with validation in additional samples.

**Results**

**Genome-wide methylation was profiled in 11 pairs of CMT and adjacent normal tissues via MBD sequencing**

Eleven pairs of CMT and adjacent normal tissues consisting of three subtypes, simple, ductal, and complex carcinoma, were subjected to MBD sequencing (Fig. 1a, Table S1A). The statistic information, including the number of reads, Q20 and 30 scores for all the raw sequence data and enrichment scores, and the CG coverage for the processed sequence data generated in this study showed good quality (Table S2). From a total of 4,655,287 bins (500 bp in size), 1,380,792 high-quality bins were obtained by filtration of no CpG, low signals (counts = <20), and bins on the X chromosome (Fig. 1b). Even signal distribution across CMT and adjacent normal in the 11 samples was representatively depicted within the genomic region (Chr 1:18,286,500–19,222,630, ~ 100 Kb) by integrative genomic viewer (IGV) [26] with peak and annotation files. Differentially methylated regions (DMRs), shown in yellow, were distributed similarly on CpG islands and tended to be enriched in gene regions (Fig. 1c). The quality of MBD enrichment was checked according to the coverage of CpGs in the dog genome. Bins with high signal depth (> 5X) covered 45–55% of the dog genome, indicating that methylated DNA was successfully enriched by MBD not only from promoter regions but also from various regulatory regions, including both genic and intergenic regions (Fig. 1d). The methylation profiles were analyzed further by focusing on the DMRs in intergenic regions for the tissue origin of CMT subtypes and the DMRs in genic regions for CMT-enriched methylation. Gene ontology (GO) enrichment analysis and OncoScore [27] were employed to elucidate the functional linkage between differential...
methylation and gene regulation. Additionally, the transcription factor (TF) binding motifs on the subtype-enriched DMRs were investigated. The CMT-enriched methylation signatures and putative regulation were furthermore comparatively investigated in HBC datasets to show how epigenetically similar these two diseases are. The analytical scheme was depicted in Fig. 1e.

Linearized mixed model (LMM) successfully clustered DMRs between CMT and adjacent normal, and among subtypes

To determine differential methylated bins as variables that respond to CMT as well as each subtype, linearized mixed model (LMM) was employed and two different thresholds, top 5% and top 10% bins based on standard variation (SD) that corresponds to p value < 0.01 and p value < 0.05, respectively, were used to obtain DMRs. A total of 137,755 bins (68,741 for CMT DMRs and 69,014 for subtype DMRs) were determined as strict DMRs (5%) of either CMT or across subtypes (Fig. 2a and Table S3, 4). Principal component analysis (PCA) using the DMRs successfully separated 22 specimens with multiple variances (CMT and adjacent normal and three different variances: simple, ductal, and complex) into corresponding groups (Fig. 2b). The sum of PC1 and PC2 in both CMT- and subtype-DMRs represented more than 50% of the total DMRs. Although no clear difference was found in the comparison of genic features consisting of CMT- and subtype-DMRs, the non-CGI (CpG island) region showed a clear difference between CMT (67.5%) and subtype (76.9%) DMRs that might occur in the alteration of repeat element regions (30.9% in CMT-DMR/41.9% in subtype-DMR). On the contrary, the proportion of CGI (7.2%) and shore (16.7%) regions encompassed in CMT-DMRs was higher than in subtype-DMRs (CGI (5.74%) and shore (10.6%)) (Fig. 2c). Interestingly, methylation profiles (hyper- and hypomethylation) showed a distinct difference between CMT- and subtype-DMRs, although, no significant difference was seen in genome-wide methylation distribution. Of note, methylation patterns were clearly biased in genic regions of CMT-DMRs. Approximately 66% of CMT-DMRs in the genetic regions were hypermethylated, while only 45% of DMRs in the intergenic region were

---

**Fig. 1** Schematic presentation of genome-wide methylation profiling in CMT using MBD sequencing. a) Sample preparation for MBD-seq. b) Sequencing data preprocessing with major parameters (window size 500 bp, filtration: bins without any CG, low signal: counts = <20, bins on Chr X). c) Overall sequencing quality is visualized by IGV showing DMRs (yellow), CGI (red), and Gene information (blue). Methylation peaks are colored in 11 cancer (purple) and adjacent normal (green) samples. The region with high density of DMRs is highlighted by the red box. d) High-quality signals (depth > 5X) cover more than 50% of the canine genome in 22 samples. e) Analytical scheme of intergenic and genic regions or subtype-DMRs and CMT-DMRs. Additional data, CMT transcriptome and HBC expression and methylation, was investigated for further analysis.

---

**Table S3, 4**

| Description | Value |
|-------------|-------|
| Top 5% DMRs | 68,741 |
| Top 10% DMRs | 69,014 |
| PC1 and PC2 | > 50% |
| CMT CGI  | 7.2% |
| CMT Shore | 16.7% |
| CMT DMRs  | 67.5% |
| Subtype CGI| 5.74% |
| Subtype Shore | 10.6% |
This bias was not seen in subtype-DMRs, which indicates that the bias is not due to the MBD sequencing (Fig. 2d). This biased genic hypermethylation in CMT fits the general features of higher methylation of genic region in cancer tissues and is similar to a previous report in human BC by Ball et al. [28].

