MicroRNA Application in Bovine Mastitis

(牛乳房炎における microRNA の応用)

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Abstract

MicroRNAs (miRNA) in tissues and liquid samples can serve as biomarkers of many diseases. The thesis hypothesis is miRNA can be the biomarker of bovine mastitis milk. The primary aim of this thesis is to identify alternatively expressed miRNAs in mastitis milk compare to normal milk.

In the first chapter, experiments are designed to decide the suitable reference miRNA in milk for qPCR assay. We chose miR-92a, miR-375, and let-7g as candidate reference genes on the basis of previous report of next generation sequencing dataset. The Normfinder software was identified miR-92a as the most stable reference gene. The candidates were then validated by normalizing the expression levels of miR-146a, the well-known inflammation associated miRNAs. The significance levels were most remarkable and reproducible when miR-92a used as the reference. Based on the results, miR-92a is the best reference gene for relative quantification of miRNA expression in bovine milk.

In the second chapter, experiments are designed to identify the miRNA biomarkers of bovine mastitis. The expression levels were analyzed using qPCR and normalized to miR-92a. Eight miRNAs (miR-21, miR-29b, miR-122, miR-125b, miR-204, miR-205, miR-222, and miR-383) were compared to normal and mastitis cows. MiR-21, miR-

122, miR-125b, miR-205, miR-222 and miR-383 were significant up-regulated and miR-29b was significant down-regulated in mastitis cows. We separated mastitis cow samples into non-inflammation quarter group (CMT−) and inflammation quarter group (CMT+). We found that MiR-21, miR-146a, miR-155, miR-222 and miR-383 were significant up-regulated in mastitis milk. These genes were further analysis using the Digital PCR System. The results of Digital PCR had a strong correlation with qPCR, and up-regulating of miR-21, miR-146a, miR-155, miR-222 and miR-383 were also confirmed in mastitis milk. MiR-21, miR-146a, and miR-155 are known to be associated with inflammation. In the second study, we discovered the new target miRNA as biomarkers of bovine mastitis milk.

In the third chapter, experiments are using next generation sequencing technic to study miRNA in bovine mastitis milk. Twenty-five miRNA were differentially expressed, with 23 miRNA being upregulated and 2 downregulated in bovine mastitis relative to the normal milk. The upregulated mature miR-1246 was likely derived from U2 small nuclear RNA instead of miR-1246 precursor. The significantly upregulated miRNA precursors and RNU2 were significantly enriched in the bovine chromosome 19. Bovine chromosome 19 is homologous to human chromosome 17, the gene related with human breast cancer. Gene ontology analysis of significantly upregulated miRNA putative mRNA targets showed that the upregulated miRNA were involved in bind to

target mRNA transcripts and regulate target gene expression, while KEGG pathway analysis showed that upregulated miRNA were mainly related to cancer and immune system pathways. Three of novel miRNA were related with bovine mastitis and relatively highly expressed in milk. We further verified that one of the mastitis related novel miRNA was significantly upregulated using a digital PCR system. The differentially expressed miRNA are known to involve in human cancer, infection and immune related diseases.

In final conclusion, our studies find that miRNA could be biomarkers of bovine mastitis. The genome-wide views of miRNA profiles provide insights into bovine mastitis and inflammatory diseases

General Introduction

Inflammation processes are controlled by miRNA, thus the hypotheses of this thesis are: Because of the regulatory functions of miRNA, miRNA play a role in bovine mastitis, and some of inflammation related miRNA are conserved between humans and cows. The primary aim is to identify alternatively expressed miRNAs in mastitis milk compare to normal milk, and use these miRNA as biomarkers for bovine mastitis. Because PCR technic needs housekeeping gene, we investigated the suitable miRNA as a housekeeping gene for bovine mastitis milk in real time PCR experiment in chapter 1. The suitability of inflammation related miRNA as bovine mastitis biomarkers will be investigated in chapter 2. To investigate genome-wide miRNA expression in bovine mastitis, we performed next generation sequencing experiment and the result will be introduced in chapter 3.

Bovine mastitis [1, 2]

Mastitis is inflammation of the parenchyma of the mammary gland, a major disease affecting dairy cattle worldwide. Mastitis is one of the important dairy animal disease because of its economic importance, the effect including: 1) loss in milk production. An estimate for the average milk-yield loss at the lactation level can be set

at 300-400 kg (4-6%) at lactation level [3]. 2) Milk composition changes. Reduction in milk fat content, resulting in dairy products with less favorable organoleptic properties. High bulk tank somatic cell counts cause reduction in price, penalty payments or unearned premiums [2, 4]. High somatic cell counts are associated with decreased productivity, shorter shelf life of fluid milk, and reduced cheese yield and quality [5, 6]. 3) Treatment and milk discard costs. Antibiotics, veterinary care, extra labor for husbandry, preventive, and medication measures. Mastitis milk and milk with antibiotics should be discarded. The withdrawal period also result in economic lost. 4) Culling. Clinical mastitis cows have an increased risk of being culled, and mastitis accounts for 5-24% of the reasons for culling [7]. The cost of mastitis has been estimated at $200/cow/yr, or $1.5 to $2 billion in the US [4].

Etiology

Although bacteria are the most common microorganism causing mastitis, fungi, yeast, algae, and virus can cause mastitis. Pathogens can be further classified as contagious, teat skin opportunistic and environmental pathogens. The most common contagious pathogens are *Staphylococcus aureus* and *Streptococcus agalactiae*. This type of mastitis is usually caused by contaminated udder washcloths, inadequate milking equipment, and residual milk in teat cups; however, the hands of milkers can

be a source of *S. aureus*. *Mycoplasma bovis* is less common but once clinical mastitis outbreaks, it does not respond to therapy and difficult to control, and thus needs culling. Coagulase-negative staphylococci (CNS) are the most common teat skin opportunistic mastitis pathogens. The skin opportunistic pathogens can ascend through the teat canal and create infection. The most common environmental mastitis pathogens are *Escherichia coli* and *Klebsiella* spp. (Coliform organisms are a common cause of clinical mastitis.), environmental *Streptococcus* spp. (most prevalent species are *Streptococcus uberis* and *Streptococcus dysgalactiae*, these pathogens usually cause a chronic subclinical infection.), and *Trueperella pyogenes* (important seasonal cause of mastitis in dry cows and late pregnant heifers). The environmental mastitis is caused by inadequate management of the environment, such as wet/dirty bedding or lots, inadequate premilking udder and teat preparation and milking processes, poor housing systems and fly control.

Epidemiology [1]

Incidence of clinical mastitis ranges from 10%-12% per 100 cows at risk per year [1]. The prevalence of bovine mastitis ranged from 29.34%-78.54% in cows [8]. Prevalence of infection with contagious pathogens ranges from 7%-40% of cows and 6%-35% of quarters [1]. Prevalence of infection with environmental pathogens: coliforms 1%-2% of

quarters; streptococci less than 5% [1]. *Staphylococcus aureus* and *Streptococcus* spp. are the chief etiological agents, the prevalence ranged from 2.89%-79.12% and 0.4%-53%, respectively in Asian country [8]. Coagulase-negative *Staphylococcus* spp. (CNS) have traditionally been considered to be minor mastitis pathogens; however, needs to be reconsidered as in many countries they have become the most common mastitis-causing agents [8], the prevalence ranged from 0.93%-97.80% [8]. Although the prevalence may differ from countries or experiment design, based our sample set from Hokkaido, Japan, the most prevalence bacteria were nonagalactiae streptococci, Coagulase-negative *Staphylococcus* spp. and *Staphylococcus aureus*.

Clinical signs [1]

Clinical signs including abnormal secretion, abnormal udder, and an abnormal cow. Gross abnormalities in secretion including discoloration, clots, flakes, and pus. Physical abnormalities of udder are in size and consistency than other quarters: Acute inflammatory is accompanied by swelling, heat, pain and marked abnormality of the secretion. Severe cases may have gangrene or abscesses. Local fibrosis and atrophy can be found in chronic cases. Systemic response including anorexia, toxemia, pyrexia, tachypnea, depression, dehydration, fever, tachycardia, ruminal stasis, recumbency and

death [1]. Because presence of localized pain in the udder, the hock-to-hock distance is increased in cattle with clinical mastitis [9].

Treatment [1]

Treatment of mastitis cow can be categorized into lactating cow and dry cow therapy. Mild cases of clinical mastitis with abnormal secretion only may not require treatment. Mastitis accompanied by an abnormal gland or systemic signs should administrate antimicrobial agents by intramammary infusion and parenterally. The considerations of making a choice of antibiotic for treatment including [10]: Antibiotic sensitivity, ability to penetrate the udder and persist at a concentration to kill bacteria, residue and withdrawal periods and cost. Commonly used antibiotics including Penicillins, Aminoglycosides, Cephalosporins, and Tetracyclines [10]. Acute and peracute mastitis cases require fluid, electrolytes supportive therapy and nonsteroidal antiinflammatory agents [1]. Intramammary infusion of long-acting antimicrobial agents at drying off prevents new infections during the early weeks of the dry period, and provides treatment for subclinical mastitis [1].

History of miRNA [11-13]

miRNA are small non-coding RNA (ncRNA) molecules with ~22 nucleotides in length and functions as a post-transcriptional regulation regulator. The hint of transcriptional regulation function of miRNAs came from work that began in early 1980s, which showed that *lin-4* gene mutation in *Caenorhabditis elegans* caused developmental abnormalities [14, 15]. Mutation in another *Caenorhabditis elegans* gene named *lin-14* also cause developmental defects [16]. In 1987, Ferguson et al. found that a suppressor mutation in *lin-14* reversed *lin-4* mutation phenotype, suggested that *lin-4* negatively regulates *lin-14* [17]. In 1993, a novel regulatory mechanism mediated by non-coding RNA has been found by researchers. Two small non-coding *lin-4* transcripts of 22 and 61 nt, and 3' untranslated region of lin-14 that had sequence complementarity to the lin-4 small RNAs [18, 19]. A second microRNA let-7 has been discovered in 2000. The function of let-7 is similarly to lin-4, controlling the L4-to-adult stage transition of larval development [20]. Unlike lin-4, let-7 family is conserved among other species from flies to humans, suggesting that small RNA regulation mechanism is not a specific phenomenon in nematodes [20]. Since then, hundreds of miRNA have been identified in almost all metazoan genomes, including worms, flies, plants and mammals [21]. In 2002, Calin et al. found that miR-15 and miR-16 are frequently deleted or down-regulated in chronic lymphocytic leukemia samples with deletions on 13q14 [22]. Within few years,

miRNA became a research hotspot of cancers, biomarkers and other diseases. In 2004, Takamizawa et al. demonstrated the prognostic value of miRNAs. They found the reduced expression of let-7 in human lung cancers in association with shortened postoperative survival [23]. In 2005, Zhao et al. reported that miR-1 are specifically expressed in cardiac and skeletal muscle precursor cells and that miR-1 regulates ventricular cardiomyocytes. MiR-1 effects of cardiac regulatory proteins to control differentiation and proliferation during cardiogenesis [24]. In 2007, Sonkoly et al. found that keratinocytes expressed miR-203 is upregulated in an autoimmune disease: psoriasis affected skin compared with healthy human skin or chronic inflammatory skin disease [25]. Stanczyk et al. found increased expression of miR-155 and miR-146 in another autoimmune disease: rheumatoid arthritis affected synovial fibroblasts and synovial tissues [26]. In 2007, Schaefer et al. suggested an involvement of miRNAs in neurodegenerative disorders [27]. In 2010, Weber et al. investigated the miRNA spectrum in 12 body fluids and expand using body fluids as biomarkers for detecting and monitoring various physiopathological conditions [28]. Because of the important roles of miRNA in cancer, miravirsen was the first miRNA which entered phase I clinical trial in 2009, miravirsen is a 15-nucleotide antisense RNA oligo with complementarity to the 5' end of miR-122, for the treatment of HCV [29].

Biogenesis [30]

miRNA biogenesis processes including 1) Gene transcription. 2) Processing by Drosha. 3) Exported into the cytoplasm by Exportin-5. 4) Processing by Dicer. 5) Loading onto AGO proteins. MiRNA genes are mainly transcribed by RNA polymerase II [31]. Transcribed primary miRNA undergoes nuclear processing by Drosha. The pre-miRNA is then exported into the cytoplasm by Exportin-5 (EXP5). In the cytoplasm, pre-miRNA is cleaved by Dicer near the terminal loop, and producing a small RNA duplex. The Dicer produced small RNA duplex is subsequently loaded onto an AGO and form RNA-induced silencing complex (RISC) assembly.

miRNA binding to mRNA and types of target sites

After miRNA incorporated into RISC, it guides the complex to target the 3' untranslated region (3' UTR) of the target mRNAs and result in mRNA repression or degradation. The fate of mRNA depends on the base pair matching degree. If the miRNA is perfectly matched to mRNA, the target mRNA will be degraded. However, miRNA usually have mismatches to mRNA, and thus repress mRNA translation while it remain intact, although target degradation also can be triggered. Imperfect centered miRNA binding sites are common and can mediate repression of target mRNA; however the functional importance relative to seed region remains unknown [32].

The most effective canonical binding site of miRNA-mRNA is conserved Watson–Crick pairing to the 5' region of the miRNA on the miRNA seed region nucleotides 2–7 [33-37]. Listed in order of decreasing preferential conservation and efficacy, the sites are: 1) 8mer site. miRNA positions 2–8 match with an A in position 1 [37]. 2) 7mer-m8 site. miRNA position 2–8 match [35-37]. 3) 7mer-A1 site. miRNA position 2–7 match with an A in position 1 [37]. 6mer sites have weaker preferential conservation and much lower efficacy, including 4) 6mer site. miRNA position 2–7 match[37]. 5) Offset-6mer. miRNA position 3–8 match [38] and 6) 6mer-A1 site, in which the efficacy is difficult to distinguish from background [38, 39]. The 3' end pairing can supplement canonical sites, however no more than 5% of the seed matched sites supplement with 3' end supplementary pairing [35, 37-39].

Canonical binding site types of miRNA. Figure from Target scan website (http://www.targetscan.org/docs/8mer.html).

Based on the miRNA-mRNA binding types and other characteristics, the targets of miRNA can be predicted by computational methods. Introduction of available target predicting tools please refer to chapter 3.

miRNA in inflammatory responses

Bovine mastitis is caused by pathogenic challenge and related with host immune response. When the immune cells expose to inflammatory mediator, the signals can cause up- or downregulation of hundreds of immune response genes. miRNA also play a role in the inflammatory processes. This section will introduce the roles for miRNA in immune system.

miR-146a and miR-155 are originally identified and best characterized as inflammatory response miRNA that are upregulated by NF-κB signaling pathway [40-42]. Protein regulators of Drosha processing in the miRNA biogenesis pathways such as p53 and SMAD can also be affected during inflammatory responses[43]. Another Drosha processing regulating protein KSRP which binds to the terminal loop assists in the rapid increase in mature miR-155 levels seen during inflammation [44]. miRNA and inflammatory conditions please see the table below [43].

miRNA and inflammatory conditions. Table is modified from [43]. PBMC, peripheral blood mononuclear cell; HSPC, hematopoietic stem/progenitor cell.

Disease	miRNA	cell
Multiple sclerosis	miR-155 miR-326 miR-124	Th1 and Th17 Th17 Myeloid
Rheumatoid arthritis	miR-155 miR-223 miR-182 miR-146a	B cell and Th17 T cells T cells T cells and Macs
Systemic lupus erythematosus	miR-146a miR-182 miR-17-92 miR-21 miR-155	T cells T cells T cells T cells B and T cells
Type 1 diabetes	miR-510	Tregs
Type 2 diabetes	miR-146a	PBMCs
Sjögren syndrome	miR-146a	Monocytes
Atopic dermatitis	miR-155	T cells
Allergic inflammation	let-7 miR-126	T cells Th2
Inflammatory bowel disease	miR-155	
IgA nephropathy	miR-155 miR-146a	Extracellular Extracellular
Endotoxemia	miR-146a miR-155	Myeloid Myeloid
Bacterial infection	miR-155	Myeloid, B, T cells
Myeloproliferative disorders	miR-125b miR-155 miR-146a	HSPCs HSPCs

miRNAs in innate immunity

The innate immune response is a cellular response, the first line of defense against pathogens and the initiator of inflammatory responses. The cells involved in innate immune response including: 1) Granulocytes. 2) Monocyte/Macrophages. 3) Dendritic cells. The Toll-like receptors (TLR) on the membrane of macrophages and dendritic cells recognize and bind to specific microbial products called pathogen-associated molecular patterns (PAMPs), such as TLR2 can recognizes lipoteichoic acid from Gram-positive bacteria and TLR4 can recognizes lipopolysaccharide from Gram-negative bacteria, then trigger downstream signaling pathway to initiate inflammatory responses. The relationship between TLR and miR-21, miR-146a and miR-155 have been extensively studied and used as bovine mastitis biomarkers in our work (Chapter 2). miR-155 downregulates SHIP1 and SOCS1, the important genes in controlling inflammatory response, result in AKT and IFN response gene expression upregulated, and promote the immune response [45, 46]. Immune responses are decreased in miR-155 deficient mice, whereas miR-155-overexpressing mice develop a myeloproliferation similar to chronic inflammation and hematopoietic cancers [47-50]. miR-146a and miR-21 are the negative regulators of the immune response. miR-146a inhibits *TRAF6* and *IRAK1* that are involved in TLR signaling transduction and lead to NF-κB activation. miR-146a reduces pro-inflammatory mediators such as IL-6 and TNF-α production [41,

[51, 52]. miR-21 targeting PDCD4 decreases NF-κB signaling and IL-10 production, and thus switch to anti-inflammatory response [53]. miRNA, TLR and their signaling molecular please see the table below [54].

miRNA, TLR and their signaling molecular. Table is modified from [54]. ND: not determined.

miRNA	TLR	Signaling molecular
Upregulated		
miR-155	TLR2, TLR3, TLR4, TLR9	MYD88, TRIF, JNK, AP1, NF-κB, KSRP
miR-146	TLR2, TLR3, TLR4, TLR5	MYD88, NF-κB
miR-132	TLR4, TLR9	ND
miR-21	TLR4	MYD88, TRIF, NF-κB
miR-223	TLR4	ND
miR-147	TLR2, TLR3, TLR4	MYD88, TRIF, NF-κB, IRF3
miR-9	TLR2, TLR4, TLR7, TLR8	MYD88, NF-κB
miR-125b	TLR4	NF-κB
let-7e	TLR4	AKT1
miR-27b	TLR4	NF-κB
Downregulated		
miR-125b	TLR4	NF-κB, AKT1
let-7i	TLR4	NF-κB, C/EBPβ
miR-98	TLR4	ND

Other important miRNA which regulate innate immunity including miR-223, which induced by the transcription factor C/EBPα and repress NFI-A and E2F1, leads to granulocytes differentiation enhanced [55, 56]. miR-223 also acts as a negative regulator of granulocyte differentiation in miR-223 knockout mice by targeting mef2c [57]. The

granulocytes in miR-223 mutant mice are hyperactivated, lead to inflammatory lung pathology and exhibit exaggerated tissue destruction after endotoxin challenge [57]. The expression of miR-223 was increased in mastitis milk, suggesting that mastitis is related to neutrophils (Chapter 3).

miR-17-5p, miR-20a, and miR-106a regulate monocyte-derived macrophages production, their expression is decreased during monocytopoiesis, which in turns upregulated target gene AML-1 to promote macrophage colony-stimulating factor receptor (M-CSFR) expression [58]. Another microRNA miR-424 enhances monocyte differentiation by NFIA repression, which also led to M-CSFR activation [59].

miRNAs in adaptive immunity

T and B lymphocytes are the major cells in adaptive immunity. The development of T and B lymphocytes is impaired by disruption of miRNA biogenesis in lymphocyte progenitors. Deletion of Dicer in early T lymphocytes impaired T cell development and aberrant T helper cell differentiation and cytokine production [60, 61]. Deletion of Dicer in early B lymphocytes affects antibody diversity and led to an arrest of development at the pro-B cell stage; miR-17-92 target, the proapoptotic molecule BIM, was highly upregulated [62]. miR-17-92 are also involved in managing T cell survival by repressing BIM and PTEN [63]. miR-155 regulates T and B cells upon activation and maintains

lymphocyte homeostasis and normal immune function. miR-155. miR-155 knockout mice have abnormal Th1/Th2 differentiation ratio with Th2 polarization and cytokine production increased, regulatory T cells, plasma cells and memory cells reduced, and decreased germinal center responses [42, 64-66]. miR-181a is another important miRNA in T and B cell development. miR-181 is preferentially expressed in the B-lymphoid cells of mouse bone marrow, and its ectopic expression in hematopoietic stem/progenitor cells led to an increased fraction of B-lineage cells in both tissue-culture differentiation assays and adult mice [67]. Increasing miR-181a expression in mature T cells augments the sensitivity to peptide antigens, while inhibiting miR-181a expression in the immature T cells reduces sensitivity and impairs positive and negative selection [68]. miR-150 is highly up-regulated during the development of mature T and B cells, and sharply up-regulated at the immature B cell stage. Premature expression of miR-150 blocked the transition from the pro-B to the pre-B stage [69]. miR-150 controls B cell differentiation by targeting the transcription factor c-Myb [70].

T cell activation is suppressed by regulatory T cells (Tregs) to maintain immune system homeostasis and self-antigen tolerance [71]. miR-155, miR-146a, miR-31 and miR-21 have been shown to regulate Treg development [66, 72-74]. Numbers of Tregs in the thymus and periphery are reduced in miR-155 deficient mice without suppress their function, suggesting that miR-155 contributes to Treg development [66]. miR-155

inhibition in CD4+ Th cells for Treg mediated suppression. miR-146a is highly expressed in Treg, and a certain optimal range of Stat1 activation maintained by miR-146a is important for Treg-mediated control of Th1 responses [73]. miR-31 and miR-21 regulate Treg development by regulating FOXP3 expression negatively and positively, respectively [74].

miR-326 and miR-155 are associated with the Th17 differentiation and induction of IL-17, a neutrophilic inflammation-inducing cytokine. miR-326 promote Th17 differentiation by targeting Ets-1, a negative regulator of Th17 differentiation [75]. miR-155 functions in the hematopoietic compartment to promote the development of Th17 cells [49]. miR-155 is also required for optimum dendritic cell production of cytokines that promoted Th17 cell formation [49]. miR-155−/− mice are defective in producing Th17 cells during autoimmune inflammation [49].

Chapter 1

Housekeeping Gene for Bovine Mastitis Related microRNA in Milk

Abstract

Our aim was to identify suitable housekeeping gene for bovine mastitis milk microRNA (miRNA) analysis by real time PCR. We chose miR-92a, miR-375 and let-7g as housekeeping gene candidates on the basis of previous Solexa sequencing results. CT values of miR-92a, miR-375, and let-7g were not changed between normal and mastitis milk. NormFinder software identified miR-92a as the most stable single housekeeping gene. The suitability of housekeeping gene candidates was evaluated by the expression levels of inflammation related gene miR-146a. The level of significance across normal and mastitis milk was the highest when using miR-92a as a housekeeping gene. These results suggested that miR-92a is suitable as a housekeeping gene for bovine mastitis milk microRNA analysis.

Introduction

To characterize the expression patterns of miRNAs, different methodologies have been applied. Low throughput techniques including quantitative real-time PCR (qPCR) and northern blotting, in situ hybridization; high throughput techniques including microarray, nCounter® nanoString technology and next generation sequencing. The expression data generated from the techniques above usually needs normalization to accurately determine the level of miRNAs. Different normalization methods or genes used among studies may lead to biases, misleading results and conclusions, and thus impair comparison and reproducibility between studies. In recent years, qPCR is the most commonly used technic for miRNA expression quantification.

Commonly used normalization methods including using endogenous reference genes, exogenous reference genes, standard curves, total RNA and global mean normalization. All of the miRNA relative expression values were normalized to reference gene and calculated by $2^{-\Delta\Delta CT}$ in this thesis. The $2^{-\Delta\Delta CT}$ method is extensively used as a relative quantification strategy for qPCR data with assumes a uniform PCR amplification efficiency of 100% across all samples. In brief, ΔCT is the difference in threshold cycle between the target and reference genes.

$$\Delta CT = CT\ (\text{a target gene}) - CT\ (\text{a reference gene})$$

The ΔΔCT is the difference in ΔCT between the target and reference samples

$$\Delta\Delta CT = \Delta CT \text{ (a target sample)} - \Delta CT \text{ (a reference sample)}$$

In our studies, we used the mean ΔCT value of all samples as a ΔCT of reference sample, thus the formula can be modified as

$$\Delta\Delta CT = \Delta CT \text{ (a target sample)} - \Delta CT \text{ (mean value of all samples)}$$

Endogenous reference gene is originated from total RNA library in the samples with low variation and diseases do not affect the gene expression. Stably expressed endogenous reference gene can reflect the total miRNA quality or concentration in the samples. Thus normalize to endogenous reference gene can reduce the differences due to sampling and quality of the samples. Small nuclear RNA (snRNA), small nucleolar RNA (snoRNA) and ribosomal RNA (rRNA) can be used as internal control gene for miRNA RT-qPCR data analysis. However non-coding RNAs other than miRNAs do not reflect the biochemical properties of miRNA, the extraction, reverse transcription, and PCR amplification efficiency of other non-coding RNAs may differ from that of

miRNAs. Previous studies suggesting that cell lines have a lower miRNA content per total RNA than tissue samples [76, 77]. When the overall abundance of miRNAs varies, in experiments affecting the miRNA processing machinery, or in comparisons involving multiple tissues or combinations of tissues and cell lines, the effect of using non-miRNA reference genes for qPCR normalization is pronounced. It has been argued that it is best to normalize genes with reference genes belonging to the same RNA class, thus miRNA qPCR needs to normalize to miRNA control genes [78].

Endogenous reference gene selection might be varying from studies. The document of TaqMan® MicroRNA Assays recommend endogenous controls for human by investigate the expression profile of endogenous control genes across 38 normal human tissues and 59 NCI-60 cell lines, including snRNA: RNU6B; snoRNA: RNU48, RNU44 and U47; miRNA: miR-26b, miR-92, miR-92N, miR-423, miR-374 and miR-16 [79]. The most frequently used reference genes are snRNA RNU6 and microRNA miR-16. Usually, non-miRNA small RNAs such as snRNAs or snoRNAs are not stably present in serum or other body fluids. Previous studies also demonstrated that expression levels of RNU6B and other snoRNA have a high variability among individual serum samples [80-83]. miR-16 is highly expressed and relatively invariant across various samples and can be used as a endogenous reference gene; however, miR-16 and miR-451 are red blood cell enriched miRNA, and the levels of miR-16 and miR-

451 vary depending on the degree of hemolysis [84-89]. The level of hemolysis in serum/plasma samples might be necessary to be assessed by spectrophotometry before miR-16 used as a control. For detailed experimental data and discussion, please refer to the Additional experiment section of chapter 2. In addition to hemolysis issue, miR-16 has been described to be deregulated in different diseases by several other studies [90-95].Thus, at the beginning of experiments, it is important to develop an ideal reference gene system based on the research needs.

Approaches to find stably expressed reference genes

Several approaches to find stably expressed reference genes has been proposed [96-102]. Most popular algorithms are GeNorm [78], NormFinder [100], and BestKeeper [97]. RefFinder is a web-based tool (http://leonxie.esy.es/RefFinder/) which integrates GeNorm, NormFinder, BestKeeper and the comparative delta-Ct method [103]. NormFinder (https://moma.dk/normfinder-software) and BestKeeper (http://www.labtools.us/bestkeeper/) are excel-based tools, researchers can get free access in their website. GeNorm is the most famous algorithm among them with more than 12000 times citation; however since 2010, the new version of GeNorm module is integrated in the qbase+ software (https://genorm.cmgg.be/) and available for 299 EUR. For this reason, NormFinder was used to analyze best reference miRNA in this study.

