수의학박사학위논문

Analysis of miRNA identification according to age and disorder in dog reproductive system

개의 연령과 질병에 따른 생식기관의 miRNA 발현 분석 연구

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이 논문을 수의학 박사학위논문으로 제출함

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Analysis of miRNA identification according to age and disorder in dog reproductive system

by Eun Pyo Kim

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENT FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

in

Veterinary Clinical Sciences (Theriogenology)

Department of Veterinary Medicine, Graduate School

Seoul National University

We accept this thesis as confirming to the required standard

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Declaration

This thesis is submitted by the undersigned for examination for the degree of Doctor of Philosophy to the Seoul National University. This thesis has not been submitted for the purposes of obtaining any other degree or qualification from any other academic institution.

I hereby declare that the composition and experiment of this thesis and the work presented in it are entirely my own.

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Analysis of miRNA identification according to age and disorder in dog reproductive system

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ABSTRACT

The expression of microRNA (miRNA) in dogs is related to aging, and environmental and genetic factors can influence the accumulation of cellular and molecular damage, leading to organ and tissue dysfunction. Epigenetic alterations, a specification of aging, are generated when DNA methylation, histone modification, or noncoding RNA regulation occurs.

miRNAs, which have 20–24 nucleotides, repress translation or cause messenger RNA (mRNA) degradation of target transcripts and play important roles in all biological pathways. However, although miRNAs are biological markers of aging, it is unknown whether they play a role in the aging of the reproductive system of dogs. As humans and dogs share many characteristics, including aging, domesticated dogs are ideal disease models for determining genetic and environmental factors. Moreover, approximately half of their hereditary diseases are similar to human diseases indicating that dogs have more genetic similarities with humans than mice.

Although miRNA studies on several diseases using dogs have been conducted, miRNA studies on the reproductive system of dogs with various ages and disease conditions have not yet been carried out. Accordingly, this study aimed to analyze differentially expressed miRNAs in dog reproductive system according to age and the disease status and identify the differences and changes in miRNA expression. For this experiment, samples were collected from dogs brought to animal hospitals and analyzed using microarray, RNA sequencing (RNA-Seq), and real-time polymerase chain reaction. To evaluate the effectiveness of miRNA regulation,

miRNA was first selected, and target genes were searched using the miRNA target prediction databases TargetScan and miRDB.

In Chapter I of this study, the changes in the miRNA expression in the ovaries, oviducts, and uteri were examined in 18 dogs—including 6 immature dogs (<1 year old), 6 mature dogs (>3 years old), and 6 dogs with uteropathy (>3 years old). As a result, two miRNAs (miR-151 and miR-708) had significantly higher expression levels in the ovaries of mature dogs than those in immature dog ovaries. Additionally, the expression levels of let-7a, let-7b, let-7c, miR-125b, and miR-26a were relatively higher in the ovaries of mature dogs with uteropathy than in those of healthy mature dogs. miR-30d and miR-140 were significantly high expressed in the oviducts of mature dogs; however, the miR-203 expression level was lower in the oviducts of dogs with uteropathy than that in those of mature, healthy dogs. The uteri of mature dogs had higher expression levels of all five miRNAs (miR-29a, miR-125a, miR-23a, miR-10a, and miR-221) than those in immature dogs, although no variations in miRNA expression were discernible between mature dogs and mature dogs with uteropathy. The findings suggest that both aging and uteropathy affected miRNA expression in reproductive organs of female dogs.

In addition, in Chapter II of this study, the miRNA and mRNA expression profiles of canine mammary tumors (CMTs) were analyzed by comparing them with human tumors considering. Humans and dogs have genetic, environmental, and physiological similarities. Tissue samples were collected from 12 dogs with benign CMT (BCMT) and 8 dogs with malignant CMT (MCMT). The changes in mRNA

levels were explored based on the type of tumor (benign or malignant) and the location of the biopsy samples (tumors and surrounding normal tissues), using differentially expressed miRNAs and RNA-Seq. With advancements in sample preparation, sequencing platforms, and bioinformatics data analysis, RNA-Seq technology emerges as an alternative to conventional Sanger sequencing- and microarray-based methods, allowing thorough and accurate examination of gene expression and alternative splicing. This technology also enables detailed transcriptome profiling and new insights into various physiological and pathological conditions. In this study, cfa-miR-503 was the only miRNA expressed corresponding to a MCMT. Cfa-miR-503 can be considered as a potential biomarker and RNA-based tumor treatment for the diagnosis and prognosis of MCMT provided that additional research is conducted.

In Chapter III, miRNA expression variations in the testis and epididymis cauda of 12 male dogs were examined, and a high-throughput miRNA array was performed to test whether age, cryptorchidism had an impact on miRNAs in the reproductive system of male dogs. The dogs were categorized into two age groups: <1 year and >3 years. The analysis revealed that only cfa-miR-503 expression was downregulated in the epididymis of older dogs, whereas the expression levels of other 64 miRNAs were elevated. cfa-miR-148a and cfa-miR-497 expression levels in the testis of dogs with cryptorchidism were significantly lower than those in age-matched normal dogs. cfa-miR-1841 levels were markedly reduced in the epididymis of cryptorchid dogs. The 26 cfa-miRNA expressions in testicular tumors and healthy tissues were

markedly different. Through this study, differences in miRNA expression due to aging, cryptorchidism were identified.

In conclusion, miRNA expression varies depending on the age and the presence of diseases in dog reproductive systems. In general, miRNA expression levels tend to increase mainly when dogs age or are affected by disease. Moreover, the identified miRNAs may be considered as potential biomarkers for aging and the various diseases of the dog reproductive system. Further investigation of miRNA should be pursued to elucidate the impact of aging and diseases on epigenetic alteration and finally establish diagnostic biomarker for reproductive diseases.

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Key words: microRNA, dogs, aging, uterine disease, mammary gland tumor, cryptorchidism, diagnostic biomarker

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TABLE OF CONTENTS

ABSTRACT..... ii

TABLE OF CONTENTS..... vi

LIST OF TABLES..... ix

LIST OF FIGURES..... x

LIST OF ABBREVIATIONS..... xii

PART I.

GENERAL INTRODUCTION..... 1

1. Literature review..... 2

2. General objective..... 23

PART II.

GENERAL METHODOLOGY..... 24

1. Tissue sample collection..... 25

2. Total RNA extraction and quality check..... 27

3. Gene microRNA hybridization, scanning, and data processing..... 28

4. Statistical analysis..... 29

PART III. **MicroRNA EXPRESSION IN FEMALE DOG REPRODUCTIVE SYSTEM..... 30**

Chapter I. **MicroRNA expression variation in female dog (*Canis familiaris*) reproductive organs with age and presence of uteropathy …..... 31**

1. Introduction..... 31

2. Materials and methods..... 33

3. Results..... 37

4. Discussion..... 45

5. Conclusions..... 49

Chapter II. **MicroRNA and messenger RNA expression profiles in canine mammary gland tumor..... 51**

1. Introduction..... 51

2. Materials and methods..... 53

3. Results..... 59

4. Discussion..... 70

5. Conclusions..... 77

PART IV. **MicroRNA EXPRESSION IN MALE DOG REPRODUCTIVE SYSTEM..... 78**

Chapter III. **Integrated miRNA in canine testis and epididymis according to age and cryptorchidism..... 79**

1. Introduction..... 79

2. Materials and methods..... 81

3. Results..... 87

4. Discussion..... 91

5. Conclusions..... 96

PART V. **GENERAL CONCLUSION..... 98**

REFERENCES..... 101

국문초록..... 137

LIST OF TABLES

Table 1. Predicted target of miRNA and primer sequence of each genes. 36

Table 2. Clinical examination of dogs included in this study. 38

Table 3. Animals used for sample collection. 55

Table 4. The mRNA expression profile of miRNA-target genes. 63

Table 5. Intersection and breast cancer related genes of miRNA and mRNA 70

Table 6. Clinical examination of dogs included in this study 84

LIST OF FIGURES

Figure 1. Volume plots of miRNAs in (A) ovary, (B) oviduct and (C) uterus of above 3 years dogs compared with below 1 years old dogs. 41

Figure 2. Volume plots of miRNAs in (A) ovary, (B) oviduct and (C) uterus of dogs older than 3 years old with uteropathy compared to healthy dogs older than 3 years old. 42

Figure 3. Validation of the differentially expressed levels of each predicted of miRNA of (A) ovary, (B) oviduct, (C) uterus and (D) ovary of dogs with uteropathy. 44

Figure 4. Volcano plot in canine mammary gland tumor for array-based miRNA. Red dots indicate upregulated miRNAs and grey dots indicate unsignificant miRNAs.....61

Figure 5. Pathway analysis in breast cancer. Red boxes indicate miRNAs detected in the pathway among DE-miRNAs. 62

Figure 6. Enrichment analysis for GO_BP, GO_CC, GO_MF, and KEGG....65

Figure 7. Venn diagrams showing GO_BP, GO_CC, and GO_MF of miRNAs 66

Figure 8. Volcano plot in canine mammary gland tumor for mRNA. Red dots indicate upregulation, blue dots indicate downregulation and grey dots indicate unsignificant miRNAs. 68

Figure 9. Venn diagram of commonly appearing genes between miRNAs and their targeted mRNAs.. 69

Figure 10. Images of hematoxylin and eosin-stained sections of testis (A, B) and epididymis (C, D) in unilateral cryptorchid dogs.. 88

Figure 11. Volume plots of miRNAs in the (A) testis and (B) epididymis of mature 3-year-old dogs (above) compared those of immature 1-year-old dogs (below).....89

Figure 12. Volume plots of miRNAs in (A) testis and (B) epididymis of cryptorchids dogs compared with dogs younger than one year of age. 90

LIST OF ABBREVIATIONS

ACTB	**Beta-actin**
AGCC	**Affymetrix GeneChip® Command Console® Software**
ALKP	**Alkaline phosphatase**
ALT	**Alanine aminotransferase**
BC	**Breast cancer**
BCMT	**Benign canine mammary gland tumor**
BP	**Biological processing**
BUN	**Blood urea nitrogen**
BWA	**Burrows– Wheeler aligner**
BWT	**Burrows– Wheeler transform**
CA	**Cancer antigen**
CC	**Cellular component**
cDNA	**Complementary DNA**
CEA	**Carcinoembryonic antigen**
CEL	**Celestia script file**
Cfa-miR	**Canis lupus familiaris microRNA**
circRNAs	**Circular RNAs**
CLL	**Chronic lymphocytic leukemia**
CMT	**Canine mammary gland tumor**
cPCa	**Canine prostate cancer**

CSC	**Cancer stem cells**
CTD	**Comparative toxicogenomics database**
CVDs	**Cardiovascular diseases**
DABG	**Detection above background**
DEGs	**Differentially expressed genes**
DE-miRNAs	**Differentially expressed miRNAs**
DNA	**Deoxyribo nucleic acid**
DSD	**Disorders of sex development**
EGFR	**Epidermal growth factor receptor**
F	**Female**
FDR	**False discovery rate**
FPKM	**Fragments per kilobase of exon per million fragments**
FS	**Spayed female**
GFM	**Graph FM index**
GO	**Gene ontology**
HBC	**Human breast cancers**
HCT	**Hematocrit**
HISAT	**Hierarchical indexing for spliced alignment of transcripts**
HPV	**Human Papillomavirus**
KEGG	**Kyoto encyclopedia of genes and genomes**
LN	**Lupus nephritis**
LPS	**Lipopolysaccharide**
lncRNAs	**Long noncoding RNAs**

MCMT	**Malignant CMT**
MF	**Molecular function**
miRDB	**MicroRNA target prediction database**
MiRNA	**MicroRNA**
ncRNAs	**Non-coding RNAs**
OHE	**Ovariohysterectomy**
piRNA	**PIWI-interacting RNAs**
PCR	**Polymerase chain reaction**
PTEN	**Phosphatase and tensin**
RBC	**Red blood cell**
RIN	**RNA integrity number**
RNA	**Ribonucleic acid**
RNA-Seq	**RNA sequencing**
RT-qPCR	**Quantitative real time PCR**
SLE	**Systemic lupus erythematosus**
SPSS	**Statistical package for the social sciences**
STK11	**E-cadherin and serine/threonine kinase11**
WBC	**White blood cell**

PART I

GENERAL INTRODUCTION

1. Literature review

1.1. MicroRNA

Since the initial identification of microRNAs (miRNAs) in 1993 as a small RNA involved in the developmental process of nematodes (Lee et al., 1993), they have been known as an essential component of the noncoding genome that influences gene regulation and important processes in many animal species, including vertebrates (Lagos-Quintana et al., 2001). Small non-coding RNAs (ncRNAs) have emerged as important post-transcriptional regulators of gene expression in organisms such as animals, plants, and protozoa. miRNAs are approximately 21 nucleotides long (Fabian et al., 2010). Their role in cellular communication can be compared to that of endogenous or synthesized short-interfering RNAs, which act by inhibiting the expression of target mRNAs with complementary sequences (Mohr & Mott, 2015). According to Friedman et al. (Friedman et al., 2009), miRNAs in mammals may control the activity of >60% of all protein-coding genes and are involved in the regulation of almost all cellular processes (Bartel, 2009; Bushati & Cohen, 2007; Ghildiyal & Zamore, 2009).

In addition to identifying differentially expressed miRNAs that may have diagnostic, prognostic, or predictive value, miRNA expression profiling in cancer has also helped elucidate the causal role of particular miRNAs related to cancer initiation, progression, and/or metastasis (Anfossi et al., 2018; Graveel et al., 2015;

Peng & Croce, 2016; Rupaimoole & Slack, 2017; Sempere, 2014; Sempere & Kauppinen, 2010).

In studies of chronic lymphocytic leukemia identifying tumor suppressors on chromosome 13q14, a frequently deleted region, Iorio and Crosce discovered two microRNA genes, miR-15a and miR-16-1, expressed in the same polycistronic RNA instead of a protein-coding tumor suppressor gene. This discovery provided the first evidence of microRNA involvement in human cancer (Iorio & Croce, 2012) as the loss of miR-15a and miR-16-1 was caused by the deletion of chromosome 13q14 (Calin et al., 2002). The overexpression of miR-205 and miR-21 in ductal adenocarcinoma, which has been known to occur before alterations in the ducts, suggests their potential use for early detection of this type of tumor (du Rieu et al., 2010). Additionally, the expression patterns of circulating miRNAs in humans with different types of tumors matched those observed in the tumor tissues, providing the intriguing possibility of using circulating miRNAs as easily detectable tumor biomarkers, especially for early diagnosis (Heneghan et al., 2010; Huang et al., 2010; Lawrie et al., 2008; Xing et al., 2010). Important studies comparing the miRNA expressions in tumor tissues and healthy controls discovered that a group of miRNAs (let-7, miR-10b, -15, -16, -17-5p, -20a, -21, -29b, -34, -126, -145, -155, and -221) had disrupted expression in multiple blood and solid tumors (Barbarotto et al., 2008; Lu et al., 2005; Peng & Croce, 2016; Sempere, 2014; Sempere et al., 2010; Volinia et al., 2006). As compared with messenger RNA expression patterns, those of miRNAs may be more accurate for identifying the cancer type as they offers more

details on the developmental lineage and differentiation of distinct cancers (He et al., 2005).

Recently, different methods including cDNA arrays, a modified invader assay, and real-time polymerase chain reaction (PCR) have been established for measuring miRNAs (Allawi et al., 2004; Babak et al., 2004; Schmittgen & Livak, 2008; Sun et al., 2008; Takamizawa et al., 2004). These techniques enable the accurate differentiation of similar members of the let-7 family of miRNA isoforms (Jiang et al., 2005). In the field of human cancer, recent studies using advanced technologies have revealed changes in miRNA expression in patients with pancreatic cancer tumors (Bloomston et al., 2007; Gironella et al., 2007; Roldo et al., 2006; Sun et al., 2008; Szafranska et al., 2007; Tsuda et al., 2006), offering the potential for differential diagnosis from other tumors. The human pancreatic cancer cell line Panc1 has the highest expression of the miR-376 precursor among other cell lines (Jiang et al., 2005; Volinia et al., 2006).

Other recent non-cancer-related studies have reported an increase in exosome-associated miRNAs (de Jong et al., 2012; Zhang et al., 2010). These miRNAs can be retained in circulation, protected by their vesicular structure, and then be transferred into target cells to regulate specific gene expressions, linked to various chronic diseases, including diabetes mellitus, fatty liver, obesity, and atherosclerosis (Yao et al., 2018).

Meanwhile, cancer-related reports of miRNA expression in dogs include studies on neoplastic illness (Heishima et al., 2017), lymphoma (Craig et al., 2019),

mammary gland tumors (Ramadan et al., 2019), and prostate cancer (Kobayashi et al., 2017). Heishima et al. (2017) used quantitative real time PCR (RT-qPCR) to obtain the profiles of circulating miRNA-214 and -126 in the blood of 181 dogs with neoplastic diseases and those of healthy controls. The study has reported high miRNA-214 levels in two epithelial and four non-epithelial tumors and high miRNA-126 levels in six epithelial and four non-epithelial tumors, with both miRNAs having diagnostic potential in different types of tumors and being prognostic predictors in various groups, although they did not demonstrate a strong correlation with other clinical parameters. These miRNAs may serve as diagnostic and prognostic biomarkers for canine neoplastic diseases and, potentially, for human cancers (Heishima et al., 2017).

Lymphoma, which is the most common hematological malignancy in dogs, is very similar to that in humans (Vail, 2007). A customized PCR array was utilized to profile 38 canine target miRNAs in lymph nodes and plasma from dogs with multicentric B or T cell lymphoma and healthy control dogs. The study identified various sets of miRNAs with altered expressions in lymph nodes and plasma for B- and T-cell lymphomas, with only eight miRNAs having significant differences in diagnosis and recurrence (Craig et al., 2019).

In the case of canine mammary cancers, elevated CA15-3 (Cancer antigen 15-3) levels were identified in all dogs with canine mammary tumors (CMTs), and miR-21 had a 12.84-fold higher upregulated expression than in dogs without tumors, especially in tumors positive for vimentin immunoreactivity. This indicates miR-21

levels may be a more sensitive and non-invasive indicator for CMT than carcinoembryonic antigen levels, which remained unchanged (Ramadan et al., 2019).

Canine prostate cancer (cPCa), which affects the male canine reproductive system, is an untreatable disease that can spread to other parts of the body (Ryman-Tubb et al., 2022). When the expression levels of 277 mature miRNAs in prostatic tissue were compared between non-tumor and tumor groups using real-time PCR, five miRNAs had upregulated expressions (miR-18a, 95, 221, and 330), whereas 14 miRNAs had downregulated expressions (miR-127, 148a, 205, 299, 329b, 335, 376a, 376c, 379, 380, 381, 411, 487b, and 495), offering potential for early diagnosis markers and opportunities for miRNA-based therapy (Kobayashi et al., 2017).

The development of cardiovascular diseases (CVDs), a non-cancer domain, has also been linked to miRNAs (Zhou et al., 2018). Certain miRNAs have been identified as new biomarkers for various CVDs owing to the growing understanding of cardiac biology (Ultimo et al., 2018). These miRNAs are involved in processes such as differentiation, growth, contractility, and heart rhythm (Islas & Moreno-Cuevas, 2018). Alterations in miRNA expression in the heart have been associated with pathological processes such as congenital abnormalities, arrhythmias, cardiac hypertrophy, and heart failure (Çakmak & Demir, 2020).

Exosomal miRNA profiles have been demonstrated to differ between patients with systemic lupus erythematosus/lupus nephritis (SLE/LN) and healthy controls, indicating their potential as biomarkers for renal injury and for exosome-delivered immunomodulatory therapy (Perez-Hernandez et al., 2015). These exosomes are

useful as potential therapies because of their stability, low toxicity, and specific targeting capabilities. However, diagnosing and treating SLE, particularly LN, in dogs is challenging (Wang et al., 2021).

Both canine and human tumor malignancies have been reported to have downregulated levels of numerous miRNAs, and several oncomiRs that contribute to the development and progression of cancers have been identified (Wagner et al., 2013). Studies have investigated miRNA in lymphoma, CMT, breast cancer, mast cell tumor, transitional cell carcinoma, urothelial carcinoma, osteosarcoma, melanoma, leukemia, hemangiosarcoma, and cancer stem cells (CSC). Further, better cancer prevention and treatment may be developed by examining the similarities and differences between canine and human miRNA function and dynamics in cancer (Sahabi et al., 2018).