Gene ontology (GO) enrichment and pathway analysis using DMRs on both genic and intergenic regions—fittingly represented the functional relationship between DMRs and CMT as well as subtypes

Extraordinary hypermethylation throughout genic regions including promoter, exon, intron, and TTS in CMT was shown (Fig. 2d). On the other hand, differential methylation on intergenic regions where enhancers or silencers exist contribute to the tissue-type specificity [29]. We first performed hierarchical clustering and heatmap plotting using the genic regions of CMT-DMRs (Fig. 3a). Hypermethylation was more enriched in CMT than adjacent normal, parallel to Fig. 2d and what was previously known (Fig. 3a). Subsequently, OncoScore [27], functional annotations, and Gene ontology (GO) enrichment analysis were performed with the list of CMT-DMGs (Fig. 3b, d and Table S5-S7) to investigate the functional linkage between DMGs and the molecular pathophysiology of CMT. As expected, many DMGs that were hypermethylated and downregulated in CMT including TP63, LIFR, PLA2G16, LRIG1, STAT5A, and AKAP12 and have been known as tumor suppressors, were identified from high scoring (OncoScore > 50) CMT-DMRs (Fig. 3b). On the contrary, some oncogenes including WT1, TFPI2, and ETV1 were also found from hypomethylated and upregulated DMGs. The methylation of 4 representative canine genes and their orthologous human genes, identified as three hypermethylated tumor suppressors (TP63, LIFR, and FOLH1) and one hypomethylated oncogene (WT1) in CMT, showed an anti-correlation with gene expression between normal and cancer in both dogs and humans (Fig. 3c and Additional file 1: Fig. S3). In addition, GO analysis with the disease perturbations from the GEO library revealed that CMT-DMGs were frequently enriched in the list of downregulated genes from various types of cancers including BC (breast cancer G006142 rat GSE1872 sample 63 (p value = 1.4E−16), breast cancer DOID-1612 human GSE26910 sample 602 (p value = 9.81E−13), and sporadic breast
cancer DOID-8029 human GSE3744 sample 979 (p value = 2.49E−11) (Fig. 3d). Furthermore, based on the methylation profiles in the intergenic regions of subtype-DMRs, the ductal subtype was distinctively separated from the simple subtype, while the complex subtype was located in between (Fig. 3e). This result may indicate that the cell type components are shared by the simple and complex subtypes of CMT but not by the ductal subtype.
Hierarchical clustering was performed using the intergenic subtype-DMRs (Fig. 3e) and the nearest genes from the intergenic DMRs were found and processed with GO analysis. The list of genes near intergenic subtype-DMRs was presented in Table S8. The top 5 GO_biological process (BP) and GO_cellular component (CC) terms found in subtype-DMRs indicated that diverse processes were enriched in each subtype. Of note, simple and complex subtypes shared some biological processes, such as extracellular matrix organization (GO:00030198, \( p \) value = 6.79E−04 (simple), \( p \) value = 2.32E−03 (complex)) and cellular response to tumor necrosis factor (GO:0071356, \( p \) value = 1.25E−03 (simple), \( p \) value = 4.56E−03 (complex)), but all terms were unique in the ductal subtype, such as vascular endothelial growth factor receptor signaling pathway all terms were unique in the ductal subtype, such as vascular endothelial growth factor receptor signaling pathway.

In brief, no relevant terms to either cell types or cancer were retrieved (Table S 9-S11). In brief, no relevant terms to either cell types or cancer were retrieved (Table S 9-S11).

**Aberration in intron methylation is associated with cancer**

A total of 10,583 CMT-DMGs were divided into 7 subgroups based on the distribution of DMRs (Fig. 4a). More than 60% of DMGs, consisting of 6745 genes, harbored DMRs only in the intron region, whereas 977 and 819 genes were identified with DMRs in only promoter and exon regions, respectively. A greater amount of intronic DMRs than either exonic or promoter DMRs could have been expected due to the large discrepancy in chromosomal coverage among the intron (26%), exon (1.5%), and promoter (< 1%) regions. Indeed, CMT-DMRs in the exon and promoter regions account for 22% and 17% of the total DMRs, respectively. This is higher than expected based on the coverage of the exon and promoter regions in the genomic sequence (less than 2%). This may mean that more CpG enrichment was done by MB-Seq in these areas (Fig. 4a).

The most interesting finding was that all terms associated with cancer in the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis were enriched in DMRs that included intron DMRs such as intron only (I), exon+intron (EI), promoter+exon+intron (PEI), and promoter+intron (PI) (Fig. 4b, c). Not only the term of "pathways in cancer (hsa05200)" but also "microRNAs in cancer (hsa05206)," "proteoglycans in cancer (hsa05205)," "PI3K-Akt signaling pathway (hsa04151)," etc., which are associated with cancer and cancer pathophysiological characteristics, were highly enriched in intron only DMGs followed by EI and PI groups (Fig. 4c). However, KEGG terms such as "HTLV-1 infection (hsa05166)," "Neuroactive ligand-receptor interaction (hsa04080)," and "Lysosome (hsa04142)" that are extrinsic to cancer and CMT were enriched in DMGs that excluded intron DMRs such as the promoter only (P), exon only (E), and promoter+exon (PE) groups (Fig. 4c). Considering that intronic regions comprise a large portion of the genome, we counted the number of genes enriched in the "hsa05205: Pathways in cancer" term from a group of 530 genes, and the proportion for each group was calculated (data not shown). The percentage of cancer-related DMGs containing intron DMRs was 22.85% (I 5.34%, EI 7.80%, PI 6.70%, PEI 3.01%), which is higher than 17.27%, the percentage of cancer-related DMGs with promoter DMRs (P 3.51%, PI 6.70%, PE 4.05%, PEI 3.01%). Consequently, these results indicate that intron methylation may have important regulatory functions that are associated with CMT. It has been reported that intron CpG methylation might be associated with gene expression in human cancer. For instance, the methylation of the first intron of the EGR2 gene, known as a tumor suppressor, affects the recruitment of proteins required for transcription [31], and anti-tumorigenic PMP24 gene is silenced by the intronic single CpG methylation in prostate cancer cells [32].

