Analysis for differential expression profiles of microRNA in canine mammary gland tumor

（犬の乳腺腫瘍におけるマイクロ RNA 発現差異の解析）

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I, Hui-Wen Chen, hereby certify that this thesis has been written by me, that it is the record of work carried by me (unless were stated), and that it has not been submitted in any previous application for higher degree.

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Date: 2023.3.8

DEDICATION

The thesis is dedicated to everyone who majors in veterinary medicine, and my beloved scientific world.

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Abstract

Canine mammary gland tumors (MGTs) are a common diagnosis in companion animal medicine and a potential model of human breast cancer. The genetics behind these tumors could thus be pivotal in enhancing diagnoses and therapies for canine patients and advancing translational medicine. The miRNAs are small noncoding-RNAs with a gene-regulating role. They have known roles as tumor suppressors and oncogenes, and have the potential as biomarkers for diagnosis and predicting response to therapy. However, to the best of my knowledge, research on miRNA expression in canine MGTs has largely been small-scale and focused on a few targets.

In the first chapter, I aimed to identify miRNAs differentially expressed in canine MGTs using next generation sequencing (NGS), with subsequent confirmatory qPCR and target gene analyses. Mammary gland tissue was collected from healthy dogs (n=7) and dogs with suspected tumors (n=80). A subset of samples was analyzed with NGS to identify differentially expressed miRNAs with CLC Genome Workbench. Normal (n=10), tumor-adjacent (n=6), and tumor-bearing (n=76) mammary gland tissue samples were analyzed for the identified miRNAs using qPCR.

I identified four miRNAs (cfa-miR-1-3p, cfa-miR-133a-3p, cfa-miR-133b-3p, and cfa-miR-133c-3p) as down regulated in canine MGTs relative to normal and tumor adjacent tissues. KEGG analysis revealed the potential target genes of cfa-miR-1-3p were related to the Rap1 signaling pathway, adherens junction, and Ras signaling pathway, and those of the miR-133 family were related to the TGF-beta signaling pathway, synaptic vesicle cycle, and sphingolipid signaling pathway. In combination, these target genes were related to the regulation of transcription and

DNA binding transcription (GO analysis), and the Hippo signaling pathway, adherens junction, and endocytosis (KEGG analysis). Accordingly, I suggest these four miRNAs are promising potential biomarker candidates for canine mammary gland tumors warranting further investigation.

In the second chapter, I investigated the association between miRNA expression patterns and histological classification. Mammary gland tissue was collected from healthy dogs (n=7) and dog patients (n=80). Further samples (n=5) were obtained from established canine MGT cell lines. I targeted miRNAs differentially expressed in metastatic tumor tissue versus non-metastatic and normal tissue. A subset of samples was analyzed using small RNA-Seq with subsequent qPCR. Six differentially expressed miRNAs (cfa-miR-187-3p, cfa-miR-202-5p, cfa-miR-424-5p, cfa-miR-450a-5p, cfa-miR-450b-5p, and cfa-miR-542-3p) were selected from the NGS analysis and submitted for large-scale qPCR analysis. The large-scale qPCR analysis revealed greater alternations in miRNA expression. Large-scale analysis, based on 79 samples, revealed five clusters in a hierarchical clustering based on selected miRNAs. Cluster A contained samples from all tumor subtypes, and resembled Cluster D in its tumor subtype composition. In contrast, Cluster C contained mostly non-tumor samples. The composition of Cluster B was similar to that of Cluster C, although it contained a smaller number than Cluster C. The most markedly distinct cluster was Cluster E, because it contained only one sample of metastasized adenocarcinoma.

The qPCR-based heatmap hierarchical clustering yielded a markedly greater scattering of samples than that based on NGS, thus indicating the classification of the hierarchical clustering did not strikingly match the histopathological subtype classification. I successfully investigated the large-scale miRNA expression

pattern in canine MGT and provided the signatures of whole miRNA expression. The selected miRNA demonstrated that there is no straightforward mapping between molecular signatures and histological classification of canine MGTs at the miRNA level.

In conclusion, I developed differential miRNA expression profiles pattern for canine MGTs, demonstrating downregulation of cfa-miR-1-3p and cfa-miR-133 family miRNAs in these tumors, and reported the whole miRNA expression pattern in metastatic and non-metastatic MGT. Then, I regard the miRNA expression diversity across histological canine MGT subtypes as the principal point of interest for this study. I believe these findings add to the state of existing knowledge of canine MGTs, and suggest future avenues of research that may lead improved diagnoses and therapies in canine and potentially human medicine.

General Introduction

Canine mammary gland tumors (MGTs) are a common diagnosis in companion animal medicine and a potential model of human breast cancer (Gordon et al., 2009; Parker et al., n.d.), and microRNA (miRNAs) are promising biomarkers and therapeutic targets for these tumors. The genetics behind these tumors could thus be pivotal in enhancing diagnoses and therapies for canine patients, and making advances in translational medicine. They have known roles as tumor suppressors and oncogenes (Hwang andMendell, 2006), and potential as biomarkers for predicting response to therapy (Baird andCaldas, 2013; Y.Lai et al., 2020; Meyerson et al., 2010; Md MahfuzurRahman et al., 2020). Canine MGTs are broadly classified as simple or complex carcinomas in malignant cases based on the number of involved components, in the same way as human breast cancers. Interestingly, miRNAs reportedly show differential expression between metastatic and non-metastatic tumors in dogs, which suggests they have potential utility as biomarkers of metastasis (Bulkowska et al., 2017).

Next generation sequencing (NGS) allows me to take investigations on miRNAs in canine MGTs a step further, by screening for molecules of interest that can then be subjected to further large-scale investigation.

Accordingly, I aimed to identify miRNAs differentially expressed in canine mammary gland tumors using NGS, with subsequent confirmatory qPCR and target gene analyses. An in silico analysis (TargetScan) was performed to predict the miRNAs' target genes using gene ontology (GO) terms and the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (DAVID). Then, I set out to elucidate miRNAs differentially expressed in canine MGTs relative to normal tissue, targeting multiple (metastatic and non-metastatic) histological types and subtypes of tumor. Furthermore, I aimed to predict the target genes for selected differentially expressed miRNAs. Ultimately, the evidence on the role of miRNAs obtained in this study is intended to aid understanding of canine MGTs at the molecular level, and provide useful information on potential biomarkers and therapeutic agents, which may ultimately prove to be of interest for combating human breast cancer.

Chapter 1

Micro RNA differential expression profile in canine mammary gland tumor by next generation sequencing

Gene, Volume 818, 15 April 2022, 146237

1.1. Abstract

Canine mammary gland tumors are very common and represent a potential model of human breast cancer, and microRNA (miRNAs) are promising biomarkers and therapeutic targets for these tumors. Accordingly, I aimed to identify miRNAs differentially expressed in canine mammary gland tumors using next generation sequencing (NGS), with subsequent confirmatory qPCR and target gene analyses. Mammary gland tissue was collected from healthy dogs (n=7) and dogs with suspected tumors (n=80). A subset of samples was analyzed with NGS to identify differentially expressed miRNAs with CLC Genome Workbench. Normal (n=10), tumor-adjacent (n=6), and tumor-bearing (n=76) mammary gland tissue samples were analyzed for the identified miRNAs using qPCR. An in silico analysis (TargetScan) was performed to predict the miRNAs' target genes using gene ontology (GO) terms and the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (DAVID). I identified four miRNAs (cfa-miR-1-3p, cfa-miR-133a-3p, cfa-miR-133b-3p, and cfa-miR-133c-3p) as down regulated in canine mammary gland tumor tissues relative to normal and tumor adjacent tissues. KEGG analysis revealed the potential target genes of cfa-miR-1-3p are related to the Rap1 signaling pathway, adherens junction, and Ras signaling pathway, and those of the miR-133 family are related to the TGF-beta signaling pathway, synaptic vesicle cycle, and

sphingolipid signaling pathway. In combination, these target genes are related to the regulation of transcription and DNA binding transcription (GO analysis), and the Hippo signaling pathway, adherens junction, and endocytosis (KEGG analysis). Accordingly, I suggest these four miRNAs are promising potential biomarker candidates for canine mammary gland tumors warranting further investigation.

1.2. Introduction

Canine mammary gland tumors (MGTs) are a common diagnosis in companion animal medicine and a potential model of human breast cancer (Gordon et al., 2009; Parker et al., n.d.). The genetics behind these tumors could thus be pivotal in enhancing diagnoses and therapies for canine patients, and making advances in translational medicine. Many clinicians and researchers are therefore seeking a better understanding of gene transcription and expression in canine MGTs, and microRNAs (miRNAs) are attracting interest in this research because they are key players in this transcription and expression.

miRNAs are small noncoding RNAs with a gene-regulating role (Ambros, 2004; Bartel, 2004; Saliminejad et al., 2019). They have known roles as tumor suppressors and oncogenes (Hwang andMendell, 2006), and potential as biomarkers for predicting response to therapy (Baird andCaldas, 2013; Y.Lai et al., 2020; Meyerson et al., 2010; Md MahfuzurRahman et al., 2020).

Canine MGTs—around half of which are malignant (Salas et al., 2015)—share many features with human breast cancer, covering tumor genetics, histological appearance, biological behavior, molecular features, and response to conventional therapies (Gordon et al., 2009; Parker et al., n.d.). For example, the MET, IGF1R, mTOR, and KIT genes have been implicated in mammary gland or breast cancers

in both female dogs and women (Paoloni andKhanna, 2008). Furthermore, histological examinations have revealed that some canine MGTs (simple carcinomas and some complex carcinomas) resemble human breast carcinomas at the molecular level (Liu et al., 2014; Paoloni andKhanna, 2008).

Histological classification is also hugely important when dealing with diagnosis, prognosis, and therapeutic approaches in canine MGT cases. Canine MGTs are broadly classified as simple or complex carcinomas in malignant cases based on the number of involved components, in the same way as human breast cancers. A formal histological classification system for canine MGTs was originally developed by the World Health Organization and most recently revised in 2011 (Goldschmidt et al., 2011). In many cases, histological classification drives diagnostic and prognostic assessments, and this suggests that differential expression of miRNAs across and within different histological types and subtypes is as a fruitful line of research. Interestingly, miRNAs reportedly show differential expression between metastatic and non-metastatic tumors in dogs, which suggests they have potential utility as biomarkers of metastasis (Bulkowska et al., 2017).

miRNAs are already receiving attention in the veterinary field, and have been established potential as biomarkers in companion animal and production animal medicine (Bai et al., 2019; Laganà et al., 2017; Y.-C.Lai et al., 2017; Y. C.Lai et al.,

2020; Muroya et al., 2016, 2013; Pokharel et al., 2018). The miRNA expression patterns in malignant canine MGTs resemble those in human breast cancers (Boggs et al., 2008, 2007; vonDeetzen et al., 2014). Reflecting developments in human medicine, canine MGT cell lines have been established and used in studies of miRNA expression and altered expression of malignancies (Osaki et al., 2016; Pang andArgyle, 2010). However, as far as I know, research on miRNA expression in canine MGTs has largely been small scale, has often involved in cell lines rather than clinical samples, and has only targeted pre-selected molecules.