1.2. Canine reproductive system

A female dog's reproductive tract consists of the female reproductive organs including the ovaries, uterus, vagina, vulva, and mammary glands (Spanel-Borowski et al., 1984). The ovaries, also known as gonads, are responsible for producing eggs and female hormones such as progesterone and estrogen. Follicle stimulation starts the estrous cycle, which results in the development of eggs, the generation of estrogen, and ovulation (Concannon, 2011). The cervix protects the uterus, where the oviducts deliver eggs, from infection. Pregnancy requires that the uterus and

cervix function properly (Aspinall, 2004a). The vagina and vulva are the final parts of the birth canal and the copulatory organs (Holumbiovska & Stefanyk, 2018), whose dysfunction can also lead to infertility (Tao et al., 2018).

The reproductive physiology of female dogs differs from that of other mammals in that it exhibits typically non-seasonal, monoestrous, spontaneous ovulation and is characterized by a spontaneous luteal phase. Canine ovulation is characterized by the release of primary oocytes, which mature for approximately 72 h in the oviduct (Lee et al., 2020). The canine reproductive cycle is considered non-seasonal and monoestrus and comprises four stages—proestrus, estrus, diestrus, and anestrus. The ovarian follicle matures during the proestrus stage with an average of 9 days, accompanied with vulvar swelling and discharge. The female dog's stand to allow mating, and a change in the color of the vulvar discharge indicate estrus, which lasts approximately 9 days. During diestrus, the female dog is no longer receptive, and a protracted luteal phase occurs with high levels of progesterone, which lasts for approximately 55–65 days. Inflammatory diseases of the uterus, such as uterine sinusitis, are frequently caused by progesterone during diestrus (Ververidis et al., 2004). Anestrus, which lasts 3–4 months, is a dormant phase. After the anestrus stage concludes, follicular development is halted until the following proestrus stage, when the serum estrogen level increases and drives follicular growth (Concannon, 2011). In female dogs, diestrus is long, and pseudopregnancy can lead to mammary gland development regardless of pregnancy (Root et al., 2018). Moreover, removing the ovary through general neutering can prevent mammary tumors (Sorenmo et al., 2000).

The reproductive system of the male dog consists of the testis, epididymis, ductus or vas deferens, prostate gland, urethra, and penis (Perez-Marin et al., 2006). The male gonads known as the testes are responsible for producing testosterone and other male sex hormones. Testosterone is required for the healthy function of the accessory sexual glands and the development of male sex traits (Aspinall, 2004b). The epididymis comprises three main parts: the caput (head), corpus (body), and cauda (tail). The cauda epididymis is connected to the vas deferens, a muscular duct that is primarily responsible for storing mature sperm. As sperm pass through the epididymis, they undergo final maturation, which lasts up to 14 days, during which they acquire motility and the ability to fertilize an egg (Ali Hassan et al., 2021). As the normal body temperature is excessively high for the synthesis of sperm, the testes must reside in the scrotum (Albrizio et al., 2013). Semen analysis and hormonal assays can be used to assess testicle functionality (Kustritz, 2007). Sperm and semen are transported to the genital canal through the epididymis, which connects the testicle to the ductus deferens and the accessory sex glands, which produce the fluid component of semen. Semen analysis, ultrasound, and physical examinations can all be performed to identify illnesses or irregularities in the genital tract (Gobello et al., 2002). In dogs, the mature sperm has a head and a tail. The acrosome is a membrane-bound lysosome with hydrolytic enzymes, and the head consists of a nucleus, an acrosome, and a post-nuclear cap (Wysokińska et al., 2021; Yanagimachi & Noda, 1970). Sperm and seminal plasma from the prostate and other accessory sex glands are combined during ejaculation. Some studies imply that prostatic fluid has a

negative impact on sperm quality, whereas other studies demonstrate an improvement (Fritsche, 2015).

Cryptorchidism is a condition in male dogs where one or both testicles fail to descend into the scrotum as they should during development (Yates et al., 2003). The testicles in dogs begin to develop near the kidneys during fetal development. As development progresses, the gubernaculum testis, a structure that pulls the testes downward, helps to guide the testes out of the abdomen and into the scrotum through the inguinal canal. This generally occurs when the animal is approximately 2 months old (Khan et al., 2018). Cryptorchidism can cause various health problems, including an increased risk of testicular cancer and infertility. Surgery is often recommended to remove the retained testis and prevent any potential complications (Jhun et al., 2022).

1.3. Prediction and diagnosis of reproductive system disorders

Every year, more people consider their companion dogs to be a member of their family (Merkouri et al., 2022). Hence, the welfare of their companion dogs becomes increasingly crucial for owners (Philpotts et al., 2019). Owing to the genetic similarity between humans and dogs, investigating whether changes in miRNA expression could also indicate probable disorders in canines is worthwhile (Leonardi et al., 2021).

1.3.1 Aging

Cancer has become more common in both humans and canines as both species' lifespans have increased (Kaszak et al., 2018). OncomiRs, which are miRNAs that are involved in the development and progression of cancer, have been detected in both human and canine cancers, demonstrating the value of dogs as a large animal model of human diseases because they not only live in the same environment as humans but also experience diseases that are similar to those of humans in terms of their clinical and pathological features, including infectious and non-infectious diseases (Boggs et al., 2008). Dogs and humans are also both diagnosed with similar cancers (Sahabi et al., 2018).

A nonlinear relationship between a dog's age and a corresponding human age has also been indicated in recent studies comparing the methylomes of humans and those of dogs. Moreover, the timing of important physiological milestones of humans and dogs is reportedly similar (Creevy et al., 2016; Sandor & Kubinyi, 2019).

Hence, especially in studies on aging, dogs can be a useful model for human health and medicine. The study of aging in companion dogs was motivated by studies on human health, making it a novel technique in translational aging research (Creevy et al., 2022). Human medicine has influenced the development of veterinary medicine (King, 2021). Long-term research on dogs can identify breeds that are predisposed to inherited illnesses, elucidate environmental risk factors that can affect both humans and dogs, and assess strategies to postpone aging-related sickness and death (Gaillard et al., 2022). By tracking illness incidence, cognitive function, frailty,

and multimorbidity, these studies also provide insightful information on the aging process in both dogs and humans (Ruple et al., 2022).

1.3.2 Uterine diseases

The development of reproductive problems in women, such as recurrent miscarriage and endometriosis in the endometrial tissue, as well as the different changes that occur during the menstrual cycle and implantation may be influenced by miRNA (Hull & Nisenblat, 2013). In one of the earliest studies in humans to analyze miRNA expression in endometrial tissues, stromal and epithelial primary cell cultures were isolated from women during the early to mid-secretory phase of their menstrual cycle, and 32 differentially expressed miRNAs were discovered (Pan et al., 2007). Large-scale miRNA profiling studies have confirmed the role of miRNA in endometrial disorders and changes in the menstrual cycle by providing the first evidence (Rekker et al., 2013). They have also experimentally validated the interactions between miRNA and target mRNA in endometrial cells, predicted their effects on target mRNA through *in silico* analyses, and identified serum and plasma endometriosis-associated miRNA, which may have potential as noninvasive diagnostic components by mediating systemic interactions with non-endocrine cells (Hull & Nisenblat, 2013).

By modifying particular miRNA levels observed in affected patients, miRNA may be involved in the development of cervical cancer (Shen et al., 2020). With

528,000 new cases and 266,000 fatalities in 2012, invasive cervical cancer has been identified as the fourth most common malignancy in women worldwide (Ferlay et al., 2015). Studies have demonstrated that cervical cancer cells with integrated human papillomavirus (HPV) have altered levels of miRNA expression (Shen et al., 2020). Additionally, cervical carcinomas have altered miRNA expression profiles (Lee et al., 2008). Understanding the clinical outcomes of cervical cancer is challenging owing to variability in results and limited tumor tissue availability. However, analyzing the impact of miRNA on HPV DNA replication and early-stage cervical cancer development may shed light on the lifecycle of HPV and the mechanisms of oncogenesis (Shen et al., 2020).

Additionally, new results demonstrate modifications in miRNA expression in pathologically damaged endometrial tissue (Pan & Chegini, 2008). Preeclampsia (Zhu et al., 2009), endometrioid endometrial adenocarcinoma (Chung et al., 2009), uterine leiomyomata (Wang et al., 2007), ovarian adenocarcinoma (Pan & Chegini, 2008; Zhang et al., 2008), endometriosis (Filigheddu et al., 2010; Teague et al., 2010), and recurrent pregnancy loss are some conditions of the human reproductive tract where dysregulated miRNA expression has been identified (Pandey et al., 2005). Recent research suggests that miRNA expression profiles in the endometrium or endometriotic lesions are biologically significant in the pathogenesis of endometrial and uterine disorders. Thus, miRNAs may play a role in regulating specific cellular processes necessary for the emergence of gynecological diseases (Santamaria & Taylor, 2014).

Although miRNA studies on human uterine diseases can be found in the literature, those on canine uterine diseases were unfortunately difficult to find. Instead, I cite a study on the variations in miRNA expression in bovine endometrial stromal cells. High-throughput RNA sequencing was used in this study to investigate how lipopolysaccharides (LPS) affects the response of bovine endometrial stromal cells to inflammatory mediators and microRNAs. The results revealed that 219 miRNAs were differentially expressed, with 113 and 106 having upregulated and downregulated expressions, respectively (Yan et al., 2021).

1.3.3 Mammary gland tumors

Studies have been conducted on the expression of miRNA in mammary gland tumors in both female dogs and women. Breast cancer is the second-leading cause of cancer-related mortality and one of the most frequently diagnosed cancers in women worldwide (Siegel et al., 2017). CMTs are a common type of cancer in dogs and are associated with high mortality rates (Valdivia et al., 2021). Recently, miRNAs have been recognized for their function in controlling the development and metastasis of breast cancer (Khordadmehr et al., 2019; McGuire et al., 2015). miR-9 expression is dysregulated in breast cancer, as demonstrated by an increasing number of studies, and while it acts as an anti-oncogene in preventing the development of breast cancer in the early stages, it plays an opposite oncogenic role in the metastasis of highly malignant breast cancer, indicating that miR-9 level and function in breast cancer vary depending on the stage of the disease (Li et al., 2020).

Moreover, in-depth research has been conducted on circulating miRNAs, which are miRNAs secreted into the bloodstream, to elucidate their functions in the detection, management, and prognosis of human breast cancer (HBC) (Chong et al., 2020). Eight distinct characteristics, including ongoing inflammation, increased invasion and spread to distant sites on the body, resistance to programmed cell death, stimulation of the growth of new blood vessels, immunological instability, and metabolic abnormalities can all be used to diagnose breast cancer (Chong et al., 2020). For instance, circulating miRNA levels are an important biomarker for determining metastasis of breast cancer to other organs (Cardinali et al., 2022) as well as for identifying the clinical and pathological stages of local disease invasion (Tito et al., 2021).

Metastatic breast cancer is a serious additional cause of cancer-related deaths in women, and it continues to rank as the second highest cause of cancer-related deaths in women in the United States (Petri & Klinge, 2020). Moreover, >30% of patients with breast cancer die from metastatic disease (Steeg, 2016). Even though breast cancer treatments have advanced, the survival rate for patients with distant metastasis remains at 27% (Siegel et al., 2019). Various ncRNAs, including circular RNAs, PIWI-interacting RNAs, miRNAs, and long noncoding RNAs, contribute to the development and spread of breast cancer (Klinge, 2018a, 2018b). Furthermore, pro-metastatic miRNAs are expressed at higher levels in advanced-stage breast cancer, which results in the reduced expression of their tumor suppressor targets (Tang et al., 2012). However, more advanced-stage breast cancers exhibit lower

quantities of miRNAs with anti-metastatic capabilities that target oncogenes (Petri & Klinge, 2020).

To evaluate plasma miR-27a for this purpose, 95 blood samples including those from 40 patients with newly diagnosed cancer, 20 patients with benign breast lesions, 20 women with a positive family history of breast cancer, and 15 healthy controls were analyzed using RT-qPCR. Thus, the miR-27a expression level could serve as a new non-invasive biomarker for improving the early diagnosis of breast cancer (Seddik et al., 2021).

Mammary gland neoplasia is the most frequently diagnosed tumor in dogs, making it an important clinical concern when considering mammary gland tumors in dogs. Although its specific etiology remains unknown (Rivera et al., 2009), CMTs typically affect older dogs, commonly between the ages of 8 and 10 years (Benavente et al., 2016; Reddy et al., 2009; Salas et al., 2015; Sorenmo, 2003). However, a few risk variables have been identified, including genetics, dietary factors, and hormone abnormalities (Beauvais et al., 2012; Chang et al., 2009; Sorenmo, 2003; Sorenmo et al., 2000).

The assessment of biomarkers provides information for clinical diagnoses, treatment options, and prognoses for female dogs suffering from mammary tumors because CMT and HBC share many similarities. Thus, cases of CMT often exhibit biomarkers that are also present in cases of HBC (Kaszak et al., 2018). As each cancer cell expresses specific tumor-associated antigens that can be considered biomarkers if identified in different concentrations compared to normal levels,

biomarkers, which are frequently proteins that can be detected in the blood, tissues such as tumors, or bodily fluids such as urine and serum, provide valuable insights on the presence of a disease, treatment success, and patient prognosis (Henry, 2010; Mobasheri & Cassidy, 2010). This is true even though CMTs share immunohistochemical traits and express the same substances as those in HBCs (Peña et al., 2014). As CMTs have a highly heterogeneous biology and morphology, selecting the best biomarker becomes challenging (Kaszak et al., 2018).

Comparative genomic research has great potential to elucidate the molecular mechanisms, important risk factors, and genetic profiles of mammary carcinomas and breast cancer, leading to the development of novel therapeutic strategies (Feng et al., 2018). However, additional research on the numerous cancer-associated genes, including cell cycle regulators and emerging miRNAs in the canine genome as well as the relationship between tumor suppressor gene expression and miRNA activity in post-transcriptional regulation, which is a central area of cancer research (Lutful Kabir et al., 2015), is necessary. This is because the genome sequence and characteristics of spontaneous tumor models are similar between humans and dogs (Liu et al., 2014).

Exosome-derived miRNAs, in addition to intracellular and cell-free miRNAs, have drawn interest as potential cancer biomarkers and offer a minimally invasive, painless, and affordable alternative to conventional tissue biopsy (Alotaibi, 2023). miRNAs proposed as diagnostic or prognostic biomarkers in CMTs include miR-21, miR-29b, miR-141, miR-429, miR-200c, miR-497, miR-210, miR-96, miR-18a,

miR19b, miR-20b, miR-93, miR-101, miR105a, miR-130a, miR-200c, miR-340, and miR-486, which are likewise altered in HBC and may serve as disease-specific biomarkers for both diseases. However, additional research is required to confirm their diagnostic or prognostic utility in CMTs (Petroušková et al., 2022).

1.3.4 Cryptorchidism and testicular tumors

Several articles have demonstrated that miRNAs play crucial roles in spermatogenesis (Chen et al., 2017; Salilew-Wondim et al., 2020). miR-210, which was significantly increased in patients with non-obstructive azoospermia (NOA), was also strongly expressed in patients with cryptorchidism, demonstrating the critical function that miRNAs play in this disorder (Duan et al., 2016). In a rat model of cryptorchidism, Moritoki et al. confirmed the downregulation of miR-135a expression in unilaterally undescended testes (Moritoki et al., 2014). However, no studies have analyzed the miRNA expression in the testes of patients with post-cryptorchidopexy and NOA, even though prior research has demonstrated the role of specific miRNAs in the control of spermatogenesis in patients with cryptorchidism (Tang et al., 2018).

Additionally, the prevalence of male infertility is rising globally and has attracted increasing attention as a severe public health concern (Agarwal et al., 2015; Jarow et al., 2002). Idiopathic illnesses of the male reproductive system, such as cryptorchidism and varicocele, frequently contribute to male infertility, which is a growing global issue (Clavijo et al., 2017; Virtanen & Toppari, 2015). A lack of the

miR-34 family has been associated with infertility in mice, and several miRNAs, including miR-17, miR-18, miR-19, and miR-25, have been discovered to be essential in spermatogenesis and play a role in early spermatogonial differentiation (Hilz et al., 2016). Researchers have reported that the miR-34c expression levels of patients with cryptorchidism were significantly decreased in the testicular tissues. The downregulation of miR-34c expression led to an increase in the protein level of Nanos2, which disrupted spermatogonial syncytium, indicating the miR-34c/Nanos2 pathway as a novel route for examining the mechanism of male infertility caused by cryptorchidism (Huang et al., 2018).

To investigate the role of miRNAs in the development of male external genitalia and the phenotypic variety of 46, XY disorders of sex development (DSD) in patients who present with undervirilized external genitalia at birth, the plasma expression of miR-210 was assessed in patients with 46, XY DSD of unknown etiology (Das et al., 2020). A significant correlation was noted between the presence of atypical genitalia and elevated plasma levels of miR-210 expression, advancing knowledge of how miRNAs are involved in male genital development and the various symptoms that patients with 46, XY DSD (Elias et al., 2022).

To the best of my knowledge, the miRNAs of dogs have not been studied for cryptorchidism and testicular tumors, although subsequent research with rats and horses has demonstrated that changes in miRNA expression indicate both cryptorchidism and testicular tumors.

A microarray analysis was performed to compare the miRNA expression of patients with undescended and descended testes and to investigate the potential role of miRNAs in the development of cryptorchidism in a rat model. Only miR-135a expression was downregulated in undescended testes, and since *FoxO1* is a critical component of stem cell maintenance, the downregulation of miR-135a expression may cause cryptorchidism, failing to maintain spermatogonial stem cells, which in turn causes spermatogenesis to change in cryptorchid testes (Moritoki et al., 2014).

In addition, a study on horses with cryptorchidism, a common congenital malformation in horses, examined the impact of variables such as high temperature and disease on the expression of testicular microRNAs in these animals. The results revealed significant differences in miRNA expression patterns, and this study also provided comprehensive expression data for equine testicular miRNAs, which is essential for future analysis (Han et al., 2020).

A previous study conducted a methodical database search to analyze the miRNAs of patients with cryptorchidism (Jia & Hao, 2021). Five articles that met the criteria were identified after the search, and they examined 185 samples of human and animal tissues (Duan et al., 2016; Han et al., 2020; Huang et al., 2018; Moritoki et al., 2014; Tang et al., 2018). The three miRNAs that were dysregulated in both animal and human testicular tissues—miR-210, miR-449a, and miR-34c—were the most frequently identified differentially expressed miRNAs. *NEAT1*, *KCNQ1OT1*, *XIST*, *AC005154.1*, and *TUG1* were the top five significant long ncRNAs that were related to these miRNAs (Jia & Hao, 2021).

Testicular cancer mostly affects those aged between the ages of 15 and 35 years, and it is a serious type of cancer that affects adolescents and young adults (Khan & Protheroe, 2007). As post-transcriptional regulators, miRNAs crucially influence cancer development (Regouc et al., 2020). The miR-371-373 cluster is identified as a potential novel tumor marker, as are other miRNAs including miR-223-3p, miR-449, miR-383, miR-514a-3p, miR-199a-3p, and miR-214 (Bezan et al., 2014). The marker with the highest potential for tracking the disease and spotting recurrence is miR-371a-3p, which has undergone significant research, including multi-center investigations (Regouc et al., 2020).

Since discovering studies on canine testicular cancer was difficult, studies on miRNA expression profiling of cPCa were extracted. The study used real-time PCR to compare the expression levels of 277 mature miRNAs in prostatic tissue from five dogs to ascertain the expression of miRNAs in cPCa tissue. Five miRNAs were upregulated, and 14 miRNAs were downregulated in cPCa ($P < 0.05$). These altered miRNAs may serve as potential early diagnostic indicators for cPCa and in miRNA-based therapy (Kobayashi et al., 2017).

1.4. RNA sequencing

A technique for profiling RNA called RNA sequencing (RNA-Seq) makes measuring and comparing the patterns of gene expression easier (Finotello & Di Camillo, 2015). Due to cost savings and the growing use of shared-resource

sequencing cores, RNA-Seq has gained popularity since its initiation in 2008 and has been adopted by more research institutes over the past decade (Koch et al., 2018). Additionally, the development of biomarkers has been one of the many applications of RNA sequencing in cancer research and therapy (Hong et al., 2020).