Aim of this study

To date, there is no suitable gene (especially miRNA) for normalizing the mastitis milk. Our aim was to identify suitable housekeeping genes for bovine mastitis milk microRNA analysis using qPCR.

Results

Identification of suitable candidates

A previous study systematically screened miRNA expression in mature milk and colostrum using Solexa sequencing [104]. We used 2 criteria to identify candidate housekeeping miRNA from these data: (1) we included miRNA with Solexa reads in mature milk and colostrum of between 3,000 and 30,000 and (2) we included only miRNA for which the difference in reads between mature milk and colostrum was less than 15%. Based on these criteria, we selected let-7g, miR-375, and miR-92a for further evaluation [104] (Table 1-1).

Table 1-1 miRNA expression in mature milk and colostrum using Solexa sequencing results in a previous study.

Name	Mature milk	Colostrum	Difference (%)
let-7g	8804	9903	11.10
miR-375	8526	8658	1.52
miR-92a	16014	18731	14.51

Milk samples from 10 mastitis-affected Holstein cows (including 11 mastitis-affected quarter samples) and 10 healthy controls were included in the qPCR candidate housekeeping gene expression validation study. The threshold cycle (CT) values for miR-92a, miR-375, and let-7g did not differ between milk from control cows and milk

from mastitis-affected cows (Figure 1-1 and Appendix 1-1 for detail CT values). We also examined miR-26b in the same experiment; its CT values were significantly lower in milk from mastitis-affected cows. The stability of the 3 candidate housekeeping genes was analyzed using NormFinder (MOMA, Aarhus, Denmark) as previously described [100] (Figure 1-2). NormFinder identified miR-92a as the most stably gene; miR-92a and miR-375 as the best combination housekeeping genes.

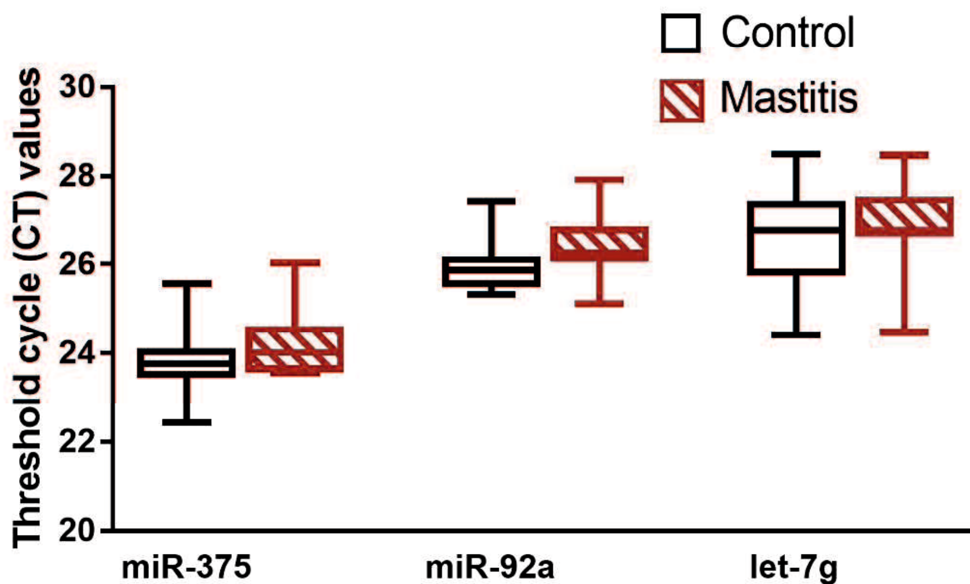


Figure 1-1 Threshold cycle (CT) values for candidate housekeeping genes in control and mastitis-affected groups.

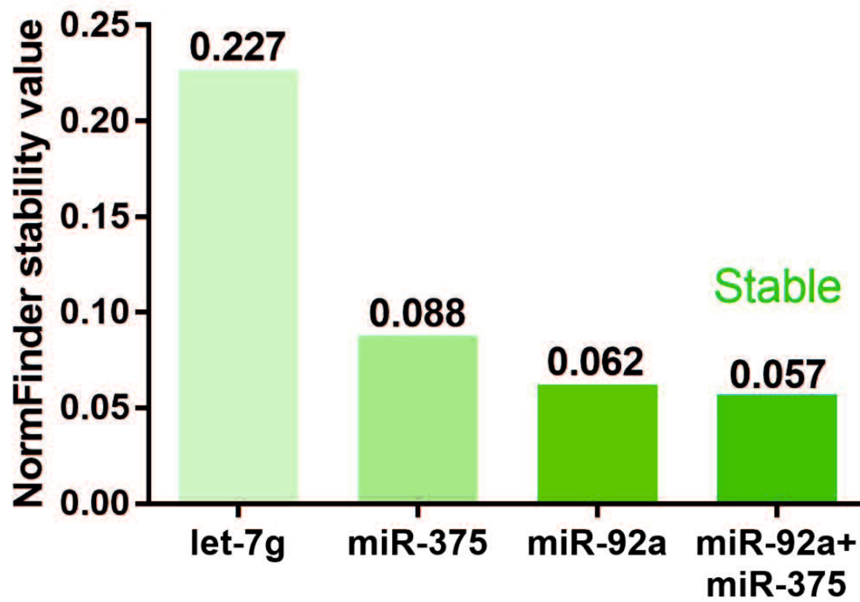


Figure 1-2 NormFinder stability values for candidate housekeeping genes.

Verification the suitability of housekeeping gene by inflammation related miR-146a

To confirm the suitability of the selected housekeeping gene candidates, we conducted a second experiment including 15 control cows (including 19 separate quarter samples from 6 cows and 9 mixed milk samples) and 14 mastitis-affected cows (including 17 mastitis affected quarter samples. miR-146a expression levels are significantly increased in bovine mammary tissues infected with subclinical, clinical, and experimental mastitis [105]. Therefore, miR-146a is a good inflammation indicator, and we used it in our study as a target gene. We used let-7g, miR-375, and miR-92a as housekeeping genes alone and in combination to evaluate their suitability. Regardless

of the housekeeping gene candidates used for normalization, relative expression levels of miR-146a in mastitis-affected samples were significantly higher than in control samples ($P < 0.05$). However, normalization with miR-92a alone ($P = 0.0001$) and in combination with miR-375 ($P = 0.0004$) generated higher significance levels for this difference than normalization with let-7g and miR-375 alone or combined ($P < 0.05$ to 0.01; Figure 1-3). Detailed miR-146a CT and normalized relative expression values please refer to Appendix 1-2.

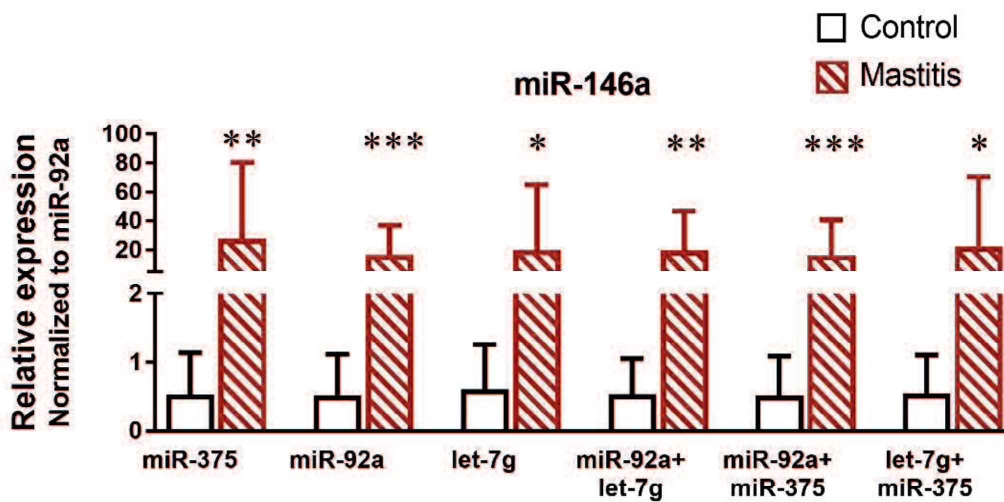


Figure 1-3 Relative expression levels of miR-146a normalized to housekeeping gene candidates. Error bars indicate SE (*P < 0.05; **P < 0.01; ***P < 0.001).

Discussion

A previous study suggested that a combination of more than one reference gene may increase normalization accuracy [78]. However, the significance level obtained when a single gene, miR-92a, was used for normalization was similar to those obtained when multiple housekeeping genes were used in our study. We recommend miR-92a as the best choice of normalization gene. Particularly when using miRNA as biomarkers for mastitis in large-scale screenings of cows and in clinical diagnosis, the absolute stability of the housekeeping gene may not be the only consideration; using a single normalization gene reduces costs. Using multiple housekeeping genes also means that screening or diagnosis takes longer to complete. The biological function regarding the miR-92a is mainly reported as oncomiR. The miR-17/92 cluster is

the first discovered oncogene and is also known as oncomiR-1 [106]. Aberrant expression of miR-92a can be observed in many kinds of tumors such as lung, breast, stomach, prostate, colon, pancreas, liver, and kidney tumors [107]. In addition, miR-92a is one of the most highly expressed miRNA in cow milk fractions including milk fat, whey, and cells [108]. It is also abundant in the human milk [109] and human milk–derived exosomes [110]. miR-92a is present in normal breast ducts and lobules and downregulated in a fraction of breast cancer [111]. This evidence suggested that miR-92a plays a physiological role in normal breast tissue and milk. As such, all of these support the potential of miR-

92a as a housekeeping gene for its constant expression in different milk components across different species.

This is the first study to demonstrate that miR-146a expression is upregulated in milk from mastitis-affected dairy cows. This result shows the potential of miRNA in milk for use as a biomarker for mastitis. To establish good biomarkers for bovine mastitis, further experiments evaluating an increased number of miRNA are necessary.

In summary, housekeeping genes play an important role in qPCR studies of miRNA gene expression. We recommend using miR-92a as a housekeeping gene for studying miRNA expression in mastitis-affected bovine milk samples.

Materials and Methods

Milk sample preparation

All of the milk samples were taken from milking Holstein-Friesian cows. The cows were kept in free-stall barn or tie-stall and pasture without grazing systems; milked twice a day. The animals were fed twice daily, and water was available ad libitum. Milk samples (approximately 5–10 ml) were collected and immediately screened in the field using a modified California Mastitis Test (CMT) with a commercial tester ("PL Tester", Nippon Zenyaku Kogyo) as previously described [112]. Cows with no CMT+ result for any quarter were defined as the normal group; cows with a CMT+ result for at least one quarter were defined as the mastitis-affected group. On the basis of the CMT results, each quarter of the mastitis-affected group was defined as CMT− or CMT+ as appropriate. Reducing sampling and quarter bias, the samples were collected from the farms of four different locations in Japan (Kagoshima, Miyazaki, and Hiroshima prefectures). The samples were stored at 4 °C after collection and transported to the laboratory, then centrifuged at 3000 × g for 15 min at room temperature to remove cell debris and fat. The supernatant was recovered and further centrifuged at 15000 × g for 15 min at 4°C. The milk whey was recovered and stored at −80 °C for RNA extraction.

RNA isolation

Total RNA was extracted from milk whey using a mirVana PARIS kit (Thermo Fisher Scientific) according to the manufacturer’s protocol.

Quantification of miRNA by qPCR

Equal volumes of RNA (1.25 microliter) were reverse transcribed to cDNA using TaqMan MicroRNA Assays (Thermo Fisher Scientific) according to the manufacturer’s protocol. qPCR was performed using a TaqMan Fast Advanced Master Mix kit and a StepOne Plus Real Time PCR system (Thermo Fisher Scientific). Thermal cycling was conducted according to the manufacturer’s recommended protocol, and all experiments were performed in duplicate. miR-92a was used as an internal control for miR-146a expriment and expression level was determined using the $2^{-\triangle\triangle CT}$ method. qPCR reactions of undetermined CT were assigned CT = 40. The TaqMan MicroRNA Assays used in qPCR of this study and their IDs are as follows: let-7g (ID: 002282), miR-375 (ID: 007627_mat), miR-92a (ID: 000431), and miR-146a (ID: 000431).

Statistical Analysis

Data analysis was performed using GraphPad Prism 6 (GraphPad Software Inc., San Diego, CA). Data were analyzed using a parametric unpaired t test, and $P < 0.05$ was considered statistically significant.

Chapter 2

MicroRNA as Biomarkers for Bovine Mastitis in Milk

Abstract

miRNA in tissue and liquid samples have been shown to be associated with many diseases including inflammation. This study aimed to identify inflammation-related miRNA expression level in the bovine mastitis milk. Expression level of inflammation-related miRNA in milk from mastitis-affected and normal cows was analyzed using qPCR. We found that expression level of miR-21, miR-146a, miR-155, miR-222, and miR-383 was significantly upregulated in California mastitis test positive (CMT+) milk. We further analyzed these miRNA using a chip-based QuantStudio Digital PCR System. The digital PCR results correlated with those of qPCR, demonstrating upregulation of miR-21, miR-146a, miR-155, miR-222, and miR-383 in CMT+ milk. In conclusion, we identified miRNA that are upregulated in CMT+ milk. These miRNA exhibited sensitivity and specificity greater than 80% for differentiating between CMT+ milk and normal milk. Our findings suggest that inflammation-related miRNA expression level in the bovine milk was affected by mastitis, and miRNA in milk have potential for use as biomarkers of bovine mastitis.

Introduction

California mastitis test (CMT)

California Mastitis Test (CMT) is the cowside test to estimate somatic cell count range on dairy farm with the advantages of quick, cheap and simple. CMT reagent disrupt the cells in milk sample and react with the DNA in those cells. After reaction, color changes of the reagent and rennet reaction are recorded to estimate somatic cell number range, indicating the severity of the inflammation. However, the interpretation can be subjective, and this might result in false positives and negatives. A sensitivity of 66.7% and specificity of 54.8% using the CMT to detect mastitis has been reported in fresh cows [113]. Because all of the samples we studied were clinical samples, modified California Mastitis Test (CMT) with a commercial tester ("PL Tester", Nippon Zenyaku Kogyo) was used in these studies [112].

Wisconsin Mastitis Test (WMT) uses the same reagent as the CMT but measured by gel height in a tube, providing objectivity results.

Stability of miRNA

Stability is one of the important characteristics of biomarker. miRNAs in serum/plasma are protected against degradation by being packaged in lipid vesicles such as exosomes, microvesicles (MVs), or apoptotic bodies, or by being associated

with high-density lipoproteins or bound to RNA-binding proteins such as Ago2 [114]. With these protection, miRNAs own high stability and resistance to storage handling. By stability test in harsh conditions such as boiling (digested with RNase A for 3 hours or overnight at 37 °C), low/high pH (pH=1 or 13), extended storage, 10 freeze-thaw cycles, a previous study has shown the stability of miRNA in serum [115]. miR-145 can be efficiently extracted and amplified from serum in archived 10-year-old human serum samples [116], and miR-16, miR-21 and miR-223 can be detected in unrefrigerated dried serum blots [117].

miRNA as biomarkers

In 2008, Lawrie et al. reported the elevated levels of tumor-associated microRNAs miR-155, miR-210 and miR-21 in serum of patients with diffuse large B-cell lymphoma [118], human realized the diagnostic and prognostic potential of miRNAs as cancer biomarkers. The communication between organs and cells is not only limited to soluble molecular such as hormone. miRNA are packaged into vesicles, export to blood stream, and leading to widespread consequences within the cells at a distance from the 'secreting' cell, exert their remote communication and hormone-like effects [83]. As such, it reminds us in 1889, Stephen Paget suggested a seed and soil hypothesis for organ-specific metastasis, and a previous study showed that tumor-derived exosomes uptaken

by organ-specific cells prepare the pre-metastatic niche [119]. Because miRNA are also possibly delivered to the metastasis sites, it is not surprising that circulating miRNAs in plasma/serum of patients can be used as biomarkers to their diagnosis and prognosis. Indeed, miRNAs can be used as biomarkers for various type of cancers such as breast cancer, cervical cancer, colorectal cancer, gastric cancer, hepatocellular carcinoma, lung cancer, hematopoietic cancer, melanoma, esophageal cancer, prostate cancer. Other diseases including infectious diseases, nervous system disorders, cardiovascular disorders, and diabetes [83, 120-122].

miRNA in milk

miRNA have been identified in 12 different body fluids from normal individuals, including plasma, saliva, tears, urine, amniotic fluid, colostrum, breast milk, bronchial lavage, cerebrospinal fluid, peritoneal fluid, pleural fluid, and seminal fluid [28]. miRNA have been identified in the milk from other animals such as cows [104, 123], pigs [124], goats [125], rats [126], mice[127], giant panda [128] and yaks [129]. Kosaka et al. used microarray to profile miRNA expression in human breast milk and detected high expression levels of immune related miRNAs in the first 6 months of lactation. These miRNA molecules are stable in very acidic conditions, indicating that breast milk allows dietary intake of miRNAs by infants [109]; however a mice study in 2015 suggests that milk miRNA are

not taken up into murine offspring tissues or blood but are rapid degradation by intestinal fluid [127]. Whether maternal milk miRNA are taken up by offspring may still need further studies, because this experiment measured whether miRNA expression increased in miRNA knockout mice after milk feeding instead of label the maternal exosome or miRNA. Without the data of maternal miRNA kinetics, we don't know if the samples from day 3 or 14 of birth are appropriate for maternal milk-derived taken up experiment. Izumi et al. investigated miRNA in bovine colostrum and mature milk, immune- and development-related miRNA are highly expressed in colostrum than in mature milk, and naturally existing miRNA in raw milk are resistant to acidic conditions and RNase treatment [123]. Immune-related miRNA are also present in the milk-derived microvesicles and exosomes [110, 130]. miRNA can be detected in the commercial products such as fluid milk and powdered formula milk from Japan and China markets [123, 131]. Chen et al. suggested that expression profile milk-specific miRNA can serve as ideal biomarkers for discriminating poor-quality or "manipulated" milk from pure raw milk and quality control of commercial milk products [131]. After identified that miR-92a can be used as a housekeeping gene in milk, we also measured miR-92a in commercial fluid milk products (Figure 2-1). We found that miR-92a was hard to be detected but existed (average CT value = 33.1) in commercial fluid milk products compare to raw milk, suggesting that milk processing leaded to miRNA degradation. Schanzenbach et al.

failed to use miRNAs in milk as biomarkers for detection of bovine pregnancy [132].

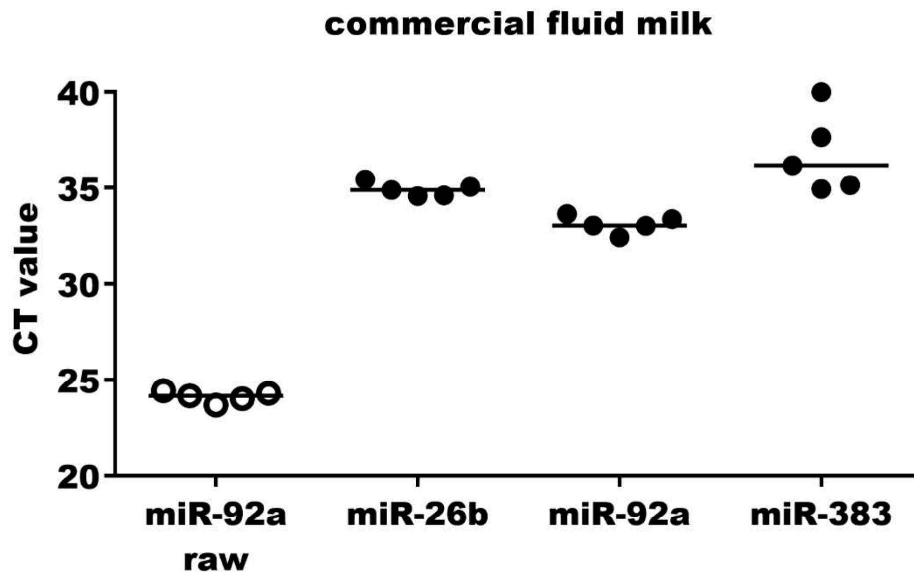


Figure 2-4 miRNA CT values of five different brands of commercial fluid milk products and raw cow milk. The raw milk samples are shown in hollow circles.

miRNA target selection in this study

In the preliminary study, we select several inflammation related miRNA. miR-21 regulates proinflammatory protein PDCD4 expression level after lipopolysaccharide (LPS) stimulation [53]. miR-26 regulates inflammation through down-regulating IL-6 production [133], and miR-26b participates in the inflammatory response of LPS stimulated bovine alveolar macrophages by enhancing the NF-κB signaling pathway [134]. miR-29b is repressed by NF-κB pathway [135], and miR-29b can represses TNFAIP3,

a negative regulator of NF-κB pathway [136]. LPS induced inflammation increases blood levels of miR-122 [137]; serum miR-122 also correlates with mortality in human sepsis patients [138, 139]. miR-125b is down-regulated in bovine CD14+ monocytes stimulated with *Staphylococcus aureus* enterotoxin B [140] and activate the NF-κB pathway by targeting TNFAIP3 [141]. miR-204 mediates vascular inflammation in high fat diet mice [142], and plays a role in the regulation of inflammation process through promoting the expression level of SIRT1 and attenuating of inflammatory factors [143, 144]. miR-205, which expression level is upregulated upon NF-kB activation, reduces COMMD1 expression level. The miR-205-COMMD1-NF-κB axis enhances inflammatory response [145]. miR-222 is involved in the pathogenesis of inflammatory diseases, such as rheumatoid arthritis, atherosclerosis and obesity-related inflammation [146, 147]. The mechanisms associate with adhesion and infiltration of inflammatory cells into the endothelial space [148]. miR-383 expression level is upregulated in LPS induced macrophage cell line RAW264.7 [149]. Among these miRNA, miR-26b, miR-29b, miR-122, and miR-205 are differentially expressed in the serum of cow with metritis [150]. We included miR-146a and miR-155 in the second phase of this study. miR-146a and miR-155 are the well characterized and first reported inflammation-related miRNA [41]. miR-146a and miR-155 expression level are induced by expose human acute monocytic leukemia cell line THP-1 to LPS [41]. miR-146a expression levels are significantly

increased in bovine mammary tissues infected with subclinical, clinical and experimental mastitis [151].

Application of Digital PCR in miRNA

The conception of digital PCR has been described in 1992, which is an end point PCR method for absolute quantification [152]. The samples are split into thousands of separated reaction chambers, which contain one or no copies of the gene, following PCR and Poisson's distribution model analysis, the results will be generated by the counting of binomial positive or negative events. There are two types of digital PCR: chips based and droplets based digital PCR. In this study, we used chips based QuantStudio 3D Digital PCR for the absolute quantification of miRNAs. Digital PCR provides a sensitive method for the direct measure gene expression and absolute quantitate the gene expression without the need for a standard curve. The droplets based digital PCR assay has a significantly higher degree of sensitivity compared to the qPCR assay [153-155]. Thus QuantStudio 3D Digital PCR was used for novel miRNA detection in chapter 3.

In miRNA research application, droplets based digital PCR has been used to quantitate plasma and sputum miRNAs from cancer-free subjects and non-small-cell lung cancer patients, combined quantification of miR-21-5p and miR-335-3p in plasma

provided 71.8% sensitivity and 80.6% specificity [156]; combined quantification of miR-31 and miR-210 in sputum provided 65.71 % sensitivity and 85.00 % specificity [157]. The chip-based QuantStudio 3D digital PCR has been used to measure miR-16-5p, miR-21-5p, miR-126-3p, miR-486-5p and miR-660-5p expression in plasma, tissue and cells [158].

Aim of this study

In this study, we used qPCR and QuantStudio 3D Digital PCR to investigate differences in inflammation-related miRNA expression level in three groups of milk samples: milk from normal cows; and milk from California mastitis test-negative (CMT−) and -positive (CMT+) quarters of mastitis-affected cows. We identified that miR-21, miR-146a, miR-155, miR-222, and miR-383 were significantly upregulated in CMT+ milk. These miRNA had sensitivity and specificity greater than 80% for differentiating between CMT+ milk and milk from normal cows. Our findings suggest that inflammation-related miRNA expression level in the bovine milk was affected by mastitis, and miRNA in milk have potential for use as biomarkers of bovine mastitis.

Results

Identification of miRNA with altered expression level in milk from mastitis-affected cows and CMT+ milk

We included six cows without mastitis (n=22) and three mastitis-affected cows (n=9; two mastitis-affected cows (n=7) for miR-21) in the preliminary study. We analyzed the expression levels of nine inflammation-related miRNA in milk by qPCR and normalized the values obtained to the expression level of miR-92a [159]. Six miRNA (miR-21, miR-122, miR-125b, miR-205, miR-222, and miR-383) were significantly upregulated and two miRNA (miR-26b and miR-29b) were significantly downregulated in milk from mastitis-affected cows, as compared with that from normal cows (Figure 2-2). The preliminary study suggests that bovine milk contains inflammation-related miRNA which expression level may affected by mastitis. Detailed CT and normalized relative expression values please refer to Appendix 2-1, Appendix 2-2 and Appendix 2-3.

To confirm and further assess the expression level of inflammation-related miRNA, we increased the number of samples and analyzed selected miRNA. We selected miR-21, miR-122, miR-222, and miR-383, which were highly upregulated in milk from mastitis-affected cows in the preliminary study. Additionally, we included miR-146a and miR-155, which are known to be related to inflammation [41], as candidates in the

second phase experiment. The second study included milk from 18 normal cows (n=42) and 14 mastitis-affected cows. To investigate if miRNA expression level in the CMT− quarters would be implicated by mastitis, we separated the milk from mastitis-affected cows into CMT− (n=18) and CMT+ (n=17) groups. The expression levels of five miRNA (miR-21, miR-146a, miR-155, miR-222, and miR-383) were significantly upregulated in the CMT+ group, compared with in the CMT− and normal groups. The expression levels of all miRNA did not differ between the CMT− and normal groups. There was no significant difference in miR-122 levels among the three groups, so this miRNA was excluded from further study (Figure 2-3). Detailed and normalized relative expression values please refer to Appendix 2-4.

Receiver operating characteristic analysis

Receiver operating characteristic curve analysis of relative expression levels of five miRNA was performed to evaluate the ability of the miRNA to distinguish between the CMT+ and normal groups (Figure 2-4). Area under the curve analysis and the Youden index were applied to determine the optimal cut-off point, sensitivity and specificity of each miRNA [160]. We found that miR-146a, miR-155 and miR-222 had high predictive values ($0.9 < AUC < 1$); and miR-21 and miR-383 had moderate predictive values ($0.7 < AUC < 0.9$). miR-21, miR-146a, miR-155, miR-222, and miR-

383 had sensitivity of 82%, 88%, 94%, 94%, and 88%, and specificity of 89%, 100%, 90%, 93%, and 83% in differentiating CMT+ milk from normal milk, respectively.

Analysis of miRNA with altered expression level in CMT+ milk by digital PCR

We selected the miRNA that were significantly upregulated in the CMT+ group (miR-21, miR-146a, miR-155, miR-222, and miR-383) for QuantStudio 3D Digital PCR System analysis. We analyzed milk from five normal cows and five mastitis-affected cows (including five CMT− quarters and five CMT+ quarters) in this validation study. The expression levels of the five miRNA were significantly higher in the CMT+ group than in the normal group. The miRNA were also significantly upregulated in the CMT+ group compared with the CMT− group, except for miR-146a ($P = 0.0509$). The expression levels of all miRNA did not differ between the CMT− and normal groups (Figure 2-5). Pearson's correlation analysis was applied to assess the relationship between the qPCR and digital PCR results. We found a strong negative correlation between the Ct values obtained via qPCR and the values for copies/μL obtained via digital PCR (the Pearson r values for miR-21, miR-146a, miR-155, miR-222, and miR-383 were −0.8433, −0.7853, −0.8849, −0.9256, and −0.8008, respectively)(Figure 2-6), and a strong positive correlation between relative expression levels obtained via qPCR and the values for copies/μL obtained via digital PCR (the

Pearson r values for miR-21, miR-146a, miR-155, miR-222, and miR-383 were 0.7897, 0.9047, 0.7660, 0.9536, and 0.7676, respectively)(Figure 2-7). These results suggest that chip-based QuantStudio 3D Digital PCR System could be a tool for quantification of miRNA in milk.