Next generation sequencing (NGS) allows me to take investigations on miRNAs in canine MGTs a step further, by screening for molecules of interest that can then be subjected to further large-scale investigation (ideally in large numbers of clinical samples). These techniques are well established in the veterinary field, where genome-wide miRNA profiling has been developed as a diagnostic tool in a range of species, as well as for practical applications in product science (Al-Husseini et al., 2016; Y. C.Lai et al., 2017; Y.Lai et al., 2020; Lawless et al., 2013; Mukiibi et al., 2020; VanHese et al., 2020).

Accordingly, in the current study, I set out to elucidate miRNAs differentially expressed in canine MGTs relative to normal tissue, targeting multiple (metastatic and non-metastatic) histological types and subtypes of tumor. Initially, I used NGS

to screen tissue for upregulated or downregulated miRNA expression, and then confirmed the expression for selected differentially expressed miRNAs in a large number of clinical samples using real-time qualitative PCR (qPCR). Furthermore, I aimed to predict the target genes for selected differentially expressed miRNAs. Ultimately, the evidence on the role of miRNAs obtained in this study is intended to aid understanding of canine MGTs at the molecular level, and provide useful information on potential biomarkers and therapeutic agents, which may ultimately prove to be of interest for combating human breast cancer.

1.3. Materials and Methods

1.3.1. Sample collection

Tissue samples were collected from the mammary glands of female dogs; normal tissue samples (n=7) were obtained from purpose-bred laboratory animals (beagles) at Shin Nippon Biomedical Laboratories (Kagoshima, Japan), and clinical samples (n=80) were obtained from female dogs receiving treatment at the Kagoshima University Veterinary Teaching Hospital (Kagoshima, Japan) or affiliated practices and animal hospitals. Demographic information on the sample donors is presented in Table ch1-1. Further samples (n=5) were obtained from metastatic malignant mammary gland tumor cell lines [CIPp (RRID:CVCL_L149), CIPm (RRID:CVCL_L148), CTBp (RRID:CVCL_L151), CHMm (RRID:CVCL_L146), CHMp (RRID:CVCL_L147)](Uyama et al., 2006), and included as metastatic malignant mammary gland tumor samples.

Collected samples were promptly submerged in RNAlater™ (Invitrogen; Thermo Fisher Scientific), and incubated overnight at 4°C followed by storage at −80°C, as previously described (Md MahfuzurRahman et al., 2020).

Histopathological specimens were prepared from the samples with hematoxylin and eosin staining (H&E stain), and examined microscopically by a certified veterinary pathologist (Japanese College of Veterinary Pathologists), who based

diagnoses on published standards for the classification and grading of canine MGTs (Goldschmidt et al., 2011).

1.3.2. Total RNA extraction and sequencing

Total RNA was extracted using a mirVana™ miRNA isolation kit (Thermo Fisher Scientific Inc.), in accordance with the manufacturer's instructions. RNA concentration was measured using NanoDrop 2000c (Thermo Fisher Scientific). The quality of RNA was assessed using the 2100 Bioanalyzer System (Agilent Technologies), as I described previously (M. MahfuzurRahman et al., 2020). Samples with an RNA Integrity Number (RIN) exceeding eight were allocated for analysis with NGS, and samples with a miRNA region in the 2100 Bioanalyzer System were allocated for analysis with qPCR.

Small RNA libraries were prepared and sequenced by Hokkaido System Science Co., Ltd (Sapporo, Japan). Small RNA libraries were constructed from 1 μg of total RNA using the TruSeq Small RNA Library Preparation Kit (Illumina, San Diego, CA, USA), in accordance with the manufacturer's instructions. The libraries were subjected to 100-bp paired-end sequencing on an Illumina HiSeq 2500 System (Illumina).

1.3.3. Processing small RNA sequencing data

Small RNA sequences were processed and analyzed (with steps including quality control, adapter trimming, extraction, counting, annotating, and normalization of reads) using CLC Genome Workbench 10.1.1 (CLC bio, Cambridge, MA, USA). The small RNAs differentially expressed in tumor tissue were identified with the Empirical Analysis of DGE tool in CLC Genome Workbench 10.1.1.

The Illumina files (forward and reverse reads) were merged during import (quality scores: NCBI/Sanger or Illumina Pipeline 1.8). A trimming process was performed to remove adapters and other contaminants. The adapter sequences were as stated below.

- Forward adapter sequence: TGGAATTCTCGGGTGGCCAAGG
- Reverse adapter sequence: GATCGTCGGACTGTAGAACTCTGAAC

Sequences shorter than 15 nucleotides or longer than 55 nucleotides were discarded. The clean reads were processed with the Extract-and-Count tool and annotated against miRBase (release 21), and the Ensembl canine ncRNA database (Canis familiris.canfam3.1.ncrna) ("Canis_lupus_familiaris - Ensembl genome browser 102," n.d.; Hunt et al., 2018; Kozomara et al., 2019; Kozomara and Griffiths-Jones, 2014, 2011; Yates et al., 2020). miRBase was prioritized

over other annotation resources.

1.3.4. Quantification of miRNAs with qPCR

qPCR was performed as I previously described (Y.Lai et al., 2020). Total RNA (2 ng/μl) was reverse-transcribed to cDNA with TaqMan miRNA assays (Thermo Fisher Scientific), in accordance with the manufacturer's protocol. qPCR was performed with a TaqMan Fast Advanced Master Mix kit and the StepOnePlus™ Real-Time PCR System (Thermo Fisher Scientific). Thermal cycling was performed in accordance with the manufacturer's instructions, and all experiments were performed in duplicate. RNU6B was used as the internal control, and expression levels were determined with the $2^{-\Delta\Delta Ct}$ method. When the cycle threshold (Ct) value in a qPCR assay was undetermined, a Ct value of 40 was assigned. The TaqMan miRNA assays used for qPCR in this study (and their assay IDs) were as follows: cfa-miR-1-3p (ID 000385), cfa-miR-133a-3p (ID 000458), cfa-miR-133c-3p (ID 002943_mat), and RNU6B (ID 001093).

1.3.5. Target gene prediction and pathway enrichment

TargetScan 7.2 was used to predict the target genes of the differentially expressed miRNAs (Agarwal et al., 2015). Genes with a cumulative weighted

context++ score below -0.6 were selected as putative targets. For annotation, I used the Visualization and Integrated Discovery (DAVID, v6.8) database for the gene ontology (GO) terms and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis for the predicted gene targets (Huang et al., 2009a, 2009b). GOTERM_ DIRECT parameters were used to analyze the biological process and cellular component and molecular function terms, respectively.

1.3.6. Statistical analysis

qPCR data were analyzed and graphically represented using GRAPHPAD PRISM 7 (GraphPad Software Inc., San Diego, CA, USA).

The qPCR data were analyzed with nonparametric, one-way analysis of variance (ANOVA), and subjected to a Kruskal-Wallis test. P-values were corrected for the false discovery rate (FDR-p value), and subjected to the two-stage linear step-up procedure described by Benjamini, Krieger and Yekutieli. Differences were considered to be significant at FDR-p < 0.05. Box & whiskers plots were drawn in accordance with Tukey's definition to exclude outliers.

1.4. Results

1.4.1. miRNA expression profiles determined with NGS

I selected 12 canine tissue samples for miRNA expression analysis with NGS. These samples comprised normal mammary gland (n=3), benign mixed tumor (n=3), adenoma (n=3), and adenocarcinoma (n=3) tissues, and the three subtypes of tumor tissue were compared with normal mammary gland tissue.

The numbers of significantly differentially expressed miRNAs (in tumor tissue vs. normal mammary gland tissue; $p<0.05$, FDR-p-value <0.05) were 43 for adenoma tissue (upregulated: n=6; downregulated: n=37), seven for benign mixed tumor (upregulated: n=2; downregulated: n=5), and nine for adenocarcinoma (upregulated: n=1; downregulated: n=8).

I then sought to identify miRNAs differentially expressed in multiple tumor types, and my findings are presented graphically in a Venn diagram (Figure ch1-1).

To describe the expression patterns in brief, four of the miRNAs (cfa-miR-1-3p, cfa-miR-133a-3p, cfa-miR-133b-3p, and cfa-miR-133c-3p) were downregulated in all three tumor tissue subtypes; three of the miRNAs (cfa-miR-99a-5p, cfa-miR-206-3p, and cfa-miR-338-3p) were downregulated in adenoma and adenocarcinoma tissues, and one miRNA (cfa-miR-9-5p) was upregulated in

adenoma and adenocarcinoma tissues. Furthermore, one miRNA (cfa-miR-208b-3p) was downregulated in benign mixed tumor and adenocarcinoma tissues.

The canine mammary gland tissue samples were then targeted for hierarchical clustering analysis based on the nine miRNAs stated above (Figure ch1-2). Two normal mammary gland samples (MG2 and MG3) were initially clustered into the same group, but the other (MG1) was clustered into a group with the benign mixed tumor tissue samples. The adenocarcinoma and adenoma tissue samples were initially clustered into distinct groups for their respective tumor types.

Regarding differential expression patterns (Table ch1-2), cfa-miR-9-5p was the only miRNA to be upregulated in a tumor tissue subtype (adenocarcinoma tissue, fold change vs. normal tissue: 60.37). The other nine investigated miRNAs were downregulated in benign mixed tumor, adenoma, and adenocarcinoma tissues.

1.4.2. Confirmation of differentially expressed miRNAs by qPCR

To confirm my NGS results in expression analysis with a large number of clinical samples, I targeted three miRNAs (cfa-miR-1-3p, cfa-miR-133a-3p and cfa-miR-133c-3p) for qPCR analysis. These three miRNAs were selected because they were differentially expressed in all three tumor tissue types in the NGS experiment (i.e., they were represented in the central intersection of the Venn

diagram), and showed normalized mean expression values exceeding 50. In this qPCR experiment, expression was investigated with 92 tissue samples, classified as normal mammary gland, tumor-adjacent tissue, adenoma, complex adenoma, benign mixed tumor, adenocarcinoma, or metastasized adenocarcinoma by veterinary pathologists.

All three miRNAs showed statistically significant differences in their expression between the tumor tissues (all subtypes) and non-tumor tissues (normal and tumor-adjacent tissue), but not between normal and tumor-adjacent tissues (Figure ch1-3a-3c). cfa-miR-1-3p and cfa-miR-133c-3p followed a similar pattern, with significantly lower expression in tumor tissue than normal and tumor-adjacent tissue. cfa-miR-133a-3p expression also differed significantly between tumor and non-tumor tissues, and showed a wider range of values for adenocarcinoma than for the other tumor subtypes; however, the differences between adenocarcinoma and the other tumor subtypes were not statistically significant.

1.4.3. Target gene prediction for cfa-miR-1-3p, cfa-miR-133a-3p, cfa-miR-133b-3p, and cfa-miR-133c-3p

I then set out to investigate the target genes for all four miRNAs differentially expressed across multiple histological types in NGS analysis, using the bioinformatics prediction tool TargetScan. I found 670 potential targets of cfa-miR-1-3p with cumulative weighted context++ scores ranked from 0 to -1.1, as opposed to 681 potential targets for the cfa-miR-133 family with cumulative weighted context++ scores ranked from 0 to -1.06. The targets with scores lower than -0.6 have been compiled into a partial list (Table ch1-3). This listing shows eight potential targets of cfa-miR-1-3p, and 26 potential targets of the cfa-miR-133 family.