Currently, RNA-Seq is an alternative to established techniques owing to its much wider coverage and better resolution of the dynamic nature of the transcriptome that RNA-Seq can provide compared to Sanger sequencing- and microarray-based approaches (Kukurba & Montgomery, 2015; Rao et al., 2018). Allele-specific expression, alternative splicing, and gene expression may all be examined in-depth and precisely using RNA-Seq (Amoah et al., 2021). Conducting in-depth profiling of the transcriptome and gaining insights on various physiological and pathological conditions are now possible owing to recent advancements in RNA-Seq workflows, including in sample preparation, sequencing platforms, and bioinformatics data analysis (Kukurba & Montgomery, 2015).

The primary objective of RNA-Seq can be cited as the identification of differentially expressed and coregulated genes as well as the inference of biological significance for more research (Koch et al., 2018).

2. General objective

This study aimed to investigate the possibility that changes in miRNA expression in the canine reproductive system with aging represent potential disorders. Thus, this thesis has five components. The general introduction or Part I, lists the sources used to select the subject for my thesis. The general methodology employed in this study is described in Part II.

In Part 3, miRNA expression in female dog reproductive system was investigated. First, changes in miRNA expression in the ovaries, oviducts, and uteri of young and elderly dogs, as well as dogs with uteropathy, were examined to determine the role of miRNA in aging and diseases in Chapter I.

Additionally, to explain the role of miRNA as a biomarker for canine mammary gland tumor detection, changes in miRNA expression level were identified based on malignancy (benign or malignant) and biopsy locations (tumors and surrounding normal tissues) in Chapter II.

In Part 4, as a Chapter III, I also evaluated changes in miRNA expression in male dog reproductive system caused by aging, cryptorchidism, and testicular cancers.

The study's general conclusion is presented in Part V.

PART II

GENERAL METHODOLOGY

1. Tissue sample collection

I analyzed the microRNA expression in the reproductive systems of male and female dogs. All the dogs used in the experiment were companion dogs. Tissue samples were collected from dogs needing OHE, mastectomy (total or partial), or castration as requested by their owners from local animal hospitals and the veterinary teaching hospital of Seoul National University Korea from 2019 to 2022. The samples were collected only after receiving informed consent form from the dog owners with a detailed explanation of the study, and the veterinarian followed the general surgical method according to ethical standards. Tissues obtained postoperatively were classified by anatomical region according to the purpose of the experiments. Most of the tissue samples collected at the local animal hospitals were approved for study by exemption by the Eulji University Ethics Committee, and the tumor data analyzed in this study was approved by the Seoul National University Institutional Animal Care and Use Committee (IACUC) (approval number: SNU-200217-3-2). The experimental process complied with the guidelines outlined in "The Guide for the Care and Use of Laboratory Animals" published by the Seoul National University IACUC. Before surgery, blood tests such as blood chemistry tests and complete blood cell counts were performed for all dogs to rule out other medical issues and examine whether they were suitable for general anesthesia. The study was conducted in three parts to identify whether aging and diseases in the reproductive systems of dogs can be explained by changes in miRNA expression. The first chapter presents an analysis of aging and diseases affecting miRNA

expression in the reproductive systems of female dogs. Therefore, ovaries, oviducts, and uteri tissue samples were collected from young and old female dogs and dogs suffering from uterine disease. The ovaries were sampled to include both the cortex and medulla. And the central uterine angle of the ovary and uterus bifurcation was used. For oviducts, all fat around the ovary were removed and only tubular tissue was used. The second chapter presents an analysis of the different miRNA and mRNA expressions in CMTs to investigate the alterations based on the malignancy and biopsy locations. For this analysis, dogs with CMT underwent surgery, and tissues were removed and visually collected by separating the normal part from the tumor. The extracted tumor tissue samples were classified into BCMT or MCMT. CMT diagnosis and malignancy classification was based on histopathological examination (IDEXX, Seongnam-si, Republic of Korea) and metastasis status. The last chapter is an investigation of male reproductive systems to identify miRNAs affected by age, cryptorchidism, and testicular tumors. For this analysis, tissue samples were obtained from male dogs, and testis and epididymis cauda tissues (tail part of the epididymis) were collected during castration or tumorectomy. All cryptorchid testes used in this experiment were unilaterally palpable in the right inguinal area, and the left testis was present within the scrotum of male dogs aged >6 months. Tissues collected according to the testicular tumor diagnosis were also based on histological examination (IDEXX). All samples for miRNA analysis were preserved in RNAlater (Thermo Fisher Scientific, Waltham, MA, USA), frozen at −80 °C, and stored until further analysis. Some of the tissues requiring histological

examination were stored in neutral buffered formalin. All chemicals were obtained from Sigma-Aldrich Co. LLC. (Missouri, USA) unless otherwise stated.

2. Total RNA extraction and quality check

Total RNA was extracted from samples of each reproductive organ using the Easy-Spin Total RNA Extraction Kit from Intron Biotechnology, Seoul, Republic of Korea, and homogenized with 1 mL of RNA lysis solution. Then, the quality and quantity of the RNA were measured using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific) and evaluated for RNA integrity and quantity using a 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA) with an Agilent RNA 6000 Nano Kit, to perform gene microarray hybridization. Only samples that met the conditions of A260/A280 > 1.0, concentration > 50 ng/μL, volume > 10 μL, total amount > 0.7 μg, rRNA ratio > 1.0, and RIN > 7.0 with visible small RNA peaks were selected for microRNA analysis, and the RNA samples were reverse-transcribed to cDNA with a total volume of 20 μL using the Maxime RT-PCR premix kit (Intron Biotechnology, Seoul, Republic of Korea). This study aimed to investigate the function of antioxidants and lipids.

3. Gene microRNA hybridization, scanning, and data processing

All protocols were performed according to the Affymetrix Expression Analysis Technical Manual (Affymetrix, Inc., Santa Clara, CA, USA) using the FlashTag™ Biotin HSR RNA Labeling Kit (Thermo Fisher Scientific) to label RNA and hybridize biotin-labeled samples to the GeneChip® Affymetrix miRNA microarray (Affymetrix). Raw data were analyzed using the Affymetrix GeneChip® Command Console® Software (AGCC) after scanning the arrays with the Affymetrix GeneChip® scanner and processing the raw data images into CEL files containing the intensities of each probe on the array. A cut-off of >2-fold change and p <0.05 was used to screen differentially expressed miRNAs. Microarray hybridization was performed using the Affymetrix GeneChip miRNA 4.0 Array (Thermo Fisher Scientific), and total RNA samples were labeled with the FlashTag™ Biotin RNA Labeling Kit (Genisphere, PA, USA), hybridized to the microarray, and analyzed using the Affymetrix® GeneChip™ Command. RNA was extracted from genital tissue samples (at least 50 mg) and homogenized with an easy-spin™ Total RNA Extraction Kit (Intron Biotechnology). The RNA was subjected to Gene Microarray hybridization after estimating its quantity and integrity using a 2100 Bioanalyzer (Agilent Technologies) and an Agilent RNA 6000 nanokit, with only samples meeting specific conditions selected for microRNA analysis. RNA quantity and quality were evaluated using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific) for cDNA synthesis. The Maxime RT-PCR premix kit (Intron

Biotechnology) was used to reverse transcribe RNA samples (500 ng) to cDNA (total volume of 20 μL).

4. Statistical analysis

The clinical data from the dogs in this study were analyzed using the unpaired t-test, and statistical analysis was performed using the Statistical Package for the Social Sciences (SPSS) software (version 25.0; SPSS Inc., Chicago, IL, USA), where significance was set at $p < 0.05$. Raw data from the Affymetrix miRNA microarray were extracted using the AGCC and processed into CEL files using Affymetrix® Power Tools software. Meanwhile, mRNA level data were preprocessed, aligned to the *Canis lupus familiaris* genome using HISAT (hierarchical indexing for spliced alignment of transcripts), and assembled using StringTie; expression abundance was calculated as read count or FPKM per sample. Differentially expressed genes (DEGs) were identified through comparative analysis and statistical hypothesis testing, and their functions were analyzed using the Kyoto Encyclopedia of Genes and Genomes (KEGG) mapper and clusterProfiler. The miRNA-gene associations were collected from miRDB, and a gene-level analysis was performed based on miRNA targets. Further analysis of miRNA target genes and DEGs at mRNA levels was performed.

PART III

MiRNA expression in female dog reproductive system

Chapter I. MicroRNA Expression Variation in Female Dog (*Canis familiaris*) Reproductive Organs with Age and Presence of Uteropathy

1. Introduction

Aging is a complex process in which environmental and genetic factors can modulate the accumulation of cellular and molecular damage, leading to the functional decline of tissues and organs. Recently, numerous studies have suggested the cellular hallmarks of aging (López-Otín et al., 2013). One of which is epigenetic alterations, which represent a mechanism of aging and age-related disorders. Epigenetic alterations are mediated by three major mechanisms: DNA methylation, histone modification, and regulation by noncoding RNAs. MicroRNAs (miRNAs) are 20–24 nucleotide-long ncRNAs that repress translation or induce mRNA degradation of target transcripts with important roles in all biological pathways (Bushati & Cohen, 2007). Approximately 60% of all human mRNAs are predicted to be controlled by miRNAs (Bates et al., 2009; Garzon et al., 2009), and the most common mode of post transcriptional gene regulation is the alteration in protein expression due to the miRNA regulation (O'Brien et al., 2018). In mice, the miRNAs of the brain, liver and skeletal muscles have been shown to be differentially expressed with age (Khanna et al., 2011), and in humans, changes in peripheral blood mononuclear cell miRNA expression have also been reported (Hunter et al., 2008; Noren Hooten et al., 2013).

It has been assumed that humans and dogs (*Canis familiaris*) share many attributes, including ageing. Therefore, it has been proposed that domesticated dogs are a powerful model to better understand genetic and environmental determinants of aging (Lee et al., 2022). It has become an accepted general rule that female mammals live longer than male mammals. However, dogs did not show age-specific mortality differences between sexes. Among intact animals, there is a pattern for male dogs to live slightly longer than females, but among neutered dogs, females live longer (Hoffman et al., 2018). One of the most common uterine diseases in intact female dogs is pyometra, which affects approximately 50% of the intact population (Egenvall et al., 2001). Pyometra is reported as one of the most common proliferative and inflammatory diseases of the canine endometrium (Hagman, 2017), and mainly affects middle-aged or older individuals. However, it has been reported in individuals from 4 months to 18 years, with a mean age of 6–8 years (Egenvall et al., 2001). In Sweden, an average of 20% of all bitches are diagnosed with pyometra before 10 years of age (Hagman, 2022). Although the complex pathogenesis of pyometra including hydrometra and mucometra is not completely understood, it involves both hormonal imbalances and bacterial infections. Resistance to bacterial infections in the reproductive tract often depends on the innate immune system of the uterus (Wira et al., 2005). A study on miRNA expression in LPS-induced endometritis bovine endometrial cells suggested that the inflammatory cascade affects miRNAs(Yan et al., 2021). Prior studies have also indicated correlation between miRNAs and inflammatory diseases such as pneumonia (Liu et al., 2015), mastitis (Wang et al., 2016) and endometritis (Hailemariam et al., 2014).

While there have been previous studies on miRNAs in dogs with diseases such as cardiac hypertrophy (Ro et al., 2021), and cervical spondylomyelopathy (Vansteenkiste et al., 2019), there has not been a study on miRNAs in female reproductive organs from individuals of varying ages and disease statuses. In this study, I aimed to compare the expression profiles of miRNAs in the ovaries, oviducts, and uteri from dogs of different ages to dogs with uteropathy. I hypothesized that (López-Otín et al., 2013) the miRNAs of reproductive organs would be differentially expressed according to age and disease status and (Bushati & Cohen, 2007) that transcript expression complementary to the miRNAs would provide similar results.

2. Materials and methods

2.1 RNA Isolation and Quality Check of RNA and cDNA Synthesis

Total RNA was extracted from samples of each reproductive organ (at least 50 mg), and was homogenized with 1 mL of RNA lysis solution using the easy-spin™ Total RNA Extraction Kit (Intron Biotechnology) per the manufacturer's instructions. For gene microarray hybridization, RNA integrity and quantity were evaluated using a 2100 Bioanalyzer (Agilent Technologies) with an Agilent RNA 6000 Nano Kit. Only samples conforming to the conditions (A260/A280 and A260/A280; >1.0, concentration; >50 ng/ μL, volume; >10 μL, total amount; >0.7 ug, rRNA ratio; >1.0, RIN; >7.0 with visible small RNA peaks) were subjected to

microRNA analysis. The quality and quantity of the RNA were measured using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific), and then the RNA samples (500 ng) were reverse-transcribed to cDNA (total volume of 20 L) using the Maxime RT-PCR premix kit (Intron Biotechnology).

2.2. Real-Time PCR Quantification

Real-time PCR analysis was performed using a StepOnePlus Real-Time PCR System (Applied Biosystems, Waltham, MA, USA, #4376600) with 2 x SYBR Green PCR Master Mix (Applied Biosystems, Waltham, MA, USA, #4309155). Relative gene expression results were normalized to the reference gene β-actin (ACTB), which has been recommended as a stable and common housekeeping gene in canine reproductive tissues (Du et al., 2016), then expressed in arbitrary units. The reactions were performed according to the manufacturer's protocol. To evaluate the effect of miRNA regulation, I first selected miRNAs and searched for target genes using the MicroRNA Target Prediction Database, TargetScan (https://www.targetscan.org/cgi-bin/targetscan/vert_71/, accessed on 1 July 2021). The gene names, accession numbers of the 14 selected genes, and their primer sequences (Bioneer, Daejeon, Republic of Korea) are listed in Table 1. The reaction parameters were as follows : initial enzyme activation step for 10 minute at 95 °C ; 40 cycles of 10 s at 95 °C, 20 s at 60 °C, and 40 s at 72 °C. Cooling was carried out for 30 s at 40 °C. Each sample was amplified three times. Relative quantification of

gene expression levels was assessed using the ΔΔCt method : relative quantity (R) = $2^{-(\Delta Ct\ sample - \Delta Ct\ control)}$.

Table 1. Predicted target of miRNA and primer sequence of each genes.

MicroRNA	Ortholog of target gene	Gene name	Ori	Primer sequence	NCBI accession number
cfa-miR-151	*APH1A*	APH1A gamma secretase subunit	F	cctactacaagctgcttaag	XM_038423042.1
			R	gataacagagaagacaccac	
cfa-miR-708	*MRPS35*	mitochondrial ribosomal protein S35	F	gcacgagtagtaaccttaag	NM_001284487.1
			R	ctgtctgttgtgatggtaag	
	SLC37A4	solute carrier family 37 (glucose-6-phosphate transporter), member 4	F	gttgtctccttcctctgt	NM_001287131.2
			R	gtaatgtactctcgtccttc	
cfa-miR-30d	*LHX8*	LIM homeobox 8	F	cacatccattctactgactg	XM_038670394.1
			R	tctgcagaggactttctc	
	CYP24A1	cytochrome P450, family 24, subfamily A, polypeptide 1	F	gtatactgctggcttacttg	XM_038434121.1
			R	ggacaggtacatttagtgac	
cfa-miR-140	*SIGMAR1*	sigma naon-opioid intracellular receptor 1	F	cagactcacataccacaag	XM_038681024.1
			R	ccagacagagtataataccc	
	HELLS	helicase, lymphoid-specific	F	gagaaagaagagaggaagag	XM_038439975.1
			R	cacagagattagaagaggag	
cfa-miR-23a	*SESN3*	sestrin 3	F	ctgtgtttccctactgtatc	XM_038429728.1
			R	ctgttgactgagaggaatac	
	AUH	AU RNA binding methylglutaconyl-CoA hydratase	F	gatatacgtgtagcagcttc	XM_038655406.1
			R	gtgcagagaagatgagct	
cfa-miR-10a	*PATL1*	PAT1 homolog 1, processing body mRNA decay factor	F	cttcctaccttctgtgttac	XM_038424237.1
			R	ctacggtctactctgaactg	
	ZNF367	zinc finger protein 367	F	cataggctgctattctgtag	XM_038654936.1
			R	ctgtactagaagtcccgtat	
cfa-miR-26a	*ARPP19*	cAMP-regulated phosphoprotein, 19kDa	F	ttggatccaggcatcttttc	XM_038442363.1
			R	gtgggtggagcaggaagata	
	STRADB	STE20-related kinase adaptor beta	F	ggacatgcacaggactcaga	XM_038447612.1
			R	tcgggaattcttcattctgg	
cfa-mi125b	*EIF1AD*	eukaryotic translation initiation factor 1A domain containing	F	ggctgagatctcctttgtgc	XM_038424930.1
			R	tcctgatgactgtggctctg	

2.5. Statistical Analysis

The clinical data of the dogs in this study are presented as mean standard deviation. Comparative analysis among the groups was performed using the unpaired *t*-test, and statistical analysis was performed using the SPSS software (version 25.0; SPSS Inc.). Statistical significance was set at the less than 0.05.

3. Results

The clinical trial results of the female dogs used in this study are shown in Table 2. The breeds of healthy young females under 1 year of age (average 8.5±1.9 months) were: Mixed(n=1), Shiba inu (n=1), Pomeranian (n=1), Maltese (n=1), and Poodle (n=2). The average hematological data was as follows: total protein (6.2±0.3 g/dL), glucose (116.3±5.9 mg/dL), blood urea nitrogen (BUN, 19.9±1.2 mg/dL), creatine (0.6±0.1 mg/dL), white blood cell (WBC, 10.3±0.8 ($x10^9$/L)), red blood cell (RBC, 7.0±0.2 ($x10^{12}$/L)), hematocrit (HCT, 47.6±1.0%), platelet (277.3±36.7 ($x10^9$/L)), alkaline phosphatase (ALKP, 198.3±24.0 U/L), and alanine aminotransferase (ALT, 52.2±5.8 U/L).

Table 2. Clinical examination of dogs included in this study.

Variables (mean± SEM)	Healthy Dogs below 1 yr (n=6)	Healthy Dogs above 3 yr (n=6)	Uteropathy (n=6)	Reference value
Age, months	8.5±1.9	78.2±29.0	104.4±15.1	
Breed	mixed, shiba inu, pomeranian, maltese, poodle	poodle, bichon frise, shih tzu, chihuahua, maltese	mixed, shih tzu, spitz, yorkshire terrier	
Total protein	6.2±0.3	6.1±0.2	7.1±0.4	5~7.2 (g/dL)
Glucose	116.3±5.9	109.3±2.3	115.0±14.1	75~128 (mg/dL)
BUN	19.9±1.2	20.5±2.9	24.1±14.8	9.2~29.2 (mg/dL)
Creatine	0.6±0.1	0.6±0.1	0.7±0.3	0.4~1.4 (mg/dL)
WBC	10.3±0.8	10.6±0.5	12.1±4.2	5~20 ($x10^9$/L)
RBC	7.0±0.2	7.1±0.2	7.2±0.8	5.5~8.5 ($x10^{12}$/L)
HCT	47.6±1.0	48.8±1.7	48.3±6.7	35~56 (%)
Platelet	277.3±36.7	348.5±36.8	324.7±195.8	100~500 ($x10^9$/L)
ALKP	198.3±24.0	176.0±9.3	359.5±211.8	47~254 (U/L)
ALT	52.2±5.8	62.3±6.7	46.8±48.9	17~78 (U/L)

*n, number of examined canine; BUN, blood urea nitrogen; WBC, white blood cell; RBC, red blood cell; HCT, hematocrit; ALKP, alkaline phosphatase; ALT, alanine aminotransferase.
*Reference value : FUJIFILM (https://www.fujifilm.com/jp/ja/healthcare/veterinary/chemical-analysis/common-slide/slide)

The breeds of healthy females above the age of 1 year (average 78.2±29.0 months) were: poodles (1), bichon frise (1), shih tzu (2), chihuahua (1), and maltese (1). The hematological data was: total protein (6.1±0.2 g/dL), glucose (109.3±2.3 mg/dL), BUN (20.5±2.9 mg/dL), Creatine (0.6±0.1 mg/dL), WBC (10.6±0.5 ($x10^9$/L)), RBC (7.1±0.2 ($x10^{12}$/L)), HCT (48.8±1.7%), Platelet (348.5±36.8 ($x10^9$/L)), ALKP (176.0±9.3 U/L), ALT (62.3±6.7 U/L).