Discussion

Dysregulation of miRNA expression level has been shown to play a role in inflammatory diseases [161]. Previous studies have demonstrated that miRNA expression level in mastitis-affected cows is altered in mammary epithelial cells [162-164], monocytes [165], milk exosome [166], and mammary gland tissue [151, 167]. Our study showed that expression level of miRNA was altered in CMT+ milk, suggesting that miRNA may play a role in bovine mastitis. Our findings suggest the potential for development of molecular biology-based biomarkers for bovine mastitis-affected milk. We further verified the miRNA upregulation using a QuantStudio 3D Digital PCR System, and obtained reproducible results. To the best of our knowledge, this is the first report to compare miRNA in milk from mastitis-affected and normal cows.

Liquid biopsy allows diagnosis of diseases in noninvasive, safe, and fast way using biomarkers isolated from body fluids, such as blood and urine [28]. Circulating miRNA have been proposed to have either diagnostic or prognostic value in various types of human cancer [120]. We evaluated the suitability of miRNA in milk as liquid biopsy biomarkers using receiver operating characteristic analysis. Our results showed that several miRNA had high predictive values (AUC greater than 0.83) and sensitivity and specificity greater than 80% in differentiating CMT+ milk from normal cow milk. These results demonstrate the potential of miRNA in milk for use as a liquid biopsy

biomarker for mastitis.

Previous studies have demonstrated that droplet digital PCR can measure expression level of miRNA in body fluids, and that these miRNA can serve as diagnostic biomarkers. For example, miRNA in serum can be used as a biomarker in breast cancer diagnosis [168], and miRNA in sputum and plasma can be used for lung cancer diagnosis [156, 157]. We used a QuantStudio 3D Digital PCR System to measure miRNA expression levels in milk, and compared the results with those of qPCR. Direct comparison of miRNA expression levels and CT values obtained via qPCR with copy numbers obtained via digital PCR for the same sample set demonstrated a strong correlation between the two methods. A previous study also indicated high correlation between copy numbers obtained via digital PCR and expression levels determined by qPCR across serially diluted samples [157]. In this study, we demonstrated that the results of qPCR were reproducible using QuantStudio 3D Digital PCR. Therefore, the chip-based QuantStudio 3D Digital PCR System could be a tool for quantification of miRNA in milk for diagnosis of bovine mastitis.

There are some of limitations to our study. First, further evaluation in large cohorts of the miRNA identified in this study is required before they could be used as robust biomarkers. Second, we used the California mastitis test (CMT), which is based on the somatic cell count of milk to detect mastitis. In addition to inflammation, there are many

other factors that could influence milk somatic cell count, such as seasonal effects and physiological or environmental stress [169, 170].

In conclusion, miR-21, miR-146a, miR-155, miR-222, and miR-383 expression levels were significantly upregulated in the CMT+ milk. Our findings suggest that inflammation-related miRNA expression level in the bovine milk was affected by mastitis, and miRNA in milk have potential for use as biomarkers of bovine mastitis.

Materials and Methods

Milk sample preparation

All of the milk samples were taken from milking Holstein-Friesian cows. The cows were kept in free-stall barn or tie-stall and pasture without grazing systems; milked twice a day. The animals were fed twice daily, and water was available ad libitum. Milk samples (approximately 5–10 ml) were collected and immediately screened in the field using a modified California Mastitis Test (CMT) with a commercial tester ("PL Tester", Nippon Zenyaku Kogyo) as previously described [112]. Cows with no CMT+ result for any quarter were defined as the normal group; cows with a CMT+ result for at least one quarter were defined as the mastitis-affected group. On the basis of the CMT results, each quarter of the mastitis-affected group was defined as CMT− or CMT+ as appropriate. Reducing sampling and quarter bias, the samples were collected from the farms of four different locations in Japan (Kagoshima, Miyazaki, and Hiroshima prefectures). The samples were stored at 4 °C after collection and transported to the laboratory, then centrifuged at 3000 × g for 15 min at room temperature to remove cell debris and fat. The supernatant was recovered and further centrifuged at 15000 × g for 15 min at 4°C. The milk whey was recovered and stored at −80 °C for RNA extraction.

Total RNA extraction

Total RNA was extracted from 300 μL milk using a mirVana PARIS kit (Thermo Fisher Scientific) according to the manufacturer's protocol. The quality of RNA was assessed using the Small RNA kit in combination with the 2100 Bioanalyzer System (Agilent).

Quantification of miRNA by qPCR

qPCR was performed as chapter 1 described. Equal volumes of RNA (1.25 microliter) were reverse transcribed to cDNA using TaqMan MicroRNA Assays (Thermo Fisher Scientific) according to the manufacturer's protocol. qPCR was performed using a TaqMan Fast Advanced Master Mix kit and a StepOne Plus Real Time PCR system (Thermo Fisher Scientific). Thermal cycling was conducted according to the manufacturer's recommended protocol, and all experiments were performed in duplicate. miR-92a was used as an internal control and expression level was determined using the $2^{-\triangle\triangle CT}$ method. qPCR reactions of undetermined CT were assigned CT = 40. The TaqMan MicroRNA Assays used in qPCR of this study and their IDs are as follows: miR-21 (ID: 000397), miR-29b (ID: 000413), miR-92a (ID: 000431), miR-122 (ID: 002245), miR-125b (ID: 000449), miR-146a (ID: 005896_mat), miR-155 (ID: 002623), miR-204 (ID: 000508), miR-205 (ID: 000509), miR-222 (ID:

002276), and miR-383 (ID: 000573).

Quantification of miRNA by digital PCR

Digital PCR was performed using the QuantStudio 3D Digital PCR System (Thermo Fisher Scientific) according to the manufacturer's protocol. In brief, 3 μL cDNA of miR-146a, miR-155, miR-222, and miR-383 were combined with QuantStudio 3D Digital PCR Master Mix and TaqMan Assay. The cDNA of miR-21 was diluted 1:30 with RNase-free water before being combined with the reagent because miR-21 was highly expressed and exceeded the instrument detection range. The samples were loaded onto chips using QuantStudio 3D Digital PCR Chip Loader (Thermo Fisher Scientific). The manufacturer's recommended digital PCR thermal cycling protocol was used. After PCR, the fluorescence data from the chips were collected using a QuantStudio 3D Digital PCR Instrument and uploaded to QuantStudio 3D Analysis Suite Cloud Software for further analysis.

Statistics

Data analysis was performed using GraphPad Prism 6 (GraphPad Software Inc., San Diego, CA). Data were compared using a parametric unpaired t-test, or one-way ANOVA followed by Tukey's test where appropriate. Differences were considered to

be significant at $P < 0.05$. The area under the curve (AUC), cut-off point, sensitivity, and specificity were analyzed by receiver operating characteristic curve. Cut-off points were determined by the Youden index [160]. Correlation analysis was performed using Pearson's correlation coefficient.

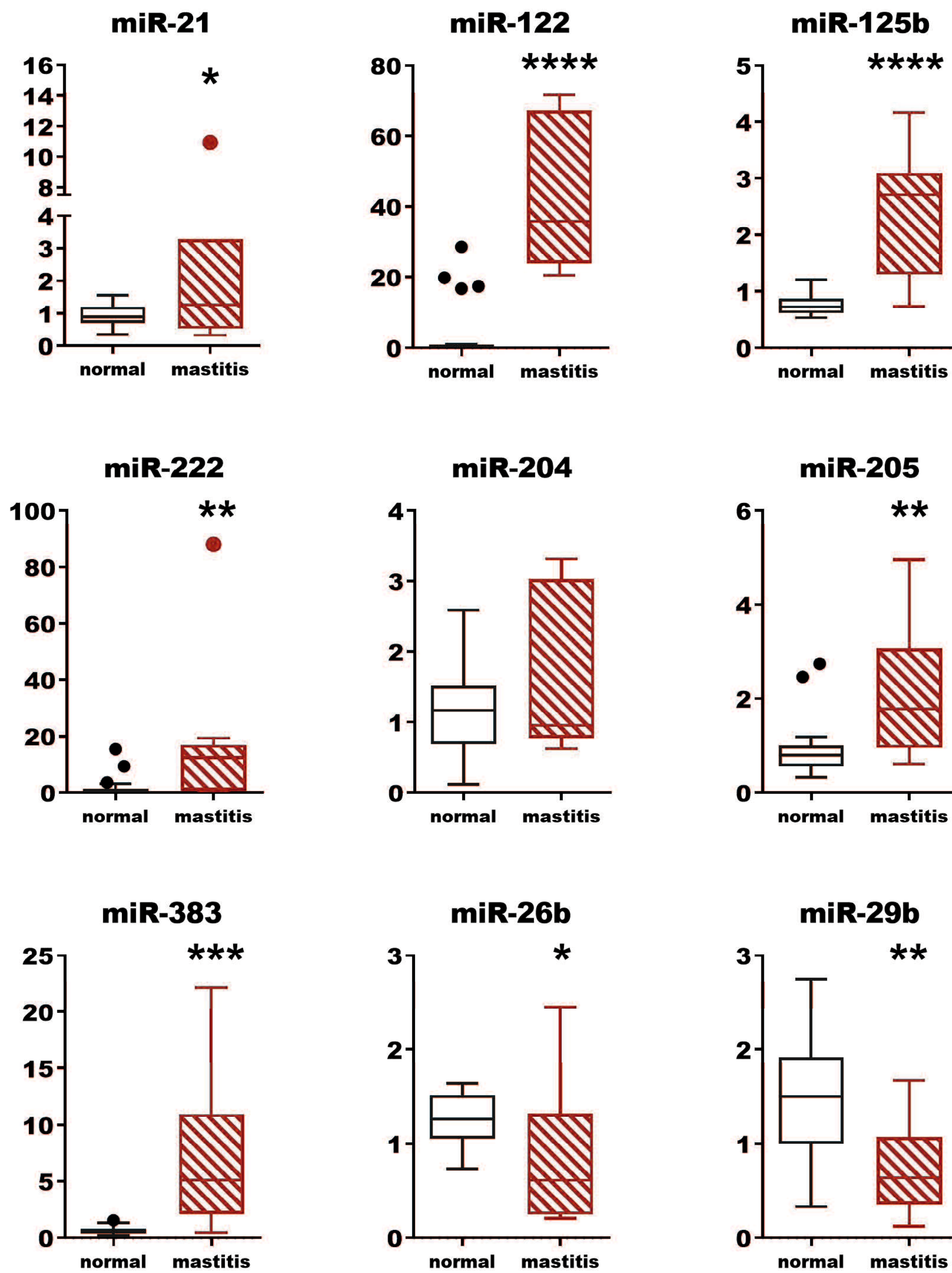


Figure 2-5 Relative expression levels of nine miRNA in milk from mastitis-affected cows and normal cows using qPCR. Boxes indicate the median, and 25th and 75th quartiles. Whiskers extend from the edge of the box to 1.5 times the interquartile range, and dots indicate data points outside this range. The y-axes represent relative miRNA expression levels in arbitrary units (parametric unpaired t-test, *P < 0.05, **P < 0.01, ***P <0.001, ****P <0.0001)

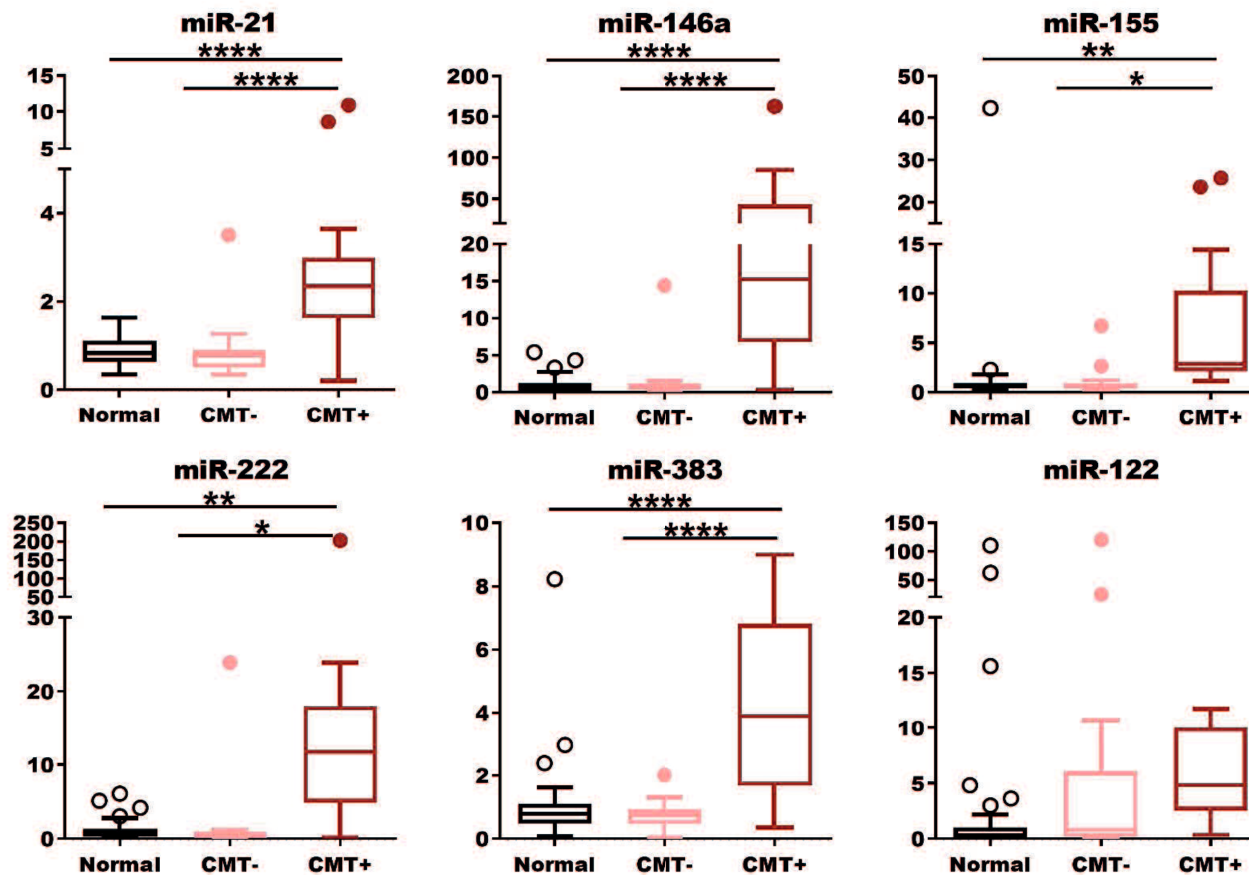
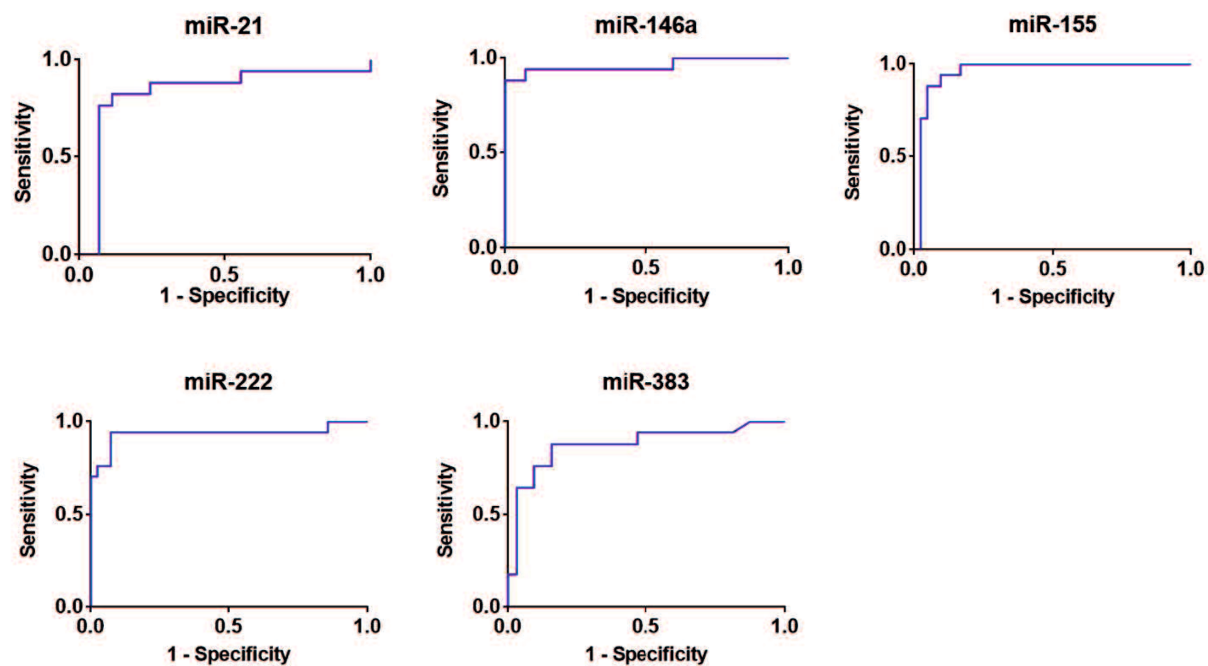


Figure 2-6 Relative expression levels of six miRNA in milk from normal cows, and from CMT− and CMT+ quarters using qPCR. Boxes indicate the median, and 25th and 75th quartiles. Whiskers extend from the edge of the box to 1.5 times the interquartile range, and dots indicate data points outside this range. The y-axes represent relative miRNA expression levels in arbitrary units (One-way ANOVA followed by Tukey's test, *P < 0.05, **P < 0.01, ****P <0.0001).



	miR-21	miR-146a	miR-155	miR-222	miR-383
AUC	0.8366	0.9608	0.9594	0.9356	0.8789
Cut-off point	1.505	5.62	1.525	3.135	1.185
Sensitivity	82%	88%	94%	94%	88%
Specificity	89%	100%	90%	93%	83%

Figure 2-7 Mastitis diagnostic values of miRNA in milk quantified using qPCR. The Youden index was applied to determine the optimal cut-off point, sensitivity and specificity. AUC: area under the curve.

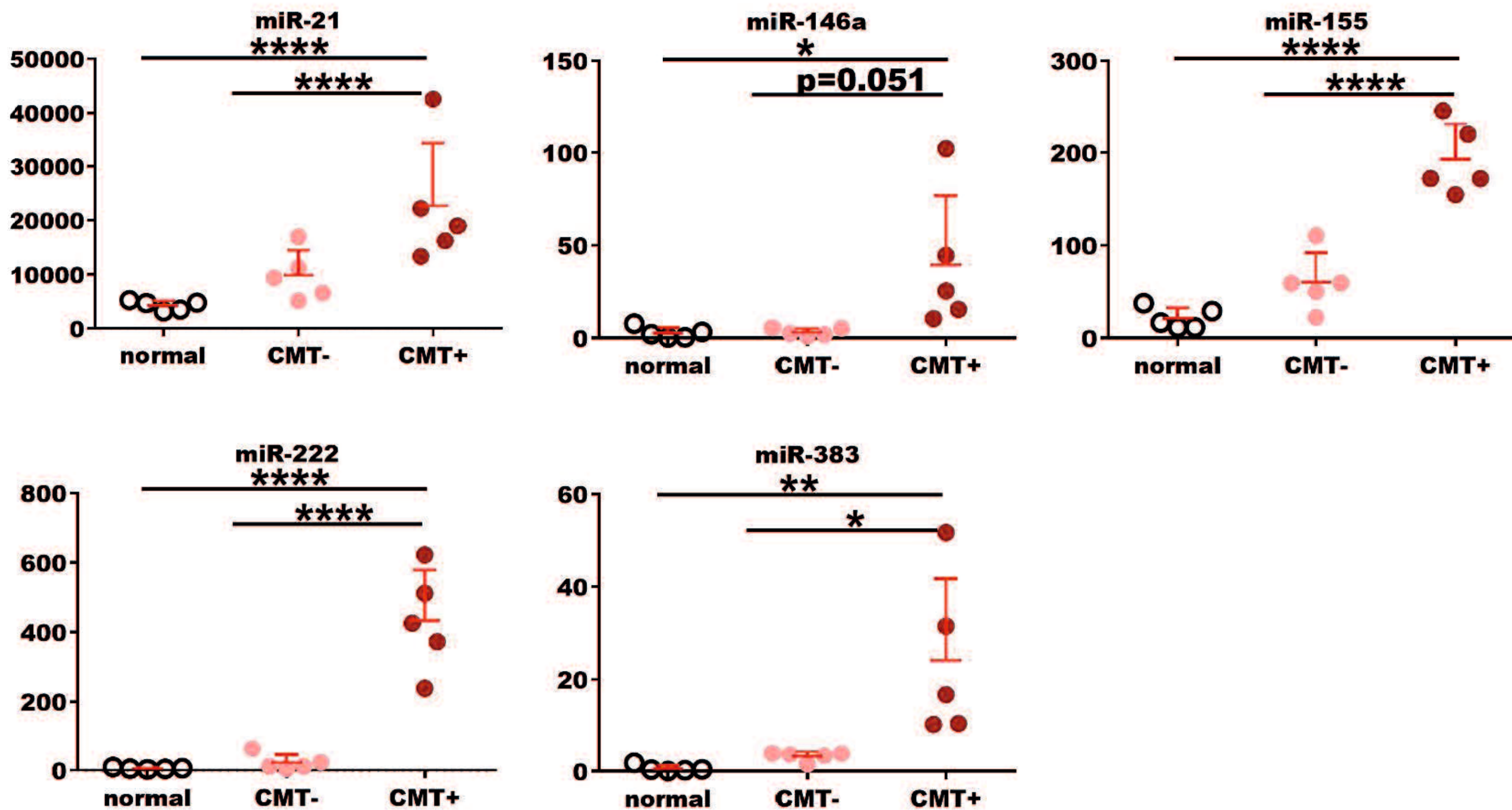


Figure 2-8 Digital PCR system quantification of expression levels of five miRNA in milk. Lower bars indicate mean values with vertical standard deviation bars. The y-axes represent copies/μL assessed by digital PCR (One-way ANOVA followed by Tukey's test, *P < 0.05, **P < 0.01, ****P <0.0001).

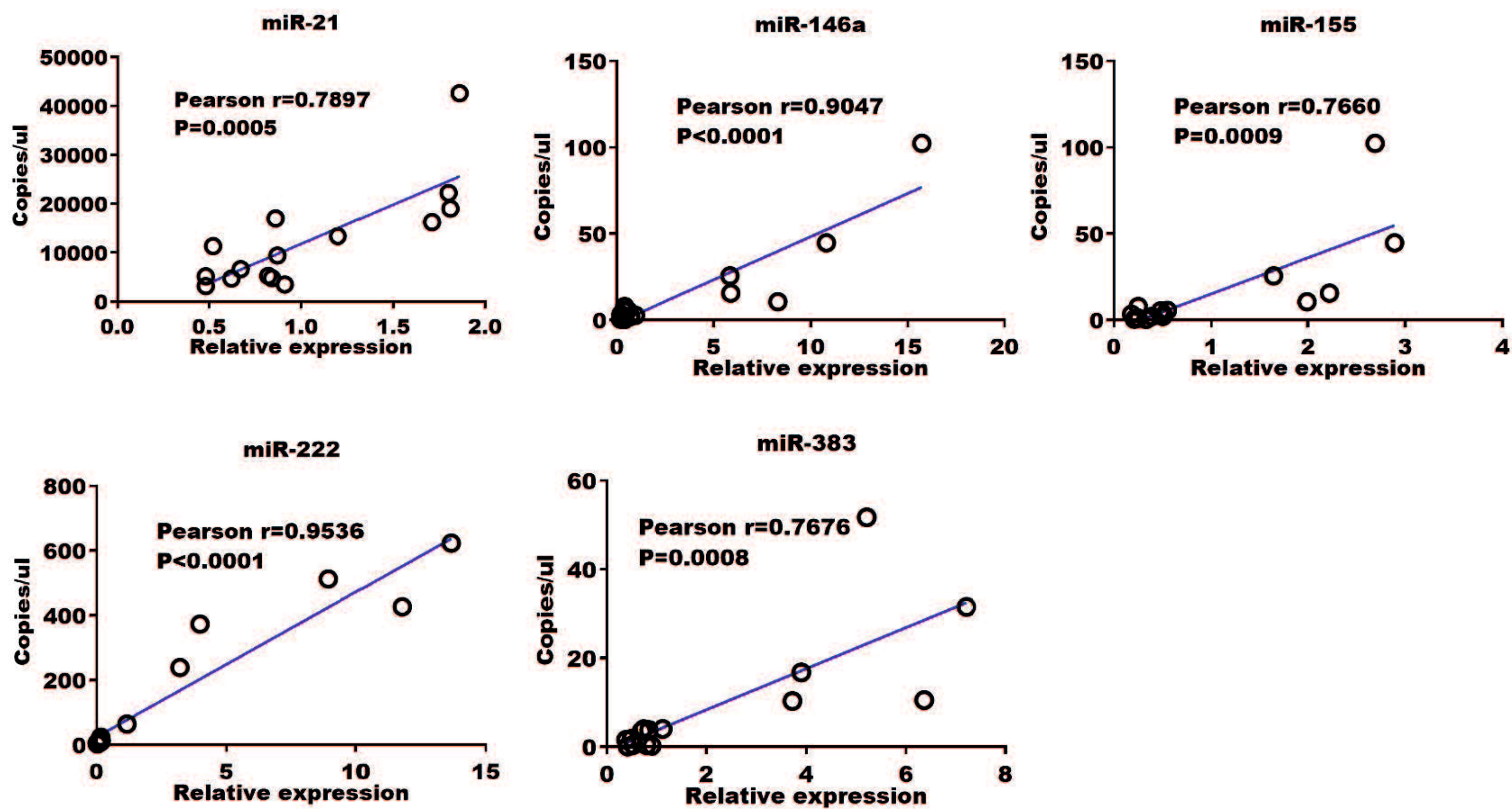


Figure 2-9 Relationship between copies/μL obtained via digital PCR and relative expression value obtained via qPCR.