GO and KEGG enrichment revealed a range of target genes for cfa-miR-1-3p and the cfa-miR-133 family miRNAs (cfa-miR-133a-3p, cfa-miR-133b-3p, and cfa-miR-133c-3p). The three most enriched terms for cfa-miR-1-3p target were Rap1 signaling pathway, adherens junction, and Ras signaling pathway. The enriched terms for cfa-miR-133 family target genes were TGF-beta signaling pathway, synaptic vesicle cycle, and sphingolipid signaling pathway (Tables 4-5). I then performed further GO and KEGG analyses using all target genes of both cfa-miR-1-3p and the cfa-miR-133 family as input, which revealed that the combined target genes were involved in the regulation of transcription from RNA polymerase II promoter, protein transport, and DNA binding transcription (GO

analysis; Table ch1-6), and the Hippo signaling pathway, adherens junction, and endocytosis (KEGG analysis; Table ch1-7).

1.5. Figures and Tables

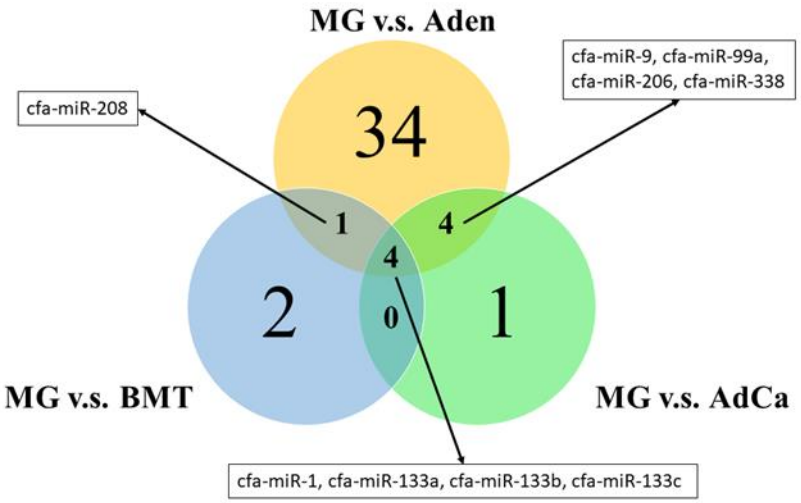

Figure ch1-1. Venn diagram for differentially expressed miRNAs in NGS profiles. (MG, mammary gland; Aden, adenoma; BMT, benign mixed tumor; AdCa, adenocarcinoma.)

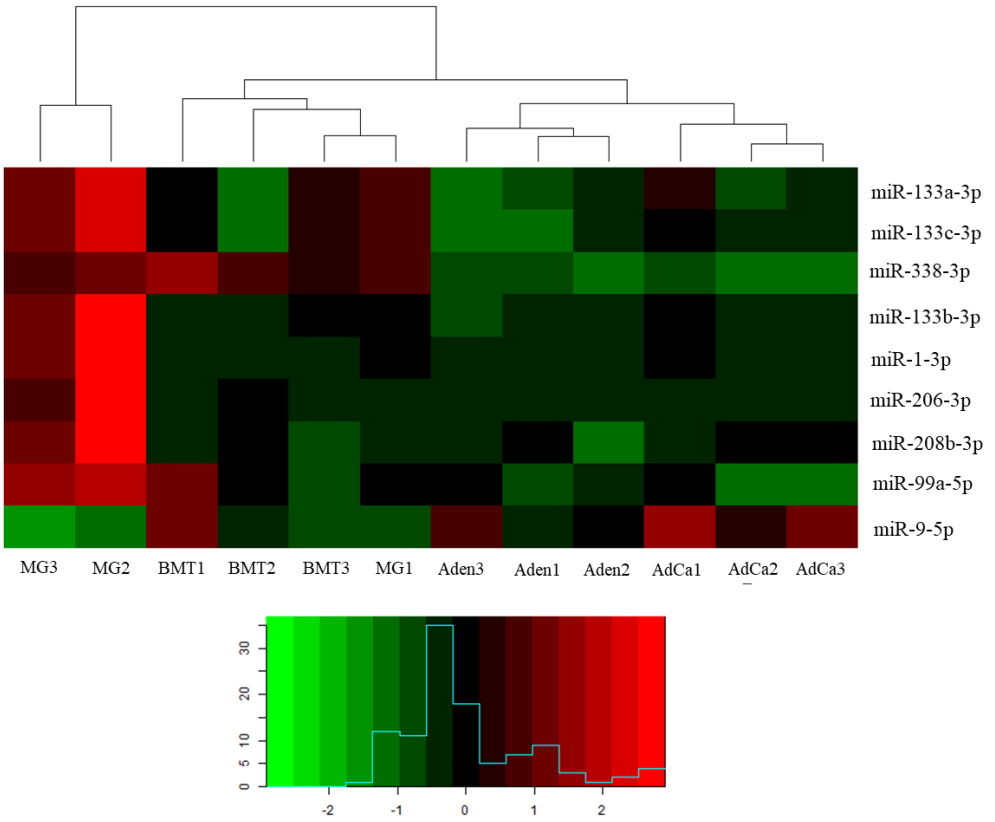

Figure ch1-2. Hierarchical clustering analysis for subtypes of canine mammary gland tumor and normal mammary gland tissues. Differentially expressed (multi-subtype) miRNAs identified in NGS were subject to this analysis. MG1 was s classified more closely to BMT, while the other canine mammary gland tumor samples were each classified into their own groups. (MG, mammary gland; TAT, tumor adjacent tissue; Aden adenoma; CA, complex adenoma; BMT benign mixed tumor; AdCa, adenocarcinoma; AdCa(meta), metastasized adenocarcinoma.)

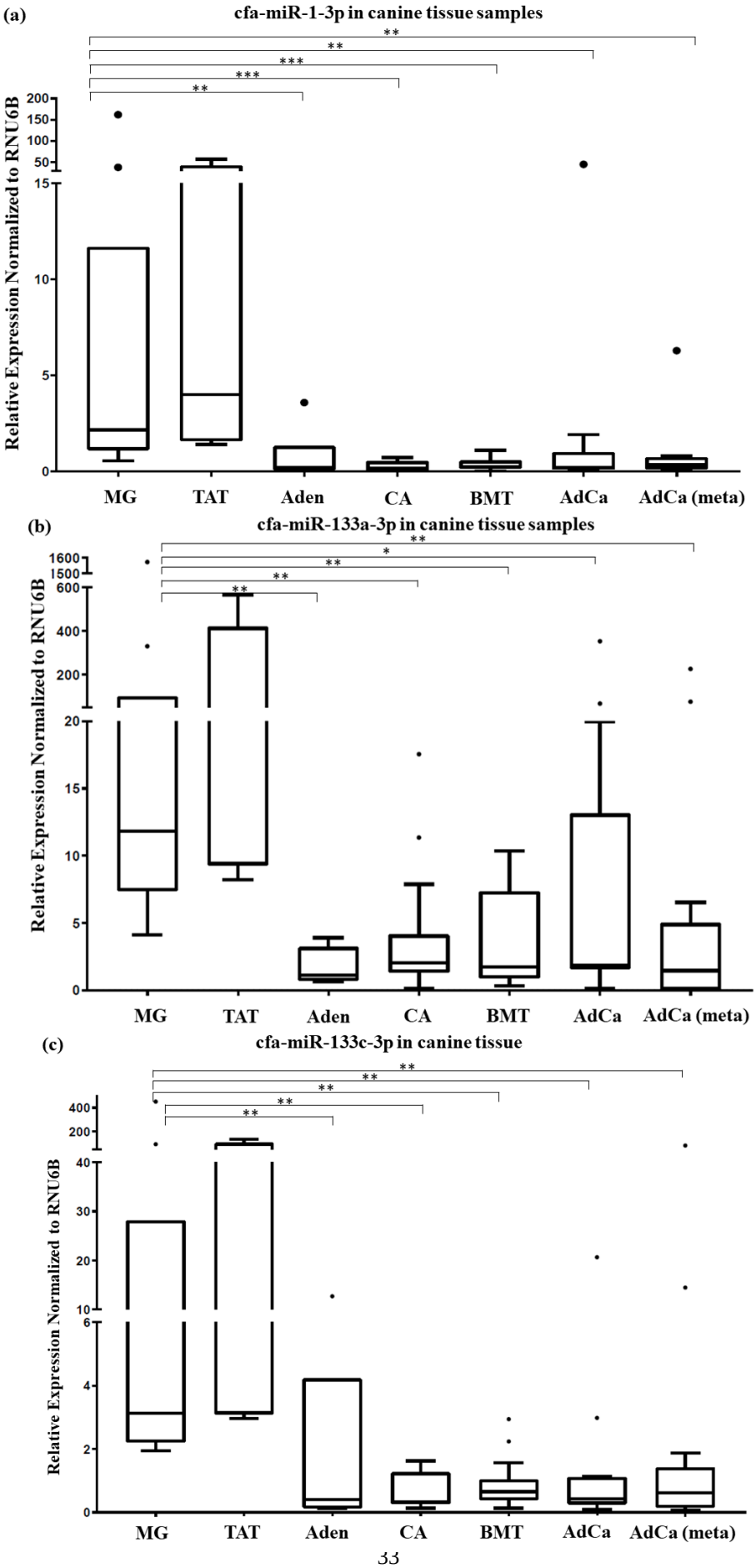
(a)
cfa-miR-1-3p in canine tissue samples
Relative Expression Normalized to RNU6B
MG
TAT
Aden
CA
BMT
AdCa
AdCa (meta)
(b)
cfa-miR-133a-3p in canine tissue samples
Relative Expression Normalized to RNU6B
MG
TAT
Aden
CA
BMT
AdCa
AdCa (meta)
(c)
cfa-miR-133c-3p in canine tissue
Relative Expression Normalized to RNU6B
MG
TAT
Aden
CA
BMT
AdCa
AdCa (meta)

Figure ch1-3. Relative expressions of cfa-miR-1-3p, cfa-miR-133a-3p and cfa-miR-133c-3p in canine mammary gland tumors by real-time PCR. * $p < 0.05$, ** $p < 0.01$, ***$p<0.001$. One-Way ANOVA (nonparametric), and Krukal-Wallis test (MG, mammary gland; TAT, tumor adjacent tissue; Aden, adenoma; CA, complex adenoma; BMT, benign mixed tumor; AdCa, adenocarcinoma; AdCa(meta), metastasized adenocarcinoma.)