Altered CG methylation surrounding transcription factor binding motifs is an important epigenetic regulation in CMT

To investigate enriched CMT-responsive transcription factor (TF) binding motifs, intron DMRs were leniently extracted from the upper 10% of covariance in an LMM analysis (mean \( p \) value < 0.05, Figure S 4A). The list of the top 10% of CMT-DMRs was also able to separately group cancer and adjacent normal (Figure S 4B). According to the alteration of methylation, a total of 56,253 intron DMRs were obtained and subsequently divided into hyper- (36,401) and hypo- (19,852) methylated intron DMRs in CMT, then subjected to motif analysis using HOMER v4.11 [33]. Motif analysis revealed that 10 putative motifs, including PAX5, USF1, ZFX, and SREBF1, were enriched in hypermethylated intron DMRs, while 6 motifs, including CREB1, ELK1, PAX6, and ELK4 motifs, were enriched in hypomethylated intron DMRs. These motifs harbor CG nucleotides the methylation of which may influence protein binding activity [34]. We indeed focused on two PAX motifs, PAX5 and PAX6 that have been known as tumor suppressive and oncogenic, respectively [35–38]. Additionally, Kaplan-Meier plot [39, 40] showed breast cancer patients with lower PAX5 expression live shorter than those with higher, while the survival rate of patients with higher PAX6 expression decreased compared to those with lower expression (Fig. S 5). It was
expected that these two genes would have reverse effects in breast cancer. PAX5 and PAX6 motifs, respectively designated by 16 bp and 20 bp consensus nucleotide sequences (PAX5—GCAGCCAAGCGTGACC, PAX6—NGTGTTCAVTSAAGCGKAAA), were significantly enriched in each DMR group (PAX5 p value $1 \times 10^{-9}$, PAX6 p value $1 \times 10^{-3}$) (Fig. 5a, b and Table S 12, S 13). An enriched heatmap successfully visualized the enrichment of hyper- and hypomethylation signals in the 5 kb surrounding PAX5 and PAX6 motifs, respectively (Fig. 5c, d). We then investigated putative target genes that harbor hypermethylated PAX5 and PAX6 motifs in their intron regions (Table S14-S16). Hypermethylation in the intron DMRs of the PAX5 motifs of CMT, relative to that in adjacent normal, was visualized in the representative genes, CDH5 and LRIG1, by IGV (Fig. 5e). On the other hand, hypomethylation related to PAX6 was found in the CDH2 and ADAM19 genes (Fig. 5f). All of these target genes, hyper- and hypomethylated in CMT, were reversely correlated to gene expression. RNA expression levels of the candidate genes were obtained from our previous transcriptome data [23] and an anti-correlation was shown by
Validation of intron DMRs and their anti-correlation to gene expression

The methylome signature in CMT identified by MBD sequencing was validated in both the 8 pairs of specimens originally subjected to high-throughput sequencing and 9 additional validation sets. Bisulfite genomic DNA conversion followed by PCR was performed in the pairs of CMT and adjacent normal samples to obtain a fine map of intron methylation surrounding PAX5 motif regions of candidate genes (Table S18). Primers used in BS-conversion PCR and sequencing are listed in Table S19. Overall, a hypermethylated intron was confirmed in two candidate genes that included the PAX5 motif, CDH5 and LRIG1, with box plots showing the DNA methylation profiles of the intron DMRs of genes (Fig. 6, Table S20). As for the CDH5 and LRIG1 genes,
respectively, a total of 16 CGs and 7 CGs surrounding PAX5 motifs, were tested in 14 and 17 pairs of CMT and adjacent normal samples. Of the 16 CGs tested in the 1st intron region of CDH5, 12 showed significant hypermethylation (Fig. 6a, upper panel). Unexpectedly, the PAX5 motif was located on the 14th and 15th CGs where no significant difference was found (Fig. S 6A). Pairwise comparison of each CG’s methylation between CMT and adjacent normal showed significant hypermethylation. In the intron-DMR tested region of LRIG1, all CG loci tended to show hypermethylation in CMT and one CG locus (1st CG, p value = 0.019, Fig. S6B), among them showed a significant difference (Fig. 6a, lower panel). In addition, differential intron methylation of CDH5 was clear in all three CMT subtypes but showed the best result in the ductal subtype (p value = 3.9E–13). The differences in LRIG1 intron methylation were more distinct in the complex subtype (p value = 3.1E–05) than in the other subtypes (Fig. 6b). These results suggest that hypermethylation of these two intron regions can be useful candidate epigenetic markers for CMT as well as subtypes.