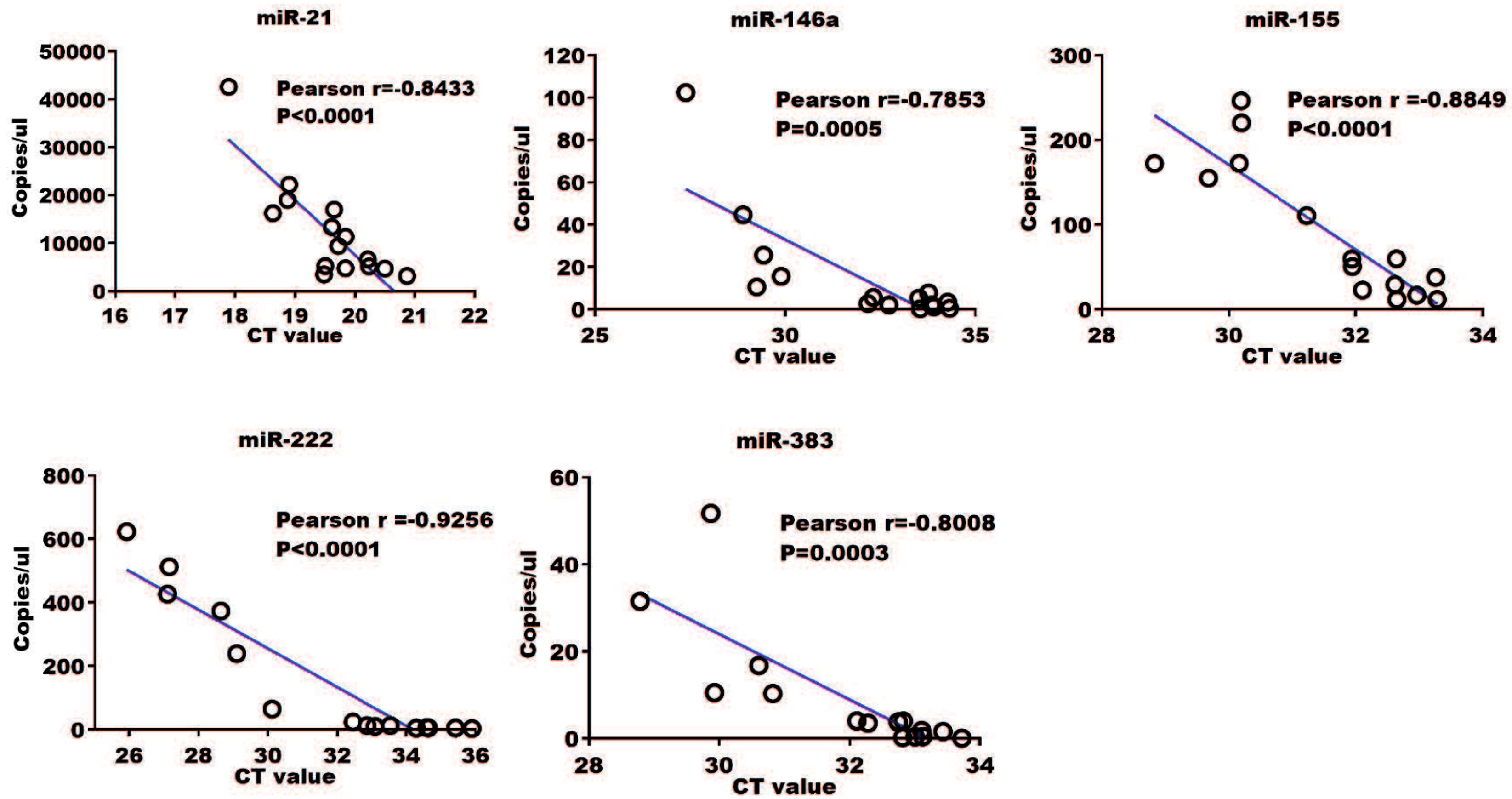


Figure 2-10 Relationship between copies/μL obtained via digital PCR and CT value obtained via qPCR.

Additional Experiment: Can Inflammation-related microRNA Expression Level in The Mastitis Cow Serum Be Affected by Mastitis?

miR-21, miR-146a, miR-155, miR-222 and miR-383 in milk can be bovine mastitis biomarkers. In this additional experiment, we investigated the expression level of these miRNA in serum from mastitis cows.

The whole blood was collected and centrifuged to recover serum from normal and mastitis cows. RNA isolation and PCR procedures were the same as mastitis milk miRNA experiment, however we added exogenous control 25 fmol of synthetic cel-miR-39 as our reference gene because we did not investigate the suitable housekeeping genes for miRNA analysis as milk did.

Exogenous and endogenous control genes

miR-16 is frequently used as an internal control gene for serum and plasma miRNA analysis, as it is highly expressed and relatively invariant across various samples [171]. However, miR-16 is highly abundant miRNA in red blood cells, hemolysis leads red blood cells release miR-16 to serum, and therefore concentration of miR-16 in serum increased substantially, which raising some concerns to use as a reference miRNA in serum or plasma studies [84-89, 172].

As humans, we found that hemolysis significantly altered cow serum miR-16 expression (Figure 2-8, Mann-Whitney test p = 0.0001). The exogenous control cel-miR-39 expression was stable (standard deviation = 0.51, Figure 2-8). However exogenous control may not always reflect true sample condition, because it was added artificially and represents only the efficiency of RNA extraction. We found that total RNA concentration might be low in one of sample (high CT value in most of miRNA), and endogenous miR-16 reflected this issue; however exogenous cel-miR-39 expression could not distinguish from other samples and can lead to normalization error (Figure 2-9). This sample was ignored for further analysis.

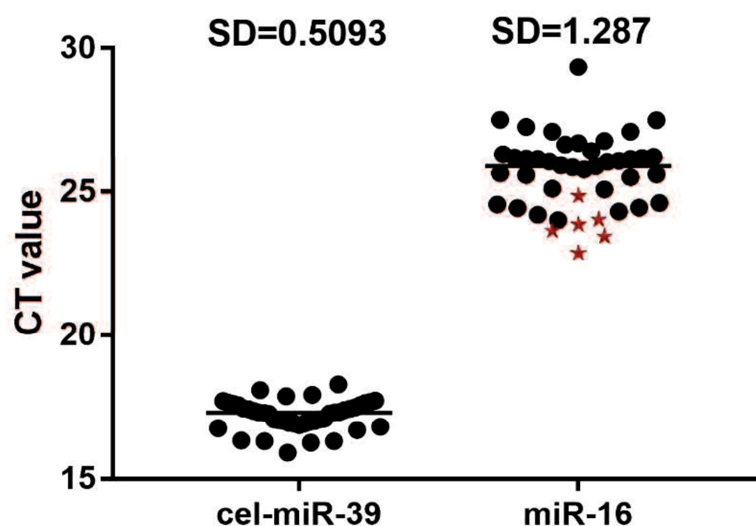


Figure 2-11 CT values of cel-miR-39 and miR-16. The CT values of miR-16 in suspected hemolytic samples are shown in red star symbol. The bars indicate median values.

Only miR-21 was significantly upregulated in serum from mastitis cow

Eleven normal and fifteen mastitis cows were included in the serum study. The expression levels normalized to cel-miR-39 among five miRNAs (miR-21, miR-146a, miR-155, miR-222, and miR-383), only miR-21 expression level was significantly upregulated in serum from mastitis cows ($p = 0.036$) compared to that of normal cows (Figure 2-10). We also used miR-16 as a control. The result was similar as cel-miR-39 to be used as a control, miR-21 had a trend to be increased ($p = 0.097$) in mastitis cow serum, other miRNAs were not significantly changed between normal and mastitis cows (Figure 2-11). Detailed CT values please refer to Appendix 2-5; relative expression values normalized to cel-miR-39 and miR-16, please refer to Appendix 2-6.

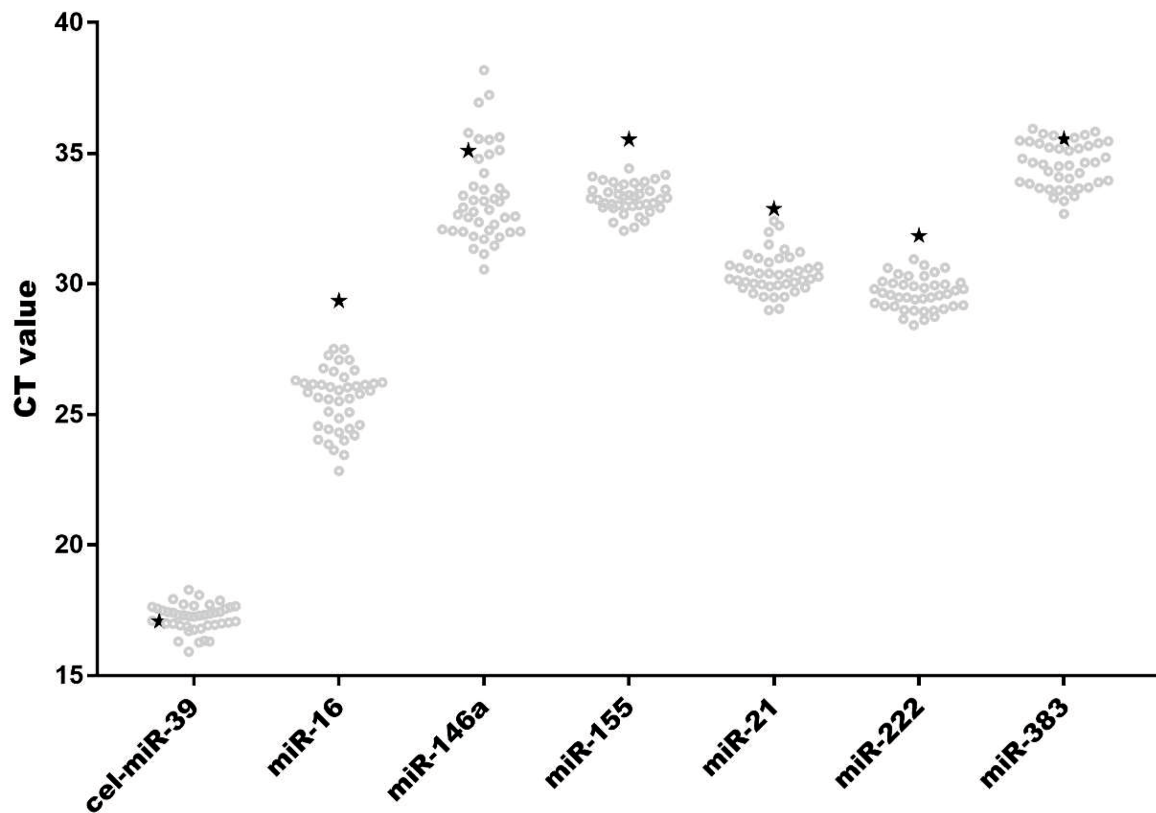


Figure 2-12 CT value distribution of all serum RNA samples. The CT values of one of low miRNA expressed sample is shown in black star. The endogenous miR-16 reflected that the black star position was high in other miRNA compared to other samples, however in the cel-miR-39 results, the black star was located near the mean value. Some of samples were not included in the analysis of this study (n = 43).

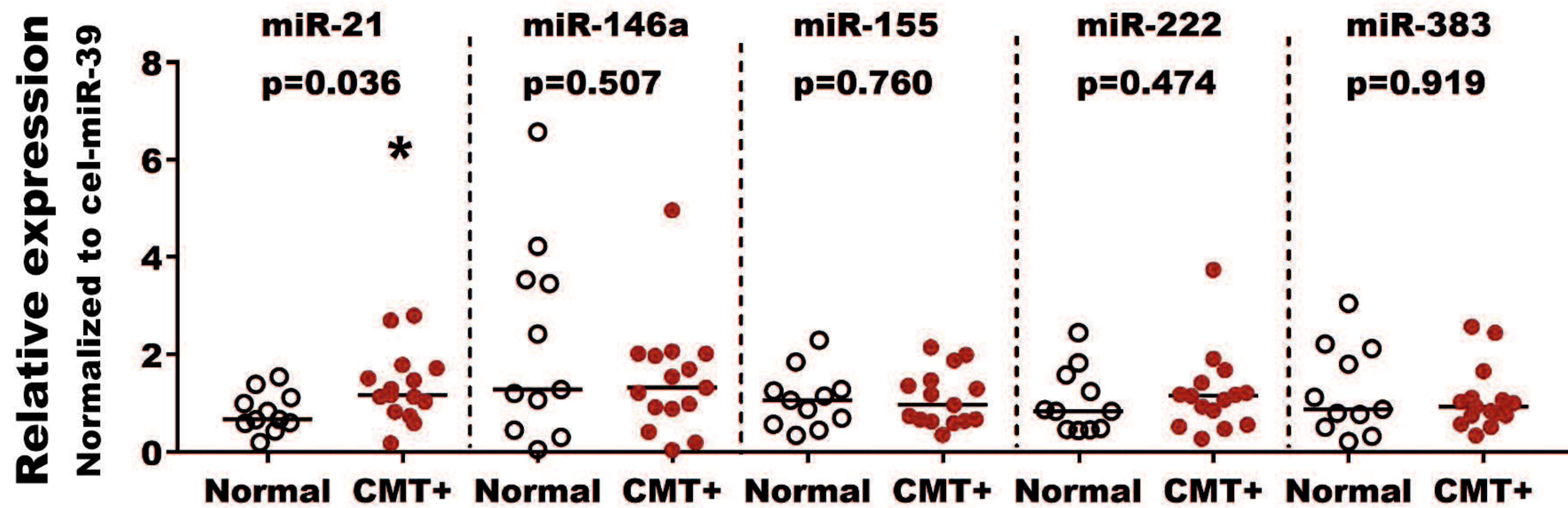


Figure 2-13 Serum miRNA relative expression normalized to cel-miR-39 (Mann-Whitney test, *p = 0.036). The bars indicate median values.

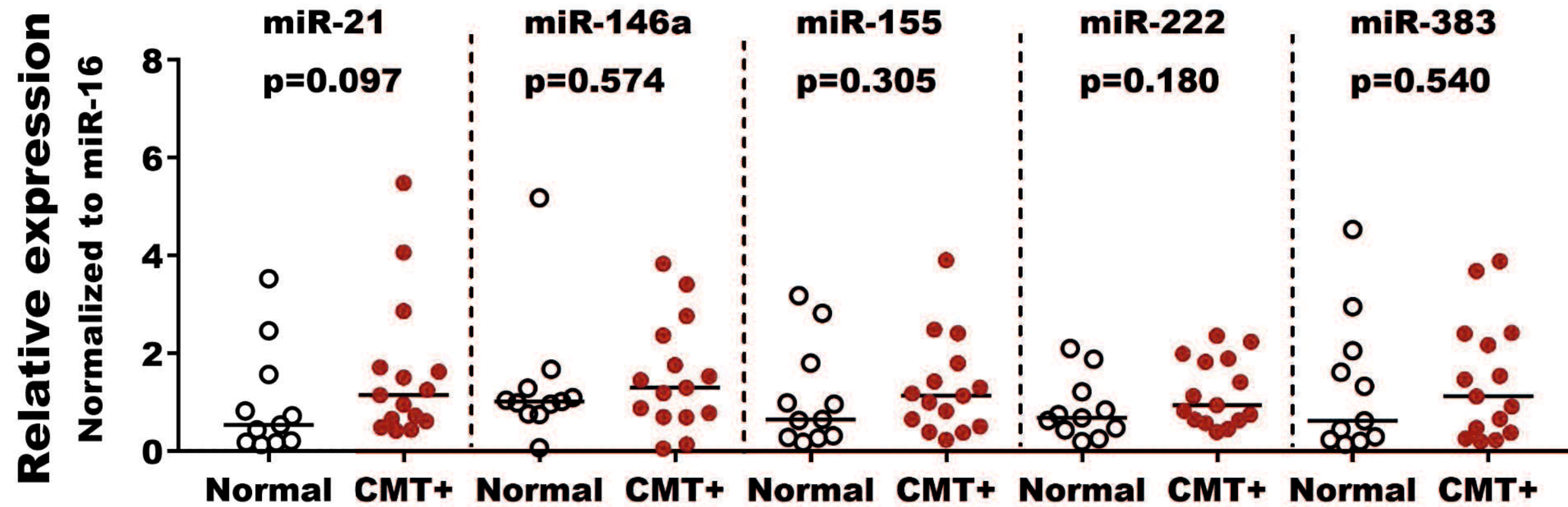


Figure 2-14 Serum miRNA relative expression normalized to miR-16 (Mann-Whitney test). The bars indicate median values. The bars indicate median values.

A previous study investigate whole blood from Holstein cows with *Staphylococcus aureus*-caused mastitis by Solexa sequencing also shows the patterns of miRNA expression differed significantly between healthy and mastitis Holstein cattles [173]. However only one library was constructed without any replicates in both healthy and mastitis groups in this study might lead their conclusions with very limited reliability. The significant differentially expressed miRNA in this study may be due to biological variation, and the expression patterns may be specific to the individual instead of mastitis cows [174].

In accordance with the previous study, we found that miR-21 was 1.63 fold increases in mastitis cow. In a biological perspective, we demonstrated that mastitis can cause systemically impaction because circulating serum miRNA expression was changed. However, compared to milk miRNA, 1.63 fold upregulation was relatively a small change, and other inflammation related miRNA such as miR-146a and miR-155 were not changed in mastitis cow serum. In a biomarker and diagnostic tool perspective, milk samples might be superior to serum samples because: 1) Inflammation related miRNAs were changed much clearly in mastitis affected milk samples. 2) Milk samples can be took without invasive venipuncture and blood taking technique, thereby increases the sample accessibility. 3) Hemolysis that impact miRNA expression and endogenous gene usability never happens in milk sample. 4) Milk samples can

recognize specific mastitis affected quarters which is impossible for serum samples.

In conclusion, we found that miR-21 expression was increased in mastitis cow serum, suggesting that focal mastitis can cause molecular biological mechanisms changed systemically.

Chapter 3

Transcriptome Analysis for Bovine Mastitis Related microRNA in Milk

Abstract

We present a genome-wide miRNA study in California mastitis test positive (CMT+) bovine mastitis milk and normal milk. Twenty-five miRNA were differentially expressed, with 23 miRNA being upregulated and 2 downregulated in bovine mastitis relative to the normal milk. The upregulated mature miR-1246 was likely derived from U2 small nuclear RNA instead of miR-1246 precursor. The significantly upregulated miRNA precursors and RNU2 were significantly enriched in the bovine chromosome 19 which homologous to human chromosome 17. Gene ontology analysis of significantly upregulated miRNA putative mRNA targets showed that the upregulated miRNA were involved in bind to target mRNA transcripts and regulate target gene expression, while KEGG pathway analysis showed that upregulated miRNA were mainly related to cancer and immune system pathways. Three of novel miRNA were related with bovine mastitis and relatively highly expressed in milk. We further verified that one of the mastitis related novel miRNA was significantly upregulated using a digital PCR system. The differentially expressed miRNA are known to involve in human cancer, infection and immune related diseases. The genome-wide views of miRNA profiles in this study provide insights into bovine mastitis and inflammatory diseases.

Introduction

Next Generation Sequencing

The next generation sequencing technologies generates millions of nucleotide short reads in parallel, faster, high throughput reduced cost, and without electrophoresis processes. The major secondary generation sequencing platform including Roche 454, Illumina Solexa, ABI SOLiD, and Ion torrent. The principles of secondary generation sequencing are as following: ligate the adapter sequences to fragmented DNA (include cDNA) for library construction. Library is amplified on a solid surface, beads or and DNA nanoball generation. Nucleotide incorporation is monitored by luminescence detection or electrical charge changes [175, 176]. In Illumina Solexa sequencing, DNAs are immobilized on a glass surface and bridge amplification to form clusters which contains clonal DNA using four differently colored fluorescently labeled chain terminators. The newly incorporated nucleotides are detected by fluorescence color imaging. The fluorophore and blocking group can then be removed and start a new cycle. Illumina provides a wide range of instruments with different throughputs suitable for industrial-level or laboratory use, including MiniSeq, MiSeq, NextSeq, HiSeq, and HiSeq X series. The industrial-level sequencing machines can produces up to 6 billion, which is more than needed for a single study, thus the libraries are indexed, normalized and combined to be run on a single flow cell in practice. Compared with 454 and SOLiD, Illumina

Solexa is cheap in sequencing ($0.07 / million bases), it is also the most successful sequencing system with a claimed > 70% dominance of the market [175]. We also used Illumina Solexa sequencing in this study.

Brief introduction of miRNA sequencing data processing and bioinformatic analysis [177]

The miRNA sequence processing can be mainly divided into: 1) Sequences processing, 2) Align sequences to reference, and 3) Differential expression analysis.In sequences processing step, we can remove low quality, ambiguous, specified number of bases at either 3' or 5' end, shorter or longer than a specified threshold, and sample-specific tag (barcode or index) sequences. The most important process for miRNA sequences is removing the adapter. Aligning the sequences with 3' adapter to the reference can impacts on the results severely. Please see the table below.

Table 3-2 Sequencing results of dog liver miRNA. Comparing the annotated and unannotated sequences before and after 3' end adaptor removing.

	With adaptor		Without adaptor	
	Sequences	**Percent**	**Sequences**	**Percent**
Total	34482309	100.00%	33732947	100.00%
Unannotated	34440413	99.88%	2411923	7.15%
miRNA	26717	0.08%	28651229	84.94%
others	15179	0.04%	2669795	7.91%

Most of the sequences with 3’ end adaptor could not be annotated to miRNA; however, 85% of sequences could be annotated to miRNA, suggesting the 3’ end adaptor must be removed when performing miRNA analysis.

The goal of mapping miRNA sequences to reference is to annotate the original source of miRNA. We used CLC Genomics Workbench 10.1.1 to map the processed miRNA to the reference downloaded from miRBase. A common mapping processes are introduced below:

1. Select preprocessed reads between 18-30 bp and map to the reference genome with allowing 1-3 bp mismatches to detect isomiRs. Discard the reads cannot map to reference genome.

2. Map the sequences to database of small RNA other than miRNA, such as Rfam or Genebank, and discard rRNA, tRNA, snRNA and snoRNA, coding and non-coding RNAs except miRNA.

3. Map the sequences to miRBase to identify known miRNAs.

For the study to investigate differential expression, genomic mapping step can be totally skipped, because known miRNA must be located somewhere in the genome. In our differential expression part of study, we ignored genomic mapping step. However genome location is important to confirm the sequences origin in novel miRNA

investigation study. The second step might be necessary to the "strict" miRNA study, because these small RNAs are not belong to miRNA category which can be confounding factors of pure miRNA study. Most of studies calculate the proportion of these fragments in the library, make a pie chart and ignore from further investigation. However accumulating evidences suggest that RNA fragments derived from other types of non-coding RNA are not just random degradation products but rather stable entities, which may have biological function [178]. In this study, we focus on the small nuclear RNA (snRNA) derived small RNA, and we found that RNU2 derived small RNA were increased in mastitis milk.

We used empirical analysis of DGE implemented in CLC workbench for differential expression analysis. Once differential expression miRNAs are obtained, downstream analysis could be performed to insight into gene regulation and function, such as miRNA-mRNA network, gene ontology, and pathway enrichment.

miRNA and mRNA network [177]

A miRNA can target hundreds of mRNAs, thus the miRNA-mRNA network can be investigated to know the regulatory function of different expressed miRNAs. On the other hand, mRNA list is necessary for gene ontology and KEGG pathway analysis.

The list of target gene can be predicted using target prediction tools. The

commonly used features of these tools including [179]: 1) Seed match; several canonical types of seed region matches that can be considered by algorithm. 2) Conservation; the maintenance of a sequence across species. 3) Free energy; the stability of miRNA binding to a candidate target mRNA and 4) Site accessibility; measure if miRNA can locate and hybridize with an mRNA target. miRNA target prediction analysis methods can be mainly categorized as: 1) Computational prediction method, 2) Artificial intelligence methods. Computational prediction method rely on the guidelines above, provide a basis to score the potential target, users can choose thresholds for targets in the trade-off between sensitivity and specificity. Artificial intelligence methods including support vector machine (SVM) and self-organizing map (SOM). Support vector machines (SVM) is a popular method in miRNA target prediction. In SVM, features such as commonly used in computational prediction method are mapped into multi-dimensional space. SVM allows to separate them into two classes by constructing a linear boundary in a large, transformed version of the feature space. SVM classifier needs to train by positive and negative examples, like miRNAs that downregulated and nonregulated mRNAs from a microarray experiment, respectively. SVM study results can be used to develop a web browser tool.

Commonly used miRNA target prediction tools are miRanda [180] and TargetScan [181]. This study use TargetScan as a tool for mRNA target prediction. TargetScan is easy

to use and actively maintained. The new version TargetScan 7.1 released in June 2016. It also includes more species than other programs. Analyzing can be done by click and enter miRNA or mRNA symbol. For advanced users, the code of TargetScan can be downloaded and run the program locally.

Gene ontology and KEGG pathway analysis

With a list of target mRNAs, we can perform enrichment analysis to understand the biological themes behind the large mRNA list indirectly. Gene ontology analysis provides defined terms representing gene properties and functions covering cellular components, molecular function, and biological processes. Some genes in disease could be imbalanced in biological pathways, and some pathways are even comprise only up- or downregulated genes in a particular disease [182]. Kyoto Encyclopaedia of Genes and Genomes (KEGG) pathways is a database resource to integrate functions and utilities of biological systems from molecular-level information [175]. GO enrichment analysis can be performed in CLC workbench platform. The Ingenuity Pathway Analysis plugin of CLC workbench allows to perform Ingenuity Pathway Analysis (IPA) of genes and expression data directly from CLC Workbench. However CLC workbench did not provide KEGG pathway analysis function. In this study we used DAVID, which is an online graphical user interface based tool. GO terms and KEGG pathways can be

analyzed by paste gene list to the website. GOseq is also applicable to KEGG pathway analysis.

Novel miRNA prediction

Novel miRNA can be predicted in the NGS small RNA reads that could not map to the miRBase. Map to the genome can confirm the origin of the unmapped small RNA sequences; the sequence reads which cannot map to genome are discarded, and then filtered the small RNA derived from known non-miRNA annotation. The remaining sequence reads are possible derived from undiscovered miRNA, to predict the novel miRNA, they should conform to some of canonical miRNA characteristics, such as their precursors can be folded as a hairpin structure, the hairpin structure do not contain large bulges and should be energetically stable. The putative hairpin duplex should be base paired and the small RNA derived from their putative precursors should correspond to guide strand, passenger strand, terminal loop, or even to the miRNA-offset RNA (moR).

miRDeep [183, 184] and miReap (https://sourceforge.net/projects/mireap/) are the most popular tools used in bovine novel miRNA prediction. This study uses miRDeep2 for novel miRNA prediction. miRDeep system is the one of most wildly accepted tools in NGS and novel miRNA prediction field. miRDeep2 is also able to count known miRNA reads for downstream differential expression analysis. Latest released version

is miRDeep2.0.0.8 on May 2016. Overall, performance of miRdeep family including first and second version in novel miRNA prediction are good. The accuracy levels of simulated data set are at 80.4 and 75.4% for miRDeep and miRDeep2, respectively [185]. miRDeep also listed as best two recommended software in predicting novel nematode (*Caenorhabditis elegans*), vertebrate (*Gallus gallus*) and mammal (*Homo sapiens*) miRNAs [186]. In another study, miRDeep2 demonstrates a high specificity in *H.sapiens* (94 (±2.7) % in 23 datasets), *M.musculus* (98.6 (±2) % in 21 datasets), and *D.rerio* (89.7 (±1.3) % in 2 datasets) miRNA prediction. The putative miRNA can be validated by common wet lab RNA technics including qPCR, Northern blot, and Knock-down experiment. In this study, we use PCR based QuantStudio 3D Digital PCR System to verify the existence and expression between normal and mastitis milk of novel miRNA.

miRNA and human disease databases

Deregulation of miRNA is associated with diseases, by analysis of deregulated miRNA list using database with miRNA associated diseases, researchers can investigate the relationship between miRNA and diseases. We used miRWalk 2.0 [187] and HMDD 2.0 [188] for miRNA enrichment analysis in this study. miRWalk2.0 supplies predicted and experimentally verified miRNA-target interactions. It also incorporates the experimentally verified information on miRNAs linked with genes, diseases, pathways,

ontologies, cell lines and organs. Testing phase of the miRWalk 3 is started in May, 2017 (http://129.206.7.150/). HMDD (Human microRNA Disease Database) is a database that curated experiment-supported evidence for human miRNA and disease associations. Currently, HMDD collected 10368 entries that include 572 miRNA genes, 378 diseases from 3511 papers. The miRNA enrichment analysis of miRWalk 2.0 and HMDD 2.0 have been implemented in the miRNA Enrichment Analysis and Annotation tool (miEAA). MiEAA is a web-based, graphical user interface tool offers over-representation analysis (ORA) and Gene Set Enrichment Analysis (GSEA) statistical tests. MiEAA includes over 14 000 miRNA set of pathways, diseases, organs and target genes.

miRNA sequencing application in bovine mastitis

Previous studies can mainly categorized into milk/mammary gland miRNA study and circulating miRNA study. J Pu et al. collected mammary gland tissues from dairy cows with *Streptococcus agalactiae*-induced mastitis to identify differentially expressed miRNAs related to mastitis [189]. They found 10 up-regulated miRNAs and 25 down-regulated miRNAs. Of these miRNAs, miR-223 exhibited the highest degree of up-regulation whereas miR-26a exhibited the most decreased expression level. J Sun et al. purified milk exosomes from control and *S. aureus* infected cows [166]. miRNA

expression from milk exosomes produced in response to infection supporting a role for delivery into milk of specific miRNA involved in immune responses. In particular, bta-miR-142-5p, and miR-223 are potential biomarkers for early detection of bacterial infection of the mammary gland. Additionally, 22 mammary-expressed genes involved in immune process regulation and response to inflammation are identified as potential binding targets of the differentially expressed miRNAs. N Lawless et al. reported a profile of mRNA and miRNA expression at multiple time points (0, 12, 24, 36 and 48 hr) in milk and blood FACS-isolated CD14+ monocytes from animals infected *in vivo* with *Streptococcus uberis* [165]. 26 miRNA are differentially expressed in milk-isolated monocytes and three are differentially expressed in blood-isolated monocytes. Pathway analysis reveals that predicted targets of downregulated miRNA are highly enriched for roles in innate immunity, particularly TLR signaling, whereas upregulated miRNA are preferentially targeted genes involved in metabolism. The authors concluded that during *S. uberis* infection, miRNA are key amplifiers of monocyte inflammatory response networks and repressors of several metabolic pathways. Another paper from N Lawless et al. reported the miRNA expression in primary bovine mammary epithelial cells at 1, 2, 4 and 6 hours postinfection with *Streptococcus uberis* [164]. 21 miRNAs were identified as significantly differentially expressed post-infection with *S. uberis*, and several of them are known to have roles in the immune system of other species. miRNA

response to the Gram-positive *S. uberis* is markedly different to lipopolysaccharide (LPS), the major component of the outer membrane of Gram-negative bacteria, induced miRNA expression. Down-regulated miRNA in bovine mammary epithelial cells following *S. uberis* infection are statistically enriched for roles in innate immunity. This study suggests that miRNA may significantly regulate the sentinel capacity of mammary epithelial cells to mobilise the innate immune system. W Jin et al. reported global expression of miRNA in bovine mammary epithelial cells challenged with and without heat-inactivated *Staphylococcus aureus* (*S. aureus*) or *Escherichia coli* (*E. coli*) bacteria at 0, 6, 12, 24, and 48 hr [162]. 17 miRNA were differentially regulated by the presence of *E. coli*, and *E. coli* initiated an earlier regulation of miRNA while *S. aureus* presented a delayed response. Differentially expressed of bta-miR-184, miR-24-3p, miR-148, miR-486 and let-7a-5p are unique to *E. coli*, while bta-miR-2339, miR-499, miR-23a and miR-99b are unique to *S. aureus*. This study provided a further confirmation of the involvement of mammary epithelia cells in contributing to the immune response to infecting pathogens. R Li et al. administer *S. aureus* to the mammary gland. A total of 77 miRNA in the *S. aureus* group are significant differences compared to that in the control group [167]. Z Li et al. reported differentially expressed miRNA in peripheral blood from healthy and mastitis Holstein cattle. 173 unique miRNA were significant differential expression between healthy and mastitis Holstein

cattle. Most differentially expressed miRNA belong to the chemokine signaling pathway involved in the immune responses [173].