Table ch1-1. Demographic information on MGT and normal tissue donors

Diagnosis	Breed	Age (year)	Sex (M/F)	Neutering (Y/N)	Number
Mammary gland	Pure Breed	Unknown	Unknown	Unknown	7
		<10.5	F	N	1
		>10.5	F	N	2
Tumor-adjacent Tissue	Pure Breed	<10.5	F	Y	1
			F	N	2
		>10.5	F	N	2
	Mixed	<10.5	F	N	1
Adenoma	Pure Breed	<10.5	F	N	2
		>10.5	F	Y	1
			F	N	2
	Mixed	>10.5	F	Y	1
Benign Mixed Tumor	Pure Breed	<10.5	F	N	10
		>10.5	F	Y	2
			F	N	16
	Mixed	<10.5	F	Y	2
		>10.5	F	N	2
Complex Adenoma	Pure Breed	<10.5	F	N	2
		>10.5	F	N	6
	Mixed	>10.5	F	N	1
Adenocarcinoma	Pure Breed	<10.5	F	Y	2
			F	N	2
		>10.5	F	Y	3
			F	N	1
Complex Carcinoma	Pure Breed	>10.5	F	Y	2
	Mixed	<10.5	F	N	1
Tubulopapillary Carcinoma	Pure Breed	>10.5	F	N	2
Adenocarcinoma (metastasized)	Pure Breed	<10.5	F	unknow	1
		>10.5	F	Y	1
	Mixed	unknow	F	Y	1
Carcinoma (metastasized)	Pure Breed	<10.5	F	unknow	1
		>10.5	F	N	2
	Mixed	>10.5	F	unknow	3
Complex Carcinoma (metastasized)	Pure Breed	>10.5	F	Y	1
Osteosarcoma (metastasized)	Pure Breed	>10.5	F	N	1
Tubulopapillary Carcinoma (metastasized)	Pure Breed	>10.5	F	Y	2
		>10.5	F	N	2
	Mixed	>10.5	F	N	1

*The record for neutering status was the status before the dog subjected to the tumor removal.

Table ch1-2. Partial list of differentially expressed miRNAs in canine mammary gland tumors

Comparator	miRNA	Fold change	p-value	FDR-p value	Expression level in comparator
Adenoma	cfa-miR-133c-3p	-123.13	1.58E-07	3.26E-05	down
	cfa-miR-133a-3p	-105.86	1.98E-07	3.26E-05	down
	cfa-miR-133b-3p	-213.98	6.45E-07	7.09E-05	down
	cfa-miR-1-3p	-127.62	4.17E-06	3.44E-04	down
	cfa-miR-377-5p	-1234.98	8.06E-05	5.32E-03	down
	cfa-miR-485-5p	-63.94	1.43E-04	7.86E-03	down
	cfa-miR-196b-5p	-15.63	2.17E-04	1.02E-02	down
	cfa-miR-338-3p	-59.06	3.56E-04	1.40E-02	down
	cfa-miR-379-5p	-40.88	3.82E-04	1.40E-02	down
	cfa-miR-208b-3p	-16.31	5.02E-04	1.66E-02	down
Benign mixed tumor	cfa-miR-133b-3p	-111.12	4.17E-06	6.88E-04	down
	cfa-miR-1-3p	-112.53	4.17E-06	6.88E-04	down
	cfa-miR-133a-3p	-46.05	6.35E-06	6.99E-04	down
	cfa-miR-133c-3p	-45.09	9.17E-06	7.57E-04	down
	cfa-miR-138a-5p	27.14	4.80E-04	3.17E-02	up
	cfa-miR-208b-3p	-15.05	6.55E-04	3.60E-02	down
	cfa-miR-216b-5p	293.15	7.88E-04	3.72E-02	up
Adenocarcinoma	cfa-miR-133b-3p	-99.82	5.85E-06	6.57E-04	down
	cfa-miR-133a-3p	-46.75	5.97E-06	6.57E-04	down
	cfa-miR-133c-3p	-52.76	4.91E-06	6.57E-04	down
	cfa-miR-1-3p	-41.69	1.05E-04	8.50E-03	down
	cfa-miR-338-3p	-89.34	1.29E-04	8.50E-03	down
	cfa-miR-99a-5p	-15.51	2.79E-04	1.49E-02	down
	cfa-miR-9-5p	60.37	3.16E-04	1.49E-02	up
	cfa-miR-1271-5p	-16.09	3.77E-04	1.55E-02	down
	cfa-miR-206-3p	-1260.38	0.00056	0.0205	down

*down: downregulated *up: upregulated

*Differentially expressed miRNAs identified with the DGE *empirical analysis* tool.

Table ch1-3. Partial list of cfa-miR-1-3p and cfa-miR-133 family gene targets with TargetScan.

miRNA	Ortholog of target gene	Representative transcript	Cumulative weighted context++ score
cfa-miR-1	SMIM14	ENST00000295958.5	-1.1
	SERP1	ENST00000239944.2	-0.92
	GJA1	ENST00000282561.3	-0.88
	MMD2	ENST00000406755.1	-0.75
	BDNF	ENST00000439476.2	-0.67
	SRSF9	ENST00000229390.3	-0.66
	RIT2	ENST00000589109.1	-0.65
	GPR137C	ENST00000321662.6	-0.6
cfa-miR-133 family	LHFP	ENST00000379589.3	-1.06
	SEC61B	ENST00000498603.1	-1.01
	CETN3	ENST00000283122.3	-0.89
	TAGLN2	ENST00000368096.1	-0.88
	PTBP1	ENST00000350092.4	-0.86
	CNN2	ENST00000263097.4	-0.82
	DAPL1	ENST00000309950.3	-0.8
	CLTA	ENST00000433436.2	-0.79
	TFAP2D	ENST00000008391.3	-0.77
	TIMM17A	ENST00000367287.4	-0.75
	VKORC1	ENST00000300851.6	-0.7
	ZNF354A	ENST00000335815.2	-0.7
	PPP2CA	ENST00000481195.1	-0.67
	STOM	ENST00000286713.2	-0.67
	CNTNAP1	ENST00000264638.4	-0.66
	PRRT2	ENST00000300797.6	-0.66
	EFNA4	ENST00000368409.3	-0.65
	C12orf43	ENST00000536407.2	-0.65
	PPP2CB	ENST00000221138.4	-0.65
	GABPB2	ENST00000368918.3	-0.64
	SUMO1	ENST00000392246.2	-0.64
	PSMD10	ENST00000372296.1	-0.63
	SIMC1	ENST00000443967.1	-0.63
	TPM4	ENST00000300933.4	-0.63
	GDNF	ENST00000326524.2	-0.62
	ZIC3	ENST00000287538.5	-0.6

*Cfa-miR-133a-3p, cfa-miR-133b-3p, and cfa-miR-133c-3p were classified into cfa-miR-133 family, sharing the same results. *Only the targets with cumulative weighted context++ scores lower than -0.6 are included in this partial list.

Table ch1-4. Partial list of cfa-miR-1-3p and cfa-miR-133 family from GO analysis.

miRNA	Category	Term	Counts	%	p-value	FDR-p
cfa-miR-1-3p	GOTERM_BP	Positive regulation of transcription from RNA polymerase II promoter	53	6.35	1.37E-06	2.38E-03
		Protein transport	15	1.80	2.14E-06	3.73E-03
		Negative regulation of transcription from RNA polymerase II promoter	40	4.79	1.42E-05	0.02
		Transcription, DNA-templated	30	3.59	2.80E-04	0.49
		Actin cytoskeleton organization	12	1.44	7.70E-04	1.34
		Osteoblast differentiation	13	1.56	8.53E-04	1.48
		Palate development	11	1.32	2.22E-03	3.80
	GOTERM_CC	Nucleoplasm	103	12.34	2.16E-07	3.02E-04
		Golgi apparatus	50	5.99	4.70E-07	6.57E-04
		Nucleus	157	18.80	3.03E-05	0.04
		Nuclear membrane	18	2.16	1.49E-04	0.21
		Membrane	64	7.66	3.98E-04	0.55
		Nuclear chromatin	18	2.16	5.25E-04	0.73
		Ruffle	10	1.20	9.76E-04	1.36
		Focal adhesion	28	3.35	1.20E-03	1.66
		Cytosol	64	7.66	2.58E-03	3.54
		Lamellipodium	13	1.56	2.93E-03	4.02
	GOTERM_MF	RNA polymerase II core Promoter proximal region sequence-specific DNA binding	32	3.83	1.11E-05	0.02
		Transcriptional activator activity, RNA polymerase II core promoter proximal region sequence-specific binding	24	2.87	5.97E-05	0.09
		Chromatin binding	31	3.71	1.62E-04	0.23
		Histone acetyltransferase Activity	8	0.96	2.91E-04	0.42
		Protein transporter activity	10	1.20	1.56E-03	2.23
		DNA binding	9	1.08	1.63E-03	2.33
cfa-miR-133-3p	GOTERM_BP	Negative regulation of transcription from RNA	28	4.18	2.89E-03	4.82
	GOTERM_CC	Nucleus	133	19.85	8.42E-06	0.01
		Cytoplasm	128	19.10	2.00E-03	2.70
	GOTERM_MF	Protein tyrosine phosphatase activity	13	1.94	9.10E-05	0.13

Table ch1-4. Partial list of cfa-miR-1-3p and cfa-miR-133 family from GO analysis.

miRNA	Category	Term	Counts	%	p-value	FDR-p
		RNA polymerase II core promoter proximal region sequence-specific DNA binding	26	3.88	9.17E-05	0.13
		Metal ion binding	55	8.21	1.52E-03	2.13

*BP, Biological Process; CC, Cellular Component; MF, Molecular Function

Table ch1-5. Partial list of cfa-miR-1-3p and cfa-miR-133 family from KEGG pathway analysis

miRNA	KEGG pathway ID	Name	Count	%	p-value	FDR
cfa-miR-1-3p	cfa04015	Rap1 signaling pathway	24	2.87	9.59E-05	0.12
	cfa04520	Adherens junction	13	1.56	9.83E-05	0.13
	cfa04014	Ras signaling pathway	24	2.87	2.09E-04	0.27
	cfa05206	MicroRNAs in cancer	18	2.16	2.52E-04	0.32
	cfa05100	Bacterial invasion of epithelial cells	13	1.56	2.80E-04	0.36
	cfa04144	Endocytosis	24	2.87	5.49E-04	0.70
	cfa04360	Axon guidance	16	1.92	6.51E-04	0.83
	cfa04390	Hippo signaling pathway	18	2.16	6.72E-04	0.86
	cfa05231	Choline metabolism in cancer	14	1.68	6.78E-04	0.86
	cfa04921	Oxytocin signaling pathway	17	2.04	1.38E-03	1.75
	cfa05030	Cocaine addiction	9	1.08	1.58E-03	2.00
	cfa05202	Transcriptional misregulation in cancer	17	2.04	1.97E-03	2.49
	cfa04022	cGMP-PKG signaling pathway	17	2.04	2.74E-03	3.45
	cfa04720	Long-term potentiation	10	1.20	3.11E-03	3.90
	cfa05203	Viral carcinogenesis	19	2.28	3.82E-03	4.78
cfa-miR-133-p	cfa04350	TGF-beta signaling pathway	12	1.79	1.37E-04	0.17
	cfa04721	Synaptic vesicle cycle	9	1.34	1.94E-03	2.45
	cfa04071	Sphingolipid signaling pathway	12	1.79	4.06E-03	5.05

Table ch1-6. Partial list (FDR <0.05) from GO analysis with input of all mRNA targets of cfa-miR-1-3p and cfa-miR-133 family