CMT-enriched differential intron methylation and its anti-correlation with gene expression was conserved in human breast cancer

To validate our CMT-enriched methylome signature findings to human breast cancer (HBC), we investigated the consistency of the aberrations of candidate gene methylation and RNA expression between CMT and HBC. The methylation status and expression profiles of 4 representative candidate genes in HBC was surveyed using the Wanderer database (Fig. 7) [41]. We determined locally corresponding CG sites and introns of the human orthologous genes from the breast cancer methylome data. Methylation levels were regionally dynamic within a target gene and there were some CGs differentially methylated between normal and HBC populations (Fig. 7, top panels of mean methylation). The scatter plots for CDH5 and LRIG1 consisting of hypermethylated intron motifs depicted the trend of increased methylation and decreased gene expression in HBC when compared to normal and thus resulted in normal being represented by the blue dots located in the top-left and HBC being represented by the red dots located in the bottom-right (Fig. 7a, b). On the contrary, CDH2 and ADAM19 showed the opposite pattern of methylation profiles and gene expression between normal and HBC (Fig. 7c, d). Methylation profiles and gene expression of two CDH genes (hypermethylation in CDH5, hypomethylation in CDH2) were well-conserved in normal and HBC populations. The 1st intron of CDH5 harboring the hypermethylated PAX5 motif in CMT was also hypermethylated and downregulated in HBC (Fig. 7a). Moreover, the 2nd intron of CDH2 which harbors a hypomethylated PAX6 motif in CMT was also hypomethylated and upregulated in HBC (Fig. 7b). Of note, LRIG1 has somewhat different gene structures in human and dog, such as different number of exons (22 in human, 25 in dog), and thus the hypermethylated intron

Fig. 6 Validation of intron hypermethylation in the candidate genes, CDH5 and LRIG1. a Comparison of overall methylation states in the surrounding regions of the intronic PAX5 motif in CDH5 and LRIG1 genes. Methylation was measured by the ratio of cytosine on each CG site. Red lines between CMT and adjacent normal indicate hypermethylation, while blue lines indicate hypomethylation. N, adjacent normal; C, CMT. Statistical p value was calculated by paired t test. b Differential methylation is depicted in three separated CMT subtypes.
with the PAX5 motif that has anti-correlation with gene expression (Fig. 7c) was found in the 3rd and 5th introns in human and dog, respectively. Similarly, hypomethylated PAX6 motifs in *ADAM19* have an anti-correlation with the gene expression even though the hypomethylated intronic PAX6 motifs are located on different introns in dog and human (13th intron in dog and 5th intron in human) (Fig. 7d).

As a whole, our data revealed that the orthologous intron regions of PAX5 and PAX6 binding motifs between human and dog have similar CG methylation alterations in breast cancers. These results thus suggest that the molecular similarity between CMT and HBC exists not only at the genomic and transcriptomic levels but also the epigenomic level.

**Discussion**

The study of CMT has gained increasing importance not only for animal welfare but also for better understanding of HBC. Over the past decade, comparative studies of CMT and HBC have been conducted at the genome and transcriptome levels using high-throughput sequencing data and have presented similarities and discrepancies existing between CMT and HBC [23, 25]. However, a comprehensive analysis of the genome-wide methylome in CMT and its comparison with the HBC methylome had not been studied yet.

We employed a linearized mixed model to classify DMRs with multiple variances and successfully determined CMT- and subtype-DMRs. Our methylome data showed that DMRs were biased towards hypermethylation...
on the genic regions represented by promoter, exon, intron, and TTS in CMT. This is consistent with the previous knowledge that the general cancer methylation pattern is represented by intergenic hypomethylation and gene body hypermethylation [28]. In addition, each DMR (CMT- and subtype-) as a methylation signature could separate either normal from CMT or among the three subtypes in principal component analysis. The OncoScore and the GO enrichment analysis results demonstrated that our CMT- and subtype-DMRs are functionally linked to CMT and subtypes.

Of further note in the present study was that most of the enriched cancer-associated pathways were from DMRs that included intron regions. Recently, the regulatory role of the intron region has been proposed in certain gene expressions, particularly the first intron closely located to the promoter [31, 42, 43]. Some studies proposed enhancer sequences in introns and showed the transcription factor (TF) binding to the sequences [44]. Although, some studies also proposed alternative splicing in RNA causing intron retention as putative roles of intron DNA methylation, this needs to be further elucidated [42, 45, 46]. Furthermore, the role of TFs and DNA methylation in intron regions also needs to be elucidated because, although DNA methylation is generally associated with transcriptional silencing, the effect of methylation on binding affinity for most TFs is still unknown [47, 48]. Yet, Yin et al. measured the TF binding affinity to the methylated motif in about half of human TFs using modified high-throughput sequencing and suggested that the affinity of individual TFs can either be increased or decreased on methylation, depending on the different positions within the binding site [34]. In this study, we identified PAX5 and PAX6 motifs, known to be tumor suppressive and oncogenic TFs that are enriched in hyper- and hypomethylated intron DMRs of CMT, respectively. Nine members are known in the paired box (PAX) gene family and some members [49] particularly PAX5 and PAX6 are known to have similar binding sites based on their crystal structure [50]. However, recent studies provided enough evidence that PAX5 and PAX6 work independently [36–38]. For instance, they are clustered in different groups (PAX5 in group 2, PAX6 in group 4) [51] and bind to different genomic loci in ChIP-seq analysis [52]. It is also known that only PAX genes from the same group are capable of complementing the loss of function in others [51]. We also identified a list of motifs, such as NR2F1, RORA, HNF4G, NR3C, MYB, and RUNX that were enriched in intron DMRs but of which the motifs lacked a CG nucleotide inside their recognition sites. The substantial putative target genes reversely regulated by intron methylation around motifs were listed in Table S16. These are also meaningful to study further since these motifs without a CG sequence in their recognition site can still be influenced by the surrounding CG methylation levels [34].