Aim of this study

In this study, we characterized miRNA sequences of normal milk and clinical mastitis milk whey samples through next-generation sequencing (NGS) technology, and aimed to investigate the miRNA involved in the molecular level pathogenesis of mastitis and the regulatory role of miRNAome in inflammatory diseases.

Results

miRNA profiles by next-generation sequencing in CMT+ group were different from that in Normal and CMT− groups

To study the miRNA differentiation in bovine mastitis milk, fifteen small RNA libraries were constructed from cow milk samples (including five samples from five normal cows, five CMT− and five CMT+ samples from five mastitis-affected cows) and subjected to Illumina small RNA sequencing. The sequencing generated total 116.1 million, 121.1 million and 103.2 million raw reads (including forward and reverse direction paired end reads) from the five libraries of normal, CMT− and CMT+ groups, respectively (Table 3-1). To investigate the overlapping rate of paired end reads, we performed two-step trimming processes. In the first trimming step, we aimed to remove low quality sequences, ambiguous nucleotides, 3' adaptor, and short (< 15 nt) or long (> 29 nt) reads, then merge the paired end reads. 94.2%, 93.8% and 93.2% paired end reads from normal, CMT− and CMT+ groups were merged, respectively (Table 3-1). In the second trimming step, we aimed to remove the contamination sequences including stop solution and 5' adaptor sequences [190], 13.6 million, 8.7 million, and 8.1 million clean reads were obtained from normal, CMT− and CMT+ groups for further small RNA analysis, respectively (Table 3-1). The length distribution of small RNA was consistent among three groups, the most common reads were ranged between 20-

24 nucleotides, 84.2%, 77.1%, and 70.3% clean reads of normal, CMT− and CMT+ groups were located within this range corresponds to the length of mature miRNA, respectively (Figure 3-1). The 82.4%, 73.1%, and 69.5% of clean reads were annotated to miRBase [191] or Ensembl cow non-coding RNA (ncRNA) databases [192], respectively (Figure 3-2A). The small RNA components in normal and CMT− groups were similar, more than 90% of annotated small RNA belonged to miRNA (Figure 3-2B). Interestingly, the remaining ncRNA classes were increased in CMT+ group, of which, small nucleolar RNA (snoRNA) and small nuclear RNA (snRNA) were relatively increased. At present, the functions of snoRNA and snRNA derived small RNA were largely unknown compared to miRNA. These small RNA were much less abundant than miRNA (account for ~2.5% of annotated small RNA in the CMT+ group), nevertheless they may played a role in bovine mastitis (Figure 3-2C).

More than 400 unique miRNA were identified in the normal, CMT− and CMT+ libraries (Figure 3-3). The top ten unique miRNA accounted for ~70% of the total miRNA reads mapped to miRBase (Figure 3-3). The top eight miRNA were overlapped among three groups (miR-148a, miR-22-3p, miR-26a, miR-30a, miR-141, miR-186, miR-27b, and miR-21-5p) (Figure 3-3). Five of these miRNA (miR-148a, miR-26a, miR-30a, miR-21-5p, and miR-186) were corresponded to the top expressed miRNA share among milk fat, whey, cells, and mammary gland tissue in previous study [108],

suggesting that these miRNA were common components in cow milk. The ranking order of top eight miRNA was similar in normal and CMT− groups, only the miRNA ranked third and fifth (miR-26a and miR-141) were different between normal and CMT− groups. However all of the miRNA ranking order was different between normal and CMT+ groups, suggesting that the composition of highly expressed miRNA could be affected by mastitis (Figure 3-3). The ranking of miR-21-5p shifted from eighth in normal group to be fourth in CMT+ group, was the most obviously moved up abundant miRNA in ranking order. miR-21-5p has been studied as one of bovine mastitis biomarkers and upregulated in CMT+ group in our previous work [193]. The principal component analysis (PCA) also indicated a separation of normal and CMT− groups from CMT+ group (Figure 3-4). Based on these results, we concluded that miRNA profiles in CMT+ group were different from that in normal and CMT− groups, and next we analyzed the differentially expressed miRNA in normal and CMT+ groups.

Differentially expressed miRNA in mastitis milk

Twenty-three miRNA were up-regulated and two miRNA were down-regulated in mastitis milk (Empirical analysis of DGE FDR < 0.05, and miRNA mean read counts in either normal or CMT+ groups > 50)(Figure 3-5 and Table 3-3). Among these miRNA, miR-146a and miR-222 have been studied as mastitis biomarkers in our

previous work [193]. Hierarchical clustering revealed the separation of the normal, CMT 2+, and CMT 4+ groups, suggesting that miRNA expression was correlated to severity of inflammation. Seven miRNA were further validated using qRT-PCR (Normal n = 55, CMT− n = 42, and CMT+ n = 41). miR-142-5p, miR-221 and miR-2284w were statistically highly significant (FDR $<10^{-5}$). miR-6529a has been reported as the most common non-human miRNA discovered in plasma-derived exosomes [194, 195]. miR-15a forms a miRNA cluster with miR-16-1 and involve in human chronic lymphocytic leukemia [22]. miR-23b-3p was one of the two downregulated miRNA. miR-1246 was statistically highly significant (FDR $=1.76 \times 10^{-7}$) and has been reported as the fragment derived from RNU2-1 snRNA in human [196]; interestingly, snRNA population was increased in CMT+ group (Figure 3-2B). RNU2 derived small RNA was the most abundant in snRNA population in both normal and CMT+ libraries, it accounted for 68.9% and 82.4% of total snRNA, respectively (Table 3-5). The small RNA derived from some of RNU2 were also significantly upregulated in CMT+ group (Table 3-6). Querying the read count distribution of miR-1246 and RNU2 derived small RNA in cow milk, we investigated the canonical miR-1246, RNU2 and their isomiR with 3' and 5' end at most 3 nucleotides addition or deletion in the pooled normal, CMT− and CMT+ libraries (Figure 3-6). The canonical RNU2 derived small RNA was considered the one with the most reads. Total read counts of canonical RNU2 derived small RNA

were much abundant than that of miR-1246 (15183 and 269 counts, respectively) (Figure 3-6). Compared to miR-1246 was almost lack of 3' end isomiR, RNU2 isomiR were highly expressed and the read count distribution of isomiR was nearly normal (Figure 3-6), which is consistent with the results of a previous study that the typical size distribution of isomiR was nearly normal, especially for highly expressed miRNA [197]. 5' end isomiR analysis showed that mature miR-1246 was not a dominant isomiR, the read counts of miR-1246 sequence with 1 nucleotide addition or deletion at 5' end were more than that of miR-1246 (Figure 3-7), suggesting that bovine miR-1246 may be a pseudo miRNA or small RNA fragment. The RNU2 isomiR were unlikely the degraded or damaged RNA and entire miR-1246 sequence was comprised within the canonical RNU2 derived small RNA sequence in cow and human [196], therefore we analyzed canonical RNU2 derived small RNA by the Custom TaqMan® Gene Expression Assay.

Consistent with the deep sequencing results, the relative expression of six upregulated miRNA was significantly higher in the CMT+ group than in the normal and CMT− groups (Figure 3-5B). However, miR-2284w had relative low read counts in deep sequencing results (on average 3.4 and 102.6 reads in normal and CMT+ libraries, respectively; Table 3-3) was also hardly detectable with qPCR. Using qPCR, miR-2284w was below the limit of detection (threshold cycle (Ct) value >40) in 78% of normal samples; 46% of CMT+ samples had high Ct values ($35 < Ct < 40$) and 5%

of samples were below the limit of detection. miR-23b-3p expression level had a trend to be downregulated in CMT+ group and significantly downregulated in CMT− group, compared with in the normal group (Figure 3-5B). Detailed CT and normalized relative expression values please refer to Appendix 3-2.

Bovine chromosome 19 was a hotspot containing bovine mastitis-related miRNA

The differentially expressed miRNA in mastitis milk were tended to be upregulated, and RNU2 were also significantly upregulated, we examined the genomic distribution of upregulated miRNA and snRNA precursors separately. Half of the miRNA precursors were located in the chromosome 19 and X (Figure 3-8).The upregulated miRNA precursors were significantly enriched in the chromosome 19 as compared to the background genome distribution of upregulated miRNA precursors in other chromosomes ($p = 0.0061$, Fisher's exact test with Benjamini–Hochberg correction; Table 3-7). Interestingly, the upregulated snRNA precursors were also significantly enriched in the chromosome 19 as compared to the background genome distribution of snRNA precursors in other chromosomes ($p=0.003$, Fisher's exact test with Benjamini–Hochberg correction; Table 3-8 and Figure 3-9), and significantly upregulated RNU2 were significantly enriched in the chromosome 19 ($p = 0.0081$, Fisher's exact test). These significantly upregulated RNU2 derived small RNA were

located in the tandemly repeated *RNU2* locus in the upstream and close proximity to the *BRCA1* gene [198] (Figure 3-9). We concluded that bovine chromosome 19 was a hotspot containing bovine mastitis-related miRNA.

miRNA-mRNA interaction network, gene ontology and KEGG pathway analysis

To investigate the global biological functions of miRNA, we used the TargetScan 7.1 algorithm [181] to predict mRNA targets of significantly different expressed miRNA. With cumulative weighted context++ score < -0.4, TargetScan algorithm generated 1189 putative target mRNA, 250 of them were immune-related genes which included in the ImmPort comprehensive list of immune related genes [199] (Table 3-9), 18 of immune-related genes co-regulated by more than one miRNA (Table 3-10). The miRNA and immune-related mRNA interaction network are summarized in Figure 3-10.

We used upregulated miRNA targets for gene ontology (GO) and KEGG pathway analysis, because significantly different expressed miRNA were mainly upregulated. The significantly upregulated miRNA had 1092 putative target mRNA genes. GO analysis of these genes in the “Biological Process” and “Molecular Functions” categories were significantly enriched for terms related to various gene binding and expression regulation processes (Table 3-11). Protein modification process was also significantly enriched in the “Biological Process” category (Table 3-11). These terms

were correspond to the common functions of miRNA, including bind to target mRNA transcripts, regulate target gene expression, and down-regulate protein expression by inhibiting target mRNA translation or induce rapid decay of target mRNA [200]. KEGG pathway analysis showed that the predicted mRNA target genes were mainly related to cancer and immune system pathways, including proteoglycans in cancer, microRNAs in cancer, FoxO, PI3K-Akt, ErbB, and MAPK signaling pathways (Table 3-11).

Identification of novel and bovine mastitis related miRNA in bovine milk

Using a miRDeep2 score cut-off point of 5 yielded a signal-to-noise ratio of 8.3 and corresponded to a true positive prediction percentage of 88 ± 3%. A total of 104 novel potential novel miRNA precursor coordinates were predicted, which generated 98 unique mature novel miRNA. Nine of predicted novel miRNA had mean read counts > 50 in either normal or CMT+ groups (Table 3-12 and Figure 3-11). Eighteen of predicted unique novel miRNA were significantly different expressed (FDR < 0.05) in CMT+ group when compared to normal group (Table 3-13), three of them were relative highly expressed novel miRNA (with mean read counts > 50 in CMT+ group)(Figure 3-11). The miRNA precursors chr26_14095, chr26_14097, chr26_14099 and chr26_14101 were located within an adjacent region (<10kb) of the introns of protein coding gene *DMBT1* (Ensembl Gene ID: ENSBTAG00000022715) on chromosome 26

(Figure 3-12). RNA sequence alignment of these novel miRNA precursors showed a highly similarity with 90% (53/59) nucleotides were identical, chr26_14099 and chr26_14101 were perfectly identical sequences (Figure 3-12). The chr26_14095 and chr26_14099_14101 precursors generated the same mature miRNA; the mature novel miRNA chr26_14095, 14097, 14099, 14101 might be a miRNA family, because they had only one nucleotide mismatch (95% (20/21) nucleotides were identical) and shared the same seed region at positions 2-8, suggesting that they were functionally identical miRNA (Figure 3-12). Six genes including *BCL6* were predicted as targets of these miRNA by TargetScan algorithm, suggesting that the ability of these miRNA to regulate immune responses mediated by *BCL6* [201, 202]. These two mature miRNA have not been annotated in miRBase 21 for any species. We further verified the expression of mature chr26_14097, because it was the most significantly different expressed novel miRNA (Empirical analysis of DGE FDR =7.9E − 05), and had been discovered by previous studies (Figure 3-13A and Table 3-12 and Table 3-13) [165, 203, 204]. We used digital PCR methods to improve detection sensitivity for the low expression level of novel miRNA. The expression level of the mature chr26_14097 was significantly higher in the CMT+ group than in the normal group ($p = 0.0006$) (Figure 3-13B). The limit of detection (LoD) [205] of mature chr26_14097 Custom TaqMan® Gene Expression Assay was 3.55 copies/µL in QuantStudio 3D Digital PCR System (Refer

to method section for detail). Ten (71.4%) of normal milk samples (n = 14) expression was below the LoD, whereas all of the CMT+ milk samples (n = 10) expression was above the LoD (Figure 3-13B). These results suggest that the mature chr26_14097 existed in bovine milk and related with bovine mastitis.

Human disease enrichment analysis of mastitis-related miRNA

To identify human diseases that are related to the differentially expressed miRNA in bovine mastitis, 19 of 32 differentially expressed miRNA with human homologues were analyzed using miRWalk 2.0 [187] and Human microRNA Disease Database (HMDD v2.0) [188]. The complete results of the miRWalk 2.0 and HMDD 2.0 are shown in Table 3-14 and Table 3-15, respectively. The mature miRNA analyzed by miRWalk 2.0 were related to 5 human disease terms which can be mainly categorized into cancer, infection and immune related diseases (Figure 3-14A). The miRNA precursors analyzed by HMDD 2.0 were related to 50 human disease terms, 37 terms can be categorized into cancer, infection and immune related diseases (Figure 3-14B). We found these miRNA were annotated to B cell related leukemia, breast and liver cancers in the cancer category, to human immunodeficiency virus (HIV), hepatitis E virus (HEV) and sepsis in the infection category, and to inflammation and hepatitis B in the immune category (Figure 3-14C, refer to "Discussion" section for the disease details). It

suggested that bovine mastitis related miRNA identified in milk were related to cancer, B cell function, and modulate infection and inflammation responses in human.

Discussion

We have shown that miR-92a is suitable for use as a housekeeping gene for analysis of bovine mastitis-related microRNA in milk [159], and miRNA in milk could be used as liquid biopsy biomarkers for bovine mastitis [193] in chapeter 1 and 2. In the present study, we comprehensively profiled genome-wide miRNA expression in bovine mastitis milk. By relative abundance of miRNA and ncRNA, top ten unique miRNA and PCA analysis, we revealed that the small RNA expression profiles of inflammation-affected (CMT+) milk samples were different from that of inflammation-unaffected (normal and CMT−) milk samples, in addition, small RNA expression profiles were similar among normal and CMT− groups, suggesting that small RNA expression was aberrant in inflammation condition.

NGS has been widely used in small RNA transcriptome bovine mastitis related studies, the common experimental designs are artificial bacterial challenge to an *in Vivo* or *in Vitro* model and investigate the different expressed miRNA [105, 162, 164-167, 173, 189]. In contrast to the bacterial challenge experiments, we used the milk samples from naturally infected cattle. Compared to the previous studies, our results were most similar to the transcriptome miRNA profile of bovine mammary glands infected with *Staphylococcus aureus* [167], 44% of significantly different expressed miRNA in our results were shared with this study, including miR-1246, miR-223, miR-142-3p, miR-142-5p, miR-21-3p,

miR-6529, miR-2284aa, miR-2284w, miR-132 and miR-130b are upregulated, miR-23b-3p is downregulated; however, miR-874 is downregulated in this study. miR-10a, miR-146a, miR-146b, miR-221, and miR-223 are associated with regulation of innate immunity and mammary epithelial cell function in tissue challenged with *Streptococcus uberis* [163]. miR-146b, miR-223 and miR-338 are upregulated after *Streptococcus uberis* infection in milk-isolated and blood-isolated monocytes [165]. miR-30 family including miR-30f is significantly decreased; miR-222 is significantly correlated with somatic cell counts and suggests utility of miR-222 as mastitis indicators in the study using the milk samples contain fat and somatic cells (without centrifugation process) [206]. miR-301a can activate NF-κB signaling and has been shown to down-regulated in blood of mastitis cows [207]. miR-2284 and miR-2285 are bovine specific families without homologs in human or mouse [208]. Although the family members have been discovered in different bovine organs including liver, rumen, jejunum [209, 210] mammary gland [167, 210], milk components [166, 211] and serum [212], their precise functions still remain largely unknown.

To the best of our knowledge, this is the first study to describe miR-147 and miR-505 were related to bovine mastitis. A previous study demonstrates a negative-feedback loop in which toll-like receptor stimulation induces miR-147 to prevent excessive inflammatory responses [213]. miR-505 is a tumor suppressive miRNA and inhibits

human mammary carcinoma cell proliferation by inducing apoptosis [214]. miR-505 also express in basal human and mouse mammary tumors [215], and significantly overexpress in plasma of patients with breast cancer and decrease after treatment [216].

We found that upregulated bovine mastitis-related miRNA were enriched on chromosome 19. Bovine chromosome 19 is highly conserved with human chromosome 17 [217-219]. Some of well-known human breast cancer related oncogenes and tumor suppressor genes such as *HER2*, *p53* and *BRCA1* are located on chromosome 17 [220]. Molecular genetic abnormality events of human chromosome 17 including monosomy or polysomy can be observed in human breast cancers [220], chromosome 17 and the locus for *HER2* are the most frequently involved in chromothripsis-like patterns in human breast cancer [221]. *BRCA1* mutations associate with worse overall survival in women with breast cancer [222]. *BRCA1* also effects on bovine mastitis resistance [223]. miR-1246 has been found to be upregulated in response to *Staphylococcus aureus* infection of bovine mammary gland and milk exosomes [166, 167]. However, miR-1246 is likely a pseudo miRNA derived from U2 snRNA fragments being the result of false mapping in human [196]. We found that total read counts of canonical and isomiR of RNU2 derived small RNA were much abundant than that of miR-1246 in bovine milk, and the read count distribution of RNU2 isomiR was also similar to the true miRNA. In 5' end isomiR analysis, the sequence read counts of RNU2 derived small RNA with deletion

in positions 1-3 (sequence: ***<u>AAA</u>***TGGATTTTTGGAGCAGGGA to TGGATTTTTGGAGCAGGGA) and miR-1246 without 5' end adenine nucleotides decreased substantially (Figure 3-7). It suggested that the "non-functional small RNA" sequences which without canonical offset 6-mer site match to position 3-8 were almost not expressed in milk. A General rules for functional miRNA targeting study has shown that third nucleotide position is the margin of matching starting point; miRNA-mRNA interactions without any matches at positions 1-3 has not been discovered [224]. The sequence read counts of RNU2 with one nucleotide, and miR-1246 with two nucleotides addition at the 5' end were also decreased substantially (Figure 3-7); the read counts of sequences started with thymine decreased substantially, suggesting that adenines in positions 1-3 may played an important role in RNU2 mature small RNA biogenesis processing (Figure 3-7). The patterns of miRNA processing suggesting that RNU2 derived small RNA may have biologically relevant roles instead of degradation fragments or sequencing error. Our isomiR analysis results were also corresponded to the results of RNU2 derived small RNA in human lung tissue as well as in serum from lung cancer patients [225]. miR-1246 (RNU2 derived small RNA) expression in ductal lavages originate from donors that had epithelial atypia and atypical ductal hyperplasia on biopsy is higher than the milk from normal donors, suggesting that miR-1246 concentrations are indicative of the presence of abnormal cells in the mammary gland

[226], we infer that RNU2 derived small RNA are associated with the pathogenic mechanisms in mammary epithelial cells, such as tumor or inflammation. RNU2 derived small RNA were significantly upregulated in mastitis milk and enriched in chromosome 19, the tandemly repeated *RNU2* locus close proximity to a major cancer susceptibility gene *BRCA1*. Copy number variations (CNV) could influence the expression of genes in their vicinity and extends up to half a megabase [227], thus the proximity of *RNU2* CNV repeat unit to the *BRCA1* raise a possibility that it could be involved in human breast cancer [198] and bovine mastitis [223] susceptibility. Based on the previous studies and our results, we inferred that human chromosome 17 and bovine chromosome 19 play an important role in breast-related diseases including breast cancer and mastitis.

Eighteen of putative unique novel miRNA were bovine mastitis related miRNA; however, only three of them fulfilled the expression criterion of mean read counts in either normal or CMT+ groups > 50. We further investigated the mature novel miRNA designated as chr26_14097 by miRDeep2 in this study. Chr26_14097 has been discovered in different bovine specimens of previous studies, including milk-isolated monocytes of the cows infected *in vivo* with *Streptococcus uberis* via the teat canal [165], alveolar macrophage response to *Mycobacterium bovis* infection [203], *Onchocerca ochengi* (a filarial parasite of cattle) infected cow (*Bos indicus*) plasma [228], whole blood

[229] and gut tissues of calves (30 min after delivery, 7, 21, and 42-day-old) [230], suggesting that chr26_14097 is present in cells of the immune system and may regulates immune functions. Indeed, chr26_14097 was significantly upregulated in the CMT+ group in our sequencing data and verified by digital PCR. Among these previous studies, chr26_14095, 14097, 14099, and 14101 miRNA family has been discovered in alveolar macrophage, whole blood and gut tissues of calves [203, 229, 230]. While it was predicted to regulate expression of *BCL6*, a transcription factor essential for T-follicular helper cell differentiation and regulates inflammatory signaling in macrophages [201, 202], further investigation is warranted to elucidate the biological functions of these novel miRNA in bovine immunity.

Differentially expressed miRNA in mastitis milk were mainly related with cancer, infection and immune system in KEGG pathway, and human related disease enrichment analysis. We reasoned that cancer has complex relationship with nutrition, immunological and inflammatory reactions and pathogen infection [231-233]. Inflammation and cancer share the similar pathways of cell survival, proliferation and angiogenesis. Various types of inflammatory cells are infiltrated to cancer tissues, and inflammation impacts every single step of tumorigenesis, from initiation through promotion, malignant conversion, invasion, and metastasis [232, 233]. In the cancer category of human disease enrichment analysis, mastitis-related miRNA were enriched

in B cell related leukemia and breast neoplasms. Bovine leukemia virus (BLV) is prevalent and causes B-cell leukemia/lymphoma in cattle globally. Recent studies have suggested that BLV DNA was present in the breast tissues from the US and Australian women with breast cancer [234-236], and raise a concern that BLV may be a risk factor for human breast cancer [237-239]. BLV miRNA mediate the expression of genes involved in cell cancer and immunity, essential to induce B-cell tumors and promote efficient viral replication [240], BLV miRNA B4 share identical seed sequences with human miR-29, suggesting that both viral and human miRNA has a role in orchestrating zoonotic-induced oncogenesis [240]. The cows repeated immunization with immunogens that antigenically mimic the HIV envelope glycoprotein elicit neutralizing antibodies to HIV in colostrum [241, 242] and serum [243]. Interestingly, these categories contained liver diseases (hepatocellular carcinoma in the cancer category, HEV in the infection category, and hepatitis B in the immune category). Gene expression profiling of liver tissue from dairy cows treated intra-mammary with *Escherichia coli* (*E. coli*) or lipopolysaccharide LPS verify that liver plays a major role in the acute phase response of *E. coli* mastitis [244-247]; hepatic failure can be caused by mastitis or metritis [248], suggesting a potential of crosstalk between liver and mastitic mammary gland. HEV is the causative agent of hepatitis E, and anti-HEV antibodies can be detected in humans, domestic and wild animals [249]. As a food borne disease, a recent study discovers that

infectious HEV can excrete into milk, and HEV contaminated cow milk is a new zoonotic source that has a high risk of human transmission [250]. The immunologic function and importance of the homologues miRNA in zoonoses and the disease characteristics either same or similar between humans and animals deserve further intensive study.

In summary, we identified 25 different expressed miRNA and RNU2 derived small RNA in the CMT+ milk, these different expressed small RNA were enriched on bovine chromosome 19. The different expressed miRNA were primarily correlated with cancer, infection and immune system, and may contribute to mastitis through the regulation of gene expression. Our study provides genome-wide views of miRNA profiles in bovine mastitis milk, and a potential to study the roles of miRNA in inflammatory diseases. With mastitis related novel miRNA, we also provide several miRNA as potential bovine mastitis biomarkers.