Category	Term	Count	%	p-value	FDR
GOTERM_BP	protein transport	20	1.40	7.59E-07	1.44E-03
	negative regulation of transcription from RNA polymerase II promoter	62	4.35	9.17E-07	1.44E-03
	positive regulation of transcription from RNA polymerase II promoter	75	5.27	8.59E-06	8.98E-03
GOTERM_CC	nucleus	271	19.03	6.06E-09	3.15E-06
	nucleoplasm	155	10.88	4.65E-07	1.21E-04
	Golgi apparatus	68	4.78	9.20E-06	1.59E-03
	cytosol	111	7.79	2.35E-05	3.05E-03
	nuclear membrane	25	1.76	1.13E-04	0.01
	cytoplasm	257	18.05	4.75E-04	4.11E-02
GOTERM_MF	RNA polymerase II core promoter proximal region sequence-specific DNA binding	52	3.65	3.50E-08	2.50E-05
	transcriptional activator activity, RNA polymerase II core promoter proximal region sequence-specific binding	36	2.53	7.57E-06	2.70E-03
	protein tyrosine phosphatase activity	20	1.40	3.56E-05	8.48E-03
	chromatin binding	45	3.16	1.70E-04	0.03

*BP, Biological Process; CC, Cellular Component; MF, Molecular Function

Table ch1-7. Partial list (FDR <0.05) from KEGG pathway analysis with input of all mRNA targets of cfa-miR-1-3p and cfa-miR-133 family

KEGG pathway ID	Term	Count	%	p-value	FDR-p
cfa04390	Hippo signaling pathway	30	2.11	3.55E-06	4.93E-04
cfa04520	Adherens junction	19	1.33	4.87E-06	4.93E-04
cfa04144	Endocytosis	39	2.74	7.23E-06	4.93E-04
cfa04350	TGF-beta signaling pathway	20	1.40	9.13E-06	4.93E-04
cfa04015	Rap1 signaling pathway	35	2.46	1.90E-05	8.19E-04
cfa04014	Ras signaling pathway	36	2.53	2.37E-05	8.51E-04
cfa05206	MicroRNAs in cancer	26	1.83	5.33E-05	1.64E-03
cfa05202	Transcriptional misregulation in cancer	26	1.83	2.36E-04	6.36E-03
cfa04721	Synaptic vesicle cycle	15	1.05	3.02E-04	7.26E-03
cfa04360	Axon guidance	22	1.54	4.90E-04	0.01
cfa04728	Dopaminergic synapse	22	1.54	7.56E-04	0.01
cfa05030	Cocaine addiction	12	0.84	8.76E-04	0.01
cfa04071	Sphingolipid signaling pathway	21	1.47	9.11E-04	0.01
cfa05100	Bacterial invasion of epithelial cells	16	1.12	9.22E-04	0.01
cfa04730	Long-term depression	13	0.91	1.85E-03	0.03
cfa04152	AMPK signaling pathway	20	1.40	2.48E-03	0.03
cfa04010	MAPK signaling pathway	33	2.32	2.72E-03	0.03
cfa05220	Chronic myeloid leukemia	14	0.98	3.17E-03	0.04
cfa04720	Long-term potentiation	13	0.91	3.73E-03	0.04
cfa05200	Pathways in cancer	46	3.23	3.96E-03	0.04
cfa05205	Proteoglycans in cancer	27	1.90	4.25E-03	0.04
cfa04912	GnRH signaling pathway	15	1.05	4.28E-03	0.04
cfa04921	Oxytocin signaling pathway	22	1.54	4.38E-03	0.04

1.6. Discussion

As a major novel finding in this study, I identified 46 differentially expressed miRNAs across histological subtypes of MGT through a comparison with normal canine mammary gland tissue in my initial NGS profiling. The histological subtypes used in this profiling were benign mixed tumor, adenoma, and adenocarcinoma, and I found four miRNAs (cfa-miR-1-3p, cfa-miR-133a-3p, cfa-miR-133b-3p, and cfa-miR-133c-3p) significantly downregulated across all three histological subtypes. I regarded them as the principal miRNAs of interest for the rest of my study.

My next step was to confirm the differential expression of these miRNAs of interest in large-scale qPCR analyses. However, I predicted that cfa-miR-133b-3p would be undetectable in qPCR analysis, based on its low normalized expression value (<50) in NGS analysis, so I focused on the other three miRNAs of interest for the qPCR confirmatory analysis. The large-scale qPCR analysis revealed significant downregulations of cfa-miR-1-3p, cfa-miR-133a-3p, and cfa-miR-133c-3p across a range of MGT tissue relative to normal and tumor-adjacent mammary gland tissue, with no significant difference in expression between the latter two (non-tumor) tissue types. The histological subtypes in the qPCR

analysis were adenoma, complex adenoma, benign mixed tumor, adenocarcinoma, and metastasized adenocarcinoma.

My miRNAs of interest can be functionally classified as members of the miR-1/206 and miR-133 families, based on their seed sequences, and are suggested to be muscle-specific miRNAs (Kusakabe et al., 2013; Xie et al., 2016). My findings for these miRNAs showed some consistencies with previous reports from both canine and human medicine. Cfa-miR-1-3p showed a similar pattern of expression in canine oral melanoma in previous research in my laboratory (Rahman et al., 2019). Reportedly downregulated miRNAs in cancer include the human homologue of miR-1 in thyroid carcinomas, squamous cell carcinomas, colorectal cancer, and osteosarcoma (Gourbault andLlobat, n.d.; Leonardo et al., 2018; Leone et al., 2011; Nohata et al., 2012, 2011; Novello et al., 2013; Pidíkova et al., 2020). Other reports of downregulation include miR-133a-3p and miR-133b-3p in colorectal cancer samples and miR-133b in osteosarcoma samples (Gourbault andLlobat, n.d.; Leonardo et al., 2018; Nohata et al., 2012; Novello et al., 2013; Pidíkova et al., 2020). MiR-1 has been identified as a tumor suppressor for thyroid carcinoma, targeting CCND2, CXCR4, and SDF-1α (Leone et al., 2011). Differential miR-1 expression is reportedly elevated in stage IV human breast carcinoma (relative to stage I–III cases) and this miRNA's association with

more aggressive phenotypes suggests it could be regarded as a prognostic factor (Minemura et al., 2015). miR-133 is reportedly downregulated in breast cancer samples (along with miR-25; based on RT-qPCR analyses of serum), and has shown significant associations with clinical stage, metastasis, and survival time (Hesari et al., 2019). Human miRNAs are considerably better described than their canine homologues, but this study represents an advance in the knowledge of the relevant miRNAs in canine MGTs.

My study also contributes to the evidence on target genes for miR-1 and miR-133 family members, although this evidence is far from complete, and much remains to be elucidated. Previous reports have identified target genes for these miRNAs and oncogenic effects of their dysregulation in both human and canine medicine, with the preponderance of evidence coming from the human field. Their target genes for the miR-1/MET pathway alone include MET and MCL1, with dysregulation implicated in canine and human osteosarcoma, as well as canine and human hepatocellular carcinomas (Datta et al., 2008; Gourbault andLlobat, n.d.; Lai et al., 2018; Leonardo et al., 2018; Nohata et al., 2012; Novello et al., 2013). miR-133a-3p reportedly regulates the LASP1 and TGF-β/Smad3 signaling pathways, with its dysregulation implicated in human lung cancer (Shen et al., 2020). miR-133b-3p also targets CXCR4, EGFR, and

HOXA9 in the human field (Wang et al., 2017; Zhang et al., 2020; Zhou et al., 2015).

My key findings from GO and KEGG pathway enrichment suggest that cfa-miR-1 and the three cfa-miR-133 family members may regulate RNA polymerase II core promoter transcription. I speculate that the downregulation of these miRNAs causes abnormalities in this transcription, with an oncogenic effect on previously normal mammary gland cells. Furthermore, an adverse effect on the initiation of transcription might explain why MGTs are more diverse histologically than other carcinomas; such a phenomenon may emerge as structural mutations are triggered for a number of proteins. In my future research, I plan to evaluate a range of histologically diverse MGTs, and further characterize the impacts of miR-1 and miR-133 family downregulation on mammary gland cells with KEGG analysis. Ultimately, I aim to identify the oncogenic mechanism for canine MGTs, thus enabling the development of new therapeutic strategies.

The other miRNAs in my NGS dataset, which were differentially expressed in only one or two histological subtypes, showed both consistencies and inconsistencies with previous reports. The only upregulated miRNA in my dataset, cfa-miR-9-5p (in adenocarcinoma) is reportedly also upregulated in canine malignant melanoma (Ushio et al., 2019). I found cfa-miR-338-3p to be

significantly downregulated in both adenoma and adenocarcinoma, but had previously found this miRNA to be upregulated in canine oral melanoma samples (Ushio et al., 2019). I found downregulations of cfa-miR-485-5p and cfa-miR-138a-5p in adenoma and benign mixed tumor samples, respectively, and these expression patterns are consistent with previously reported expression patterns in exosomes collected from cell cultures of canine mammary epithelial cell and canine MGT cell lines (Fish et al., 2018). However, Fish et al. reported upregulated cfa-miR-9-5 and downregulated cfa-miR-99a in adenocarcinoma samples, and downregulated cfa-miR-196b-5b in adenoma samples (Fish et al., 2018), the opposite of the expression patterns I found. Direct comparisons with my study may be difficult because of the different designs, and further studies designed to answer carefully constructed research questions are required to throw further light on this area.

NGS sequencing allows genome transcriptomes and miRNA profiles to be developed, with concomitant breakthroughs in the field of oncology (Amini et al., 2019; Ettlin et al., 2017; Huskey et al., 2020; Kim et al., 2019; Lee et al., 2018). Human-breast-cancer-associated miRNAs in canine MGTs have featured in a range of previous studies (Bulkowska et al., n.d.; Fish et al., 2020, 2018; Jeong et al., 2019; Yu andCheah, 2017). My study builds upon this previous knowledge

and is the first to generate an NGS dataset for miRNAs differentially expressed in canine MGTs.

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Chapter 2.

NGS-identified miRNAs in Canine Mammary Gland Tumors Show Unexpected Expression Alterations in qPCR Analysis

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2.1. Abstract

Canine mammary gland tumors (MGTs), as a potential model of human breast cancer, have a well-defined histological classification system. MicroRNA (miRNA) expression is a key part of the molecular signatures of both MGTs and human breast cancer, although the signatures alone do not yet provide a sufficient basis for definitive diagnosis. In this study, I investigated the association between miRNA expression patterns and histological classification.

Mammary gland tissue was collected from healthy dogs (n=7) and dog patients (n=80). Further samples (n=5) were obtained from established MGT cell lines. I targeted miRNAs differentially expressed in metastatic tumor tissue versus non-metastatic and normal tissue. A subset of samples was analyzed using small RNA next generation sequencing (NGS) with subsequent qPCR. Six differentially expressed miRNAs were selected from the NGS analysis and submitted for large-scale qPCR. The large-scale qPCR analysis revealed greater alternations in miRNA expression. Large-scale analysis, based on 79 samples, revealed a hierarchical clustering based on selected miRNAs that did not strikingly match the histopathological subtype classification. To conclude, I successfully investigated the large-scale miRNA expression pattern in canine MGT and provided the whole miRNA expression. The selected miRNA demonstrated that

there is no straightforward mapping between molecular signatures and histological classification of canine MGTs at the miRNA level.