There exists some limitation in directly comparing our CMT methylation profile to the HBC methylome database since the methylation profiling for HBC provided by TCGA was generated from an Infinium Human Methylation450 BeadChip array (Illumina, USA), not MBD sequencing. Nonetheless, the result showing the correlation between methylation in the intron region and gene expression may support the importance of intron methylation, at least in regard to these candidate genes, CDH5 and LRIG1 with PAX5 motifs and CDH2 and ADAM19 with PAX6 motif in both CMT and HBC (Fig. 7).

**Conclusion**

In the present study, we first comprehensively profiled CMT methylation and inspected its correlation with the HBC methylome. We successfully separated CMT-DMRs and subtype-DMRs, and showed their biological relevance by GO and pathway enrichment analysis. We also suggested that changes in intron methylation play an important role in CMT by altering TF binding affinity. The importance of the intron methylation was further confirmed in the HBC data by anti-correlation of selected gene expression with intronic hypermethylated PAX5 and hypomethylated PAX6 motifs. This study allows us to better understand both HBC and CMT at the epigenomic level, yielding new insight into cross-species mechanisms of cancer initiation and progression by DNA methylation alteration and also into the development of cancer biomarkers.

**Materials and methods**

**Tissue samples**

Based on the methods reviewed and approved by the Seoul National University Institutional Review Board/Institutional Animal Care and Use Committee (IACUC SNU-170602-1), a total of 11 dog patients with clinically diagnosed CMT were enrolled in the present study. Tumor and adjacent normal tissue samples of spontaneously occurred canine mammary gland cancer were obtained and freshly frozen. The information for CMT dogs is provided in Table S1.

**Genomic DNA isolation and MBD sequencing**

Genomic DNA was extracted from 11 pairs of CMT and adjacent normal tissues and sheared into 100–300 bp lengths using Bioruptor® Pico (Diagenode, Belgium). Methylated DNA fragments were captured by MBD-beads using the MethylMiner™ Methylated DNA Enrichment Kit (Cat# ME10025) from Invitrogen (CA, USA) according to the manufacturer’s protocol (Invitrogen,
Carlsbad, CA). To obtain more highly methylated DNA, MBD-captured DNA was eluted step-wise with different NaCl concentrations (200, 300, 400, 600, and 800 mM) and ethanol precipitated. After that, we confirmed that methylated DNA was highly enriched in the 600 and 800 mM fractions using real-time PCR. We pooled the 600 and 800 mM fractions and then conducted paired-end sequencing (read length, 101 bp) on the Illumina Hiseq 4000 next-generation sequencing platform (Illumina, CA, USA) after library construction using the TrueSeq Nano DNA Sample Preparation Guide (Part # 15041110 Rev. D) as the manufacturer’s guide.

**MBD-sequencing data processing**

Both per base sequence quality and per sequence quality scores were checked with FastQC v0.11.8 (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/) [53] and sequencing reads with low quality were trimmed using Trim Galore v0.5.0 [54]. Processed reads were mapped to the dog reference genome (CanFam3.1) with Bowtie2 v2.3.4.3 [55] and complete BAM files were obtained after converting SAM to BAM and removing duplicated reads in Linux OS. Using MEDIPS v.1.38.0 (R Bioconductor) [56], MBD reads were calculated in every bin, dividing the whole genome into user-defined window sizes (500 bp, total 4,655,287 bins). Each read per bin was quantile normalized to reduce experimental difference, followed by an estimation of genomic CpG coverage by sequencing depth (Fig. 1d), sequencing reproducibility (Fig. S1A), and enriched methylated fragments according to the number of CpGs in bins (Fig. S1B). Read counts across the total bins showed high correlation between each sample (Fig. S1C, S2A). The entire process is summarized in Fig. 1b.

**DMR identification using LMM (linear mixed model)**

Bins located in chromosome X were excepted for downstream analysis because some CMT patients were spayed females, which could affect the methylation difference on sex chromosome. Low-signal bins with ~ < 20 counts throughout all samples and also bins with no CG dinucleotides had been removed to obtain only valuable signal peaks. Finally, a total of 1,380,792 bins were used for DMR identification. Covariance between “CMT vs. adjacent normal” and “between subtypes” respectively, were calculated for the entirety of the bins using R package “lme4” and we chose the upper 5% of the bins in each comparison group (between “CMT vs. adjacent normal” and “between subtypes”) following prioritizing variance by descending order from 0 to 1. After this, we defined bins whose priority between CMT vs. adjacent normal was higher than that between subtypes as “CMT-DMRs.”

**Motif analysis**

Highly enriched known motifs in hypermethylated and hypomethylated intron DMR sequences were respectively identified using the “HOMER – findMotifsGenome.pl” command. The CpG normalization option was used since genome-wide methylation changes in CMT
usually occur in CpG-rich regions. The \( p \) value for each motif was estimated by comparing the percentage of target sequence with motifs with the percentage of background sequence with motifs. We considered motifs relevant when the \( p \) value was \( < 0.01 \). After that, we found loci where the PAX5 and PAX6 motifs exist across the dog reference genome “CanFam3” (or “hg19” for human) using a motif scanning tool, “FIMO” (matched \( p \) value \( < 0.01 \)) (http://meme-suite.org/doc/fimo.html).