There were six breeds of females with pyometra (104.4±15.1 months): mixed (3), shih tzu (1), spitz (1), and yorkshire terrier (1). The mean hematological data were: total protein (7.1±0.4 g/dL), glucose (115.0±14.1 mg/dL), BUN (24.1±14.8 mg/dL), creatine (0.7±0.3 mg/dL), WBC (12.1±4.2 ($x10^9$/L)), RBC (7.2±0.8 ($x10^{12}$/L)), HCT (48.3±6.7 %), platelet (324.7±195.8 ($x10^9$/L)), ALKP (359.5±211.8 U/L), ALT (46.8±48.9 U/L). The reference range of each hematological level is as follows (FUGIFILM). Total protein : 5~7.2 (g/dL); glucose : 75~128 (mg/dL); BUN : 9.2~29. 2(mg/dL); creatine : 20.4~1.4 (mg/dL); WBC : 25~20 ($x10^9$/L); RBC : 5.5~8.5 ($x10^{12}$/L); HCT (Hematocrit) : 35~56 (%); platelet :100~500 ($x10^9$/L); ALKP : 47~254 (U/L); and ALT : 17~78 (U/L). The hematological results of healthy bitches both under and over 1 year of age did not deviate from the reference ranges. However, bitches with pyometra presented with high levels of BUN, platelets, ALKP, and ALT, though they were still considered for general anesthesia during OHE surgery at a local animal hospital. Patients diagnosed with pyometra were those whose pus was discharged outside of the vagina and only those who were clearly diagnosed by the ultrasonography and then checking the pus in the uterine cavity during OHE were included in this study.

The miRNA volume plots for comparison between immature dogs younger than 1 year and mature dogs older than 3 years of age are illustrated in Figure 1. With a fold change cut-off of >1.5 (up or down regulation) and a *p*-value of <0.05, two miRNAs (miR-151 and miR-708) were found to be significantly upregulated in the ovarian tissue of mature dogs compared to that of immature dogs. In the oviducts, miR-30d and miR-140 were highly expressed in mature dogs. In the uterus, the top 5 significant miRNAs were identified by considering the volume value, fold change, and *p*-value. All five miRNAs (miR-29a, miR-125a, miR-23a, miR-10a, and miR-221) were overexpressed in the uterus of elderly dogs compared to in young dogs.

The volume plots of miRNA for comparison between mature dogs above 3 years of age and older dogs with uteropathy are shown in Figure 2. Using the same aforementioned criteria, 49 significant miRNAs (1 downregulated, 48 upregulated miRNAs— navy dots) were identified in the ovaries. The expression of let-7a, let-7b, let7c, miR-125b, and miR-26a in the ovaries of mature dogs with uteropathy was relatively higher than in those of healthy dogs over 3 years of age. However, the expression of miR-203 was lower in the oviducts of dogs with uteropathy than in those of healthy dogs over 3 years of age. No significant differences were found in the miRNA expression in the uteri.

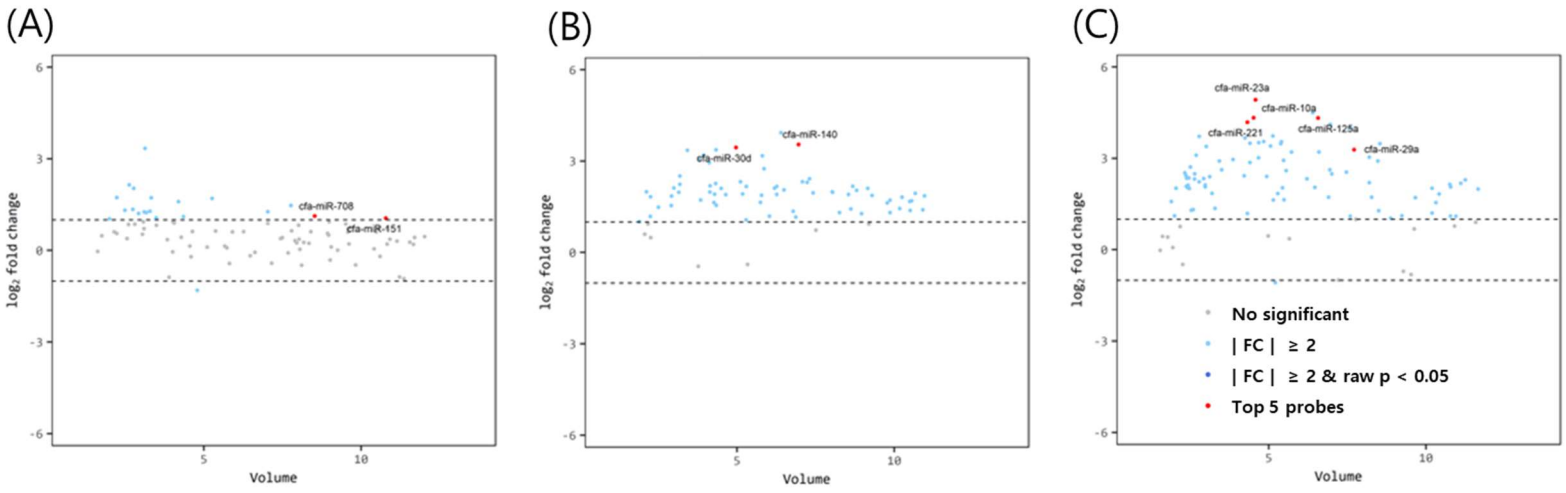

Figure 1. Volume plots of miRNAs in (**A**) ovary, (**B**) oviduct and (**C**) uterus of above 3 years dogs compared with below 1 years old dogs. The volcano plot shows the fold change and according volume of the miRNAs between the dogs with healthy young and old dogs. The horizontal line displayed a 2-fold change differences. Gray dots mean no significance for comparison between volume and fold change value. Sky blue dots mean above 2-fold change differences without $p < 0.05$. cfa-miR = *Canis lupus familiaris* microRNA. Red dots means significant top five miRNAs for comparisons.

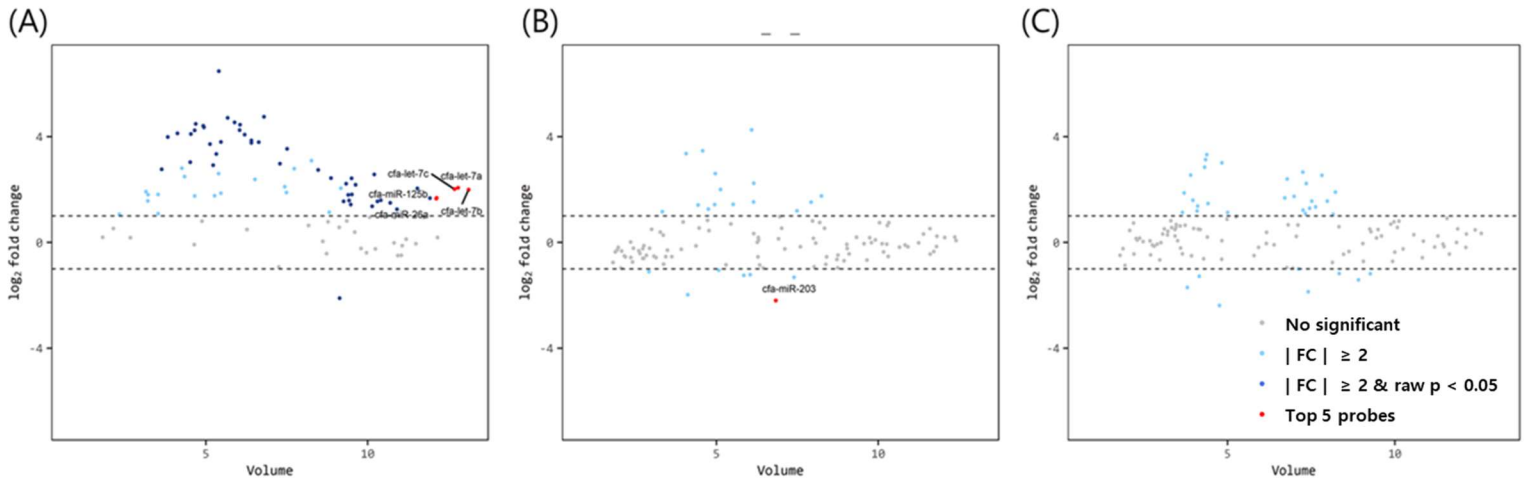

Figure 2. Volume plots of miRNAs in (A) ovary, (B) oviduct and (C) uterus of dogs older than 3 years old with uteropathy compared to healthy dogs older than 3 years old. The volcano plot shows the fold change and according volume of the miRNAs between the two groups. The horizontal line displayed a 2-fold change differences. Gray dots mean no significance for comparison between volume and fold change value. Sky blue dots mean above 2-fold change differences without $p < 0.05$. Navy blue represents significant fold change and $p < 0.05$. cfa-miR = *Canis lupus familiaris* microRNA. Red dots mean significant top five miRNAs for comparisons.

Finally, to identify potential target molecules that might be directly affected by the upregulation of miRNAs, I determined *in silico* common targets of the upregulated miRNAs. Fourteen genes were identified, 14 gene primer pairs for real-time PCR gene quantification analysis were designed, then the gene expression levels were compared (Figure 3). The relative expression levels of the predicted target *APH1A* of miR151 and *MPRS35* of miR708 was significantly lower in mature ovaries than in immature ovaries. In the oviducts, there were no significant differences between the predicted target genes of miR30d and miR140. In uteri, miR-23a predicted the target *AUH*, whose expression in mature uteri was lower than in immature uteri. To determine whether miRNA expression changes were correlated with their targeted mRNA dysregulation, three targeted genes were selected. Although *ARPP19* expression as predicted by miR-26 was higher in uteropathy afflicted ovaries, *STRADB* predicted by miR26a, and *E1F1AD* predicted by miR125b presented with no significant differences between the groups.

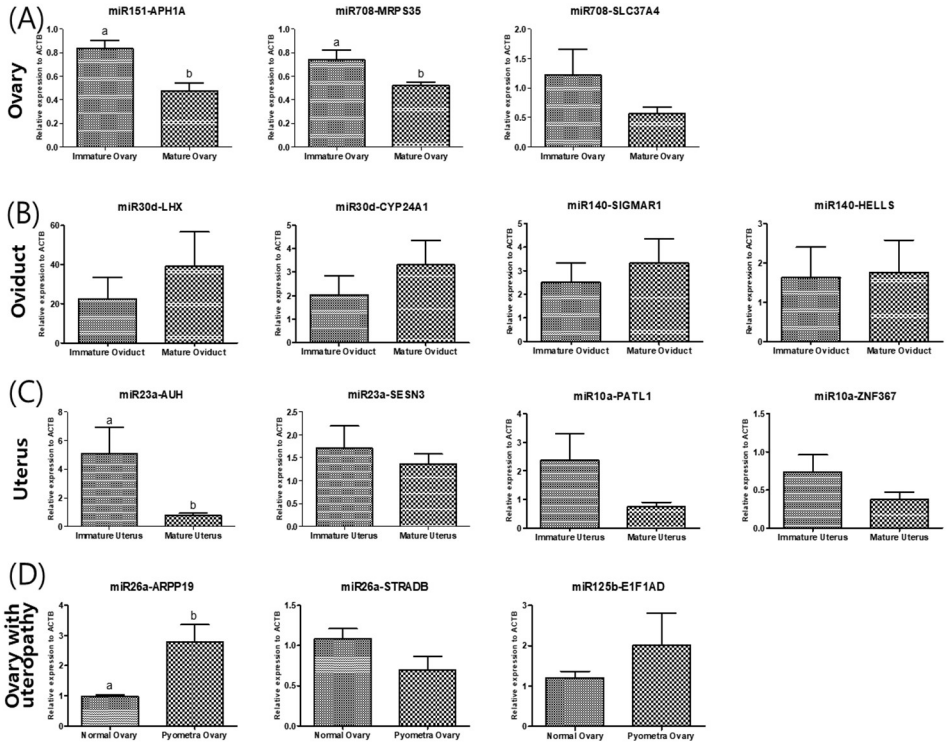

Figure 3. Validation of the differentially expressed levels of each predicted of miRNA of (A) ovary, (B) oviduct, (C) uterus and (D) ovary of dogs with uteropathy. (A) The differentially expressed miR in ovary was cfa-miR-151 and cfa-miR-708. The cfa-miR-151 is expected to target *APH1A*, cfa-miR-708 target *MRPS35* and *SLC37A4*. (B) The differentially expressed miR of oviduct is cfa-miR-30d and cfa-miR-140. Their predicted target is *LHX8* and *CYP24A1* of cfa-miR-30d and *SIGMAR1* and *HELLS* of cfa-miR-140. (C) The differentially expressed miR in uterus was cfa-miR-23a and cfa-miR-10a. The cfa-miR-23a is expected to target *SESN3* and *AUH*, cfa-miR-10a target *PATL1* and *ZNF367*. (D) The differentially expressed miR of ovary with uteropathy is cfa-miR-26a and cfa-miR-125b. Their predicted target is *ARPP19* and *STRADB* of cfa-miR-26a and *E1F1AD* of cfa-miR-

125b. The gene expression was validated by performing quantitative real-time PCR. The data is shown as mean ± SD, $p < 0.05$. cfa-miR = *Canis lupus familiaris* microRNA.

4. Discussion

Although it is widely recognized that aging is a key factor in explaining infertility, to the best of my knowledge, no data on the miRNA expression profiles of reproductive organs of dogs of various ages have been published. Therefore, I aimed to explore the miRNA profiles of reproductive organs including the ovaries, oviducts, and uteri from immature, mature, and uteropathy afflicted dogs. The age-related differences in miRNA expression in each tissue were analyzed by dividing the dogs into two age groups: younger than 1 year, and older than 3 years. In addition, to examine the effect of uteropathy on miRNA expression, the same reproductive organs of individuals with and without uteropathy were compared. The diagnostic criterion for uteropathy was whether there was an increase in echo in the sonographs of dogs who came to the local veterinary hospital with vaginal discharge.

Cellular senescence from aging presents as various epigenetic changes, for which there are three main mechanisms: DNA methylation, histone modifications, and regulatory microRNAs (miRNAs) (McCulloch et al., 2017). MiRNAs are classified according to their size— some are small ncRNAs involved in gene regulation as post-transcriptional regulators, and others are elements of chromatin-modifying

complexes. My study confirmed that the expression of various types of miRNAs in female reproductive organs are affected by age and uterine diseases.

The miR-151 predicted target, APH1A (anterior pharynx-defective 1), is a component of γ -secretase. γ -secretase is not only able to cleave type-I transmembrane proteins, but also the amyloid precursor protein and Notch receptors (Ran et al., 2017). Alterations in the Notch pathway are significantly associated with poor clinical outcomes in patients with ovarian cancer (Hu et al., 2014), and therefore I can speculate that the expression of miR-151, *APH1A* and the notch3 pathway may be correlated with the ovaries and associated with aging. One human study examined miRNAs in serum from young and old individuals. The small RNA sequencing and real-time RT-PCR results showed increased levels of miR-151 in old individuals(Noren Hooten et al., 2013). In my study, the results of miR-151 also showed a significant increase, as expected from the target gene *APH1A* transcript expression.

The microRNA-30 (miR-30) family contains five members and six mature miRNA molecules (miR-30a, miR-30b, miR-30c-1, miR30c-2, miR-30d, and miR-30e), and affects the expression of recombinant proteins by regulating the ubiquitin E3 ligase-Skp2-induced ubiquitin pathway, thereby affecting the development of the reproductive system (Fischer et al., 2015). Interestingly, one study using canine oviductal cells and their microvesicles found that miR- 30b with miR-375 and miR-503, which target factors such as WNT, MAPK, neurotrophin, and ubiquitin, could be detected and were involved in follicular growth and oocyte maturation (Lange-

Consiglio et al., 2017). Similarly, my study found that miR-30d was among the significant miRNAs that were more highly expressed in the oviducts of older dogs.

It has been shown that the levels of miR-7, miR-468, miR-542, and miR-698 increased in mouse muscles due to skeletal muscle aging, whereas miR-124a, miR-181a, miR-221, miR-382, miR-434, and miR-455 decreased (Hamrick et al., 2010). Interestingly, miR-221 was more highly expressed in the uterus, which is the representative muscle tissue of the female organ in dogs older than 3 years of age. It has is known that miR-221 along with miR-222 in mice can promote vascular smooth muscle cell calcification (Mackenzie et al., 2014), and I thus assumed that miR-221 regulates smooth muscle aging. Furthermore, aged uteri in dogs showed upregulated miR-125a in fold changes and volcano plots in my study. It has been shown that miR-125, a well-conserved homolog of lin-4, and co-transcribed in Drosophila, has been shown to modulate lifespan in a sex- and tissue-specific manner (Chawla et al., 2016). Moreover, my miR-125 results from uteri of dogs appears consistent with those of a study showing that miR-125 overexpression in neurons reduces female Drosophila lifespan (Chawla et al., 2016).

When miRNA expression in younger (31 ± 2 years old) and older (73 ± 3 years old) human skeletal muscle was compared, 18 miRNAs were found to be differentially expressed, with let-7b and let-7e being upregulated in the older muscles (Drummond et al., 2011). However, let-7a, let-7b, and let-7c in the ovaries of dogs with uteropathy were upregulated compared to in healthy dog ovaries. Interestingly, in an *in vitro* inflammatory model using bovine endometrial epithelial

cells, let-7c was found to play an important role for regulating inflammation (Yan et al., 2021). One study found that overexpression of this miRNA reduced uterine inflammation (Zhao et al., 2019) and another study showed that let-7c reduced the release of proinflammatory cytokines (Jiang et al., 2014). Unlike my findings, the results of these previous studies suggest that the let-7 family serves as an anti-inflammatory mimic. However, if the function of let-7 is assumed to be also anti-inflammatory in the ovaries of dogs, it can be assumed that the ovarian let-7 family, including let-7a, let-7b, and let-7c, increases as a compensatory response to reduce or repair uterine inflammation or pathological status. According to prior literature, the role of ovaries in controlling inflammatory diseases in the uterus is unclear. However, my study demonstrated that the let-7 expression in the ovaries of dogs with an inflammatory uterine disease was significantly higher than in healthy ovaries, presumably to provide a specific signal for the inflammation of the uterus. These results indicate that further studies on the effects of let-7 on the uterus are needed.

This study had several limitations. Firstly, the breeds of healthy dogs across the age groups did not match. Secondly, the estrous cycle of each dog was not evaluated. In human studies, miRNA expression has been shown to fluctuate, such as in the levels of the two main classes of endocrine hormones from the follicular, ovulation, and to the luteal phase (Toms et al., 2017). Thirdly, only dogs with vaginal discharge for uteropathy were included in this study. On histological examination, cystic endometrial hyperplasia—defined as proliferation of endometrial glands—endometrial hyperplasia, and cyst formation, can present with or without fluid accumulation in the uterus. Uterine diseases can also be classified into mucometra,

hydrometra, and pyometra according to the type of fluid present in the uterus and the degree of mucin. Although pus in the uterus found from the OHE indicated pyometra and thus which individual's tissue to use, the etiological pathogen could be either *Escherichia coli*, *Staphylococcus spp*., or *Streptococcus* (Hagman, 2022). The exact pathogens and associated miRNAs need to be clarified in further studies. The last limitation is the small sample size. Large-scale follow-up studies are required to examine the effects of age and the most common female reproductive disease, pyometra, on miRNA expression.

5. Conclusions

In this study, the expression of miRNAs in female dog reproductive tissues was investigated based on aging and the presence of uteropathy. Results showed that miR-151 and miR-708 had higher expression levels in mature dog ovaries compared to immature dog ovaries. The expression levels of let-7a, let-7b, let-7c, miR-125b, and miR-26a were relatively higher in the ovaries of mature dogs with uteropathy compared to healthy mature dogs. In the oviducts, miR-30d and miR-140 exhibited significantly higher expression levels in mature dogs, while miR-203 was lower in dogs with uteropathy compared to healthy mature dogs. The expression levels of miR-29a, miR-125a, miR-23a, miR-10a, and miR-221 were higher in the uteri of mature dogs compared to immature dogs. However, there were no discernible differences in miRNA expression between mature dogs and those with uteropathy. These findings indicate that both aging and uteropathy influence miRNA expression

in the reproductive organs of female dogs. Thus, these miRNAs may serve as biological markers for aging and reproductive diseases in female dogs. With further research based on my study, the identified miRNAs will provide clues and therapeutic targets for delaying aging, improving fertility, and preventing uterine diseases.

Chapter II. MicroRNA and Messenger RNA Expression Profiles in Canine Mammary Gland Tumor.