2.2. Introduction

Canine mammary gland tumors (MGTs) are attracting attention in genetic research, with microRNAs (miRNAs) regarded as a fruitful line of investigation. Canine MGTs share many features with human breast cancer, including histological and biological behavior as well as molecular features, thus they should be fully established as a suitable model of human breast cancer (Gordon et al., 2009; Parker et al., n.d.). Molecular signatures encompass miRNAs, small noncoding RNAs that play a role in gene regulation through mRNA silencing (Ambros, 2004; Bartel, 2004; Saliminejad et al., 2019). Identifying miRNAs that are differentially expressed in certain disease conditions may reveal molecules of interest for the diagnosis and treatment of these conditions.

In veterinary oncology, miRNAs have been examined in other tumor types. Furthermore, similarities in miRNA expression pattern between human breast cancers and canine MGTs have been noted (Boggs et al., 2008, 2007; vonDeetzen et al., 2014), and research on miRNAs in human breast cancer has identified many potential therapeutic and diagnostic targets.

In my previous study, I used NGS followed by large-scale qPCR to investigate miRNAs differentially expressed in canine MGTs relative to normal mammary gland tissue (Chen et al., 2022). Furthermore, differences in microRNA

expression between non-metastatic and metastatic canine MGTs have already been reported in a microarray-based study (Bulkowska et al., 2017), suggesting their expression profiles differ between at least some histological subtypes and indicating their potential as histological-subtype-specific biomarkers.

In this study, I identified miRNAs differentially expressed between non-metastatic and metastatic mammary gland tissue by using NGS. I then proceeded to investigate the relevant miRNA expression patterns through real-time PCR and hierarchical clustering.

2.3. Materials and Methods

2.3.1. Sample collection

The canine mammary gland samples targeted for real-time PCR and NGS analyses in this study were from the same pool of tissue samples described in Chapter 1 (Chen et al., 2022), and relevant information on these samples covering the breed and age of the relevant dog, and the diagnosis for the relevant case is presented in Table ch2-1.

Pathological diagnosis. Histopathological specimens were prepared by hematoxylin and eosin (H&E) staining of collected tissue, and H&E-stained specimens were submitted for microscopic examination by a certified veterinary pathologist. All pathologists were engaged in examining samples for this study have a diploma from the Japanese College of Veterinary Pathologists), and used common, published standards for the classification and grading of canine MGTs and Surgical Pathology of Tumors of Domestic Animals Volume 2: Mammary Tumors in making their diagnoses (Goldschmidt et al., 2011; Zapulli et al., 2019). Specifically, these standards were used to classify the tumor tissue samples by type and/or subtype. Furthermore, tumor was regarded as metastasized when tumor cells were found in the removed lymph nodes, and/or

imaging of the lung revealed regions of mass. Tumor tissue samples not meeting these criteria were regarded as non-metastatic for the purposes of this study. Each dog in this study underwent imaging with radiography and/or ultrasound, and in most of cases full-body Computed Tomography, for veterinary assessment.

2.3.2. Total RNA extraction and sequencing

Total RNA was extracted evaluated by using the same approach as Chapter 1. Briefly to describe, total RNA was extracted using a mirVana™ miRNA isolation kit. RNA concentration was measured using NanoDrop 2000c. The quality of RNA was assessed using the 2100 Bioanalyzer System. Samples with an RNA Integrity Number (RIN) exceeding eight were allocated for analysis with NGS, and samples with a miRNA region in the 2100 Bioanalyzer System were allocated for analysis with qPCR. Small RNA libraries were prepared and sequenced by Hokkaido System Science Co., Ltd. The libraries were subjected to 100-bp paired-end sequencing on an Illumina HiSeq 2500 System (Illumina).

2.3.3. Availability of data

Sequence reads for mammary gland (n=3), adenomas (n=3), adenocarcinomas (n=3), were submitted to the sequence read archive (SRA) (www.ncbi.nlm.nih.gov/sra) under the Bioproject accession number; PRJNA716131. Sequence reads for metastasized adenocarcinomas (n=6) were submitted to the SRA under the Bioproject accession number; PRJNA738308.

2.3.4. Processing small RNA sequencing data

Small RNA sequences were processed and analyzed using CLC Genome Workbench 10.1.1 (CLC bio, Cambridge, MA, USA). The sequence reads were annotated against miRBase (release 21) (Kozomara et al., 2019; Kozomara andGriffiths-Jones, 2014, 2011), Ensembl canine ncRNA database (Canis familiris.canfam3.1.ncrna) ("Canis_lupus_familiaris - Ensembl genome browser 102," n.d.; Hunt et al., 2018; Yates et al., 2020). miRBase was prioritized over other annotation resources. Small RNAs differentially expressed in the tumor tissue were identified with the empirical analysis of DGE tool in CLC Genome Workbench 10.1.1.

2.3.5. Quantification of miRNAs with qPCR

Total RNA (2 ng/µl) was reverse-transcribed to cDNA with TaqMan miRNA assays (Thermo Fisher Scientific), in accordance with description in Chapter 1. TaqMan miRNA assays used for qPCR in this study and their assay IDs were as follows: cfa-miR-187-3p (ID: 001193), cfa-miR-202-5p (ID: 002362), cfa-miR-424-5p (ID: 002309), cfa-miR-450a-5p (ID: 001031), cfa-miR-450b-5p (ID: 006407_mat), and cfa-miR-542-3p (ID: 001284).

2.3.6. Statistical analysis

qPCR data were analyzed to create a graphical representation using GraphPad Prism 7 (GraphPad Software, San Diego, CA, USA). The qPCR data were analyzed by One-Way ANOVA (nonparametric) and were subjected to a Kruskal–Wallis test. Furthermore, box & whiskers plots were drawn in accordance with Tukey's definition to exclude outliers. Heatmap hierarchical cluster analysis was performed using the R statistical environment.

2.4. Results

2.4.1. miRNA expression profiles determined with NGS

In order to identify miRNAs that are potentially differentially expressed between canine MGT histological subtypes, I compared miRNA expression values between non-metastatic and metastatic canine mammary gland tissue, using NGS analysis. The samples targeted in this analysis were obtained from four different histological subtypes. The non-metastatic tissue comprised nine samples from my previous study (Chen et al., 2022); specifically, normal mammary gland (n=3; Sample Nos.: MG1-3), and adenoma (n=3; Aden1-3), and adenocarcinoma (n=3; AdCa1-3). The metastatic tissue consisted of six samples; specifically metastasized adenocarcinoma [n=6; AdCa(Meta)1-6].

A total of 39 miRNAs were significantly differentially expressed in metastatic tissue *vs*. non-metastatic tissue ($p<0.05$), of which 17 were upregulated and 22 were downregulated. The 39 miRNAs are listed in Table ch2-2.

Of these 39 miRNAs, I targeted those with a false-discovery-rate-adjusted *p*-value (FDP-*p*) <0.05 and detectable normalized expression value >50 in the NGS for inclusion in subsequent investigations and qPCR analysis.

2.4.2. NGS-based heatmap hierarchical clustering of differentially expressed miRNAs

To visualize patterns in miRNA expression elucidated by NGS, the canine mammary gland tissue samples were then submitted for heatmap hierarchical clustering analysis. Metastasized adenocarcinoma was the most widely scattered subtype across the dendrogram for mammary gland tumor tissues, and mammary gland tissue was the most tightly clustered within the dendrogram (Figure ch2-1).

2.4.3. qPCR-based investigation of differential expression patterns of target miRNAs

I targeted some of the relevant miRNAs for further investigation in my full pool of tissue samples using qPCR analysis. The sample pool comprised 92 samples covering seven histological subtypes—mammary gland, tumor-adjacent tissue, adenoma, complex adenoma, benign mixed tumor, adenocarcinoma, and metastasized adenocarcinoma. This analysis included target miRNAs meeting the selection criterion for NGS results described above (normalized mean expression value>50 and FDR-p<0.05). Of these eight miRNAs, two (cfa-miR-133a-3p and cfa-miR-133c-3p) had already undergone

the relevant analysis in my previous study (Chen et al., 2022). The other six target miRNAs (cfa-miR-187-3p, cfa-miR-202-5p, cfa-miR-424-5p, cfa-miR-450a-5p, cfa-miR-450b-5p, and cfa-miR-542-3p) were included in this analysis. The results obtained for each miRNA are presented graphically in box-and-whisker plots in Figure ch2-2.

cfa-miR-187-3p yielded similar results to cfa-miR-133a-3p and cfa-miR-133c-3p. However, cfa-miR-424-5p, cfa-miR-450a-5p, and cfa-miR-450b-5p showed more diverse expression patterns, with some sporadic differences between normal tissue and non-metastatic MGTs. miR-450b-5p and miR-542-3p showed a wider range of values for metastasized adenocarcinoma than other subtypes. No miRNA showed a significant difference in expression between normal mammary gland tissue and tumor-adjacent tissue.

2.4.4. qPCR-based heatmap hierarchical clustering of target miRNAs (cfa-miR-187-3p, cfa-miR-202-5p, cfa-miR-424-5p, cfa-miR-450a-5p, cfa-miR-450b-5p, and cfa-miR-542-3p)

To visualize patterns in miRNA expression elucidated in real-time PCR, data from 79/92 samples in this study were then submitted for heatmap hierarchical clustering analysis based on adjusted threshold cycle (-ΔΔCt) values for the

target. In the resultant dendrogram for the target miRNAs, clusters are labeled from A to E (Figure ch2-3).

The numbers of samples in the five clusters were not uniform: Cluster A contained 32 samples, Cluster B 7 samples, Cluster C 22 samples, Cluster D 17 samples, and Cluster E one sample (Table ch2-3). Some samples were also described in Figure ch2-1, which were marked in grey. The annotations for the samples were provided in Table ch2-4. These sample numbers imposed limitations on direct percentagewise comparisons between the subtypes in each cluster, so my evaluation focused on the absence of particular subtypes from a cluster (instances of n=0 for a particular miRNA).

Cluster A contained samples from all tumor subtypes, but no samples of normal or tumor-adjacent tissue, and resembled Cluster D in its tumor subtype composition, apart from the small proportion of benign mixed tumor samples, and the single mammary gland sample. In contrast, Cluster C contained mostly non-tumor samples (normal and tumor adjacent tissue) but lacked adenoma and complex adenoma samples. The composition of Cluster B was similar to that of Cluster C, although it contained seven samples, a smaller number than Cluster C. The most markedly distinct cluster was Cluster E, because it contained only one sample of metastasized adenocarcinoma.

The qPCR-based heatmap hierarchical clustering yielded a markedly greater scattering of samples than that based on NGS, with both non-tumor and tumor tissue subtypes represented across a wider range of clusters and in different proportions within clusters, thus indicating the existence of molecular diversity across the histological classification system for canine MGTs.