Targeted BS-conversion sequencing
A total 17 pairs of CMT and adjacent normal tissue were used for validation, including the same 8 sets used in MBD sequencing (Table S1B). Bisulfite conversion was done on 500 ng of genomic DNA using the EZ DNA Methylation-Lightning Kit (Zymo Research, USA). Primers were designed using MethPrimer (http://www.urogene.org/methprimer/index1.html) [60] and are listed in Table S19. After PCR, amplicons were purified from the agarose gels using the QIAquick Gel Extraction Kit (Qiagen, Germany) and directly sequenced at Macrogen Co. Ltd. (Macrogen Co. Ltd., Seoul, Korea).

Human TCGA (BRCA) expression and methylation data
RNA sequencing and Infinium Human Methylation 450 K BeadChip array were performed in various human cancer types, such as human invasive breast cancer patients, and in normal people. Wanderer (http://maplab.imppc.org/wanderer/) grants access to a large dataset and offers an interactive viewer to show expression and methylation levels for interesting genes in BRCA (data for other cancer types also provided) [41]. We could thus obtain the methylation beta value for the interesting CGs near PAX motif regions of target genes (CDH5, LRIG1, CDH2, and ADMA19) and their transcription level changes in BRCA patients (Wilcoxon’s test).

Statistical analysis
To estimate the methylated CpG level between CMT and adjacent normal tissues, we calculated the ratio of \( C/(C+T) \) from the BS-sequencing data. For validating methylation changes between them in the target motif DMR regions, statistical significance was assessed on \( p \) values obtained by paired \( t \) test using R basic command.

Supplementary information
Supplementary information accompanies this paper at https://doi.org/10.1186/s13148-020-08884-4.
Authors’ contributions

J.Y. Cho conceived and developed the entire study and revised the manuscript. A.R. Nam worked out MBD-sequencing library preparation, mainly performed computational analysis, and wrote the first draft of the manuscript. K.H. Lee developed the analytical scheme and wrote the first draft of the manuscript. H.J. Hwang and J. Schabot validated methylation. J.H. An and S.H. Won provided statistical and analytical advice. All the authors discussed the results and contributed to the final manuscript.

Funding

This research was supported by the Bio and Medical Technology Development Program of the National Research Foundation (NRF) funded by the Ministry of Science, ICT and Future Planning (2016MSA986027711).

Availability of data and materials

All MBD-seq data generated in this study have been deposited with links to BioProject accession number PRJNA601533 in the NCBI BioProject database (https://www.ncbi.nlm.nih.gov/bioproject/).

Ethics approval and consent to participate

All experiments utilizing animals were approved by SNU IACUC (approval#SNU-170602-1, July 26, 2016) and were performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals.

Consent for publication

Not applicable

Competing interests

The authors declare that they have no competing interests.

Author details

1Department of Biochemistry, BK21 Plus and Research Institute for Veterinary Science, School of Veterinary Medicine, Seoul National University, Gwanak-ro1, Gwanak-gu, Seoul, Korea. 2Department of Public Health Sciences, Graduate School of Public Health, Seoul National University, Seoul, Korea.

Received: 11 February 2020 Accepted: 23 June 2020

References

1. Torre LA, Islami F, Siegel RL, Ward EM, Jemal A. Global cancer in women: burden and trends. Cancer Epidemiol Biomark Prev. 2017;26:444–57.
2. Weiss A, Chavez-MacGregor M, Lichtensztajn DY, Yi M, Tadros A, Hortobagyi GN, Giordano SH, Hunt KK, Mittendorf EA. Validation study of the American Joint Committee on Cancer eighth edition prognostic stage compared with the anatomic stage in breast cancer. JAMA Oncol. 2018;4:203–9.
3. Johnson KC. Risk factors for breast cancer; genetic penetrance and current status with BRCA1 and BRCA2 genes in early-onset breast cancer patients. Adv Exp Med Biol. 2018.
4. Mahdavi M, Nassiri M, Kooshay MM, Valik-Azghandi M, Awan A, Sandry R, Piliis S, Lam AK, Gopalavan V. Hereditary breast cancer; genetic penetration and current status with BRCA1 and BRCA2 genes in early-onset breast cancer patients. Adv Exp Med Biol. 2018.
5. Salleh M, Ghazali MB, Wahab M, Yusoff NM, Mahsin H, Khalid IA, Rahman MNG, Yahaya BH. The BRCA1 and BRCA2 genes and core pathways. Nature. 2008;455:1061–8.
6. Willner AI, Hruban RH, Skolnik ME, Papadopoulos N. CpG island hypermethylation. Brief Funct Genomics. 2003;2:174–79.
7. Li Q, Meng C, Wang T, Liu J, Ji T, Liang M, et al. Targeted and genome-scale strategies reveal gene-body methylation signatures in human cells. Nat Biotechnol. 2009;27:361–7.
8. Mohammadi A, Farzaneh F, Davalos V, Onciul L, Kolodner RD, Helt S, et al. A comprehensive DNA methylation profile of epithelial-to-mesenchymal transition. Cancer Res. 2014;74:5606–19.
9. Sproul D, Meehan RR. Genomic insights into cancer-associated aberrant CpG island hypermethylation. Brief Funct Genomics. 2013;12:2174–90.
10. Han M, Jia L, Lv W, Wang L, Cui W. Epigenetic enzyme mutations: role in tumorigenesis and molecular inhibitors. Front Oncol. 2019;9:194.
11. Torano EG, Petrus S, Fernandez AF, Fraga MF. Global DNA hypomethylation in cancer: review of validated methods and clinical significance. Clin Chem Lab Med. 2012;50:1733–42.
12. Ehrlich M. DNA hypomethylation in cancer cells. Epigenomics. 2009;1:239–59.
13. Wang LH, Wu CF, Rajasekaran N, Shin ’YK. Loss of tumor suppressor gene function in human cancer: an overview. Cell Physiol Biochem. 2018;51:2647–93.
14. Nusbaum C, Maller JB, Hardison RC, Li W, Haussler D. Ancestral sequence and genome-wide patterns of CpG island methylation. Science. 2001;294:1003–6.

