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Summary

Canine malignant melanoma is one of the common cancers, accounting for 7% of all malignant tumors. They are highly aggressive and metastatic causing poor prognosis. While human malignant melanoma most commonly occurs in the skin, the most frequent location of canine malignant melanoma is the oral cavity including gingiva with less predominantly lingual, buccal, pharyngeal, tonsillar, and palatine epithelium. Although previous studies have investigated various etiological factors including genetic mutations, exact mechanism of tumorigenesis remains unknown.

In human medicine, epigenetic mechanisms that refer to changes in phenotype without changes in genotype have been focused to be important factors in many diseases including neoplasia. One of the epigenetic modifications, DNA methylation is the conversion of cytosine to 5-methylcytosine at cytosine-guanine (CpG) dinucleotides, and DNA methylation at promoter region of genes represses gene transcription. Although CpG sites are generally scattered throughout the genome, there are CpG dense regions known as CpG islands (CGIs). Whereas CpG sites in CGIs are typically unmethylated in normal tissues, it gets methylated in neoplasia. CGIs are located at promoter regions of about half of the genes in mammals and de novo hypermethylation of tumor suppresser-

genes in neoplastic tissues has been identified. On the other hand, majority of CpG sites in non-CpG islands (NCGIs) are methylated in normal tissues, and DNA methylation is decreased in neoplasia.

In veterinary medicine, the number of studies for DNA methylation in canine neoplasia has been increasing. Although there are several reports for DNA methylation in canine malignant melanoma, DNA methylation status in canine malignant melanoma is still unclear. Therefore, the aim of following studies is to reveal DNA methylation change in canine malignant melanoma from three different perspectives.

In chapter 1, I analyzed genome-wide DNA methylation status in canine malignant melanoma based on the next-generation sequencing (NGS) technique, called DREAM. While genome-wide analysis of DNA methylation revealed hundreds of genes with aberrant methylation in a variety of cancers in human medicine, most of studies for DNA methylation in canine malignant melanoma analyzed single gene that was reported in human medicine. Therefore, these studies could not clarify the extent to which genomic DNA methylation status is changed. The purpose of this chapter is to reveal widespread DNA methylation changes in canine malignant melanoma.

Genomic DNA extracted from 15 samples (six samples of normal oral mucosa, four cell lines and five clinical samples of malignant melanoma) was sequentially

digested with two restriction enzymes SmaI and XmaI that recognize same DNA site of CCCGGG, but cut in different manner depending on methylation status. SmaI is sensitive to methylation status, meaning it cuts all unmethylated sites, but does not cut methylated sites. On the other hand, XmaI cuts the remaining methylated sites. Methylation levels at unique SmaI/XmaI sites are calculated based on the numbers of methylated and total signatures. De novo hypermethylation of CpG sites in CGIs was tentatively defined as a more than 20% increase from the basal DNA methylation level (0–15%) and de novo hypomethylation of CpG sites in non-CpG islands (NCGIs) was defined as a greater than 50% decrease from the basal DNA methylation level (80–100%).

Approximately 124,000–180,000 CpG sites with more than 20 reads were selected in each sample, and the 76,213 CpG sites in common across 15 samples were used for the analyses. Of the 76,213 CpG sites, 29,482 were located in CGIs and 46,731 were located in NCGIs. First, the differences in genome-wide DNA methylation patterns between normal tissues and malignant melanomas were assessed and malignant melanoma showed DNA methylation patterns clearly distinct from those of normal oral mucosa. Next, CpG sites in CGIs and NCGIs were analyzed separately to examine the differences in DNA methylation changes in each fraction. The four malignant melanoma cell lines and five malignant melanoma clinical cases were found to have 1054–4527

hypermethylated CpG sites in CGIs in comparison with normal tissues, which accounted for 9–37% of CpG sites with basal methylation levels in normal tissues. On the other hand, 201-5597 hypomethylated CpG sites were found in NCGIs which accounted for 1–25% of CpG sites with a basal methylation level in normal tissues. To gain insights into the characteristics of these hypermethylated CGI sites, their genomic coordinates were assessed to address the biological relevance of gaining DNA methylation in melanoma. Consequently, 221–393 genes in melanoma cell lines and 81-120 genes in melanoma clinical samples had de novo hypermethylated sites at their promoter regions. Of these hypermethylated genes, 23 genes were hypermethylated in all malignant melanoma samples., including six genes annotated with "sequence-specific DNA binding" that were significantly enriched by gene ontology analysis.

In this chapter, genome-wide DNA methylation analysis in malignant melanoma cell lines and clinical samples was performed by using DREAM. Increased DNA methylation levels were observed in CGIs and reduced DNA methylation levels were noted in NCGIs, which corresponds to the findings in human cancer. In addition, a large number of hypermethylated genes were identified. Although the functional effects of the observed DNA methylation changes need to be assessed, these signatures of aberrant DNA methylation could be used as early diagnostic or prognostic markers in canine

malignant melanoma, similar to their utilisation in several cancers in human medicine.

In chapter 2, I examined DNA methylation status of the Long Interspersed Nucleotide Element-1 (LINE-1) repetitive elements in canine mucosal malignant melanoma. LINE-1 is the most well recognized repetitive elements that account for about 17 % of human genome and could be used as a surrogate marker of genome-wide methylation changes. It has been reported that LINE-1 show hypomethylation and LINE-1 hypomethylation is associated with prognosis in various cancers. Global hypomethylation occurs frequently in tumors and reduced DNA methylation levels in NCGIs was detected in chapter 1. Furthermore, given the abundance of LINE-1 elements in the genome, minimum amounts of DNA are required for their amplification and analysis. Accordingly, the purpose of this study was to reveal DNA methylation status of LINE-1 in dogs with malignant melanoma and possible relationship of their DNA methylation level and survival of the patients.