1. Introduction

Cancer is the most common cause of death in dogs (Gardner et al., 2016). Among various cancers, canine mammary gland tumor (CMT) is the most frequently occurring tumor in intact female dogs (Dorn et al., 1968; Sorenmo, 2003; Thumser-Henner et al., 2020). Approximately 50% of the CMT cases lead to malignancy (Gilbertson et al., 1983); owing to its high incidence and mortality rates, CMT is of significance in canine medicine. To date, several studies have focused on CMT owing to its highly complex pathogenicity (Biondi et al., 2021; Khand et al., 2020; Kowal et al., 2022; Nassiri et al., 2021; Thumser-Henner et al., 2020).

In addition to its clinical significance to dogs, studies on canine cancer are also applicable to medical research targeting human diseases, as dogs have been considered to be ideal animal models (Gardner et al., 2016). Dogs contract clinically similar diseases to humans; they also share similar organ sizes with humans (Hong et al., 2009; Tsai et al., 2007). The fact that dogs share similar living spaces with their human owners, minimizes gaps in environmental factors between the two (Hong et al., 2009; Tsai et al., 2007). Since dogs age faster than humans and usually experience minimal genetic variations owing to breeding, they are valuable animal models of human disease (Rowell et al., 2011). The variety of studies conducted on

dogs to date have continuously proven the value of dogs as model species connecting mice and humans.

Dogs have greater genetic similarity with humans than mice (Tsai et al., 2007). About half of the hereditary diseases in dogs are remarkably similar to those in humans (Tsai et al., 2007). CMT in dogs share similarities with breast cancer (BC) in humans: In dogs, diverse genes including Tumor protein 53 (*p53*), Breast Cancer1 (*BRCA1*), Breast Cancer 2 (*BRCA2*), Phosphatase and Tensin Homolog (*PTEN*), as well as E-cadherin and Serine/Threonine kinase11 (*STK11*) are known contributors of CMT (Huskey et al., 2020), which have also been attributed to human BC.

The microRNAs (miRNAs), also known as small ncRNAs, are usually composed of about 22 nucleotides. In addition, miRNAs play key roles in post-transcriptional gene silencing by pairing with complementary messenger RNA in diverse animal clades (O'Brien et al., 2018). Thanks to their functional characteristics, miRNA studies have been actively conducted to gain new insights in human diseases. In BC, studies involving miRNA include using it as a biomarker, profiling miRNA for cancer diagnosis, as well as developing miRNAs as tools to understand disease prognosis, therapy response, and resistance mechanisms (Iorio et al., 2008; Mar-Aguilar et al., 2013; Zou et al., 2021).

Mature canine miRNAs show high homology to those of humans, which opens possibilities for comparative studies (Wagner et al., 2013). The importance of miRNA in canine cancer studies has been identified to be of significance leading to a number of studies (Craig et al., 2019; Kobayashi et al., 2017; Leonardi et al., 2021;

Sahabi et al., 2018). A miRNA study on CMT, targeting 10 types of human oncomirs identified nine miRNAs (let-7f, miR- 15a, miR-16, miR-17-5p, miR-21, miR-29b, miR-125b, miR-155, miR-181b) which showed expression patterns similar to miRNAs in human BC (Boggs et al., 2008). However, systemic miRNA profiling of CMT is yet to be conducted.

The objective of this study was to identify the expression patterns of miRNAs in CMT tissues. Benign and malignant CMTs determined based on histopathology and metastasis patterns would be compared with miRNA expression profiles of normal tissues. Similar comparative analysis would also be performed between benign and malignant CMTs. Differentially expressed miRNAs (DE-miRNAs) from the above comparative analyses would be listed, and their functions and expression patterns in both dogs and humans would be discussed based on the existing literature.

2. Materials and Methods

2.1. Collection of Tissue Samples

Tissue samples were collected from dogs at Seoul National University Veterinary Medical Teaching Hospital as well as from over 10 other local animal hospitals in the Republic of Korea during 2020–2021. Eight normal tissues and twelve BCMT tissues were collected from twelve dogs with BCMT (Table 3). Similarly, four normal tissues and eight MCMT tissues were collected from eight

dogs with MCMT (Table 3). Blood tests were performed for all animals to rule out other medical issues. Normal, unaffected tissues were also collected adjacent to the CMT mass.

2.2. Total RNA Extraction and Quality Check

Total RNA was extracted from each tissue sample using Easy-Spin Total RNA Extraction kit (Intron Biotechnology) according to the manufacturer's protocol. RNA purity and integrity were evaluated using ND-2000 Spectrophotometer (NanoDrop,Wilmington, DE, USA) and Agilent 2100 Bioanalyzer (Agilent Technologies), respectively.

Table 3. Animals used for sample collection.

Tumor Classification	ID	Breed	Sex	Age (Years)	Histopathological Features	Normal Tissue
BCMT	BCMT-1	Shih-tzu	F *	7	Benign mixed mammary tumor	Y
	BCMT-2	Alaskan malamute	F	6	Benign mammary complex adenoma	Y
	BCMT-3	Shih-tzu	FS **	11	Benign mixed mammary tumor	Y
	BCMT-4	Toy poodle	FS	12	Mammary adenoma	Y
	BCMT-5	Maltese	F	10	Simple adenoma	Y
	BCMT-6	Bichon fries	F	6	Benign mammary lobular hyperplasia	Y
	BCMT-7	Dachshund	F	11	Complex mammary adenoma	Y
	BCMT-8	Mixed	FS	16	Benign mammary adenoma	Y
	BCMT-9	Maltese	FS	11	Complex mammary adenoma	N
	BCMT-10	Cocker spaniel	F	10	Complex mammary tubular adenoma	N
	BCMT-11	Maltese	FS	14	Benign mammary complex adenoma	N
	BCMT-12	Poodle	FS	11	Benign mammary complex adenoma	N
MCMT	MCMT-1	Pomeranian	F	12	Mammary ductular adenocarcinoma, low grade (grade 1)	Y
	MCMT-2	Maltese	F	12	Mammary adenocarcinoma, high grade	Y
	MCMT-3	Shih-tzu	FS	11	Tubulopapillary mammary gland carcinoma, low grade (grade 1)	Y
	MCMT-4	Beagle	FS	11	Mammary ductular adenocarcinoma, low grade (grade 1)	N
	MCMT-5	Mixed	F	8	Multinodular mammary gland adenocarcinoma	Y
	MCMT-6	Poodle	FS	11	Mammary ductular adenocarcinoma, low grade (grade 1)	N
	MCMT-7	Poodle	FS	13	Mammary adenocarcinoma, high grade	N
	MCMT-8	Shih-tzu	F	11	Mammary carcinoma, complex type (grade 1)	N

* F: Female; ** FS: Spayed Female; Y: Collected; N: Not Collected.

2.3. Microarray Hybridization and Scanning for miRNA

Microarray hybridization was performed using the Affymetrix GeneChip miRNA 4.0 Array (Thermo Fisher Scientific) according to the manufacturer's protocol. Total RNA samples were labeled using FlashTag™ Biotin RNA Labeling Kit (Genisphere). The labeled RNA samples were then quantified, fractionated, and hybridized to the microarray according to the manufacturer's protocol. RNA-array hybridization was performed on an Affymetrix® 450 Fluidics Station (Thermo Fisher Scientific). The arrays were stained using a GeneChip Fluidics Station 450 (Affymetrix) and scanned using an Affymetrix GCS 3000 scanner (Affymetrix). The miRNA-mRNA hybridization signals were analyzed using the Affymetrix® GeneChip™ Command Console.

2.4. Raw Data Preparation and Statistical Analysis

Raw data were extracted automatically through the Affymetrix data extraction protocol using the AGCC version 6.1 (Thermo Fisher Scientific). The CEL files import, miRNA level RMA + DABG-All analyses and results were exported using Affymetrix® Power Tools (APT) Software version 2.11.4 (Thermo Fisher Scientific). Array data were filtered using probes of annotated species.

2.5. RNA-Seq Library Construction and Sequencing

Total RNA concentration was calculated using Quant-IT RiboGreen (Invitrogen, Thermo Fisher Scientific, MA, USA, #R11490). To assess the integrity of the total RNA, samples were carried out on the TapeStation RNA ScreenTape (Agilent Technologies). Only high-quality RNA preparations with RIN > 7.0 were used for RNA library construction. A library was independently prepared with 1 g total RNA of each sample using the Illumina TruSeq Stranded mRNA Sample Prep Kit (RS-122-2101, Illumina, Inc., San Diego, CA, USA). The first step in the workflow involved purifying the poly-A containing mRNA molecules using poly-T-attached magnetic beads. Following purification, the mRNA samples were fragmented into small pieces using divalent cations under an elevated temperature. The cleaved RNA fragments were then used as templates to generate first strand cDNA using SuperScript II reverse transcriptase (Invitrogen,MA, USA)) with random primers. This was followed by second strand cDNA synthesis using DNA Polymerase I, RNase H, and dUTP. Next, the generated double stranded cDNA fragments were subjected to an end repair process involving adenylation followed by adapter ligation. Thereafter, the products were purified and enriched with PCR to create the final cDNA library. The libraries were quantified using KAPA Library Quantification kits for Illumina Sequencing platforms according to the qPCR Quantification Protocol Guide (KAPA BIOSYSTEMS,Wilmington, DE, USA) and qualified using the TapeStation D1000 ScreenTape (Agilent Technologies). Finally, indexed libraries were submitted to Illumina NovaSeq (Illumina, Inc., San Diego, CA, USA) to perform paired-end (2 × 100 bp) sequencing.

2.6. Data Analysis

At the miRNA level, raw data were extracted automatically in Affymetrix data extraction protocol using the software provided by AGCC. The CEL files import, miRNA level RMA + DABG-All analyses and results were exported using Affymetrix® Power Tools (APT) Software. Array data were filtered using probes of annotated species.

At the mRNA level, I preprocessed the raw reads from the sequencer to remove low quality and adapter sequences before analysis and aligned the processed reads to the *Canis lupus familiaris* (CanFam3.1) database using HISAT v2.1.0 (Kim et al., 2015). HISAT utilizes two types of indexes for alignment (a global, whole-genome index and tens of thousands of small local indexes). These two types of indexes are constructed using the same Burrows– Wheeler transform (BWT), which is a graph FM index (GFM), such as Bowtie2. Due to its use in efficient data structures and algorithms, HISAT generates spliced alignments several times faster than Bowtie and BWA. The reference genome sequence of *Canis lupus familiaris* (CanFam3.1) and annotation data were downloaded from the NCBI database. Then, the transcript assembly of known transcripts was processed by StringTie v2.1.3b(Pertea et al., 2015). Based on the results, expression abundance of transcripts was calculated as read count or Fragments Per Kilobase of exon per Million fragments mapped (FPKM value) per sample. The expression profiles were used for additional analysis, such as DEGs. In groups with different conditions, DEGs or transcripts can be filtered through statistical hypothesis testing.

A comparative analysis between test and control samples was carried out using independent *t*-test and fold change with the null hypothesis, which indicates that no difference exists among groups. False discovery rate (FDR) was controlled by adjusting *p*-value using Benjamini-Hochberg algorithm. All statistical tests and visualization of DEGs were conducted using R statistical language (v3.3.2).

KEGG Mapper (Kanehisa et al., 2022) and clusterProfiler v3.18.1(Yu et al., 2012) in R were used to perform the functional analysis of miRNAs and genes. Based on KEGG Mapper, the roles of miRNAs and genes were discovered. Biological functions of DEGs were analyzed based on clusterProfiler. I collected miRNA-gene associations from miRDB(Wong & Wang, 2015) and performed gene level analysis based on miRNA targets. Moreover, DEGs were identified by comparisons at the mRNA level; additional analysis was performed between the miRNA target genes and DEGs at mRNA levels.

3. Results

DE-miRNAs based on microarray were identified using three comparisons: (1) BCMTT vs. BCMT-N, (2) MCMT-T vs. MCMT-N, and (3) MCMT-T vs. BCMT-T; T means tumor and N means normal. The cut-off values are |fold change| $\geq$ 2, *p*-value < 0.05 (*t*-test), and DABG *p*-value < 0.05. DABG *p*-value was computed based on the probability that the signal intensity is part of the null distribution. It could be useful in the detection and removal of low intensity signals. Eighteen

upregulated miRNAs in BCMT-T vs. BCMT-N, twenty upregulated miRNAs in MCMT-T vs. MCMT-N, and one upregulated miRNA in MCMT-T vs. BCMT-T were identified. Downregulated miRNAs were not detected in all comparisons. The volcano plot illustrates the identification of DE-miRNAs for each comparison based on fold change and *p*-value (Figure 4).

I performed the pathway analysis from KEGG based on DE-miRNAs. Among the various pathways in KEGG, I identified the role of miRNAs as “MicroRNAs in cancer”. This category contains sub-pathways for nine different types of cancer. Among these nine types, I focused on the breast cancer pathway. Since only one miRNA was detected in the MCMT-T vs. BCMT-T, this comparison was excluded from the pathway analysis. The result of the breast cancer pathway in "MicroRNAs in cancer" for the miRNAs of BCMT-T vs. BCMT-N and MCMT-T vs. MCMT-N is illustrated in Figure 5. Among the 18 array-based DE-miRNAs of BCMT-T vs. BCMT-N, breast cancer related miRNAs were identified as cfa-miR-21, cfa-miR-129b, cfa-miR-155, and cfa-miR-222.

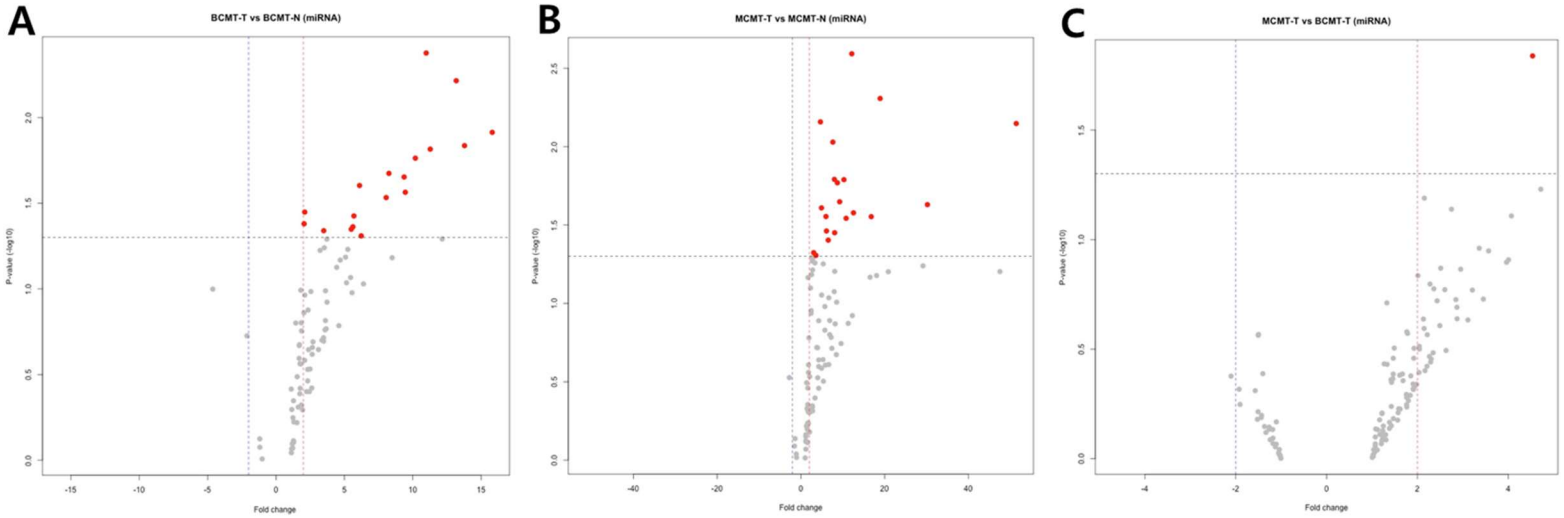

Figure 4. Volcano plot in canine mammary gland tumor for array-based miRNA. Red dots indicate upregulated miRNAs and grey dots indicate unsignificant miRNAs. Red and blue dotted line are threshold of fold change and black dotted line is threshold of *p*-value. (A) BCMT-T vs. BCMT-N; (B) MCMT-T vs. MCMT-N; (C) MCMT-T vs. BCMT-T (Figure 2).

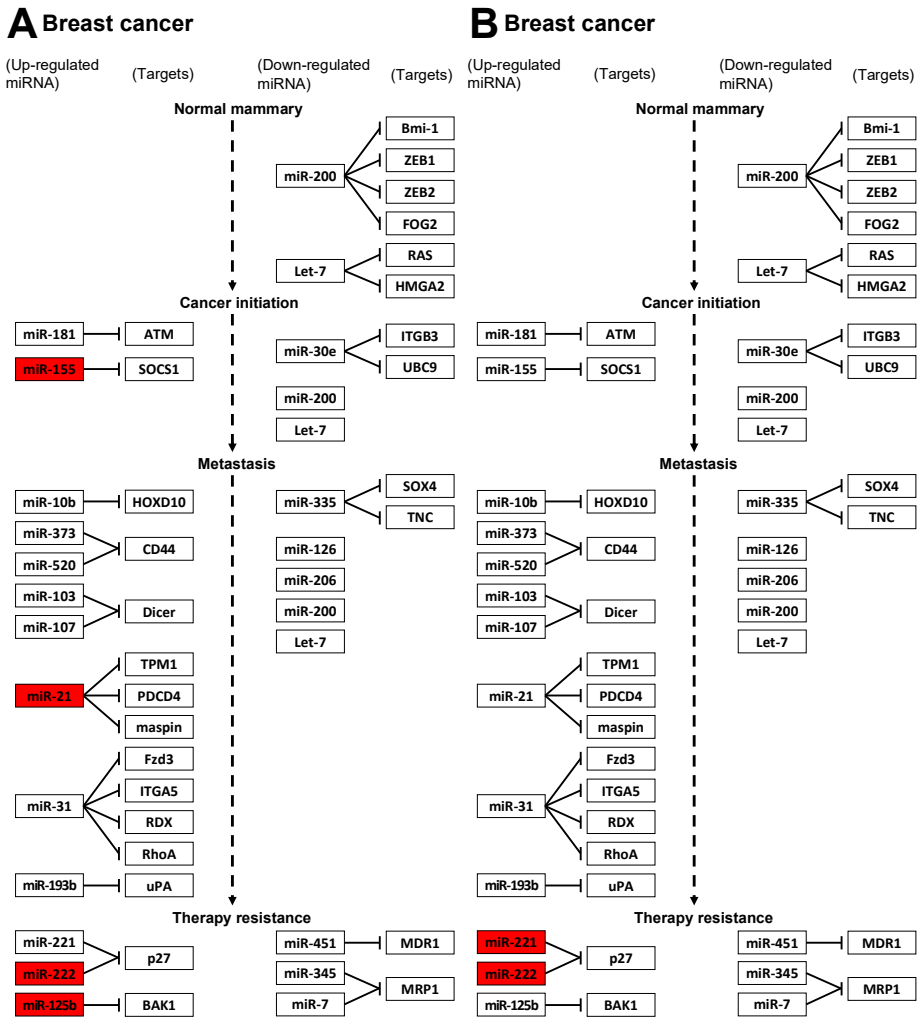

Figure 5. Pathway analysis in breast cancer. Red boxes indicate miRNAs detected in the pathway among DE-miRNAs. (A) BCMT-T vs. BCMT-N; (B) MCMT-T vs. MCMT-N.

Among the 20 miRNA of MCMT-T vs. MCMT-N, breast cancer related miRNAs were identified as cfa-miR-221 and cfa-miR-222. Moreover, I discovered

the expression pattern of target genes of breast cancer related miRNAs identified in the pathway at the mRNA level. The target genes in the BCMT group were *BAK1*, *CDKN1B*, *PDCD4*, *SERPINB5*, *SOCS1*, and *TPM1*, and the target gene in the MCMT group was *CDKN1B*. Table 4 shows the fold change and *p*-value of target genes at the mRNA level. It was found that the target genes of miRNA were downregulated in the breast cancer pathway; the genes with significant *p*-value under 0.05 at the mRNA level were downregulated (*PDCD4*, *SOCS1*, and *CDKN1B*).

Table 4. The mRNA expression profile of miRNA-target genes.