Mesirov JP. Integrative genomics viewer. Nat Biotechnol. 2011;29:24–6.
21. Abdelmegeed SM, Mohammad S. Canine mammary tumors as a model for human disease. Oncol Lett. 2018;15:8195–205.
22. Fraigomeni SM, Scallis A, Jerris JS. Molecular subtypes and local-regional control of breast cancer. Surg Oncol Clin N Am. 2018;27:95–120.
23. Lee KH, Park HM, Son KH, Shin TJ, Cho JT. Transcriptome signatures of canine mammary gland tumors and its comparison to human breast cancers. Cancers (Basel). 2018;10.
24. Fish EJ, Irizarry KJ, Delinotescent P, Ellis CJ, Prasad N, Moss AG, Curt Bird R. Malignant canine mammary epithelial cell lines exosomes containing differentially expressed microRNA that regulate oncogenic networks. BMC Cancer. 2018;18:832.
25. Kim KK, Seung BI, Kim D, Park HM, Lee S, Song DW, Lee G, Cheong JH, Nam H, Sur JH; Kim S. Whole-exome and whole-transcriptome sequencing of canine mammary gland tumors. Sci Data. 2019;6:147.
26. Robinson JT, Thorvaldsson DT, Winckler W, Gutman D, Landre ES, Getz G, Mesirov JP. Integrative genomics viewer. Nat Biotechnol. 2011;29:24–6.
27. Piazza R, Ramazzotti D, Spinelli R, Prola A, De Sano L, Ferrari P, Magistroni V, Cordani N, Sharma N, Gambacorti-Passerini C. OncoScore: a novel, internet-based tool to assess the oncogenic potential of genes. Sci Rep. 2017;7:46290.
28. Ball MP, Li JB, Gao Y, Lee JH, LeProut EM, Park IH, Xie B, Daley GQ, Church GM. Targeted and genome-scale strategies reveal gene-body methylation signatures in human cells. Nat Biotechnol. 2009;27:361–8.
29. Loker K, Modhukur V, Rajashekar B, Mänters K, Mägi R, Kolde R, Kolbitsa M, Nilsson TK, Villo J, Salumets A. DNA methylation profiling of human tissues identifies global and tissue-specific methylation patterns. Genome Biol. 2014;15:3248.
30. Chen EY, Tan CM, Kou Y, Duan Q, Wang Z, Meirelles GV, Clark NR, Ma’ayan A. Enrichr: interactive and collaborative HTSeq gene list enrichment analysis tool. BMC Bioinformatics. 2013;14:128.
31. Unoki M, Nakamura Y. Methylation at CpG islands in intron 1 of EGR2 confers enhancer-like activity. FEBS Lett. 2003;554:67–72.
32. Zhang X, Wu M, Xiao H, Lee MT, Levin L, Leung YK, Ho SM. Methylation of a CpG island hypermethylated in breast cancer. Int J Mol Sci. 2018;19.
33. Heintz S, Benner C, Spallholz JE, Weickert M, Gerlach M, et al. A comprehensive DNA methylation profile of epithelial-to-mesenchymal transition. Cancer Res. 2014;74:5606–19.
34. Yin Y, Morgunova E, Jolma A, Kaasinen E, Sahu B, Khund-Sayeed S, Mesirov JP. Integrative genomics viewer. Nat Biotechnol. 2011;29:24–6.
35. Torre LA, Islami F, Siegel RL, Ward EM, Jemal A. Global cancer in women: burden and trends. Cancer Epidemiol Biomark Prev. 2017;26:444–57.
36. Weiss A, Chavez-MacGregor M, Lichtensztajn DY, Yi M, Tadros A, Hortobagyi GN, Giordano SH, Hunt KK, Mittendorf EA. Validation study of the American Joint Committee on Cancer eighth edition prognostic stage compared with the anatomic stage in breast cancer. JAMA Oncol. 2018;4:203–9.
37. Johnson KC. Risk factors for breast cancer; genetic penetration and current status with BRCA1 and BRCA2 genes in early-onset breast cancer patients. Adv Exp Med Biol. 2018.
38. Rajendran BK, Deng CX. Characterization of potential driver mutations involved in human breast cancer by computational approaches. Oncotarget. 2017;8:50525–72.
39. Korkola J, Gray JW. Breast cancer genomes—form and function. Curr Opin Genet Dev. 2010;20:4–14.
40. Cancer Genome Atlas Research N. Comprehensive genomic characterization defines human glioblastoma genes and core pathways. Nature. 2008;455:1061–8.
41. Tate JS, Barmford S, Jubb HC, Sondka Z, Beare DM, Bindal N, Boutsalakhs H, Cole GS, Creatore C, Dawson E, et al. COSMIC: the catalogue of somatic mutations in cancer. Nucleic Acids Res. 2019;47:5041–7.
42. Cava C, Bertoli G, Castiglioni I. Integrating genetics and epigenetics in breast cancer: biological insights, experimental, computational methods and therapeutic potential. BMC Syst Biol. 2015;9:62.
43. Pfleger GP. Defining driver DNA methylation changes in human cancer. Int J Mol Sci. 2018;19.
37. Zong X, Yang H, Yu Y, Zou D, Ling Z, He X, Meng X. Possible role of Pax-6 in promoting breast cancer cell proliferation and tumorigenesis. BMB Rep. 2011;44:595–600.