Four malignant melanoma cell lines, 41 malignant melanoma clinical samples and four normal oral mucosa from two healthy dogs were utilized. Bisulfite pyrosequencing was used to quantitatively assess DNA methylation for promoter CGI of canine LINE-1. The pyrosequencing assay interrogates two adjacent CpG sites and DNA methylation levels were calculated by the average of these two sites.

Methylation status of normal mucosae was found to be ranging from 74% to 76%, whereas melanoma cell lines showed 58% to 64%. DNA methylation status of the spontaneous melanoma samples ranged from 23% to 82% and statistically lower methylation levels compared with normal mucosae. Next, the relationship between LINE-1 methylation and survival duration was analyzed. A threshold was temporarily defined to be 65%, which is 10% lower methylation levels than those in normal mucosae. As a result, LINE-1-low patients showed worse overall survival compared with LINE-1-high patients, though the difference did not reach statistical significance. Although, underlying biological mechanism through which hypomethylation of LINE-1 is associated with a poorer survival was not investigated in this study, global hypomethylation may facilitate chromosome instability, leading to the formation of abnormal chromosomal structures.

In this chapter, quantitative analysis of LINE-1 DNA methylation level was conducted by bisulfite-pyrosequencing in canine oral malignant melanoma samples. Malignant melanoma showed hypomethylation of LINE-1 compared to normal tissue corresponding to the reduced DNA methylation levels in NCGIs that was noted in chapter 1. In addition, lower methylation level of LINE-1 is associated with poorer survival in canine malignant melanoma. These results indicated that LINE-1 DNA methylation level could be used as a surrogate marker of global methylation level and prognosis in canine

oral malignant melanoma.

In chapter 3, I focused on the relationship between DNA methylation and epithelial mesenchymal transition (EMT). Canine oral malignant melanoma show various morphological patterns such as epithelioid, fusiform and mixed of them. These findings indicate that neoplastic cells change their morphology during tumor progression. Epithelial–mesenchymal transition (EMT) has been considered an important mechanism underlying morphological changes. EMT is a reversible biological process in which epithelial cells gain mesenchymal properties, including the loss of intracellular adhesion. In tumors, EMT plays a crucial role in tumor progression by inducing enhanced migratory capacity, invasiveness, and elevated resistance to apoptosis or therapeutic agents. Although EMT is the most well-known process in epithelial cell tumors, similar processes have been described in non-epithelial cancers, including melanoma. As EMT is a reversible process, epigenetic mechanisms that refer to a reversible modification are considered to influence EMT contributing to the reversible nature. In this study, I investigated the difference in DNA methylation status between epithelial and mesenchymal phenotypes in canine malignant mucosal melanoma.

This study was performed using six samples of normal oral mucosa from four healthy dogs and 28 clinical malignant melanoma samples. For classifying malignant

melanoma samples into epithelial and mesenchymal phenotypes, E-cadherin expression, which is considered the most characteristic epithelial phenotypic marker in the process of EMT, was examined using immunohistochemistry and/or western blotting for available samples. Finally, 16 samples were classified into the epithelial phenotype and 8 into the mesenchymal phenotype.

Approximately 81,000–180,000 CpG sites with more than 20 reads were selected for each sample, and the 46,673 CpG sites common across the 34 samples were used for downstream analyses. Of the 46,673 CpG sites, 18,489 were located in CGIs and 28,184 in NCGIs. First, the average DNA methylation level was compared for determining the differences in global DNA methylation status between the epithelial and mesenchymal phenotypes. Remarkable differences were not detected between the two phenotypes with respect to the average DNA methylation levels. Next, the average DNA methylation for each site in CGI promoters was analyzed for identifying the differentially methylated genes between epithelial and mesenchymal phenotypes owing to the correlation between DNA methylation and gene expression in EMT. Of 3089 sites in CGI promoters, the epithelial phenotype exhibited more methylated sites (2,291 sites) than the mesenchymal phenotype (798 sites). When differentially methylated CpG sites (DMCs) were defined as a difference of more than 10% with a p-value less than 0.05, the epithelial and

mesenchymal phenotypes exhibited 17 and 6 hypermethylated sites respectively. These 23 DMCs were included in 23 genes, and several of these genes were annotated with "ECM-receptor interaction" by gene ontology analysis. Next, I focused on de novo hypermethylation and found that 12 out of 23 genes showed de novo hypermethylation. To consider the DNA methylation level of individual samples, I determined the number of patients with de novo hypermethylation of these genes in both epithelial and mesenchymal phenotypes. Two genes, *ITAGV* and *NEUROG*, that are related to mesenchymal marker were found to be significantly hypermethylated in the epithelial phenotype.

In this chapter, differences in the DNA methylation status between epithelial and mesenchymal phenotypes were revealed in canine mucosal malignant melanoma. Evaluation of DNA methylation can be used for assessing the effect of the demethylating agent on methylation status in tumors. Demethylating agents have been reported to reverse resistance to cancer therapy acquired via an EMT-mediated process. The results from this study suggest the prospect of using demethylating agents as therapeutic agents in combination with other therapies.

In these studies, drastic DNA methylation changes in canine malignant melanoma were revealed. Although continuous studies with a greater number of clinical

cases or investigation of functional effects of the observed DNA methylation changes are required, these results should contribute to elucidation of epigenetic mechanisms in canine malignant melanoma.