BCMT-T vs. BCMT-N		
Gene	**Fold Change**	***p*-Value**
BAK1	1.134	0.390
CDKN1B	−1.256	0.167
PDCD4	−2.486	<0.001
SERPINB5	1.081	0.838
SOCS1	−2.183	0.005
TPM1	−1.249	0.177
MCMT-T vs. MCMT-N		
Gene	**Fold Change**	***p*-Value**
CDKN1B	−1.612	0.025

I performed gene-level analysis of DE-miRNAs based on the miRNA-gene associations collected from microRNA target prediction database (miRDB). The miRDB contains information regarding predicted target genes and predicted score for miRNAs. I filtered associations with a predictive score of >90 for miRNA-gene relationships. Since the same gene is associated with multiple miRNAs, the collected target genes were used after de-duplication. 370 target genes in DE-miRNA of

BCMT-T vs. BCMT-N, 648 target genes in DE-miRNA of MCMT-T vs. MCMT-N, and 3 target genes in DE-miRNA of BCMT-T vs. MCMT-T were collected. Functional analysis was performed using clusterProfiler based on predicted genes. Among the various biological functions, I focused on specific biological functions: Autophagy, response to oxidative stress, and *p53* signaling pathway. As few target genes were detected in the MCMT-T vs. BCMT-T, this comparison was excluded from the enrichment analysis. The results for the top 10 categories in Gene Ontology and KEGG enrichment analysis are presented in Figure 6.

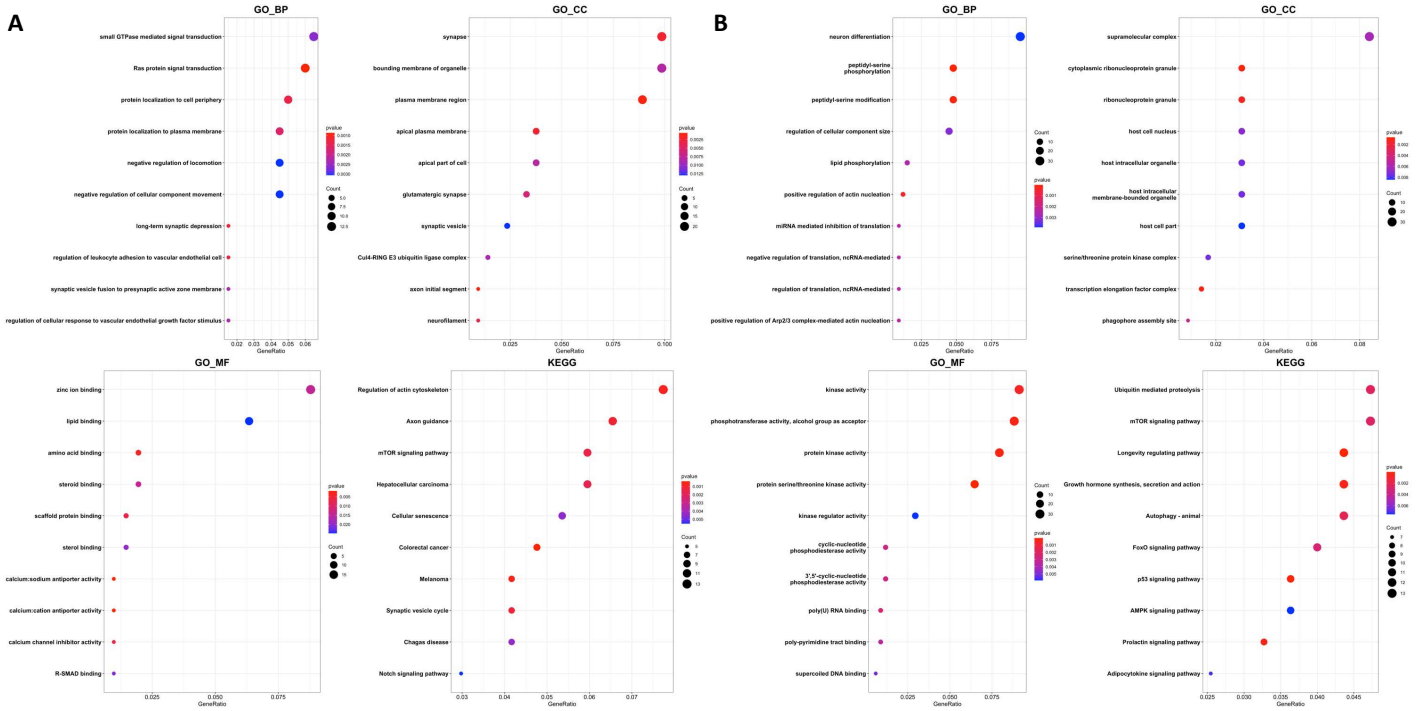

Figure 6. Enrichment analysis for GO_BP, GO_CC, GO_MF, and KEGG. GO_BP: Biological processing; GO_CC: Cellular component; GO_MF: Molecular function of Gene Ontology; KEGG: Biological pathway. (A) BCMT-T vs. BCMT-N; (B) MCMT-T vs. MCMT-N.

To evaluate the results of clusterProfiler, I collected disease related Gene Ontologies from the Comparative Toxicogenomics Database (CTD) (Davis et al., 2021). I used phenotype (GO)- disease inference networks among various information in CTD, which contains a list of diseases associated with Gene Ontology. I collected a list of GO categories associated with breast cancer using the MESH ID (D001943). In addition, GOs that do not have information on genes from the collected list were removed. I filtered 5282 categories in GO_BP, 560 in GO_CC, and 1060 in GO_MF. I compared GOs with p-value < 0.05 among clusterProfiler and filtered CTD (Figure 7).

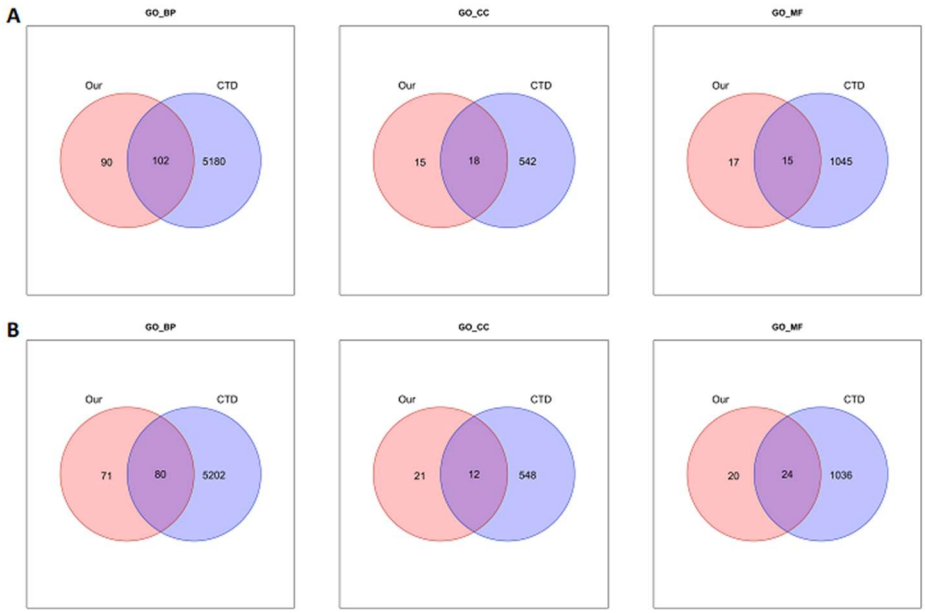

Figure 7. Venn diagrams showing GO_BP, GO_CC, and GO_MF of miRNAs. GO_BP: Biological processing; GO_CC: Cellular component; GO_MF: Molecular function of Gene Ontology; (A) BCMT-T vs. BCMT-N; (B) MCMT-T vs. MCMT-N.

It was found that the common function of the array-based DE-miRNAs' target genes was similar to those associated with breast cancer. In particular, categories related to angiogenesis were detected in the intersection. According to Nishida et al., the onset of cancer is related to angiogenesis (Nishida et al., 2006). Moreover, it was identified that angiogenesis is closely related to progression in breast cancer (Madu et al., 2020; Nishida et al., 2006). Significant pathways related breast cancer, such as melanoma (p-value < 0.001), breast cancer (p-value = 0.009), and microRNAs in cancer (p-value = 0.009) in the BCMT group, *p53* signaling pathway (p-value < 0.001), autophagy, and EGFR tyrosine kinase inhibitor resistance in MCMT group, were observed from KEGG analysis results.

I extracted mRNA level DEG for a similar comparative analysis as performed using miRNAs. The cut-off values were |fold change| ≥ 2 and p-value < 0.05. About 962 genes (392 upregulated and 570 downregulated) in BCMT-T vs. BCMT-N, and 1541 genes (856 upregulated and 685 downregulated) in MCMT-T vs. MCMT-N were identified. The volcano plot illustrates the identification of DEGs for each comparison based on fold change and p-value (Figure 8).

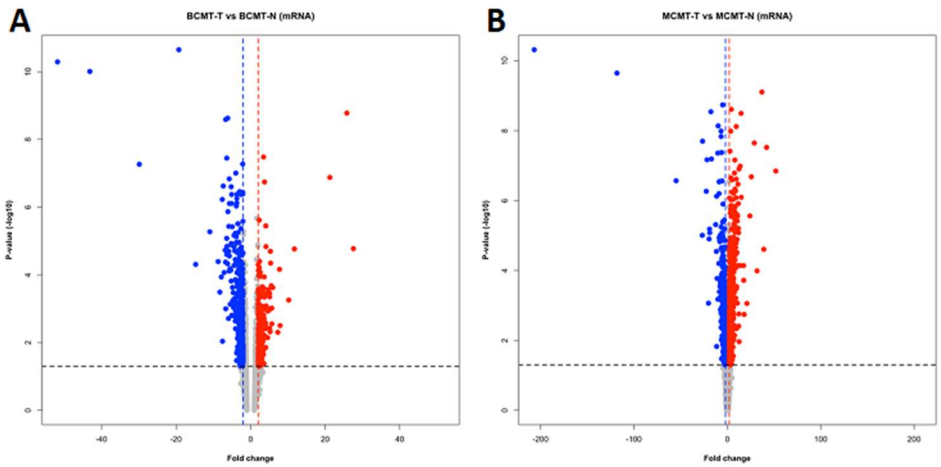

Figure 8. Volcano plot in canine mammary gland tumor for mRNA. Red dots indicate upregulation, blue dots indicate downregulation and grey dots indicate unsignificant miRNAs. Red and blue dotted line are threshold of fold change and black dotted line is threshold of *p*-value. (A) BCMT-T vs. BCMT-N; (B) MCMT-T vs. MCMT-N.

The relationship between identified DEGs and target genes of miRNA was analyzed. Genes corresponding to protein coding were filtered from DEGs. The Venn diagram illustrates the overlapping genes between DE-miRNAs target genes and DEGs based on mRNA analysis (Figure 9).

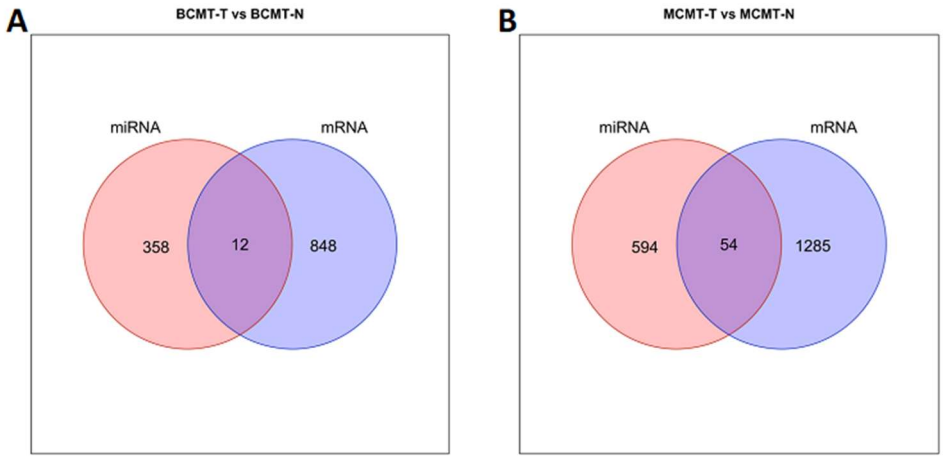

Figure 9. Venn diagram of commonly appearing genes between miRNAs and their targeted mRNAs. A) BCMT-T vs. BCMT-N; (B) MCMT-T vs. MCMT-N.

The number of genes commonly appearing between miRNA and mRNA was 12 in BCMT group and 54 in MCMT group. It was found that a small number of genes appeared as intersections compared with the number of detected genes in the miRNA and mRNA DEG analysis. To evaluate whether the genes corresponding to the intersection are breast cancer related genes, I collected disease-gene associations from DisGeNet (Piñero et al., 2015). DisGeNet is a database containing disease-gene associations for humans. Due to the lack of information on disease related genes for dogs, DisGeNet was used to verify genes corresponding to intersections. I identified 10 breast cancer related genes among 12 corresponding to the intersection in BCMT-T vs. BCMT-N (83%), and 25 related genes among 54 corresponding to the intersection in MCMT-T vs. MCMT-N (46%) (Table 5). I detected that the target

genes of miRNA showed a significant expression pattern even at the mRNA level. All of the analyzed data are addressed in Supplementary Data S2 (https: //www.mdpi.com/article/10.3390/ijms24032618/s1).

Table 5. Intersection and breast cancer related genes of miRNA and mRNA. Bold typed gene indicates breast cancer related gene.

Comparison	Genes
BCMT-T vs. BCMT-N	*ACSM5*, ***ALDH1A1***, ***ANTXR2***, ***F2RL2***, ***FOS***, ***KLF4***, ***LEF1***, ***NOVA1***, ***PDGFRB***, ***RORA***, *SDK1*, ***TRIM46***
MCMT-T vs. MCMT-N	***ALDH1A2***, ***ANK2***, ***APLN***, *B3GNT7*, *BEND6*, ***CHEK1***, *CHST1*, ***CKAP4***, *DCBLD2*, ***DCLK1***, *ECSCR*, ***ELF5***, *ELL2*, *FAM81A*, ***FBXO5***, ***FHL1***, ***GJA1***, *GJC1*, *GPAM*, *HSPA4L*, ***HUNK***, ***IGF1***, ***JPH1***, *KCNQ5*, *KLHDC1*, ***LONRF3***, ***MAP3K1***, *MEGF10*, ***MMD***, ***MMP13***, *MYBL1*, *NCKAP5*, ***NOVA1***, *NUAK2*, *PCOLCE2*, ***PGAM1***, *PRUNE2*, ***PTPRD***, *RIMS3*, ***RORA***, *RTKN2*, *SEC23A*, *SEMA6D*, ***SEMA7A***, *SESN1*, *SIMC1*, ***SLC6A6***, ***SLC7A11***, *ST6GALNAC3*, *TEF*, *TMEM51*, *TMOD2*, *VGLL3*, ***WASF3***

4. Discussion

When comparing BCMT-N and BCMT-T, 18 miRNAs were classified as DE-miRNAs with |fold change| $\geq$ 2 and p-value < 0.05. Moreover, cfa-miR-21 and cfa-miR-502 were already discovered for their overexpression in CMT and are diagnostic targets of CMT (Boggs et al., 2008; Fish et al., 2018; Piñero et al., 2015; REN Xiaoli, 2020). In particular, cfa-miR-21 is related to the inhibition of tumor cell apoptosis in dogs (Boggs et al., 2008). Human homolog genes of the five DE-

miRNAs viz. hsa-miR-21, hsa-miR-185, hsa-miR-125b, hsa-miR-500, and hsa-miR-502 are all related to human cancers, whereas all of the miRNAs except for hsa-miR-500 are related to human BC (Kalinina et al., 2020; Sun et al., 2014; Wang et al., 2020).

Hsa-miR-21 is a well-known oncomir (Sahabi et al., 2018; Si et al., 2007) that shows an increase in copy number in human tumor tissues (Czubak et al., 2015); it is indicated in tumorigenesis, apoptosis, cell proliferation, and cancer progression in human cancers (Si et al., 2007). Since hsa-miR-21 is the only miRNA overexpressed in six types of human cancers, it qualifies as an important candidate for cancer studies (Wang et al., 2020). Furthermore, hsa-miR-21 contributes to the maintenance of malignant phenotypes in certain cancers; therefore, it could be used as a biomarker for malignancy (Medina et al., 2010). Most importantly, the role of hsa-miR-21 in BC has also been studied; it is correlated with the presence and progression of BC, as it targets the tumor suppressor protein, Programmed Cell Death 4 (Asaga et al., 2011; Frankel et al., 2008). The overexpression of cfa-miR-21 is observed in both canine (CMT) and human tissue (BC) (Boggs et al., 2008). In dogs, cfa-miR-21 is attributed to the inhibition of tumor cell apoptosis (Boggs et al., 2008); it is quite natural that one of the top five DE-miRNAs was found to be cfa-miR-21 in this study (Supplementary Data S1A ; https: //www.mdpi.com/article/10.3390/ijms24032618/s1.). However, in contrast to hsa-miR-21 which is identified as a biomarker for tumor malignancy in humans, cfa-miR-21 was found only in the DE-miRNA list of BCMT and not from the MCMT tissue (Data S1A,B).

The function of hsa-miR-185 as a tumor suppressor has been established by various studies; it inhibits the proliferation of human colon cancer cell as well as the development of glioma by inhibiting global DNA methylation (Zhang et al., 2018; Zhang et al., 2011). The role of hsa-miR-185 in human BC has also been identified as a tumor suppressor since it inhibits BC by regulating S100A8/A9, NF-B/Snail signaling pathway, and programmed cell death. Although cfa-miR-185 was found to be related to IL-7R expression in dogs (Holder et al., 2018), its association with tumors has not yet been established. As observed in this study, BMGT individuals showed significantly higher expression of cfa-miR-185 in tumor tissue than normal tissues, which is contrary to the role of tumor suppression shown by hsa-miR-185 in humans.

Moreover, cfa-miR-125b is attributed to host cell resistance against canine influenza virus in (Xie et al., 2021) and to testicular retinoic acid induced spermatogenesis (Kasimanickam et al., 2014). However, the role of cfa-miR-125b in canine tumors has not yet been identified; this study is the first to record the overexpression of cfa-miR-125b in MGMT dogs. On the contrary, hsa-miR-125b has been known to act as both an oncogene and a tumor-suppressor gene. Hsa-miR-125b contributes to the regulation of glycolysis, apoptosis, metastasis, and CSCs (Peng et al., 2021). Decreased hsa-miR-125b contributes to prostate tumorigenesis via tumor cell behavior alteration as it works as a tumor suppressor (Budd et al., 2015). Furthermore, hsa-miR-125b plays a role in γ-irradiation sensitivity in BC; its increased expression results in enhanced apoptotic activity and senescence after irradiation of BC cells (Metheetrairut et al., 2017).

In this study, tumor tissue of MCMT patients showed a significant upregulation of cfa-miR-500 compared with the normal tissue (Data S1B). Since there are no existing reports on the expression patterns or the function of cfa-miR-500 in dogs, the results obtained in this study are of significance to CMT research. In humans, the expression pattern and function of hsa-miR-500 have been studied extensively. Jiang et al. revealed that hsa-miR-500 suppresses the proliferation and metastasis of non-small cell lung cancer. However, most of the studies show the function of the hsa-miR-500 as an oncomir, such as the cfa-miR-500 in this study (Jiang et al., 2018). Furthermore, hsa-miR-500 has been highly correlated with the malignant progression of gastric cancer (Zhang et al., 2015) and is found to be upregulated in human hepatocellular carcinoma and prostate cancer tissues (Yamamoto et al., 2009; Zhang et al., 2019).

Hsa-miR-502 is known for its suppressive action on the proliferation of BC (Liu et al., 2016). Moreover, it plays a role in inhibiting proliferation, tumor growth, invasion, and metastasis in hepatocellular carcinoma (Chen et al., 2015). On the other hand, hsa-miR-502 is observed to function as an oncomir as it promotes cancer cell proliferation and inhibits apoptosis in esophageal cancer (Xu et al., 2018). According to Xiaoli et al., cfa-miR-502 is significantly upregulated in CMT compared with normal tissue (REN Xiaoli, 2020). Since overexpression of cfa-miR-502 was detected in this study, the role of cfa-miR-502 could be further explored in future studies (Data S1A).