38. Eccles MR, Li CG. PAX genes in cancer; friends or foes? Front Genet. 2012;3:36.

39. Nagy Á, Lánzczek A, Ményhart O, Györrffy B. Validation of miRNA prognostic power in hepatocellular carcinoma using expression data of independent datasets. Sci Rep. 2018;8:9227.

40. Györffy B, Lanczky A, Eldund AC, Denkert C, Budcizes J, Li Q, Szallas Z. An online survival analysis tool to rapidly assess the effect of 22,277 genes on breast cancer prognosis using microarray data of 1,809 patients. Breast Cancer Res Treat. 2010;123:725–31.

41. Díez-Villanueva A, Mallona I, Peinado MA. Wanderer, an interactive viewer to explore DNA methylation and gene expression data in human cancer. Epigenetics Chromatin. 2015;8:22.

42. Gallegos JE, Rose AB. Intron DNA sequences can be more important than the proximal promoter in determining the site of transcript initiation. Plant Cell. 2017;29:843–53.

43. Holvik E, Bjonessy TE, Mai O, Okamoto S, Minokoshi Y, Shimada Y, Morohashi K-I, Boehm U, Bakke M. DNA methylation of intronic enhancers directs tissue-specific expression of steroidogenic factor 1/adrenal 4 binding protein (SF-1/Ad4BP). Endocrinology. 2011;152:2100–12.

44. Blattler A, Yao L, Witt H, Guo Y, Nicolet CM, Berman BP, Farnham PJ. Global loss of DNA methylation uncovers intronic enhancers in genes showing expression changes. Genome Biol. 2014;15:469.

45. Jezierska DM, Murray RJS, De Gobbi M, Gaentzsch R, Garrick D, Ayyub H, Chen T, Li E, Telenius J, Lynch M, et al. DNA methylation of intragenic CpG islands depends on their transcriptional activity during differentiation and disease. Proc Natl Acad Sci U S A. 2017;114:E7526–35.

46. Kim D, Shivakumar M, Han S, Sinclair MS, Lee YJ, Zheng Y, Olopade OI, Kim D, Lee Y. Population-dependent intron retention and DNA methylation in breast cancer. Mol Cancer Res. 2018;16:461–9.

47. Keshet I, Yissael J, Cedar H. Effect of regional DNA methylation on gene expression. Proc Natl Acad Sci. 1985;82:2560–4.

48. Magdinier F, Billard L-M, Wittmann G, Frappart L, BENCHAÏB M, LENOIR GM, Guerin JF, Dante R. Regional methylation of the 5′ end CpG island of BRCA1 is associated with reduced gene expression in human somatic cells. FASEB J. 2000;14:1585–94.

49. Strachan T, Read AP. PAX genes. Curr Opin Genet Dev. 1994;4:427–38.

50. Czerny T, Busslinger M. DNA-binding and transactivation properties of Pax-6: three amino acids in the paired domain are responsible for the different sequence recognition of Pax-6 and BSAP (Pax-5). Mol Cell Biol. 1995;15:2858–71.

51. Lang D, Powell SK, Rummer RS, Young KP, Ruggeri BA. PAX genes: roles in development, pathophysiology, and cancer. Biochem Pharmacol. 2007;73:1–14.

52. Oki S, Ohta T, Shioi G, Hatanaka H, Ogawara O, Okuda Y, Kawaji H, Nakaki R, Sese J, Meno C. ChiP-Atilas: a data-mining suite powered by full integration of public ChIP-seq data. EMBO Rep. 2018;19.

53. Andrews S. FastQC: a quality control tool for high throughput sequencing reads. Babraham Bioinformatics, Babraham Institute, Cambridge, United Kingdom; 2010.

54. Martin M. Cutadapt removes adapter sequences from high-throughput sequencing reads. EMNet journal. 2011;1:70–2.

55. Langmead B, Salzberg SL. Fast gapped-read alignment with Bowtie 2. Nat Methods. 2012;9:357.

56. Kuleshov MV, Jones MR, Roell AD, Fernandez NF, Duan Q, Wang Z, Kopple S, Jenkins SL, Jagodinik KM, Lachmann A. Enrichr: a comprehensive gene set enrichment analysis web server 2016 update. Nucleic Acids Res. 2016;44:W90–7.

57. Huang DW, Sherman BT, Tan Q, Kir J, Liu D, Bryant D, Guo Y, Stephens R, Baseler MW, Lane HC. DAVID Bioinformatics Resources: expanded annotation database and novel algorithms to better extract biology from large gene lists. Nucleic Acids Res. 2007;35:W169–75.

58. Kinsella RJ, Kähäri A, Haider S, Zamora J, Proctor G, Spudich G, Almeida-King J, Staines D, Derwent P, Kerhornou A. Ensembl BioMarts: a hub for data retrieval across taxonomic space. Database. 2011;2011.

59. Li L-C, Dahlia R. MethPrimer: designing primers for methylation PCRs. Bioinformatics. 2002;18:1427–31.