Materials and Methods

Milk sample preparation

Milk samples were prepared as chapter 1 described. All of the milk samples were taken from milking Holstein-Friesian cows. The cows were kept in free-stall barn or tie-stall and pasture without grazing systems; milked twice a day. The animals were fed twice daily, and water was available ad libitum. Milk samples (approximately 5–10 ml) were collected and immediately screened in the field using a modified California Mastitis Test (CMT) with a commercial tester ("PL Tester", Nippon Zenyaku Kogyo) as previously described [112]. Cows with no CMT+ result for any quarter were defined as the normal group; cows with a CMT+ result for at least one quarter were defined as the mastitis-affected group. On the basis of the CMT results, each quarter of the mastitis-affected group was defined as CMT− or CMT+ as appropriate. Reducing sampling and quarter bias, the samples were collected from the farms of four different locations in Japan (Kagoshima, Miyazaki, Hiroshima and Hokkaido prefectures). The samples were stored at 4 °C after collection and transported to the laboratory, then centrifuged at 3000 × g for 15 min at room temperature to remove cell debris and fat. The supernatant was recovered and further centrifuged at 15000 × g for 15 min at 4°C. The milk whey was recovered and stored at −80 °C for RNA extraction.

RNA isolation, Library construction and Small RNA sequencing

Total RNA was extracted from milk whey using a mirVana PARIS kit (Thermo Fisher Scientific) according to the manufacturer's protocol. Small RNA library preparation and sequencing was performed by Hokkaido System Science Co., Ltd. Concentration of total RNA was measured using the NanoDrop Spectrophotmeter (Thermo Fisher Scientific)(Appendix 3-1), and quality of RNA was assessed using the Small RNA kit in combination with the 2100 Bioanalyzer System (Agilent) (Appendix 3-1). Small RNA libraries were constructed by 1 μg of total RNA using the TruSeq Small RNA Library Preparation Kit (Illumina) following the manufacturer's protocol. The libraries were subjected to 100 bp paired end sequencing on an Illumina HiSeq 2500 system (Illumina). All miRNAseq files were submitted to the sequence read archive (SRA) with the BioProject number: PRJNA421075, and SRA Study number: SRP126134.

Small RNA sequencing data processing

CLC Genomics Workbench 10.1.1 (CLC bio) was used to processing and analysis small RNA sequencing. To investigate the overlapping rate of paired end reads, we performed two-step trimming processes. In the first trimming step, low quality sequences (Trim using quality scores limit = 0.05), ambiguous nucleotides (maximal

number of ambiguous = 2), and 3’ adaptor sequences of forward and reverse strands were removed using trim sequences tool (Action frame: discard when not found. Alignment scores costs frame: mismatch cost = 2; gap cost = 3. Match thresholds frame: Allowing internal matches with a minimum score = 10; Allowing end match with a minimum score = 4), the sequence length shorter than 15 or longer than 29 nucleotides were discarded. The overlapping paired forward and reverse reads were merged using merge overlapping pairs tool (Alignment scores: mismatch cost = 2; gap cost = 3; max unaligned end mismatches = 0; Minimum score = 8). In the second trimming step, stop solution and 5’ adaptor sequences were removed using trim sequences tool with “discard when found” action, low quality sequences and ambiguous nucleotides trimming parameters were same as the first trimming step. The clean reads were processed using “extract and count” tool and annotated to miRBase (release 21) [191], Ensembl cow ncRNA database (release 85) [192], and novel miRNA precursors predicted by miRDeep2 algorithm (Refer to novel miRNA prediction section) [184] with the default parameters (additional upstream bases, additional downstream bases, missing upstream bases, missing downstream bases and maximum mismatches = 2 with strand specific alignment) using “Annotating and merging small RNA samples” tool, miRBase was prioritized over the additional annotation resource.

Novel miRNA prediction

Clean reads were annotated to miRBase (release 21) [191] and Ensembl cow ncRNA database (release 85) [192] using the same parameters described in "Small RNA sequencing data processing and analysis" section. The unannotated small RNA reads were pooled and used the miRDeep2 algorithm (version 2.0.0.8) to predict potential novel miRNA [184] with the default parameters. The targets of mature novel miRNA were predicted by TargetScan 5.2 custom algorithm.

Differential expression analysis of small RNA

The differentially expressed small RNA between normal and CMT+ groups were identified by Empirical analysis of DGE tool [251] in CLC Genomics Workbench 10.1.1 (CLC bio) using raw small RNA counts with the default parameters (Total count filter cut-off = 5.0 with estimate tag-wise dispersions). The sequences annotated to known mature miRNA and ncRNA/novel miRNA precursors without known miRNA were analyzed separately. Differences were considered to be significant at $FDR < 0.05$, and mean read counts in either normal or CMT+ groups > 50.

Quantification of miRNA by qPCR

qPCR was performed as chapter 1 described. Equal volumes of RNA (1.25

microliter) were reverse transcribed to cDNA using TaqMan MicroRNA Assays (Thermo Fisher Scientific) according to the manufacturer's protocol. qPCR was performed using a TaqMan Fast Advanced Master Mix kit and a StepOne Plus Real Time PCR system (Thermo Fisher Scientific). Thermal cycling was conducted according to the manufacturer's recommended protocol, and all experiments were performed in duplicate. miR-92a was used as an internal control and expression level was determined using the $2^{-\triangle\triangle CT}$ method. qPCR reactions of undetermined CT were assigned CT = 40. The TaqMan MicroRNA Assays used in qPCR of this study and their IDs are as follows: miR-15a (ID: 005892_mat), miR-23b-3p (ID: 245306_mat), miR-142-5p (ID: 000465), miR-92a (ID: 000431), miR-221 (ID: 001134), miR-2284w (ID: 465051_mat), miR-6529a (ID: 476810_mat); the Custom TaqMan® Gene Expression Assay RNU2 sequence: AAATGGATTTTTGGAGCAGGGA.

Target gene prediction, Gene Ontology (GO) term and KEGG pathway analysis

The TargetScan 7.1 was used to predict the gene targets of different expressed miRNA [181]. The genes with a cumulative weighted context++ score < −0.4 were selected as putative targets. GO term and KEGG pathway enrichment analysis for the predicted gene targets was performed using the Annotation, Visualization and Integrated Discovery (DAVID) v6.8 [252, 253]. The GOTERM_BP_FAT and GOTERM_MF_FAT parameters were used for biological process and molecular

function terms analysis, respectively.

miRNA and immune related genes interaction network analysis

The immune related genes were defined by the target mRNA which cross-referenced with ImmPort comprehensive list of immune-related genes [199]. The miRNA and immune related genes interaction network was visualized using Cytoscape (version 3.4.0) [254].

Quantification of novel miRNA by digital PCR

Digital PCR was performed as previously described [193]. In brief, using the QuantStudio 3D Digital PCR System (Thermo Fisher Scientific) according to the manufacturer's protocol. 3 μL cDNA of were combined with QuantStudio 3D Digital PCR Master Mix and TaqMan Assay. The samples were loaded onto chips using QuantStudio 3D Digital PCR Chip Loader (Thermo Fisher Scientific). The manufacturer's recommended digital PCR thermal cycling protocol was used. After PCR, the fluorescence data from the chips were collected using a QuantStudio 3D Digital PCR Instrument. The Custom TaqMan® Gene Expression Assay chr26_14097 sequence: CCGAGCCUGACAGAUCACACA. The limit of detection (LoD) of assay was established according to previous study: limit of blank (LoB) is estimated by

measuring replicates of a blank sample and calculating the mean result and the standard deviation (SD) [205].

$$\text{LoB} = \text{mean}_{\text{blank}} + 1.645\ (\text{SD}_{\text{blank}})$$

The RNA solvent Elution Solution provided by mirVana PARIS kit (Thermo Fisher Scientific) was used as a blank sample and five replicates were measured. 0.692, 1.274, 1.799, 0.835, and 2.186 copies/μL were generated, LoB was 2.40 copies/μL in which mean and standard deviation of blank were 1.357 and 0.6333 copies/μL, respectively.

LoD is determined by LoB and test replicates of a sample known to contain a low concentration of analyte. The mean and SD of the low concentration sample is then calculated [205].

$$\text{LoD} = \text{LoB} + 1.645\ (\text{SD}_{\text{low concentration sample}})$$

The sample known to contain a low concentration of analyte was selected from a normal milk sample which concentration was measured by a pilot screening study. Five replicates were measured and generated 1.067, 2.323, 2.041, 0.776, and 2.151

copies/μL, LoD was 3.55 copies/μL in which standard deviation of low concentration sample was 0.6997 copies/μL.

Human disease enrichment analysis

miRWalk 2.0 [187] and Human microRNA Disease Database (HMDD v2.0) [188] were used to identify human diseases that are related to the differentially expressed miRNA using miRNA Enrichment Analysis and Annotation tool (miEAA) [255] over-representation analysis with threshold level = 2. FDR adjustment was used for p-value calculations, a significant enrichment was considered to be at FDR < 0.05.

Statistics

Data analysis was performed using GraphPad Prism 7 (GraphPad Software Inc.). qPCR data were compared using an unpaired, two-tailed Mann-Whitney test, or one-way ANOVA followed by Tukey's test where appropriate. Genomic location enrichment analysis data were compared using two-tailed Fisher's exact test with or without Benjamini–Hochberg correction where appropriate, differences were considered to be significant at P or Benjamini–Hochberg corrected P value < 0.05.

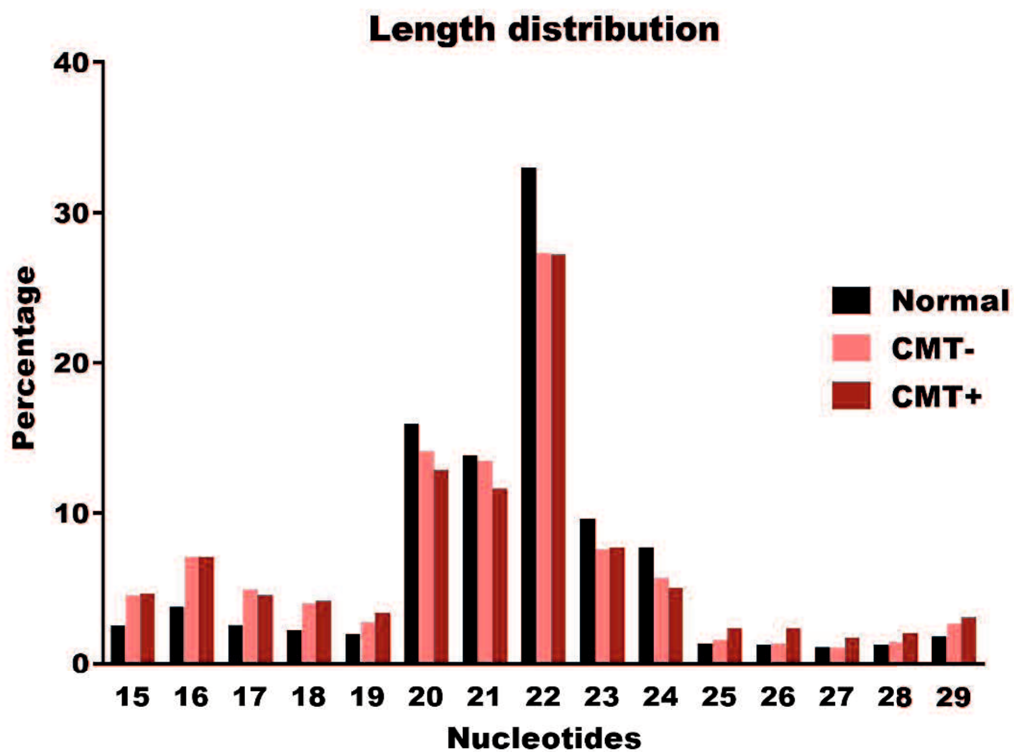
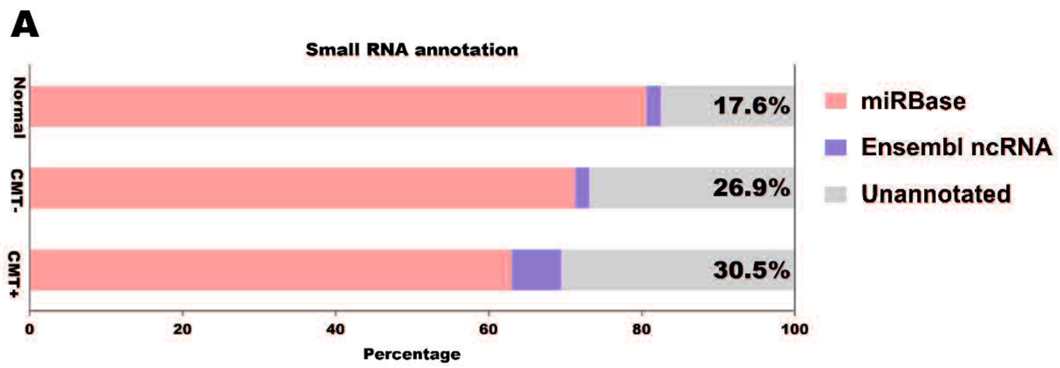


Figure 3-15 Length distribution of clean read count percentage in normal, CMT− and CMT+ libraries.


A
Small RNA annotation
Normal
CMT-
CMT+
17.6%
26.9%
30.5%
miRBase
Ensembl ncRNA
Unannotated
0
20
40
60
80
100
Percentage

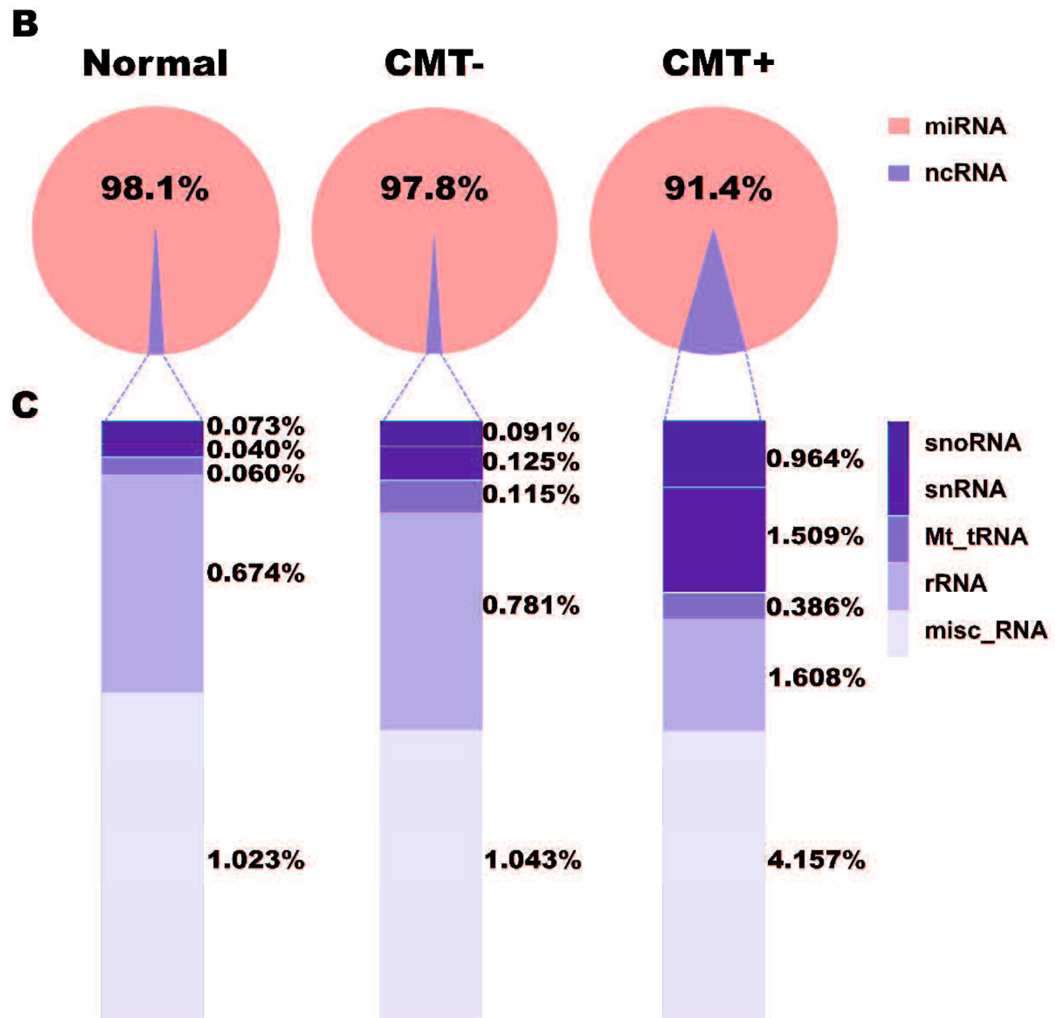

B
Normal
CMT-
CMT+
98.1%
97.8%
91.4%
miRNA
ncRNA
C
0.073%
0.040%
0.060%
0.674%
1.023%
0.091%
0.125%
0.115%
0.781%
1.043%
0.964%
1.509%
0.386%
1.608%
4.157%
snoRNA
snRNA
Mt_tRNA
rRNA
misc_RNA

Figure 3-16 Small RNA annotation analysis. **(A)** Portions of the annotated and unannotated small RNA clean reads. The number in bar graphs indicates percentage of unannotated reads. **(B)** Relative abundance of miRNA and ncRNA in normal, CMT− and CMT+ groups. The number in pie charts indicates percentage of miRNA. **(C)** Relative abundance of different ncRNA classes except miRNA in normal, CMT− and CMT+ groups. The numbers indicate percentage of ncRNA in figure 2B. snoRNA: small nucleolar RNA, snRNA: small nuclear RNA, Mt_tRNA: transfer RNA located in the mitochondrial genome, rRNA: ribosomal RNA, miscRNA: miscellaneous other RNA.

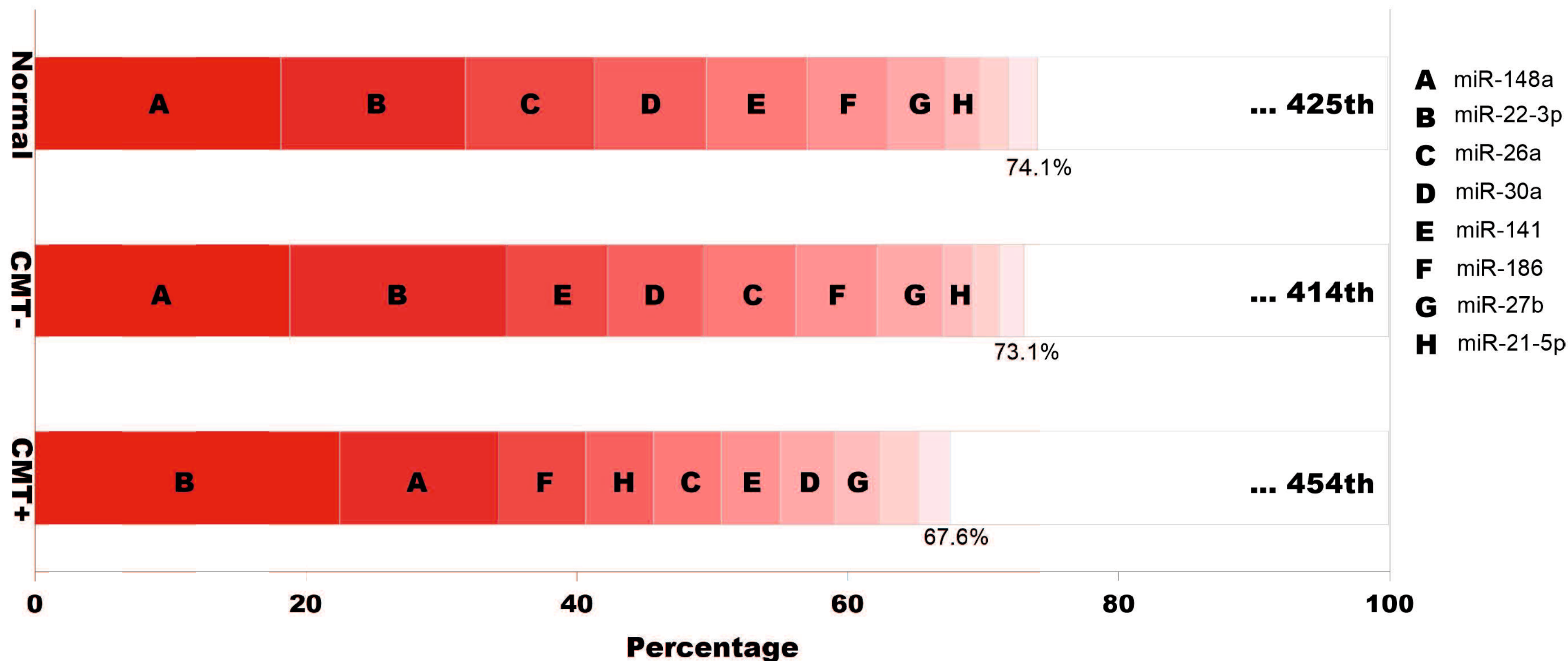


Figure 3-17 Relative abundance of unique miRNA in normal, CMT− and CMT+ libraries. The cells filled up with red color represent top ten unique miRNA. The number under each bar graph indicates accumulative percentage of the top ten unique miRNA in total read counts of all unique miRNA. The numbers in the white cells indicate total unique miRNA numbers. The top eight miRNA were overlapped among three groups and labeled with English alphabet symbols.

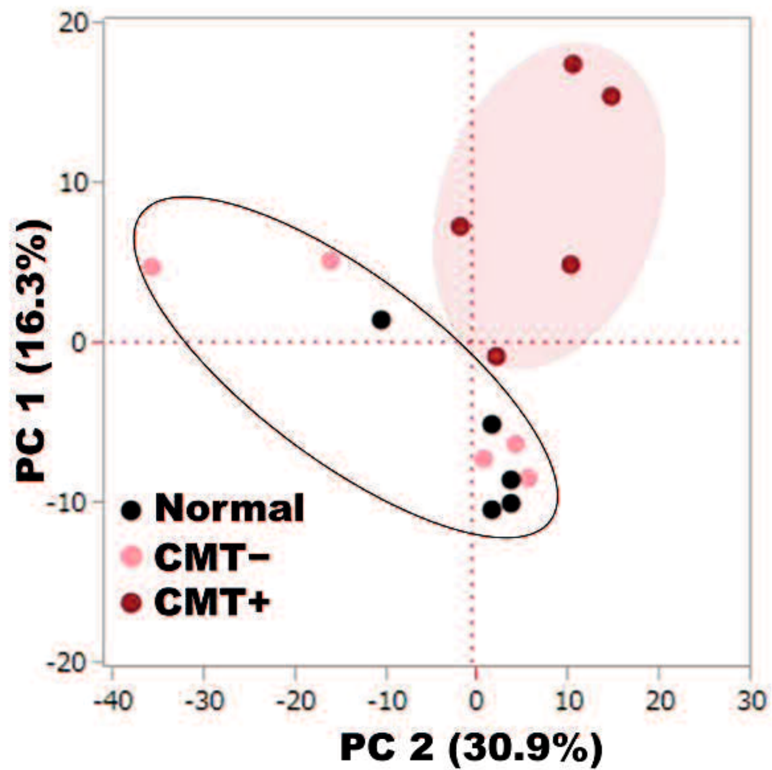
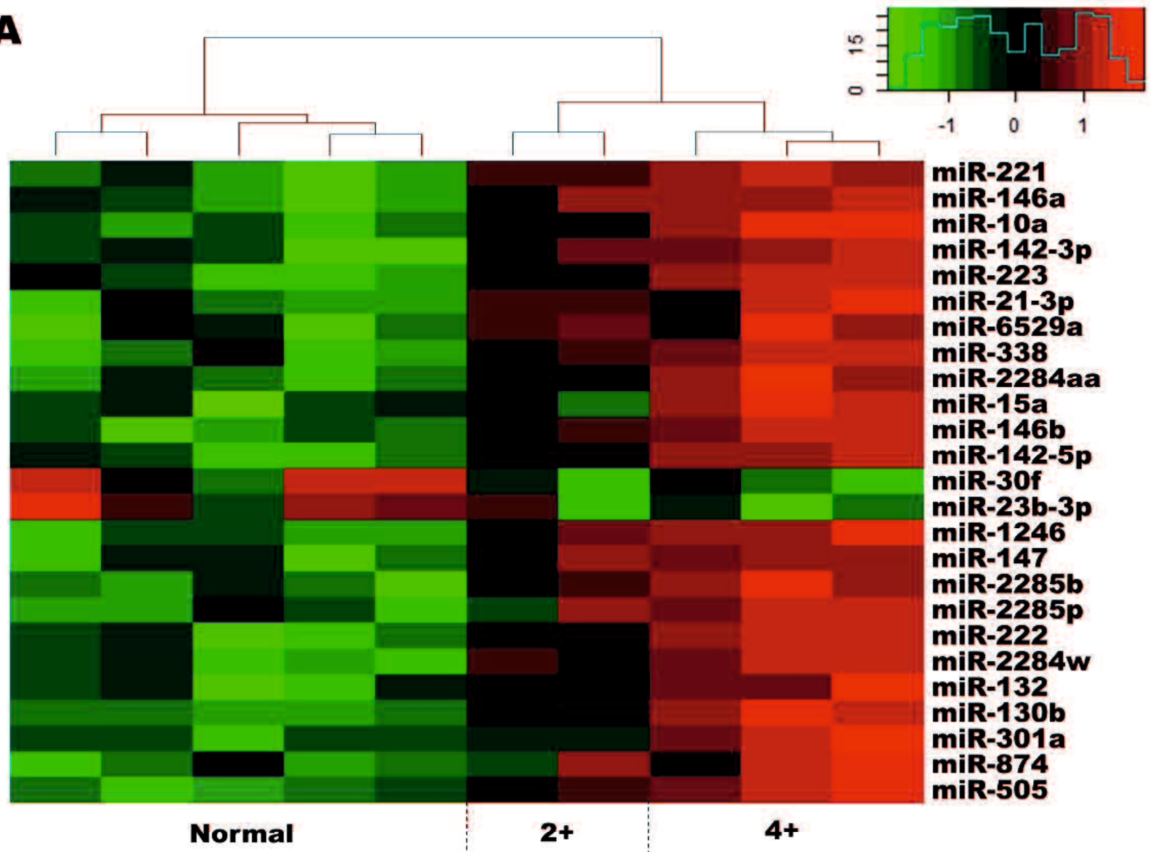


Figure 3-18 Principal component analysis (PCA) of normal, CMT− and CMT+ libraries. The mapped miRNA read counts were normalized to reads per million mapped (RPMM) and log (RPMM + 1) transformed before PCA. The pink ellipse indicates distribution area of CMT+ libraries, and the ellipse with black border indicates distribution area of normal and CMT− libraries.