In addition, hsa-miR-146a is associated with diverse tumors in humans, and it works as tumor suppressor miRNA or oncomiR depending on the target gene (Iacona et al., 2018). Moreover, hsa-miR-146a showed significantly lower expression than normal tissue in gastric cancer tissue, which makes the miRNA an independent prognostic factor for cancer patients (Kogo et al., 2011). As a tumor suppressor gene, it plays an important role in the proliferation and oncogenic transformation of myeloid cells; it is also found to be downregulated in hepatocellular carcinoma tissue (Boldin et al., 2011; Karakatsanis et al., 2013). Furthermore, cfa-miR-146a in dogs is expressed in response to a tumor (Data S1A,B) and inflammation similar to hsa-miR-146a in humans; it is one of the overexpressed miRNAs in canine meningioma and is related to cell proliferation and migration (Foiani et al., 2021). Significantly increased expression of cfa-miR-146a in tumor tissues of MCMT was observed in this study (Data S1B); cfa-miR-146a also contributes to the inflammatory response in canine meningioma and peri-implantitis, as similarly found in humans (Foiani et al., 2021; Wu et al., 2017).

Nevertheless, cfa-miR-23b has not been mentioned in officially published studies, which makes its increased expression in tumor tissues of MCMT in this study the first to be reported in dogs (Data S1B). However, hsa-miR-23b has been discussed in various studies in relation to human disease; hsa-miR-23b is an oncomir in human BC (Hannafon et al., 2019), while it exhibits decreased expression in pituitary adenoma as hsa-miR-23b inhibits proliferation by cell cycle arrest (Leone et al., 2014). In addition, hsa-miR-23b is more often identified as a tumor suppressor in human biology and hsa-miR-23b is related to cell aggressiveness inhibition, which

indicates its potential use as a biomarker for diagnosis and prognosis of cancer (Grossi et al., 2017). Hsa-miR-23b inhibits cell proliferation and invasion in prostate cancer, thereby affecting the epithelial-mesenchymal transition process (Zhou et al., 2020). The downregulation of hsa-miR-23b is related to the poor prognosis of colorectal cancer (Kou et al., 2016) and cervical cancer (Campos-Viguri et al., 2015).

Hsa-miR-221 and hsa-miR-222 miRNAs affect proliferation, differentiation, and invasion of cancer cells, and are upregulated in human BC, multiple myeloma, malignant melanoma, glioma, colorectal cancer, etc. (Chen et al., 2013; Song et al., 2017; Yang et al., 2011). The abnormal expression of hsa-miR-221 and hsa-miR-222 is attributed to the development of malignant tumors (Song et al., 2017). Their functions have been studied in detail in human BC. Hsa-miR-221 and hsa-miR-222 affect cancer development and progression as they are related to the telomerase activity, apoptosis, angiogenesis, proliferation, autophagy, and epithelial-mesenchymal transition; they also affect anticancer drug resistance in BC (Chen et al., 2013; Zhong et al., 2013). However, in contrast to humans, only a limited number of studies were conducted with cfa-miR-221 and cfa-miR-222. These miRNAs are upregulated in cPCa, contribute to cell proliferation, and exhibit increased expression in the pituitary when exposed to chronic stress stimulation (Luo et al., 2016; Zhou et al., 2020). To date, no study has been conducted in relation to CMT, and the significant upregulation of cfa-miR-221 and cfa-miR-222 in MCMT observed in this study is the first one to be recorded (Data S1B).

A comparison of malignant and benign tumor tissues (MCMT-T vs. BCMT-T) identified cfa-miR-503 as the only DE-miRNA (Figure 1C and Data S1C). In the previous studies, cfa-miR-503 has been found to contribute to follicular growth and oocyte maturation in dogs (Lange-Consiglio et al., 2017), and to the doxorubicin sensitivity in tumor tissue (Fowles et al., 2016). On the other hand, in humans, hsa-miR-503 is indicated as a tumor suppressor and oncogenic miRNA. Hsa-miR-503 inhibits tumorigenesis, progression, proliferation, metastasis in hepatocellular carcinoma, lung cancer, BC, colorectal cancer, ovarian cancer, cervical cancer, etc., and functions as a tumor suppressor (Fu et al., 2019; Gupta et al., 2018; Sun et al., 2019). However, these characteristics are not yet studied in dogs.

In summary, among the miRNAs discussed here, I first reported that cfa-miR-503 was related to the malignancy. Although not evaluated in this study, cfa-miR-503 could not only be a novel short RNA-based drug for MCMT that functions as a tumor suppressor with anti-metastasis activity, but could also be a novel biomarker for malignancy diagnostic methods in CMT, while the histopathological assessments are vague. Nevertheless, further studies on the mechanism of action should be conducted for the evaluation and validation of cfa-miR-503; this miRNA may be a promising candidate both for a novel drug and a biomarker.

5. Conclusions

In this study, I identified multiple BC-related DEGs in CMT samples. Although several miRNAs were significantly altered by tumorigenesis, only cfa-miR-503 was differentially expressed by malignancy. Along with my results, I conclude that cfa-miR-503 could be used as a potential biomarker for diagnosis and prognostic evaluation of MCMTs. Moreover, it could be suggested as a novel RNA-based drug to alleviate metastasis and proliferation of MCMTs.

PART IV

MiRNA expression in male dog reproductive system

Chapter III. Integrated miRNA in Canine Testis and Epididymis according to Age and Cryptorchidism

1. Introduction

Male reproductive function declines with age in both humans and dogs. It is well-known that testis volume, germ cell and Sertoli cell number, serum testosterone levels, and sperm viability decrease with age in humans (Gunes et al., 2016). Reproductive aging is associated with a reduction in the number of Leydig cells and seminiferous tubules, as well as oxidative stress (Beattie et al., 2015; Nguyen-Powanda & Robaire, 2020). In addition, changes in the expression of genes involved in the regulation of apoptosis and DNA repair have been observed in aging testis (Paul et al., 2011). A negative correlation has been reported between age and normal ejaculated sperm characteristics in dogs (Rijsselaere et al., 2007). A previous study has shown that epididymal sperm quality and fertility decrease with age in male dogs (Bhanmeechao et al., 2018). Furthermore, fresh and thawed semen from old male dogs display decreased sperm motility and mitochondrial function (Sampson et al., 2007). Therefore, age is one of the most important factors influencing infertility in males animals, including dogs.

Cryptorchidism is the most common pathological condition in dogs, in which the testis fails to descend to the base of the scrotum, with an informed morbidity rate of 0.8-10% (Birchard & Nappier, 2008). Hereditary defects caused by sex-limited autosomal recessive genes are considered an etiology of cryptorchidism in male dogs.

Although cryptorchidism is often considered a mild malformation, it can seriously affect the health of dogs, as a very high-risk factor for sterility and testicular tumor. As cryptorchid testes generally have considerably higher temperatures than normally descended testes, unilateral cryptorchids reduce semen quality or cannot produce normal sperm (Mieusset et al., 1993). Cryptorchidism is associated with testicular tumors in dogs (Stokowski et al., 2016; Withers et al., 2016). The incidence of testicular tumors in undescended testes is approximately 13 times greater than that in normally descended testes (Birchard & Nappier, 2008). Although testicular tumors are typically malignant, the excess production of endogenous estrogen by the tumor cells can lead to a condition known as feminization syndrome and bone marrow suppression, which could be fatal (Sontas et al., 2009).

MicroRNAs (miRNAs) are a group of short ncRNAs molecules, around 20–24-nucleotides long; they function by epigenetically downregulating gene expression. miRNAs play a crucial role in controlling both mRNA stability and protein synthesis, thereby influencing the processes of spermatogenesis (Kotaja, 2014) and sperm maturation during sperm passage through the epididymis (Jerczynski et al., 2016). Although the differentially expressed miRNAs in the testicular tissue in cryptorchidism have been investigated in humans and various animal species, including horses, rats, and mice (Jia & Hao, 2021), direct correlation studies between miRNAs and cryptorchidism in dogs are limited. miRNAs can be utilized as biomarkers to elucidate changes in tissues affected by several disorders. In my previous study (Kim et al., 2022), I identified several miRNAs associated with uteropathy and age markers in female dogs. To the best of my knowledge, there are

no reports of alterations in miRNA expression within the testes and epididymis of canines of different ages, and with cryptorchidism and testicular tumors. The purpose of this study was to (i) assess the relationship between aging and disease status and expression of miRNAs in male dog testis and epididymis and (ii) determine the potential of miRNAs as biomarkers and therapeutic targets for infertility and various diseases in male dogs.

2. Materials and Methods

2.1. Collection of tissue sample

Male reproductive organ tissues were collected from two local animal hospitals and the veterinary teaching hospital of Seoul National University, Korea, at the request of the dog owners when general neutralization surgery or castration was required according to clinical findings, such as cryptorchidism, Sertoli cell tumor, and seminoma, with owners' informed consent. Testicular tumor diagnosis was based on histological examination (IDEXX) of the surgically removed tissues. Furthermore, testicular tumor tissue collection was approved only by the Seoul National University Institutional Animal Care and Use Committee (approval number: SNU-200217-3-2). The dogs with testicular tumors were not in the cryptorchidism state. The testes were obtained from 19 male dogs and epididymis cauda tissues (tail part of the epididymis) were collected from 17 male dogs, excluding two dogs with testicular tumor, during castration for the prevention and treatment of disease at the

request of owners, with informed consent from the owners. In dogs, the final diagnosis of cryptorchidism can only be made after 6 months of age (Memon, 2007). All cryptorchid testes were palpable in the right inguinal area and the left testis was present within the scrotum. Before anesthesia for castration, all dogs underwent appropriate clinical evaluations such as blood chemistry tests for total protein, glucose, blood urea nitrogen (BUN), creatinine, alkaline phosphatase (ALKP), and alanine aminotransferase (ALT), to identify any potential pathological conditions and to determine their suitability for systemic anesthesia. The serum chemistry results for the dogs used in this clinical examination are shown in Table 1. This study involved 8 healthy male puppies under the age of 1 year, 4 healthy male dogs over the age of 3 years, 5 dogs with cryptorchidism under the age of 1 year, 1 dog with Sertoli cell tumor, and 1 dog with seminoma over the age of 10 years. The average results and reference ranges of each parameter of the serum chemistry are presented in the Table 6. Before the tissues dried or were damaged, each testis and tail of the epididymis were promptly separated after surgery and collected. As the testis and epididymis are surrounded by the tunica vaginalis, removal of the tunica vaginalis was performed after scrotal incision. The immature spermatozoa within the testes gain the ability to move and become capacitated as they pass through the epididymis. Therefore, I collected the testes and tail parts of the epididymis cauda to compare miRNA expression in all tested dogs. To collect the epididymis cauda tissue, the surrounding tissues such as the ligament of the epididymis were excised and separated from the testis. Some of the cryptorchid and descended tissues of the testis and epididymis were stored in neutral buffered formalin for histological examination.

In addition, the remaining testis and epididymal cauda tissues from each male dog were collected in RNAlater (Thermo Fisher Scientific) and stored at -80 °C for miRNA array analysis.

Table 6. Clinical examination of dogs included in this study.

Variables (mean ± SEM)	Healthy dogs, below 1 year of age (n = 8)	Healthy dogs, above 3 years of age (n = 4)	Dogs with cryptorchidism (n = 5)	Dogs with Sertoli cell tumor (n = 1)	Dogs with seminoma (n=1)	Reference value
Age, months	5.9±0.7	80.0±13.4	7.8±1.0	120	144	
Breed	papillon (1), poodle (2), Maltese (1), bichon fries (1), Pomeranian (2), shih tzu (1)	poodle (1), mixed (2), Maltese (1)	Maltipoo (1), poodle (3), Maltese (1)	chihuahua	Pomeranian	
Total protein (g/dL)	5.6±0.2	6.9±0.4	5.8±0.1	6.27	6.8	5~7.2
Glucose (mg/dL)	115.6±3.4	120.5±9.4	114.2±5.2	97	154	75~128
BUN (mg/dL)	22.0±2.2	15.8±1.9	20.5±4.3	17.5	18	9.2~29.2
Creatinine (mg/dL)	0.5±0.1	0.7±0.1	0.5±0.1	0.77	0.5	0.4~1.4
ALKP (U/L)	494.7±137.4	108.0±25.0	365.4±47.6	46	83	47~254
ALT (U/L)	45.8±3.1	84.5±32.7	66.0±7.8	50	171	17~78

*n, number of examined canine; BUN, blood urea nitrogen; WBC, white blood cell; RBC, red blood cell; HCT, hematocrit; ALKP, alkaline phosphatase; ALT, alanine aminotransferase.
*Reference value : FUJIFILM (https://www.fujifilm.com/jp/ja/healthcare/veterinary/chemical-analysis/common-slide/slide)

2.2 Hematoxylin and eosin staining

Both cryptorchid and non-cryptorchid sides of the testes and epididymis of dogs with unilateral cryptorchidism were collected for H&E staining and analysis. The testis and epididymis cauda were fixed in neutral buffered formalin, dehydrated with a gradient series of alcohol from 60% to 90%, and embedded in paraffin. According to the standard protocol, H&E-stained 4-µm sections (Leica Microsystems GmbH, Wetzlar, Germany) placed on silane-coated slides were observed under a microscope (BX53; Olympus, Tokyo, Japan).

2.3. RNA isolation and quality check, and cDNA synthesis

The total RNA was extracted following the protocol provided by the manufacturer. To extract the total RNA from each tissue sample, weighing a minimum of 50 mg, an easy-spin™ Total RNA Extraction Kit (Intron Biotechnology) was used to homogenize the samples with 1 mL of RNA lysis solution. To perform Gene Microarray hybridization, the Agilent RNA 6000 nano kit and 2100 Bioanalyzer (Agilent Technologies) were used to assess both the quantity and quality of the RNA. Only samples that fulfilled the following criteria were selected for microRNA analysis: A260/A280 and A260/A280 > 1.0, concentration > 50 ng/µL, volume > 10 µL, total amount > 0.7 ug, rRNA ratio > 1.0, and RIN > 7.0 with visible small RNA peaks. Before cDNA synthesis, the NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific) was used to assess the quantity and quality of the RNA.

The Maxime RT-PCR premix kit (Intron Biotechnology) was used to reverse transcribe the RNA samples (500 ng) to cDNA, with a reaction mixture of total volume 20 μL.

2.4. MicroRNA hybridization, scanning, and data processing

All procedures were performed in accordance with the guidelines in the Affymetrix Expression Analysis Technical Manual (Affymetrix). Total RNAs were labeled using the FlashTag Biotin HSR RNA Labeling Kit (Thermo Fisher Scientific). The GeneChip® Affymetrix miRNA microarray (Affymetrix) was used to hybridize the biotin-labeled samples. The Affymetrix GeneChip® scanner was used to examine all arrays, and raw data analysis was carried out using the AGCC. The measured intensity of each array probe was obtained from CEL files, which contained the raw data images developed by the scanner. A p-value of <0.05 and fold change of >2 served as cut-off criteria to restrict a broad range of differentially expressed microRNAs.

2.5. Statistical Analysis

The clinical data of the dogs are presented as mean ± standard deviation. An unpaired t-test was used to compare the groups. All statistical analyses were conducted with SPSS software (version 25.0; SPSS Inc.). The level of statistical significance was defined as $p < 0.05$.

3. Results

3.1. H&E staining of cryptorchid testis and epididymis

The cryptorchid side testis showed severe diffuse tubular atrophy of the seminiferous tubules and only Sertoli cells with germ cell depletion (Figure 10A). However, the other (non-cryptorchid) side testis showed tubular atrophy, which was less severe than that of the cryptorchid-side testis (Figure 10B). Degeneration of germ cells and depletion of elongating spermatids were observed. In the cryptorchid-side epididymis, no spermatozoa showed epithelial tubule degeneration (Figure 10C). Cell debris and germ cell exfoliates were observed; however, no spermatozoa were observed in the lumen of the non-cryptorchid side epididymis (Figure 10D).

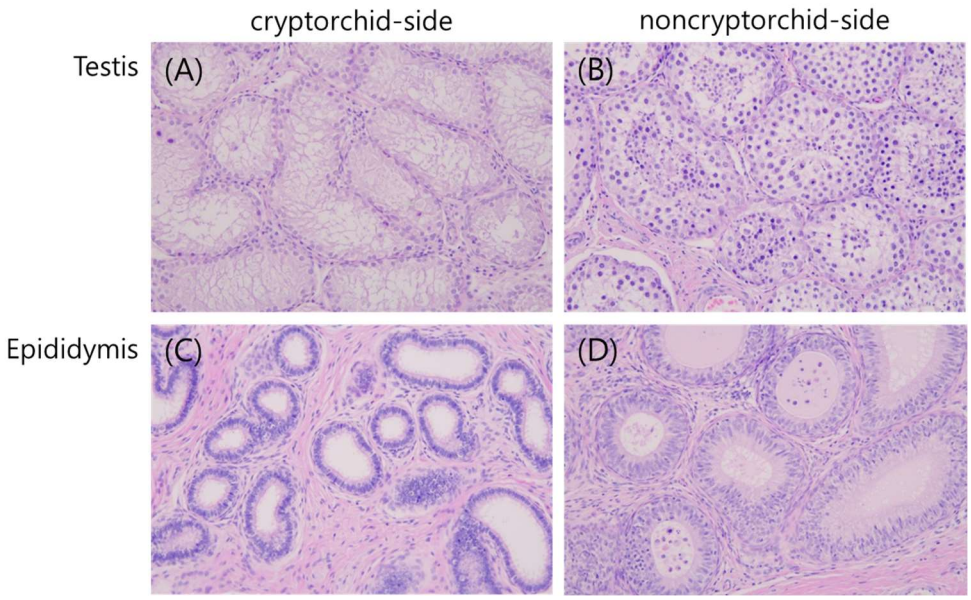

Figure 10. Images of hematoxylin and eosin-stained sections of testis (A, B) and epididymis (C, D) in unilateral cryptorchid dogs. The reproductive organ from the cryptorchid (A, C) and non-cryptorchid side (C, D) was compared.

3.2. MiRNA expression in testis and epididymis cauda in male dogs according to age

The testis and epididymis cauda miRNA volume plots for the comparison of immature male dogs younger than 1 year of age versus mature dogs older than 3 years of age are presented in Figure 11. According to the fold change cut-off of >2 (upregulation or downregulation) and *p*-value of <0.05, there were no significantly differentially expressed miRNAs in the testes between immature and mature dogs. In the epididymis, the top five miRNAs with noticeable differential expression were

confirmed by considering the fold change, *p*-value, and volume value. Sixty-five significant miRNAs, which were differentially expressed, were identified. Among them, the top five meaningful miRNAs, considering the fold change and volume values, were cfa-miR-26a, cfa-miR-200c, cfa-let-7c, cfa-let-7b, and cfa-let-7a. The only significant miRNA that was less expressed in mature dogs compared with that in immature dogs was miR-503.

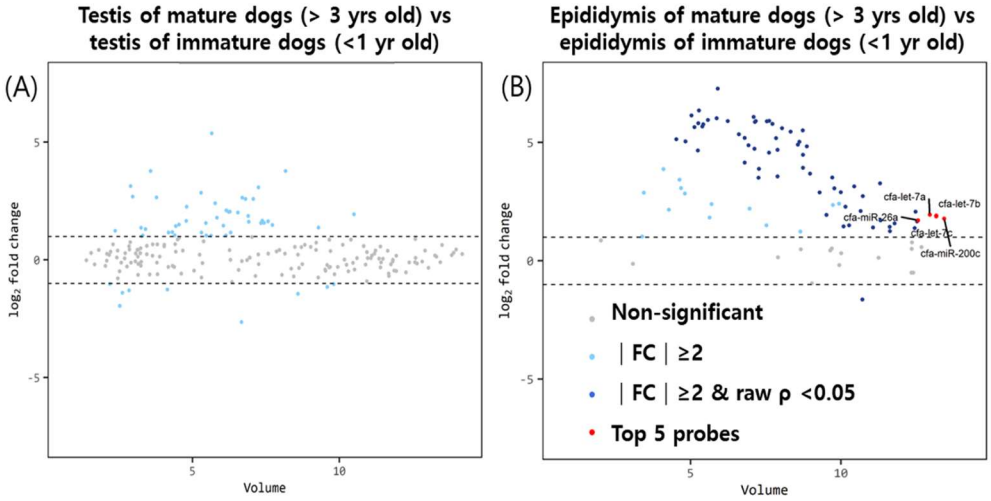

Figure 11. Volume plots of miRNAs in the (**A**) testis and (**B**) epididymis of mature 3-year-old dogs (above) compared those of immature 1-year-old dogs (below). The volcano plot illustrates the fold change and volume between the healthy immature and mature dogs. The horizontal line indicates a 2-fold difference. The red dots indicate significance of the top five miRNAs for comparisons. The blue and sky-colored dots represent significant differences above a 2-fold change without a *p*-

value of <0.05. Cfa-miR = *Canis lupus familiaris* microRNA. Gray dots signify no meaningful correlation between volume and fold change.