2.5. Figures and Tables

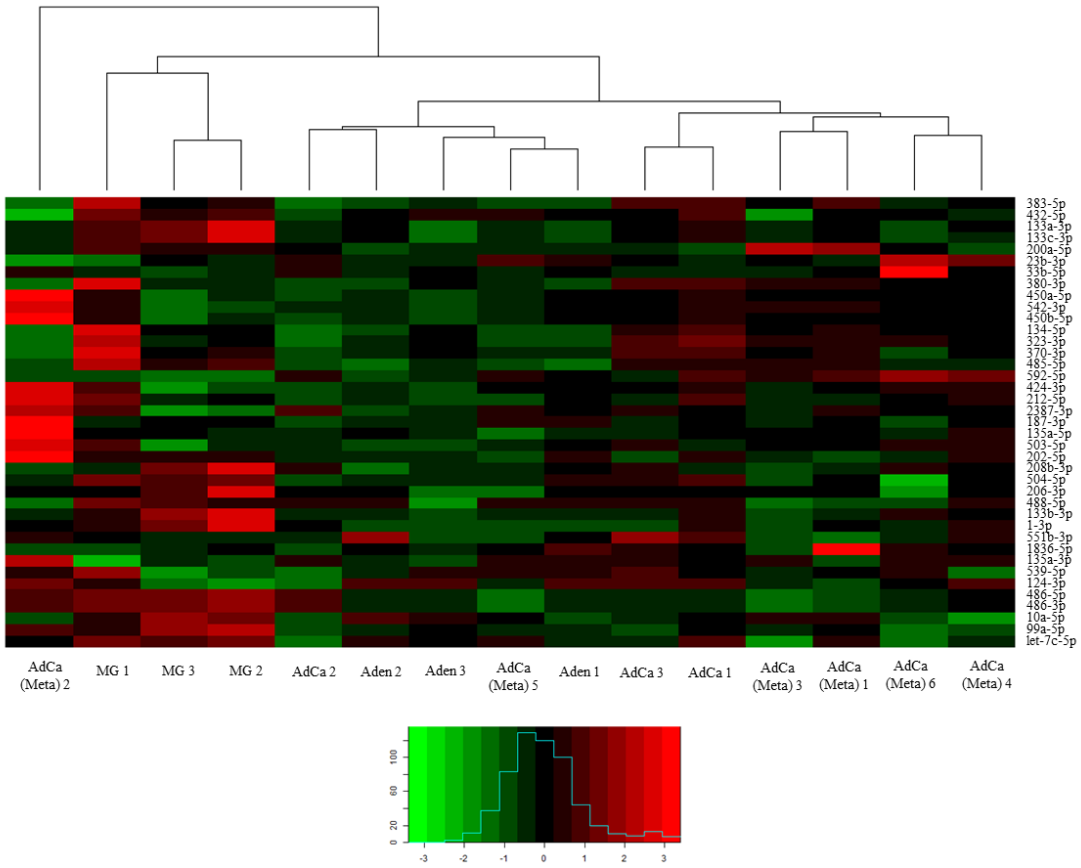

Figure ch2-1. Heatmap hierarchical clustering of differentially expressed miRNAs in canine mammary gland tumor tissue samples using Next Generation Sequencing. The numbers on the Y axis are miRNAs in Table ch2-2. The samples on the X axis are tissue samples used in Non-Meta vs. Meta comparison. AdCa(meta) scatters in the different clusters, while all the mammary gland samples (MG 1, MG 2, MG 3) are classified into the same cluster. The expression values were normalized (reads per millions) and transformed by log for graphing this figure in R. MG: Mammary gland; Aden: adenoma; AdCa: adenocarcinoma; AdCa(meta): metastasized adenocarcinoma.

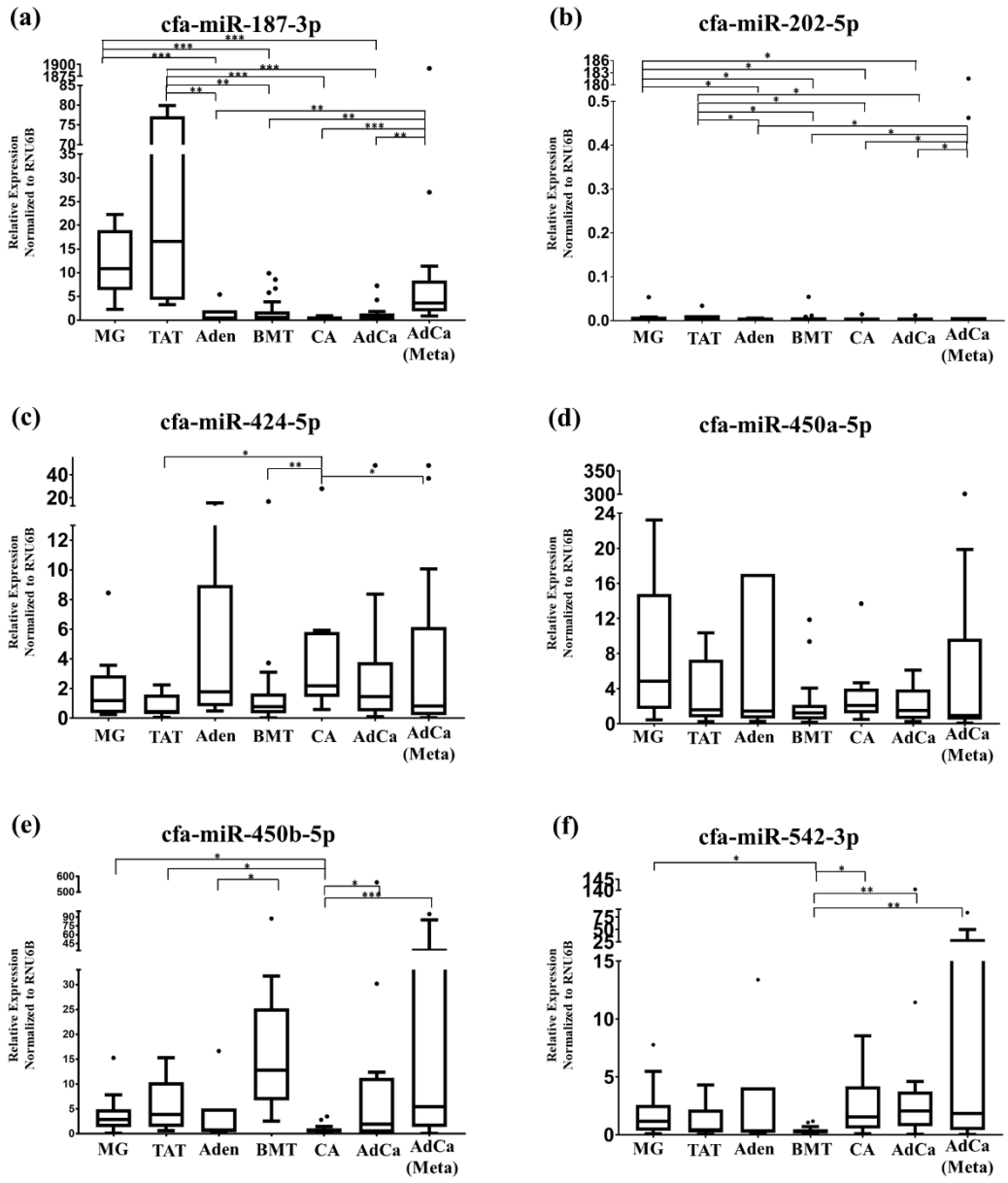

Figure ch2-2. Relative expression values of cfa-miR-187-3p, cfa-miR-202-5p, cfa-miR-424-5p, cfa-miR-450a-5p, cfa-miR-450b-5p, and cfa-miR-542-3p in canine mammary gland tumors by real-time PCR. The six target miRNAs were found to be upregulated in the metastatic group using NGS. However, real-time PCR revealed more diverse expression across tumor subtypes that was predicted from the NGS data. *p<0.05, **p<0.01, ***p<0.001. MG: Mammary gland; TAT: tumor adjacent tissue; Aden: adenoma; CA: complex adenoma; BMT: benign mixed tumor; AdCa: adenocarcinoma; AdCa(meta); metastasized adenocarcinoma.

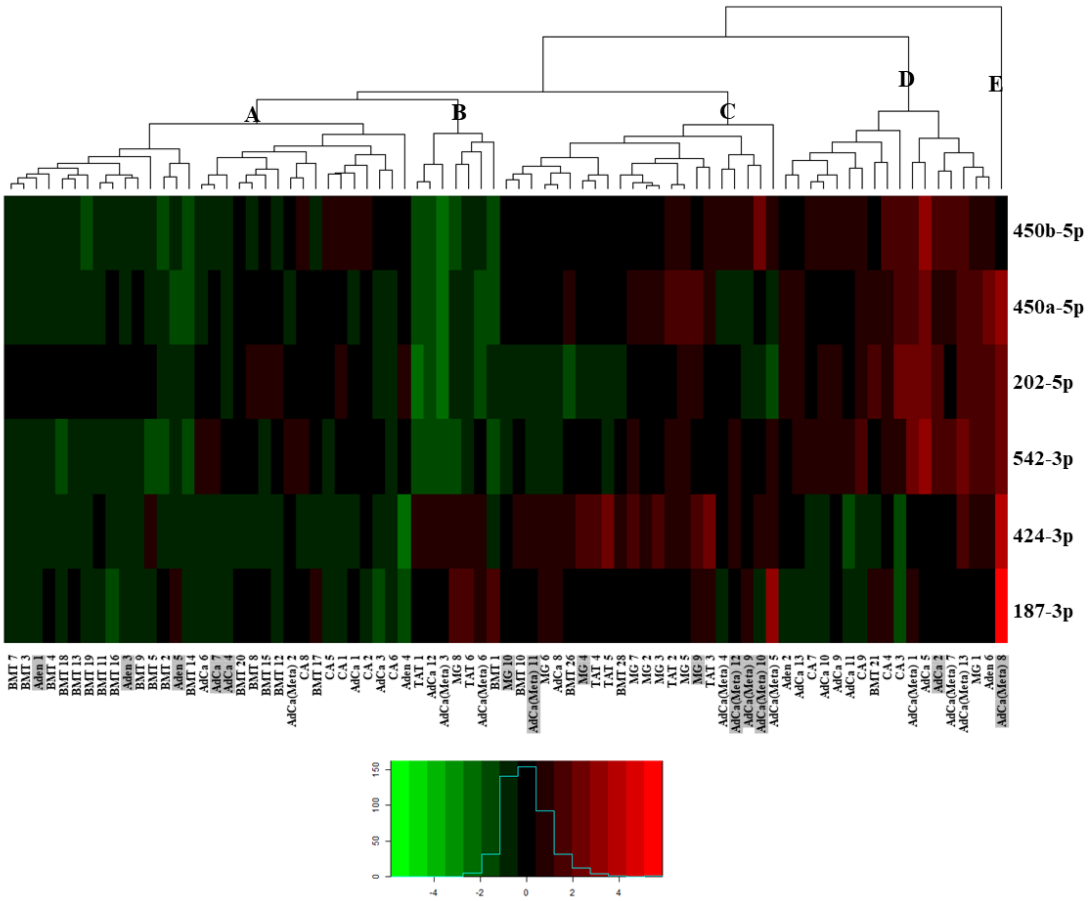

Figure ch2-3. Heatmap hierarchical clustering of miRNAs in canine mammary gland tumor tissue samples analyzed using real-time PCR. The five clusters identified in the hierarchy were designated as Clusters A-E. The number of samples in each cluster is stated in Table ch2-3. The Ct values were adjusted using RNU6B, and the -ΔΔCt values were used for graphically representing figures in R. The samples that are also described in Figure ch2-1 are marked in grey (the annotation for the samples is provided in Table ch2-4). MG: Mammary gland; TAT: tumor adjacent tissue; Aden: adenoma; CA: complex adenoma; BMT: benign mixed tumor; AdCa: adenocarcinoma; AdCa(meta): metastasized adenocarcinoma.