A
15
0
-1
0
1
miR-221
miR-146a
miR-10a
miR-142-3p
miR-223
miR-21-3p
miR-6529a
miR-338
miR-2284aa
miR-15a
miR-146b
miR-142-5p
miR-30f
miR-23b-3p
miR-1246
miR-147
miR-2285b
miR-2285p
miR-222
miR-2284w
miR-132
miR-130b
miR-301a
miR-874
miR-505
Normal
2+
4+

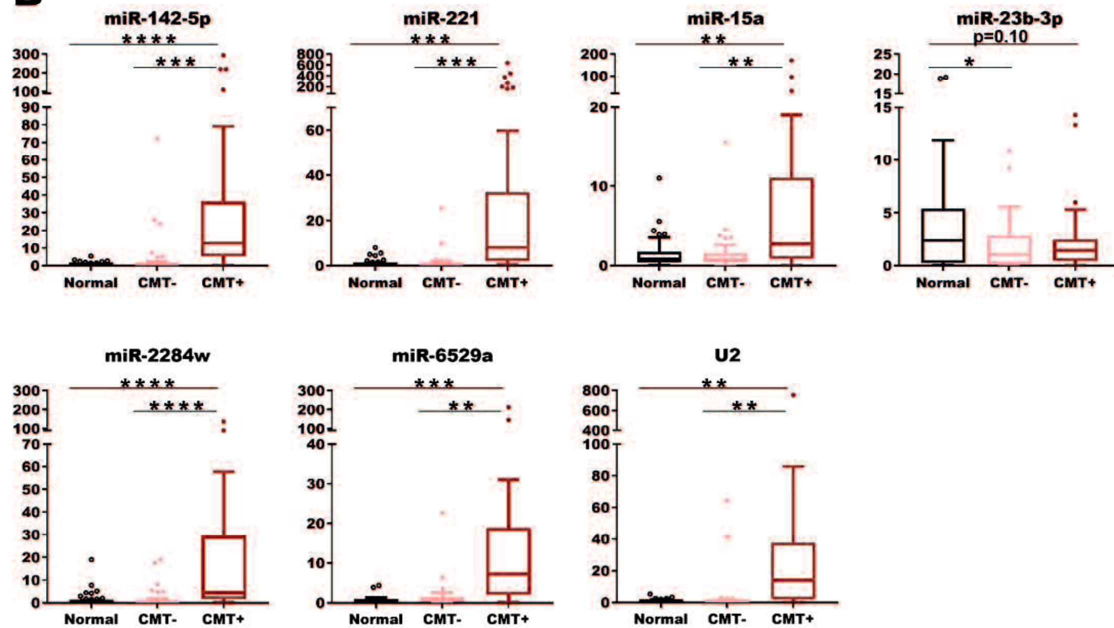

B
miR-142-5p
miR-221
miR-15a
miR-23b-3p
p=0.10
miR-2284w
miR-6529a
U2
Normal
CMT-
CMT+

Figure 3-19 Differentially expressed miRNA analysis. **(A)** Heatmap of the differentially expressed miRNA in mastitis milk based on log (RPMM + 1) transformed values (Table 3-4). Hierarchical clustering revealed the separation of the normal, CMT 2+, and CMT 4+ groups and indicated under the heatmap. **(B)** qRT-PCR relative expression of seven miRNA selected from differentially expressed miRNA of sequencing results. The y-axes represent relative miRNA expression levels normalized to miR-92a (Normal n = 55, CMT− n = 42, and CMT+ n = 41; One-way ANOVA followed by Tukey's test, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$).

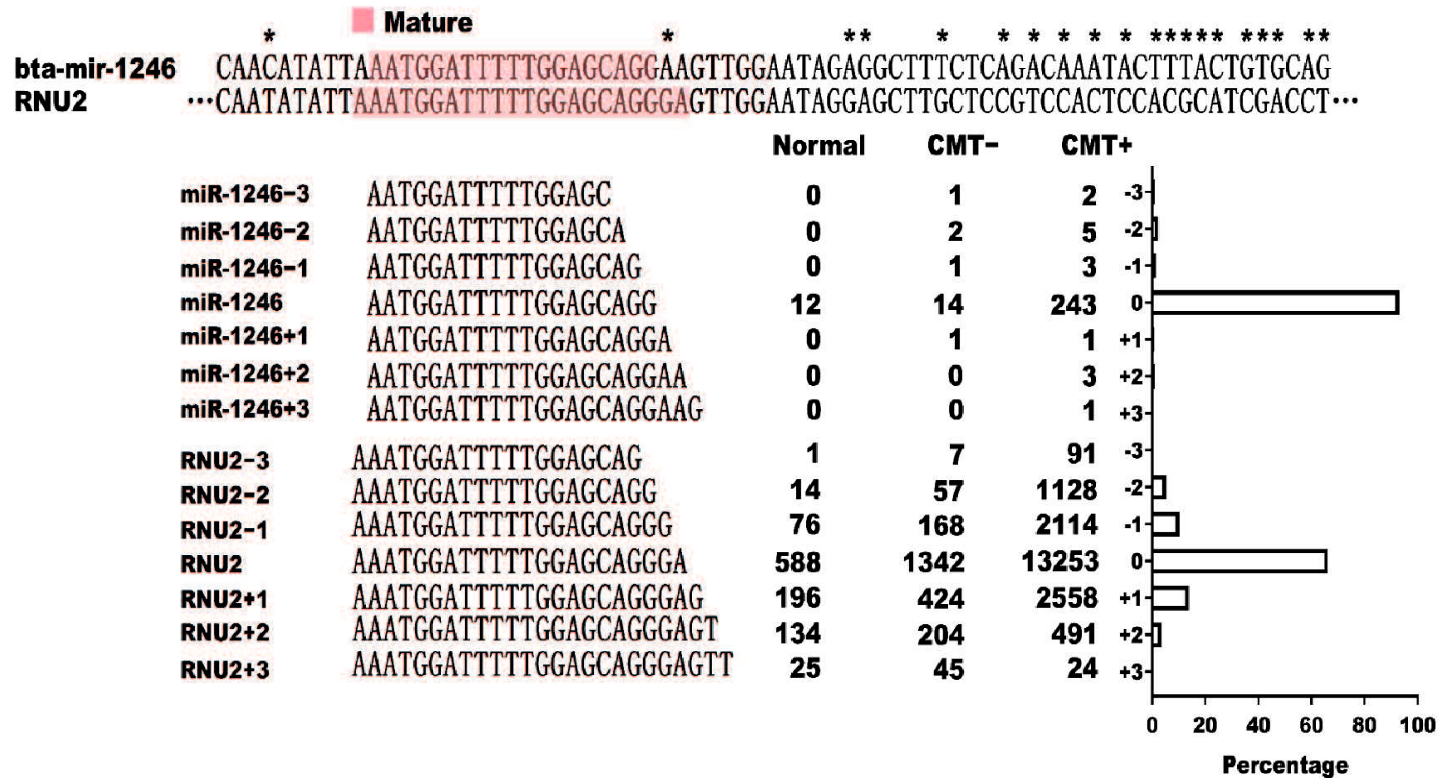


Figure 3-20 miR-1246 and RNU2 3' end variation isomiR in the pooled normal, CMT− and CMT+ libraries. bta-mir-1246 precursor sequence was downloaded from miRBase, RNU2 precursor (ENSBTAT00000063731.1) sequence was downloaded from Ensembl cow non-coding RNA (ncRNA) database. The canonical mature miR-1246 and RNU2 derived small RNA sequences were highlighted with pink. Sequence mismatches between bta-mir-1246 and RNU2 were marked with asterisks. The numbers represent read counts in each group. The bar graph represents the percentage of each isomer in total observed isomiR. −1, −2 and −3 represent 1, 2 and 3 nucleotides deletion from the 3' end; +1, +2 and +3 represent 1, 2 and 3 nucleotides addition from the 3' end, respectively.

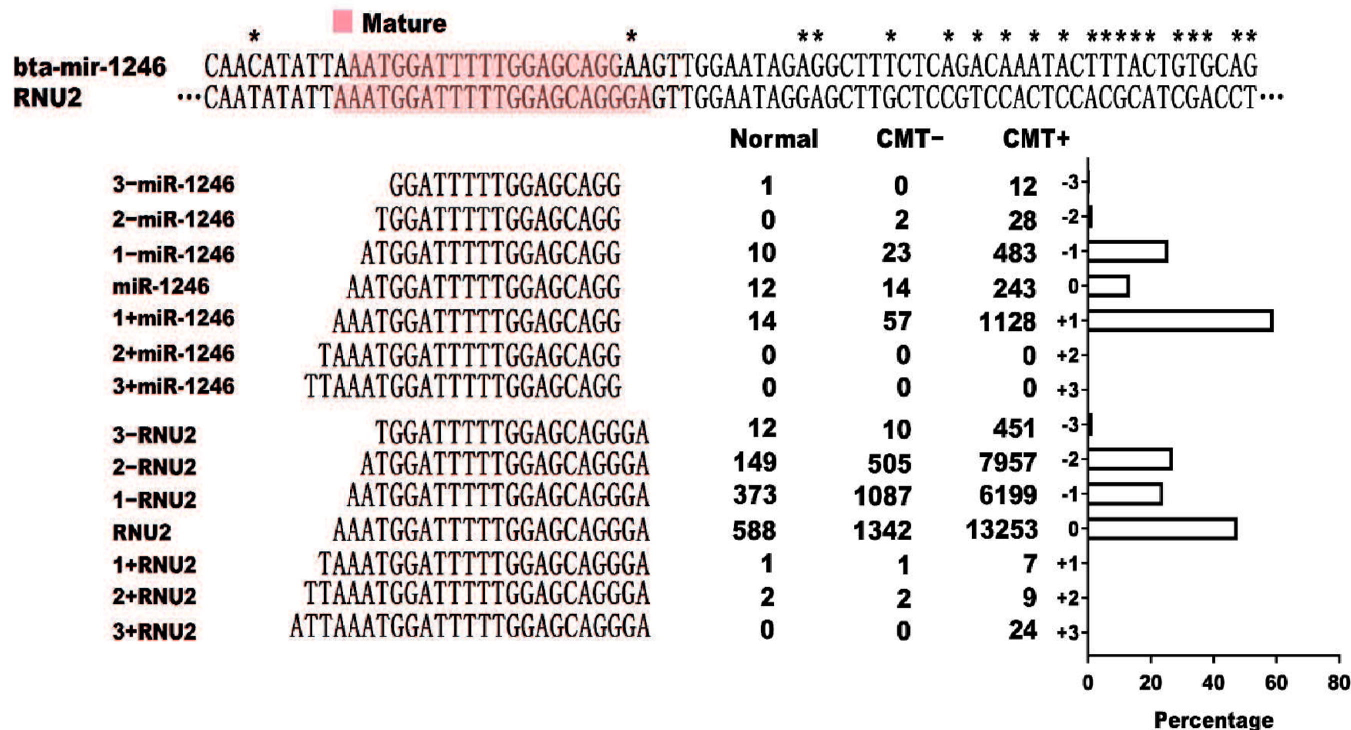


Figure 3-21 miR-1246 and RNU2 5’ end variation isomiR in the pooled normal, CMT− and CMT+ libraries. bta-mir-1246 precursor sequence was downloaded from miRBase, RNU2 precursor (ENSBTAT00000063731.1) sequence was downloaded from Ensembl cow non-coding RNA (ncRNA) database. The canonical mature miR-1246 and RNU2 derived small RNA sequences were highlighted with pink. Sequence mismatches between bta-mir-1246 and RNU2 were marked with asterisks. The numbers represent read counts in each group. −1, −2 and −3 represent 1, 2 and 3 nucleotides deletion from the 5’ end; +1, +2 and +3 represent 1, 2 and 3 nucleotides addition from the 5’ end, respectively. The sequence of 1+miR-1246 was the same as RNU2−2. The read counts of sequences started with thymine decreased substantially, suggesting that adenines in positions 1-3 may played an important role in RNU2 mature small RNA biogenesis processing.

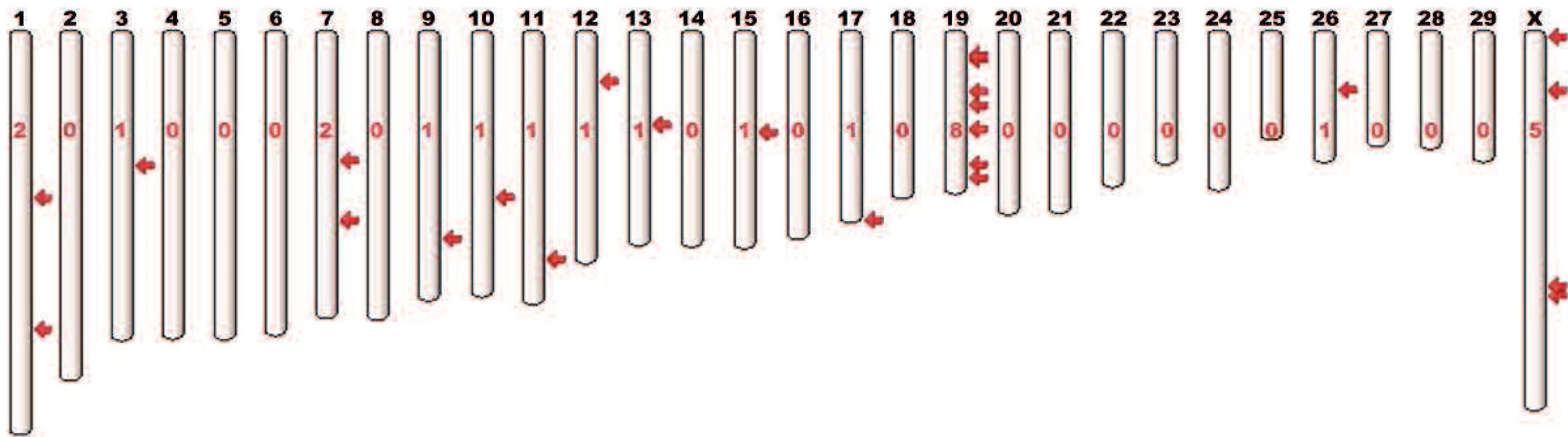


Figure 3-22 Distribution of the significantly upregulated miRNA precursors in cow chromosome ideogram. The characters at the above of chromosome schematic bars indicate the chromosome names. The red numbers in the chromosome schematic bars indicate the amount of significantly upregulated miRNA precursor numbers in each chromosome. Red arrows at the right of the chromosomes indicate the location of upregulated miRNA precursors in chromosomes.

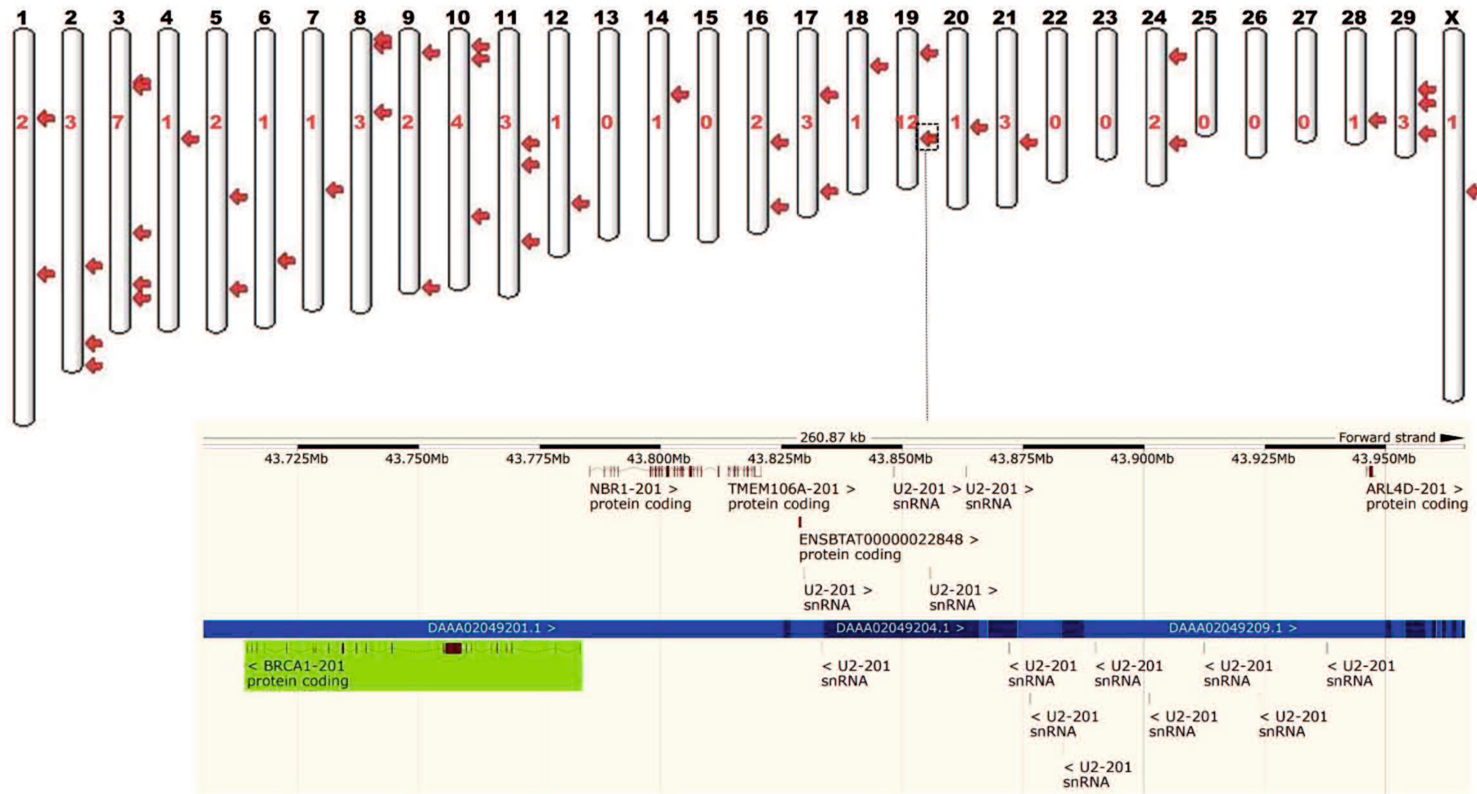


Figure 3-23 Distribution of the significantly upregulated snRNA in cow chromosome ideogram. The characters at the above of chromosome schematic bars indicate the chromosome names. The red numbers in the chromosome schematic bars indicate the amount of significantly upregulated snRNA precursor numbers in each chromosome. Red arrows at the right of the chromosomes indicate the location of upregulated snRNA precursors in chromosomes. The figure at the bottom of the chromosome schematic bars indicates the location of upregulated U2 were located in the tandemly repeated *RNU2* locus as shown in the Ensembl browser. *RNU2* locus was close proximity to the *BRCA1* gene which highlighted with green color.

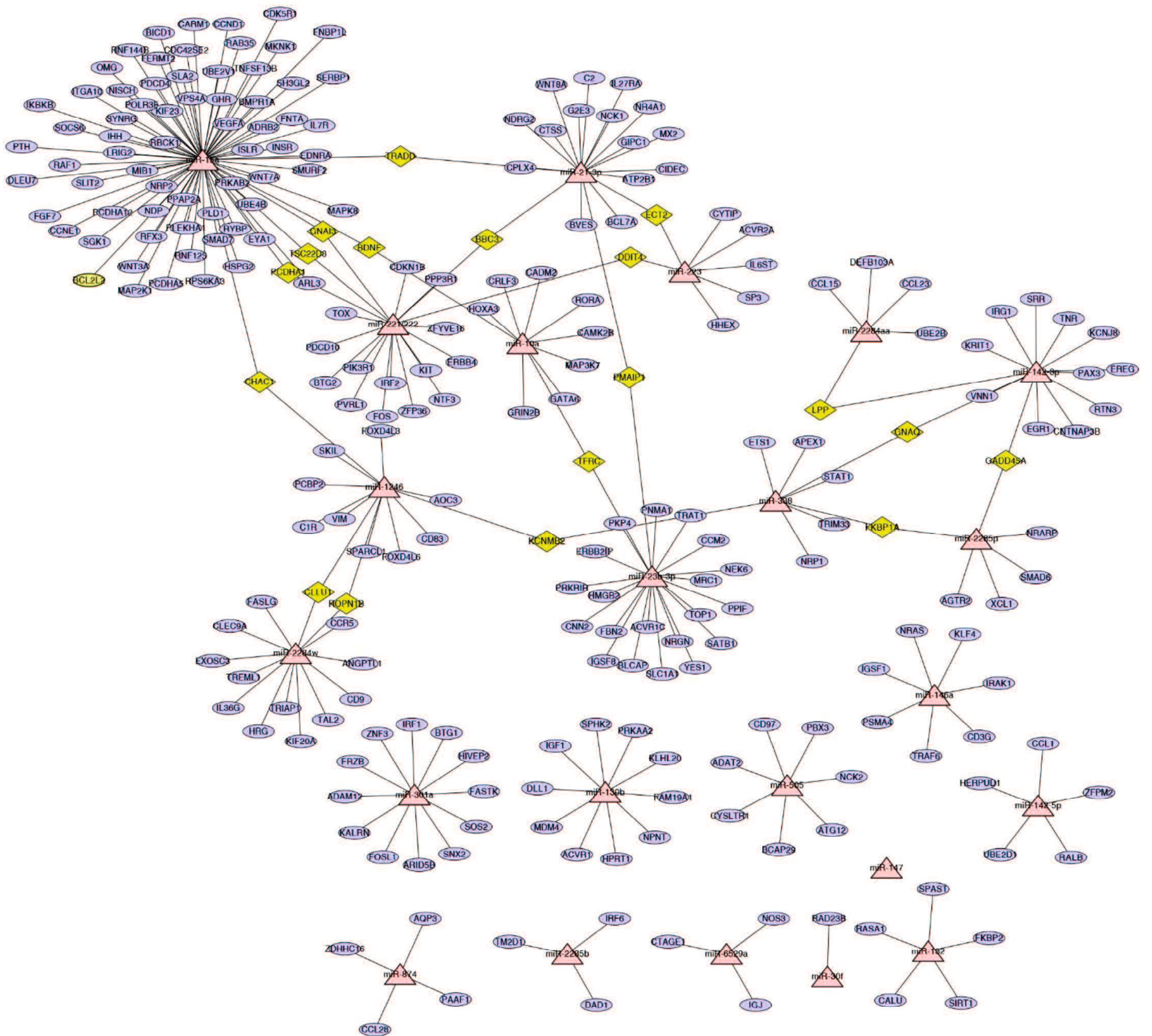


Figure 3-24 Summary of miRNA and immune-related mRNA interaction network. miRNA are shown in triangle, immune-related mRNA are shown in oval. Immune-related mRNA co-regulated by more than one miRNA are shown in diamond and highlighted with yellow.

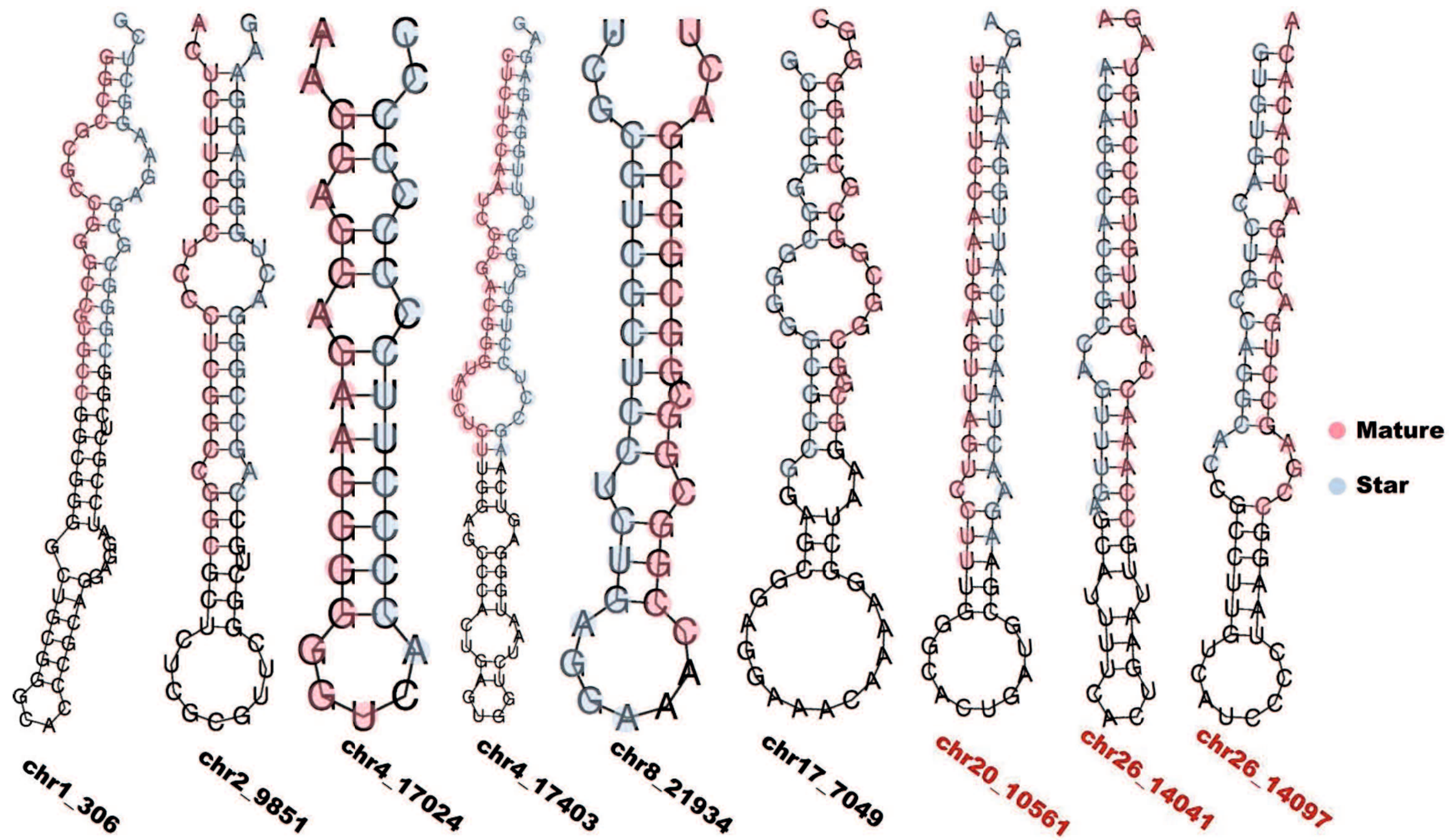


Figure 3-25 Analysis of bovine mastitis related novel miRNA. The secondary structures of nine novel miRNA had mean read counts > 50 in either normal or CMT+ groups. The name of three bovine mastitis related novel miRNA which significantly different expressed (FDR < 0.05) were marked in red. The Sequences were highlighted according to mature (pink) and star (pale blue).

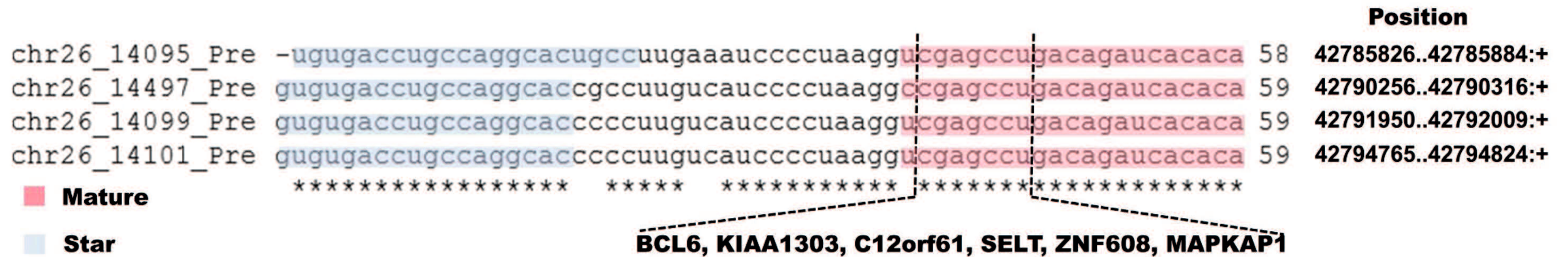


Figure 3-26 Analysis of a bovine mastitis related novel miRNA family. Conservation of four significantly different expressed novel miRNA precursors located on chromosome 26. Asterisk indicates conserved sequence. The sequences were highlighted according to mature (pink) and star (pale blue). The gene names under asterisk line were predicted targets of mature miRNA seed regions.