3.3. MiRNA expression of testis and epididymis cauda in cryptorchid dogs

As shown in Figure 12A, two significantly downregulated miRNA genes, cfa-miR-148a and cfa-miR-497, were found in the testes of cryptorchid dogs compared with age-matched normal dogs. As shown in Figure 12B, cfa-miR-1841 was significantly downregulated in the epididymis of cryptorchid dogs.

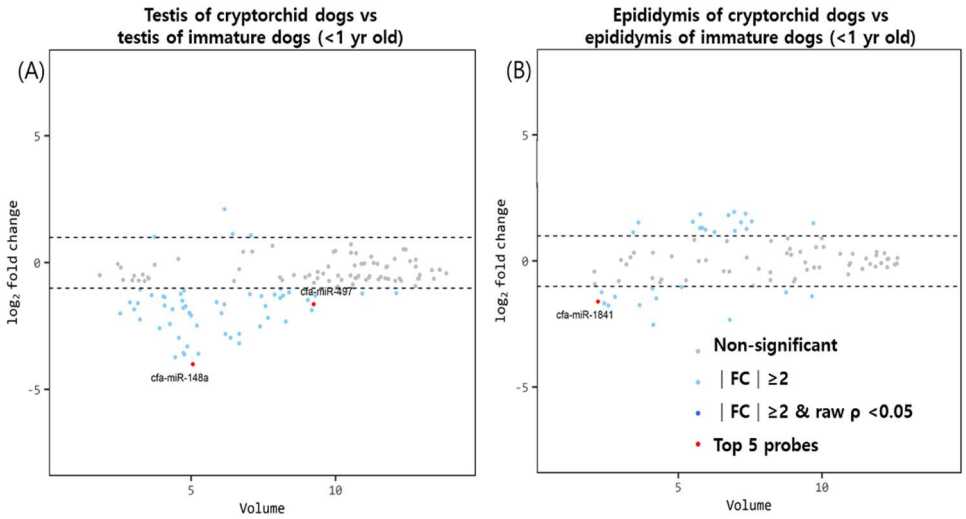

Figure 12. Volume plots of miRNAs in (A) testis and (B) epididymis of cryptorchids dogs compared with dogs younger than one year of age. The volcano plot illustrates the fold change and volume between the groups. The horizontal line indicates a 2-

fold change difference. The red dots indicate the top five significant miRNAs for comparisons. The blue and sky-blue dots represent significant differences above a 2-fold change without a *p*-value of <0.05. Cfa-miR = *Canis lupus familiaris* microRNA. Gray dots signify no meaningful correlation between volume and fold change.

4. Discussion

The results of this study provide conclusive evidence regarding the effect of age and cryptorchidism on miRNA expression in the male reproductive system. The miRNA expression in the epididymis observed in this study was affected by age, with increased expression of cfa-let-7 members, cfa-miR-26a, and cfa-miR-200c in dogs older than 3 years of age. I focused on miRNA expression in dogs with cryptorchidism and testicular tumors, as 1) cryptorchidism is the most common hereditary disorder in male dogs and 2) it may be a risk factor for infertility and testicular cancer. My results showed that in cryptorchid dogs, the expression of cfa-miR-148a and cfa-miR-497 was downregulated in the testes and that of cfa-miR-1841 was downregulated in the epididymis compared with those in age-matched dogs. In addition, testicular tumors showed different miRNA expression levels compared with those in age-matched dogs.

In addition to advanced maternal age, paternal age tends to be significantly associated with a decline in undesirable embryonic development and poor pregnancy outcomes, because semen volume, sperm motility, and normal morphology can

significantly decline with age in humans (Johnson et al., 2015). Similarly, in various animals, including dogs, ferrets, and cats, age negatively correlates with male reproductive capacity (Bhanmeechao et al., 2018; Elcock & Schoning, 1984; Rijsselaere et al., 2007; Wolf et al., 2000). To the best of my knowledge, there is no report on the miRNA expression patterns in the reproductive organs of dogs across different age groups. In this study, the dogs were categorized into two distinct age groups (<1 year old and >3 years old) for the purpose of evaluating miRNA expression in the testes and cauda epididymis. Interestingly, the miRNA levels were not significant in the testis, but were significant in the epididymis with higher expression of cfa-miR26a, miR-200c, and let 7 family members (let 7a, let7b, and let 7c). These results suggest that the testis and epididymis are different in terms of miRNA expression and processing.

The epididymis serves as the location for post-maturation of testicular sperm, resulting in the acquisition of sperm motility and the capacity to recognize and fertilize oocytes. In this study, epididymal tissue was harvested from the cauda epididymis, where mature sperm are stored until ejaculation. To minimize the influence of prostatic fluid and immature sperm, the cauda epididymis may be a more appropriate tissue to examine the effects of age and cryptorchidism on miRNA expression in dogs. Furthermore, as the incidence of canine prostatic disorders markedly increases with age, the miRNA expression results may be affected by a mixture of prostatic fluid.

miR-26a is a functional miRNA that regulates sperm metabolism and apoptosis. It has been reported that miR-26a can have a major effect on the quality of semen in Holstein bulls, as it plays a negative regulatory role in the expression of phosphoenolpyruvate carboxykinase-1 (*PCK1*) (Huang et al., 2016). Additionally, it is suggested that miR-26a is involved in the regulation of bull sperm motility (Bissonnette et al., 2009). In boars, miR-26a is associated with decreased sperm motility (Ma et al., 2018). A previous demonstrated a considerable increase in the expression of miR-26a in highly motile frozen-thawed sperm compared with that in low-motile frozen-thawed sperm (Capra et al., 2017). Furthermore, the sperm transcript level of miR-26a-5p is lower in men with unexplained infertility than in fertile control, and high phosphatase and tensin homolog (*PTEN*) expression is associated with ejaculated spermatozoa (Dorostghoal et al., 2020). *PTEN* signaling is a major negative regulator of *PI3K* signaling and is involved in the maintenance of spermatogonial stem cells in mouse (Zhou et al., 2015). Interestingly, my study showed that cfa-miR-26a was highly expressed in the epididymis of mature dogs compared with that in immature dogs. These findings offer compelling evidence supporting the hypothesis that miR-26a may play a role in the regulation of epididymal aging and, consequently, have an effect on sperm metabolism or motility.

Let-7 (Lethal-7) is among the earliest identified miRNAs. As the expression of let-7 family members gradually increases during development, it is not surprising that high levels were observed in the cauda epididymis of mature dogs compared with those of younger dogs. However, in a previous study, female germ cells did not show changes in let-7 miRNA expression, but male germ cells showed increased

expression during development (Hayashi et al., 2008). Boars with low sperm motility and a high percentage of abnormal sperm showed higher levels of let-7a, let-7d, and let-7e miRNAs in their spermatozoa (Curry et al., 2011) indicating that the let-7 family members may be markers for infertility. Interestingly, although the expression of miR-26a and let-7 family members was increased in the ovaries of dogs with uteropathies in my previous study, significant differences in the expression of these miRNAs in the epididymis of mature dogs in this study. Therefore, miR-26a and the let-7 family members may be related to female reproductive organ disease in dogs as well as aging of male reproductive organs. In Yorkshire boars, endogenous miR-26a and let-7 have anti-apoptotic and pro-survival functions in sperm cells by targeting *PTEN* and *PMAIP1* (Ma et al., 2018). The most abundant miRNAs in the epididymis of bovine species are let-7 family members and miR-200a/b tumor suppressors (Belleannée et al., 2013). miRNA-mediated inactivation of cellular oncogene products could play a role in maintaining the stability of the epididymis, which is an organ with a unique ability to evade tumorigenicity. Consistent with these findings, the let-7 family members and miR-200a/b tumor suppressors, whose expression was confirmed in the epididymis of younger dogs, were not identified in the testicular tumors in the present study.

The miR-200 family, which includes miR-141, miR-200a, miR-200b, miR-200c, and miR-429, is the most prevalent family in the miRNA system, and all members are highly conserved. It has been reported that the miR-200 family members regulate epithelial–mesenchymal transition, which is an important step for breast cancer infiltration and metastasis (Gregory et al., 2008). Furthermore, miR-200 has a

functional role in the regulation of cell invasion and migration by targeting *PTEN* (Suo et al., 2018). The expression of miR-200a, miR-200c, and miR-141 in both male and female mouse germ cells is downregulated gradually during development (Hayashi et al., 2008). In a human study, miR-200a-3p and miR-200c-3p have been identified as potential biomarkers for male subfertility (Trzybulska et al., 2017). As miR-200b and miR-200c accumulate in spermatozoa during passage through the epididymis (Reilly et al., 2016; Sharma et al., 2018), the present study results indicate that sperm maturation in the epididymis cauda may be differentially regulated according to dog age.

Cryptorchidism is a congenital defect commonly found in dogs, where one or both testicles fail to descend normally into the scrotum. The retained testicle(s) may remain in the abdomen or become lodged in the inguinal canal, causing potential health issues such as infertility, testicular tumors, and torsion. In recent years, several studies have focused on miRNA expression in the testes of various animals with cryptorchidism, including horses (Han et al., 2020), rats (Moritoki et al., 2014), and mice (Huang et al., 2018). miR-148a was identified as differentially expressed in seminal plasma extracellular microvesicles of men with oligoasthenozoospermia subfertility compared with that in men with normozoospermic fertility, demonstrating that it may be a marker for male infertility (Abu-Halima et al., 2016). Similarly, the present study suggests that cryptorchid-side testes and epididymis do not have spermatozoa and cryptorchid-side testes display differential expression of miR-148a. In agreement with the fact that miR-497-5p has been identified only in the testicular tissues of spermatozoa and seminal plasma, and in humans (Abu-

Halima et al., 2020), cfa-miR-497 was found to be expressed at low levels in the testes of cryptorchid dogs.

In testicular tumors, miR27b was highly expressed in both Sertoli cell tumors and seminomas; it has been found to be differentially expressed in mature spermatozoa of infertile men. The high expression of cysteine-rich secretory protein 2 (CRISP2), which is predominantly expressed in the testis, is correlated with the expression of miR-27 in humans (Zhou et al., 2017). miR-27b expression is downregulated during sheep fetal testis development from D42 to 75 and plays an important role in regulating cellular differentiation (Torley et al., 2011).

A major limitation of this study is that the miRNA expression was analyzed in a limited number of dogs, especially in one case each of Sertoli cell tumor and seminoma. The present analysis of miRNA expression in testicular tumor tissues lays a foundation for more extensive and larger-scale studies. The results of this study should be interpreted with caution. Furthermore, age and breed predisposition may have affected the miRNA expression results of this study. In order to reduce this expected bias, I classified the dogs into two age categories, those under 1 year old and those over 3 years old; evaluated tissue samples obtained only from small dog breeds; and analyzed age-matched dogs as a control group for the comparison of cryptorchidism testes and testicular tumors.

5. Conclusions

miRNA expression in the male reproductive tissue of dogs with cryptorchidism and testicular tumors was comprehensively analyzed, and the effect of age on miRNA expression on male reproductive organs was also examined. The present study demonstrated that epididymal cfa-miR-26a, cfa-miR-200c, cfa-let-7c, cfa-let-7b, and cfa-let-7a expression regulated via *PTEN* may be involved in aging in dogs. Furthermore, cfa-miR-148a and -497 expression was consistently lower in the testes and cfa-miR-1841 expression was lower in the epididymis of dogs with cryptorchidism, suggesting that these miRNAs may be useful biomarkers for cryptorchidism and male infertility. This study provides insights into the development and causes of cryptorchidism and testicular tumor. I hope that my study will help develop new diagnostic methods and preventive medication for spontaneous cryptorchidism and testicular tumor, and improve male fertility with aging in dogs.

PART V

GENERAL CONCLUSION

This study was conducted to analyze the expression of miRNA according to age and disorder in the reproductive system of dogs.

First, the expression of miRNAs in the ovaries, oviducts, and uteri of female dogs was influenced by both aging and uteropathy. The age-related DE-miRNAs analysis revealed that the expressions of miR-151 and miR-708 in the ovary; miR-30d and miR-140 in the oviducts; and miR-29a, miR-125a, miR-23a, miR-10a, and miR-221 in the uterus were all upregulated. Thus, these miRNAs may serve as biological markers for aging and reproductive diseases in female dogs.

Second, DEGs associated with breast cancer in CMT samples were discovered in this study. While multiple miRNAs demonstrated significant alterations due to tumorigenesis, only miR-503 exhibited a differential expression specifically related to malignancy. The findings suggest that miR-503 could serve as a potential biomarker for the diagnosis and prognostic evaluation of MCMT and as a potential RNA-based anti-tumor drug.

Third, aging, cryptorchidism, and testicular tumors were associated with miRNA expression. The age-related DE-miRNAs analysis revealed no significant differences in the testis, and miR-26a, miR-200c, let-7a, let-7b, and let-7c expression levels were upregulated in the epididymis. Meanwhile, the cryptochidism-related DE-miRNAs analysis indicated that the miR-148a and miR-497 expression levels were upregulated in the testis, while those of the miR-1841 was downregulated in the epididymis. Therefore, miRNAs may potentially serve as candidate genes to understand male reproductive traits for molecular breeding programs.

In conclusion, the findings of this study strongly suggest that miRNA expression in the reproductive systems of dogs is influenced by aging and disease states, and specific miRNAs may serve as potential biomarkers or therapeutic targets for diagnosis, prognostic evaluation, and treatment of reproductive diseases and tumors in dogs.

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국문초록

개의 연령과 질병에 따른 생식기관의 miRNA 발현 분석 연구

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개에서 miRNA(microRNA)의 발현은 노화와 연관성이 있으며, 노화 과정에서 환경적, 유전적 요인이 세포 및 분자 손상의 축적에 영향을 미쳐 장기 및 조직 기능 장애를 초래할 수 있다. 노화의 특이적 원인 중 하나인 후성유전적 변화(epigenetic alterations) 는 DNA methylation 또는 histone modification 또는 noncoding RNAs 에 의한 조절이 일어날 때 신체에서 발생한다.

MiRNA 는 noncoding RNAs 중 하나이며, 이는 번역을 억제하는 등 모든 생물학적 기전에서 중요한 역할을 하는 표적 전사체의 mRNA 분해를 유도하는 20-24 개의 뉴클레오타이드로 구성되어 있다. MiRNA 가 노화 및 생식기계 질환의 생체표지자(biomarker)로서 노화를 매개하는 데 사용될 수도 있지만, 개의 노화에 따른 생식기계 내 miRNA 발현 변화는 거의 연구된 바가 없다. 개와 인간은 노화를 포함한 공통적인 속성을 공유하기에, 개는 인간의 노화의 유전적,

환경적 결정 요인을 연구하기 위한 이상적인 모델이다. 특히, 개들은 마우스보다 인간과 더 밀접한 유전적 유사성을 가지고 있고 개들의 유전적 질병의 약 절반정도가 인간의 질병과 현저하게 비슷하다고 알려져 있다.

개의 특정 질병에서의 miRNA 관련 연구는 일부 이루어졌지만, 생식기계에서 다양한 연령과 질병 상태에 대한 miRNA 연구는 아직 접할 수 없었다. 이에 본 연구는 개에서 연령과 생식기계의 질병 상태에 따라 다양하게 발현되는 miRNAs 를 분석하여 그 차이와 발현변화를 규명하기 위해 수행되었다. 이를 위해 동물병원에 내원한 환자를 대상으로 시료를 채취하여 microarray, RNA sequencing (RNA-Seq) 그리고 real-time PCR 을 이용하여 분석하였다. 그리고 miRNA 발현으로 인한 유전자 조절의 효과를 평가하기 위해 먼저 miRNA 표적 예측 데이터베이스인 TargetScan 과 miRDB 를 사용하여 표적 유전자를 검색하였다.

본 연구의 Chapter I 에서는 암컷 개의 생식기계에서 miRNA 발현의 변화를 분석하기 위해 미성숙견 6 마리(1 세 미만), 성숙견 6 마리(3 세 이상), 자궁에 질병이 있는 6 마리를 포함한 총 18 마리 개의 난소, 난관, 자궁 내 miRNA 발현 변화를 분석했다. 그 결과 미성숙견의 난소에 비해 성숙견의 난소에서 두 개의 miRNA (miR-151 및 miR-708)가 유의적으로 상향조절(upregulation) 된다는 것을 확인하였다. 그리고 건강한 성숙견의 난소보다 자궁에 질병이 있는 성숙견의 난소에서 let-7a, let-7b, let-7c, miR-125b, miR-26a 의 발현이 상대적으로 높음을 발견하였다. 성숙견의 난소에서 miR-30d 와 miR-140 이 높게 발현되었으나, miR-203 의 발현은 건강한 성숙견의 난관보다 자궁 질환을 가진 개의 난관에서 더 낮았다. 5 개의 miRNA(miR-29a, miR-125a, miR-23a, miR-10a, miR-221)는 모두 성숙견의 자궁에서 미성숙견에 비해 상향조절 되었으나, 건강한

성숙견과 자궁병증이 있는 성숙견의 자궁에서는 miRNA 발현의 유의미한 차이가 발견되지 않았다. 따라서 miRNA 의 발현 변화는 노화와 자궁병증과 같은 염증성 질환 모두에 영향을 미치는 것으로 판단된다.

본 연구의 Chapter Ⅱ 에서는 개 유선종양의 miRNA 및 messenger RNA 발현 프로파일을 인간의 유선종양과 비교하여 분석하였다. 유선종양은 중성화 하지 않은 암컷 개에게서 가장 많이 진단되는 종양이며, 양성유선종양을 가진 12 마리의 개와 악성유선종양을 가진 8 마리의 개에서 조직 샘플을 수집하여 연구에 사용하였다. 기존의 전통적인 생어염기서열분석법 (Sanger sequencing) 및 마이크로어레이 (microarray) 기반 방법에 대한 대안으로서 RNA-Seq 기술은 유전자 발현 및 선택적 스플라이싱 (alternative splicing)에 대한 포괄적이고 정확한 검사 및 샘플 준비, 시퀀싱 플랫폼 및 생물정보학 데이터 분석의 전반적 향상을 통해 전사체의 상세한 프로파일링과 다양한 생리학적 및 병리학적 조건에 대한 통찰이 가능하다. 따라서 RNA-Seq 기법을 활용하여 종양의 악성도(양성, 악성) 및 생검 위치(종양부, 주변 정상 조직부)에 따른 조직내 mRNA 발현 수준의 변화를 조사하기 위해 차등적으로 발현된 miRNA 를 분석했다.

본 연구에서 cfa-miR-503 이 악성유선종양에서 발현하는 유일한 miRNA 임을 발견하였다. 추후 추가적인 연구를 통해, cfa-miR-503 을 개에서 악성유선종양의 진단 및 예후 평가를 위한 잠재적 생체표지자 및 잠재적인 RNA 기반 종양 치료제로 활용할 수 있는 가능성을 제시하였다.

Chapter Ⅲ 에서는 수컷 개 생식기계의 miRNA 가 노화 및 잠복고환과 연관성이 있는지를 알아보기 위해 12 마리의 개들을 두개의 연령 그룹(1 세 미만, 3 세 이상)으로 나누어 고환과 부고환의 miRNA

발현 변화를 마이크로어레이를 통해 분석했다. 분석 결과, 나이든 개의 부고환에서 cfa-miR-503 만 하향조절(downregulation)된 반면 64 개의 miRNA 는 상향조절된 것으로 나타났다. 잠복고환의 고환조직에서 cfa-miR-148a 와 cfa-miR-497 의 발현은 정상 개의 발현보다 현저히 낮았다. 잠복고환의 부고환에서 cfa-miR-1841 발현이 유의하게 감소했다. 이 연구를 통해 노화와 잠복고환에서 miRNA 발현이 차이가 있음을 발견할 수 있었다.

결론적으로 노령 및 질병 상태의 개에서 miRNA 발현이 전반적으로 증가하는 경향을 확인함으로써, 본 연구들을 통해 개의 생식기계의 노화 및 다양한 질병에서 miRNA 발현의 차이가 있음을 입증하였다. 본 연구에서 발견된 miRNA 는 개의 생식계통의 노화와 다양한 질병의 biomarker 로 사용될 수 있으며, 추가적인 연구를 통하여 후생유전학적 변화의 주요 요인 중 하나로서 생식기계의 노화와 질병에 대한 이해와 질병의 예측 및 진단에 기여할 수 있다고 제시하는 바이다.

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주요어: 마이크로 RNA, 개, 노화, 자궁의 질병, 유선종양, 잠복고환, 진단적 생체표지자

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