Table ch2-1. Demographic information on MGT and normal tissue donors

Diagnosis	Breed	Age (year)	Sex (M/F)	Neutering (Y/N)	Number
Mammary gland	Pure Breed	Unknown	Unknown	Unknown	7
		<10.5	F	N	1
		>10.5	F	N	2
Tumor-adjacent Tissue	Pure Breed	<10.5	F	Y	1
			F	N	2
		>10.5	F	N	2
	Mixed	<10.5	F	N	1
Adenoma	Pure Breed	<10.5	F	N	2
		>10.5	F	Y	1
			F	N	2
	Mixed	>10.5	F	Y	1
Benign Mixed Tumor	Pure Breed	<10.5	F	N	10
		>10.5	F	Y	2
			F	N	16
	Mixed	<10.5	F	Y	2
		>10.5	F	N	2
Complex Adenoma	Pure Breed	<10.5	F	N	2
		>10.5	F	N	6
	Mixed	>10.5	F	N	1
Adenocarcinoma	Pure Breed	<10.5	F	Y	2
			F	N	2
		>10.5	F	Y	3
			F	N	1
Complex Carcinoma	Pure Breed	>10.5	F	Y	2
	Mixed	<10.5	F	N	1
Tubulopapillary Carcinoma	Pure Breed	>10.5	F	N	2
Adenocarcinoma (metastasized)	Pure Breed	<10.5	F	unknow	1
		>10.5	F	Y	1
	Mixed	unknow	F	Y	1
Carcinoma (metastasized)	Pure Breed	<10.5	F	unknow	1
		>10.5	F	N	2
	Mixed	>10.5	F	unknow	3
Complex Carcinoma (metastasized)	Pure Breed	>10.5	F	Y	1
Osteosarcoma (metastasized)	Pure Breed	>10.5	F	N	1
Tubulopapillary Carcinoma (metastasized)	Pure Breed	>10.5	F	Y	2
		>10.5	F	N	2
	Mixed	>10.5	F	N	1

*The record for neutering status was the status before the dog subjected to the tumor removal.

*This table was referred from *Micro RNA differential expression profile in canine mammary gland tumor by Next Generation Sequencing* (Chen et al., 2022).

Table ch2-2. List of differentially expressed miRNAs of Non-Meta *vs*. Meta comparison in canine mammary gland tumor samples analyzed using Next Generation Sequencing.

	p-Value	FDR-*p*	Fold change	Non-Meta Mean*	Meta Mean*
cfa-miR-202-5p	<0.001	<0.001	8375.97	0.06	478.79
cfa-miR-450a-5p	<0.001	<0.001	66.96	116.88	5898.46
cfa-miR-450b-5p	<0.001	<0.001	60.04	301.03	13227.17
cfa-miR-187-3p	<0.001	<0.001	54.47	1.68	78.88
cfa-miR-542-3p	<0.001	<0.001	21.65	67.89	1101.16
cfa-miR-503-5p	<0.001	0.001	22.14	1.65	27.04
cfa-miR-424-3p	<0.001	0.001	18.08	6.67	88.80
cfa-miR-135a-5p	<0.001	0.01	11.81	1.43	15.66
cfa-miR-133c-3p	<0.001	0.01	-33.96	202.03	7.90
cfa-miR-133a-3p	<0.001	0.01	-29.90	380.23	16.98
cfa-miR-1836-5p	<0.001	0.01	12.42	0.40	6.20
cfa-miR-133b-3p	<0.001	0.01	-47.55	14.07	0.42
cfa-miR-1-3p	<0.001	0.06	-17.94	5.45	0.38
cfa-miR-370-3p	<0.001	0.06	-8.70	37.56	4.00
cfa-miR-33b-5p	0.01	0.14	3.46	32.07	133.09
cfa-miR-592-5p	0.01	0.16	4.62	3.28	14.99
cfa-miR-206-3p	0.01	0.16	-61.67	2.04	0.05
cfa-miR-383-5p	0.01	0.16	-3.88	58.99	17.84
cfa-miR-504-5p	0.01	0.16	-5.19	5.29	1.21
cfa-miR-208b-3p	0.01	0.16	-5.67	11.13	2.66
cfa-miR-551b-3p	0.01	0.19	-8.65	3.58	0.39
cfa-miR-124-3p	0.01	0.19	5.89	0.07	0.42
cfa-miR-134-5p	0.02	0.23	-5.94	45.70	7.24
cfa-miR-432-5p	0.02	0.26	-5.21	148.49	27.70
cfa-miR-488-5p	0.02	0.26	-9.55	0.71	0.08
cfa-miR-485-5p	0.02	0.30	-6.16	15.50	2.36
cfa-miR-323-3p	0.03	0.33	-4.96	30.16	5.45
cfa-miR-380-3p	0.03	0.36	-4.30	518.50	107.57
cfa-miR-200a-5p	0.03	0.36	3.02	12.32	42.91
cfa-miR-10a-5p	0.04	0.36	-2.47	41509.74	21464.78
cfa-let-7c-5p	0.04	0.36	-2.44	3316.92	1582.67
cfa-miR-486-3p	0.04	0.36	-2.81	16879.97	6678.89
cfa-miR-486-5p	0.04	0.36	-2.80	16974.90	6727.59
cfa-miR-2387-3p	0.04	0.38	3.20	3.91	10.95
cfa-miR-135a-3p	0.04	0.38	6.94	0.16	1.10
cfa-miR-212-5p	0.04	0.39	2.77	6.29	14.85
cfa-miR-99a-5p	0.05	0.40	-3.21	6895.19	2593.07
cfa-miR-23b-3p	0.05	0.40	2.37	37.71	104.46
cfa-miR-539-5p	0.05	0.40	-5.75	0.80	0.11

*The mean expression values are normalized by Reads per Million.

Table ch2-3. Number of samples in the clusters in Figure ch2-3.

	A	B	C	D	E	Total Sample Number
MG	0	1	8	1	0	10
TAT	0	2	4	0	0	6
Aden	4	0	0	2	0	6
BMT	17	1	3	1	0	22
CA	5	0	0	4	0	9
AdCa	5	1	1	6	0	13
AdCa(Meta)	1	2	6	3	1	13
Total	32	7	22	17	1	79

*MG: Mammary gland; TAT: Tumor adjacent tissue; Aden: adenoma; CA: Complex adenoma; BMT: Benign mixed tumor; AdCa: adenocarcinoma; AdCa(meta): metastasized adenocarcinoma.

Table ch2-4. Sample names annotations in Figure ch2-1 and Figure ch2-3.

Sample name	
in Fig. ch2-1	**in Fig. ch2-3**
MG 1	MG 9
MG 2	MG 4
MG 3	MG 10
Aden 1	Aden 1
Aden 2	Aden 3
Aden 3	Aden 5
AdCa 1	AdCa 2
AdCa 2	AdCa 4
AdCa 3	AdCa 7
AdCa(Meta) 2	AdCa(Meta) 8
AdCa(Meta) 3	AdCa(Meta) 12
AdCa(Meta) 4	AdCa(Meta) 10
AdCa(Meta) 5	AdCa(Meta) 11
AdCa(Meta) 6	AdCa(Meta) 9

2.6. Discussion

To my knowledge, this is the first study to investigate whole alternative miRNA expression in a range of histological subtypes of canine MGT by NGS. I used NGS to establish an initial profile of miRNA expression in tissue from dogs histologically diagnosed with MGT and sought to confirm these expression patterns using qPCR with a large number of clinical samples.

As the major novel finding of this study, I identified unexpectedly diverse levels of miRNA expression across tumor subtypes, based on the results for large-scale profiling with qPCR analysis. With the origin of the cancer metastasis yet to be fully elucidated and considering that metastasis may occur in any cellular stage (Fares et al., 2020; Seyfried andHuysentruyt, 2013), I initially set out to identify miRNAs for analysis by histological subtype through a comparison of metastatic MGT (metastasized adenocarcinoma) tissue with non-metastatic MGT (adenoma and adenocarcinoma) and normal mammary gland tissue using NGS. However, when comparing expression patterns of the identified miRNAs using qPCR with a large number of the clinical samples, I found a much more diverse expression than could be predicted from the smaller dataset obtained through NGS analysis (with a wider range of histological subtypes; normal mammary gland and tumor-adjacent tissue as non-tumor tissue, adenoma, complex adenoma,

benign mixed tumor, and adenocarcinoma as non-metastatic tumors, in addition to metastasized adenocarcinoma). The qPCR results were suggestive of a high level of miRNA expression diversity across a range of canine MGT subtypes.

Reports on similar variations in molecular expression already exist. Human breast cancer, a condition for which there is some consensus on molecular classification (ranging from luminal A, luminal B to HER2), reportedly shows such variation(Lusa et al., 2007; Weigelt et al., 2010), highlighting the importance for standardization of analytical methods for microarray-based breast cancer classification systems (Bombonati andSgroi, 2011). In the veterinary field, a microarray-based study identified distinct metastasis-related differences in the miRNA profile of canine mammary gland tumors, but the differential expression was not as marked in qPCR as it was in the microarray analysis (Bulkowska et al., 2017).

The limitation concerns the classification of non-metastatic tissue. I used a definition of “non-metastatic” in this study that applies only to what I could see in the mammary gland tissue samples. Each dog in this study underwent radiography and ultrasound examinations, and full-body CT scanning was also performed in the majority of cases. The results of these image-based veterinary assessments were fully consistent with the pathological diagnoses of non-

metastatic tissue. Therefore, my results can be regarded as valid for a comparison of metastatic-tumor-bearing and apparently non-metastatic-tumor-bearing mammary gland tissue, even without conclusively demonstrating the absence of metastasis.

Conclusion

In conclusion, I confirmed the relevant profiling through large-scale qPCR analyses with clinical samples. The four miRNAs, cfa-miR-1, cfa-miR-133a, cfa-miR as promising candidates for developing novel diagnostic and therapeutic approaches to canine MGTs. Furthermore, I reported the whole miRNA expression pattern in metastatic and non-metastatic MGT. Then, I regard the miRNA expression diversity across histological canine MGT subtypes as the principal point of interest. My findings support future studies of miRNA analysis comparing canine and human breast cancers, and shed some light on the issue of molecular classification systems for canine MGTs established through miRNA profiling.

Further investigations are required to clarify their molecular functions, and more broadly to determine whether the expression profiles of mRNAs match predictions based on these findings.

Compliance with ethical standards

The study design and experimental protocols were approved by the university and the Kagoshima University Veterinary Teaching Hospital Ethics Committee (Approval No. KV0004). All procedures in this study accorded with the ethics by laws and regulations of Kagoshima University. All samples were collected after obtaining the owners' informed consent.

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