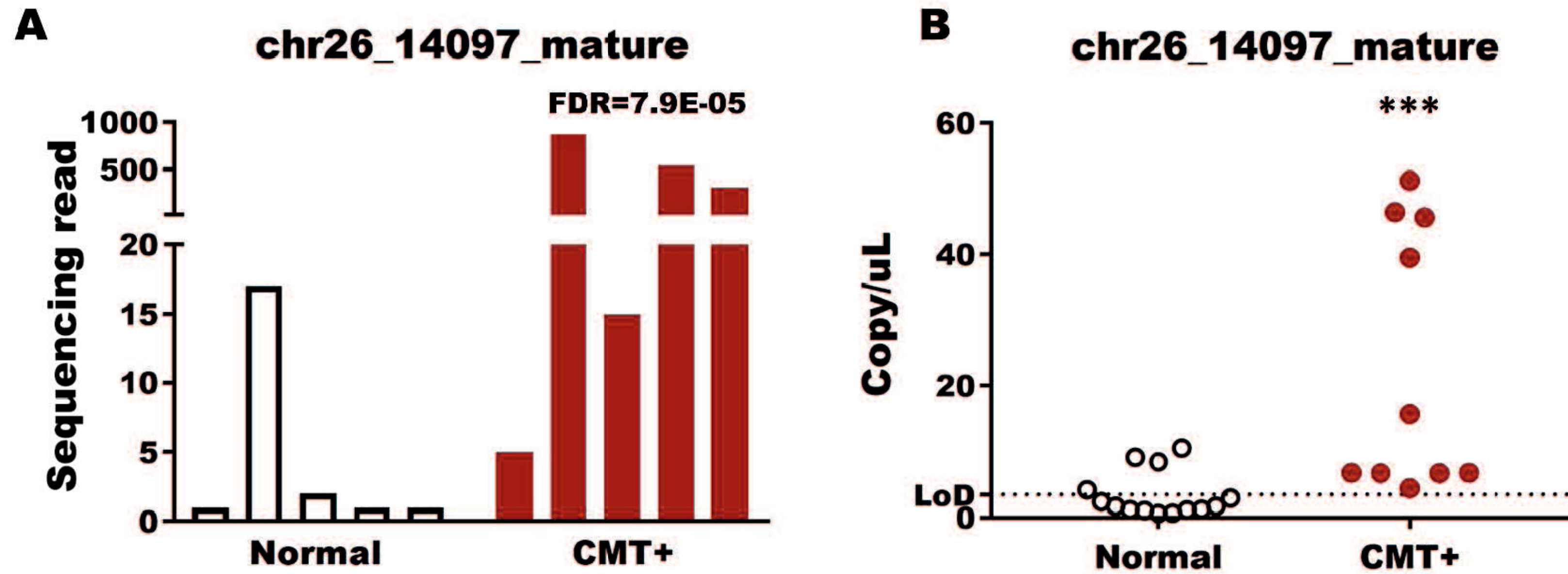
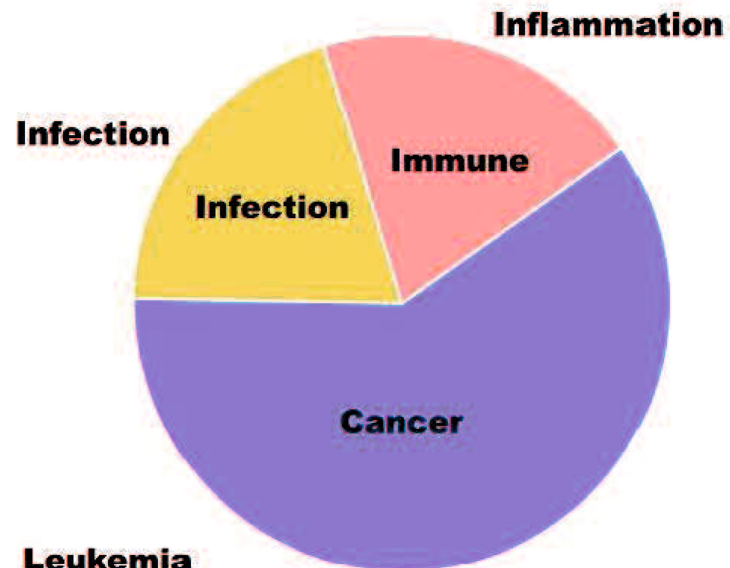


Figure 3-27 Validation of novel miRNA chr26_14097. **(A)** Total sequencing reads of mature chr26_14097 among normal and CMT+ libraries. **(B)** Digital PCR system quantification of expression level of mature chr26_14467 in normal (n = 14) and CMT+ milk (n = 10) samples (LoD: limit of detection; Mann-Whitney test, ***p = 0.0006).

A

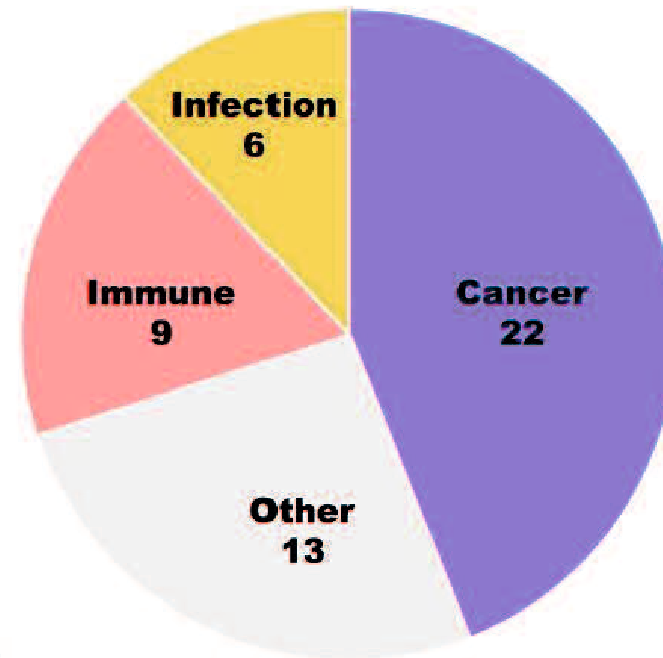
miRWalk 2.0



Leukemia
Carcinoma Non-Small-Cell Lung
Leukemia Myelogenous Chronic BCR-ABL Positive

B

HMDD 2.0



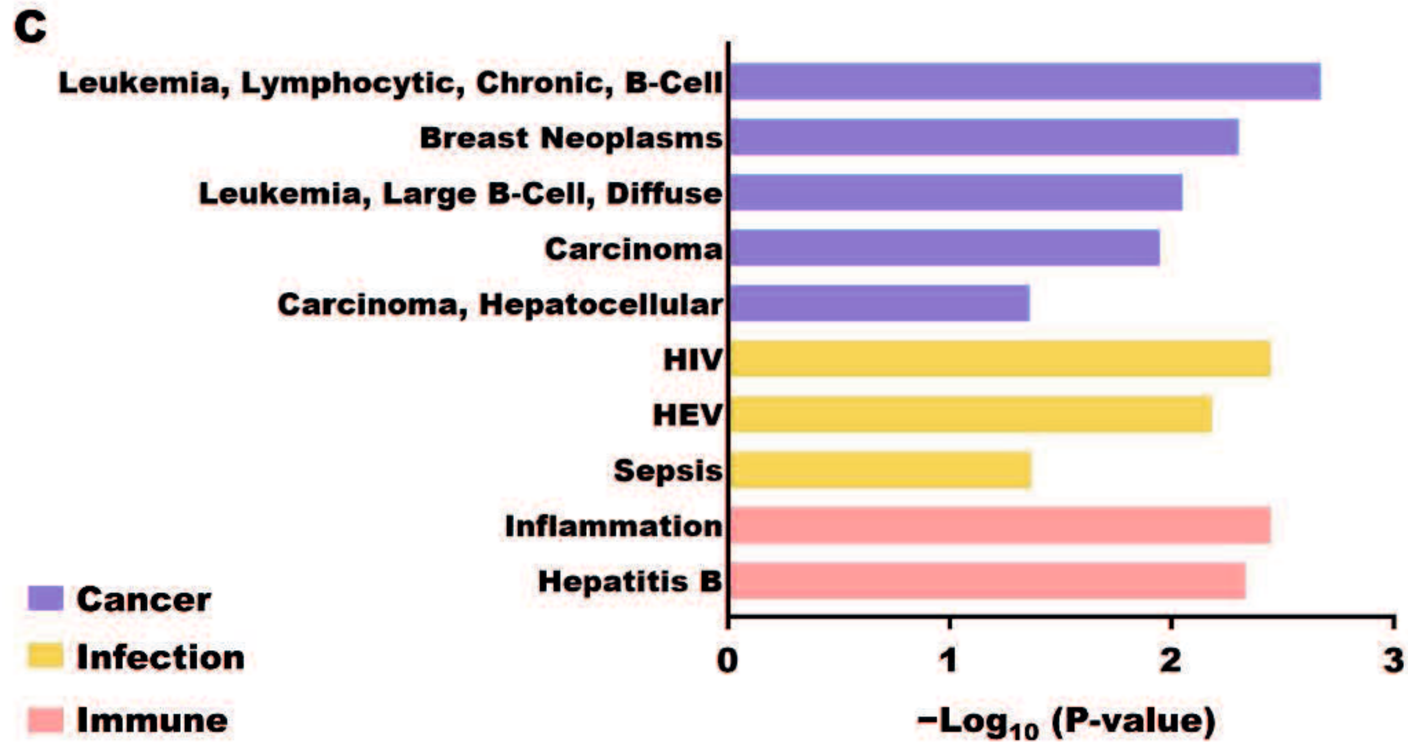


Figure 3-28 Human disease enrichment analysis of differentially expressed bovine mastitis related miRNA. **(A)** Analysis results of miRWalk 2.0. The terms in the pie chart indicate disease categories, and the terms out of the pie chart indicate the annotated diseases by miRWalk 2.0. **(B)** Analysis results of HMDD 2.0. The terms indicate disease categories, and the numbers were the number of diseases annotated to the category by HMDD 2.0. **(C)** Bar plot of the partial diseases from cancer, infection and immune categories of HMDD 2.0 results.

Table 3-3 Summary of two step trimming processes of small RNA sequencing reads from normal, CMT− and CMT+ groups. The first trimming step was aimed to remove low quality sequences, ambiguous nucleotides, 3' adaptor, and short (< 15 nt) or long (> 29 nt) reads, then merge the paired end reads. The second trimming step was aimed to remove the contamination sequences including stop solution and 5' adaptor sequences. The value in brackets indicates the number of discarded reads.

Step 1

	Normal	**CMT−**	**CMT+**
Raw reads	116,102,702	121,131,414	103,228,956
Removal of low quality sequences	6,636,202	6,804,210	5,982,143
Removal of ambiguous nucleotides	17,968 (613)	18,377 (684)	15,955 (564)
Removal of adapter sequences	50,507,034 (65,595,055)	45,103,255 (76,030,475)	47,151,069 (56,077,323)
Keep 15-29 nucleotide reads	31,490,856 (19,016,178)	22,401,262 (22,701,933)	20,025,082 (21,725,987)
Overlappable reads	30,500,582	20,931,114	18,962,722
Paired end overlapping rate	94.2%	93.8%	93.2%

Step 2

Overlapped reads	14,364,499	9,811,910	8,835,714
Removal of low quality sequences	311,908	220,037	206,448
Removal of ambiguous nucleotides	56 (6)	42 (3)	32 (3)
Removal of contamination sequences	(681,791)	(1,075,978)	(728,680)
15-29 nucleotide reads (Clean reads)	13,626,099	8,689,205	8,059,195

Table 3-4 Differentially expressed miRNA in read counts of each library of normal and CMT+ groups. The miRNA with mean read counts < 50 in both groups were ignored. Fold change and FDR were computed by Empirical analysis of DGE based on tagwise dispersions using CLC Genomics Workbench 10. N: Normal; P: CMT+; FC: fold change; Avg: Mean read counts.

miRNA	FC	FDR	N1	N2	N3	N4	N5	Avg	P1	P2	P3	P4	P5	Avg
miR-221	56.971	3.8E-11	87	113	19	14	9	48.4	693	5977	437	3602	4219	2985.6
miR-1246	43.830	1.8E-07	9	6	17	2	6	8	110	1932	25	385	195	529.4
miR-146a	31.316	3.8E-07	64	335	18	12	5	86.8	1309	5037	157	2383	1450	2067.2
miR-10a	38.821	4.8E-07	14	138	28	11	13	40.8	82	4590	63	1181	1914	1566
miR-142-3p	37.520	6.6E-07	34	116	3	1	12	33.2	456	3490	131	1128	1155	1272
miR-142-5p	63.429	6.6E-07	257	1863	96	60	7	456.6	1111	68222	1690	32715	15792	23906
miR-222	26.397	3.3E-06	13	19	3	3	0	7.6	28	630	20	345	182	241
miR-2284w	27.629	5.4E-06	7	9	1	0	0	3.4	14	284	20	93	102	102.6
miR-146b	7.838	1.3E-05	57	980	964	96	47	428.8	810	5044	530	2463	2208	2211
miR-130b	7.305	2.1E-05	27	90	79	15	6	43.4	54	553	58	333	334	266.4
miR-2285b	18.362	2.6E-05	1	10	12	0	4	5.4	26	165	10	110	136	89.4
miR-223	132.236	2.6E-05	13	343	1	2	0	71.8	229	17952	202	4430	7404	6043.4
miR-147	12.536	1.2E-04	19	8	2	3	7	7.8	105	301	31	145	135	143.4
miR-21-3p	5.395	6.0E-04	250	211	285	42	34	164.4	340	2366	293	557	842	879.6
miR-132	10.335	1.4E-03	5	15	3	3	0	5.2	7	226	10	53	21	63.4

miRNA	FC	FDR	N1	N2	N3	N4	N5	Avg	P1	P2	P3	P4	P5	Avg
miR-301a	6.881	2.3E-03	24	93	91	16	3	45.4	23	1014	25	234	182	295.6
miR-505	4.472	3.3E-03	6	38	36	8	3	18.2	25	170	18	66	61	68
miR-2284aa	3.757	4.7E-03	67	152	115	26	17	75.4	64	424	84	370	325	253.4
miR-2285p	7.897	4.7E-03	2	8	19	1	6	7.2	43	107	4	65	53	54.4
miR-874	4.238	2.5E-02	15	38	43	11	16	24.6	87	305	15	82	91	116
miR-338	3.951	2.7E-02	37	107	107	18	66	67	147	739	86	416	340	345.6
miR-30f	-2.924	3.3E-02	1498	9448	10913	1573	359	4758.2	485	1108	761	2082	617	1010.6
miR-15a	3.682	3.6E-02	488	1846	1626	390	38	877.6	225	6151	563	3628	2932	2699.8
miR-23b	-3.117	3.6E-02	495	3667	3104	410	127	1560.6	129	391	316	530	104	294
miR-6529a	2.990	4.2E-02	237	251	227	87	76	175.6	380	925	250	447	664	533.2

Table 3-5 Differentially expressed miRNA in log (RPMM + 1) transformed values of each library of normal and CMT+ groups. N: Normal; P: CMT+.

miRNA	N1	N2	N3	N4	N5	P1	P2	P3	P4	P5
miR-221	2.02	1.54	0.8	1.41	1.43	3.09	3.69	2.91	3.47	3.89
miR-1246	1.07	0.44	0.76	0.66	1.26	2.29	3.2	1.67	2.5	2.56
miR-146a	1.89	2	0.78	1.35	1.19	3.36	3.61	2.46	3.29	3.43
miR-10a	1.25	1.62	0.95	1.31	1.59	2.16	3.57	2.07	2.99	3.55
miR-142-3p	1.62	1.55	0.26	0.44	1.55	2.9	3.45	2.39	2.97	3.33
miR-142-5p	2.49	2.74	1.45	2.03	1.33	3.29	4.74	3.49	4.43	4.46
miR-222	1.22	0.82	0.26	0.8	0	1.7	2.71	1.58	2.45	2.53
miR-2284w	0.97	0.56	0.11	0	0	1.41	2.37	1.58	1.89	2.28
miR-146b	1.84	2.46	2.43	2.23	2.14	3.15	3.61	2.99	3.3	3.61
miR-130b	1.52	1.44	1.36	1.44	1.26	1.98	2.65	2.03	2.44	2.79
miR-2285b	0.34	0.6	0.64	0	1.1	1.67	2.13	1.29	1.96	2.4
miR-223	1.22	2.01	0.11	0.66	0	2.61	4.17	2.57	3.56	4.13
miR-147	1.37	0.53	0.19	0.8	1.33	2.27	2.39	1.77	2.08	2.4
miR-21-3p	2.48	1.8	1.91	1.88	2	2.78	3.29	2.73	2.66	3.19
miR-132	0.84	0.73	0.26	0.8	0	1.12	2.27	1.29	1.65	1.6
miR-301a	1.47	1.45	1.42	1.47	0.99	1.62	2.92	1.67	2.28	2.53
miR-505	0.91	1.09	1.04	1.18	0.99	1.65	2.14	1.53	1.74	2.05
miR-2284aa	1.91	1.66	1.52	1.67	1.7	2.06	2.54	2.19	2.48	2.78
miR-2285p	0.53	0.53	0.8	0.44	1.26	1.88	1.95	0.92	1.73	1.99

miRNA	N1	N2	N3	N4	N5	P1	P2	P3	P4	P5
miR-874	1.28	1.09	1.12	1.31	1.67	2.19	2.4	1.46	1.83	2.23
miR-338	1.65	1.51	1.49	1.52	2.28	2.41	2.78	2.2	2.53	2.8
miR-30f	3.25	3.45	3.49	3.45	3.02	2.93	2.96	3.15	3.23	3.06
miR-15a	2.77	2.74	2.66	2.84	2.05	2.6	3.7	3.02	3.47	3.73
miR-23b	2.77	3.03	2.94	2.86	2.57	2.36	2.5	2.77	2.64	2.28
miR-6529a	2.45	1.88	1.81	2.19	2.34	2.83	2.88	2.67	2.56	3.09

Table 3-6 snRNA derived small RNA profiles in normal and CMT+ groups.

snRNA	Normal	percentage	CMT+	percentage
U1	671	14.98%	6533	7.74%
U11	48	1.07%	605	0.72%
U12	72	1.61%	437	0.52%
U2	3084	68.84%	69610	82.42%
U4	43	0.96%	2479	2.94%
U4atac	5	0.11%	63	0.07%
U5	352	7.86%	4009	4.75%
U6	161	3.59%	329	0.39%
U6atac	16	0.36%	64	0.08%
U7	28	0.63%	329	0.39%

Table 3-7 Differentially expressed (FDR < 0.05) *RNU2* transcript ID and read counts of each library of normal and CMT+ groups. Fold change and FDR were computed by Empirical analysis of DGE based on tagwise dispersions using CLC Genomics Workbench 10. N: Normal; P: CMT+; FC: fold change; Avg: Mean read counts.

Transcript ID	FC	FDR	N1	N2	N3	N4	N5	Avg	P1	P2	P3	P4	P5	Avg
ENSBTAT00000051125.1	24.27	7.26E-07	6	12	21	1	2	8.4	77	1058	49	513	332	405.8
ENSBTAT00000055683.2	13.48	3.86E-05	46	51	114	5	10	45.2	209	2909	117	1525	1035	1159
ENSBTAT00000060635.1	12.89	3.86E-05	44	41	113	7	13	43.6	197	2812	112	1484	1000	1121
ENSBTAT00000064337.1	12.35	3.86E-05	42	49	127	8	13	47.8	201	2907	120	1526	1042	1159.2
ENSBTAT00000065942.1	12.57	3.86E-05	45	57	116	8	10	47.2	196	2900	119	1514	1051	1156
ENSBTAT00000066208.1	12.38	3.86E-05	52	56	115	7	12	48.4	207	2945	119	1533	1061	1173
ENSBTAT00000066262.1	13.05	3.86E-05	42	51	124	5	13	47	203	2926	120	1528	1055	1166.4
ENSBTAT00000042796.2	12.69	4.03E-05	3	14	11	1	3	6.4	28	402	19	182	197	165.6
ENSBTAT00000056314.2	12.90	4.03E-05	45	56	121	4	13	47.8	203	2903	123	1515	1050	1158.8
ENSBTAT00000040476.1	11.52	4.10E-05	48	61	124	10	12	51	204	2922	123	1532	1047	1165.6
ENSBTAT00000060864.1	14.03	4.28E-05	43	41	122	5	7	43.6	198	2875	116	1506	1038	1146.6
ENSBTAT00000064993.1	14.11	4.74E-05	28	21	71	3	6	25.8	120	1930	68	1016	477	722.2
ENSBTAT00000063731.1	12.34	6.36E-05	49	46	128	9	8	48	202	2920	116	1524	1048	1162
ENSBTAT00000051458.1	7.63	1.19E-03	3	3	1	0	1	1.6	26	48	5	23	29	26.2
ENSBTAT00000048223.1	9.66	4.06E-03	8	11	28	1	2	10	17	669	7	132	178	200.6
ENSBTAT00000066043.1	6.98	6.49E-03	4	8	7	3	2	4.8	8	185	10	91	100	78.8

Transcript ID	FC	FDR	N1	N2	N3	N4	N5	Avg	P1	P2	P3	P4	P5	Avg
ENSBTAT00000051159.1	8.18	1.03E-02	4	22	35	3	1	13	15	700	11	143	202	214.2
ENSBTAT00000060739.1	7.48	1.29E-02	5	25	33	3	2	13.6	16	694	8	137	191	209.2
ENSBTAT00000051509.1	3.45	2.96E-02	8	11	11	1	5	7.2	75	80	7	33	55	50
ENSBTAT00000062479.1	13.13	4.32E-02	0	0	0	0	1	0.2	3	22	1	7	9	8.4

Table 3-8 Statistical analysis results of significantly upregulated miRNA precursors chromosomal distribution. P value was obtained by Fisher's exact test; FDR was obtained by P value with Benjamini–Hochberg correction. The values lower than 0.05 were highlighted with color. Chr., chromosome.

Chr.	p value	FDR	Chr.	p value	FDR
1	0.1774	1	**16**	1	1
2	1	1	**17**	0.3893	1
3	0.5912	1	**18**	1	1
4	0.6188	1	**19**	0.0002	0.0061
5	0.4003	1	**20**	1	1
6	1	1	**21**	0.1626	1
7	0.3287	1	**22**	1	1
8	0.6208	1	**23**	1	1
9	0.3893	1	**24**	1	1
10	0.3893	1	**25**	1	1
11	0.5164	1	**26**	0.4092	1
12	0.5912	1	**27**	1	1
13	0.5478	1	**28**	1	1
14	1	1	**29**	1	1
15	1	1	**X**	0.0392	0.5968

Table 3-9 Statistical analysis results of significantly upregulated snRNA precursors chromosomal distribution. P value was obtained by Fisher's exact test; FDR was obtained by P value with Benjamini–Hochberg correction. The values lower than 0.05 were highlighted with color. Chr., chromosome.

Chr.	p value	FDR	Chr.	p value	FDR
1	0.7643	1	**16**	0.6685	1
2	1	1	**17**	0.4497	1
3	0.0771	0.771	**18**	1	1
4	1	1	**19**	0.0001	0.003
5	1	1	**20**	1	1
6	1	1	**21**	0.4288	1
7	0.7202	1	**22**	0.4003	1
8	0.4424	1	**23**	0.1681	1
9	1	1	**24**	0.6621	1
10	0.5251	1	**25**	0.6195	1
11	0.4996	1	**26**	1	1
12	1	1	**27**	1	1
13	0.395	1	**28**	1	1
14	1	1	**29**	0.0279	0.419
15	0.395	1	**X**	0.25	1

Table 3-10 Significantly different expressed miRNA and their immune-related target mRNA. The immune related genes were defined by the putative target mRNA which cross-referenced with ImmPort comprehensive list of immune-related genes.

miRNA	Immune-related target mRNA
miR-221/222	CDKN1B, PVRL1, NTF3, BTG2, KIT, TSC22D3, ZFP36, DDIT4, ERBB4, ZFYVE16, PPP3R1, PIK3R1, IRF2, BBC3, FOS, PCDHA1, GNAI3, PDCD10, TOX
miR-1246	SPARCL1, VIM, C1R, CD83, CLLU1, CHAC1, ROPN1B, PCBP2, AOC3, FOXD4L6, SKIL, KCNMB2, FOXD4L3
miR-146a	NRAS, IRAK1, IGSF1, PSMA4, TRAF6, CD3G, KLF4
miR-10a	BDNF, TFRC, HOXA3, GATA6, CRLF3, RORA, CADM2, CAMK2B, MAP3K7, GRIN2B
miR-142-5p	HERPUD1, ZFPM2, CCL1, UBE2D1, RALB
miR-142-3p	CNTNAP3B, KRIT1, LPP, TNR, GADD45A, SRR, VNN1, PAX3, KCNJ8, EGR1, EREG, IRG1, GNAQ, RTN3
miR-2284w	TREML1, TRIAP1, FASLG, ROPN1B, CLEC9A, IL36G, CCR5, CD9, EXOSC3, KIF20A, ANGPTL1, HRG, TAL2, CLLU1
miR-130b	MDM4, ACVR1, IGF1, HPRT1, KLHL20, NPNT, DLL1, PRKAA2, FAM19A1, SPHK2
miR-2284aa	LPP, DEFB103A, CCL15, CCL23, UBE2B
miR-2285p	NRARP, GADD45A, FKBP1A, AGTR2, SMAD6, XCL1
miR-2285b	TM2D1, DAD1, IRF6
miR-21-3p	ECT2, MX2, PMAIP1, ATP2B1, CIDEC, CTSS, C2, G2E3, NDRG2, CPLX4, GIPC1, NCK1, TRADD, IL27RA, BBC3, NR4A1, WNT8A, BVES, BCL7A
miR-23b-3p	ACVR1C, TFRC, PKP4, ERBB2IP, YES1, TOP1, PNMA1, IGSF8, SLC1A1, HMGB2, SATB1, CCM2, CNN2, NEK6, BLCAP, PMAIP1, FBN2, MRC1, PPIF, TRAT1, NRGN, PRKRIR
miR-301a	IRF1, BTG1, FRZB, FOSL1, SOS2, KALRN, ZNF3, HIVEP2, ARID5B, FASTK, SNX2, ADAM12

miR-505	CD97, NCK2, ADAT2, CYSLTR1, BCAP29, ATG12, PBX3
mir-874	CCL28, ZDHHC16, AQP3, PAAF1
mir-6529a	CTAGE1, IGJ, NOS3
mir-147	
mir-132	RASA1, FKBP2, SPAST, CALU, SIRT1
mir-15a	BCL2L2, CCNE1, WNT7A, PTH, WNT3A, IL7R, UBE2V1, OMG, GNAI3, PLD1, DLEU7, MKNK1, CCND1, FGF7, LRIG2, TRADD, SMAD7, RBCK1, VEGFA, POLR3F, ARL3, CDC42SE2, RAB35, IKBKB, BICD1, KIF23, MAP2K1, SH3GL2, PPAP2A, INSR, EDNRA, TNFSF13B, PCDHA1, TSC22D3, SYNRG, PRKAB2, FNBP1L, SOCS6, CARM1, RFX3, RNF144B, SLIT2, CDK5R1, ISLR, NDP, PLEKHA1, RNF125, PDCD4, RAF1, FNTA, RPS6KA3, SGK1, UBE4B, NISCH, PCDHA5, PCDHA12, NRP2, FERMT2, BDNF, ADRB2, ITGA10, RYBP, EYA1, VPS4A, CHAC1, GHR, SLA2, SMURF2, MAPK8, MIB1, BMPR1A, HSPG2, IHH, SERBP1
mir-338	FKBP1A, ETS1, STAT1, NRP1, TRIM33, APEX1, KCNMB2, GNAQ
mir-30f	RAD23B
mir-223	DDIT4, IL6ST, ECT2, CYTIP, ACVR2A, HHEX, SP3

Table 3-11 Immune-related mRNA co-regulated by more than one miRNA.

mRNA	**miRNA**
TSC22D3	miR-221/222, miR-15
DDIT4	miR-221/222, miR-223
BBC3	miR-221/222, miR-21-3p
PCDHA1	miR-221/222, miR-15
GNAI3	miR-221/222, miR-15
CLLU1	miR-1246, miR-2284w
CHAC1	miR-1246, miR-15
ROPN1B	miR-1246, miR-2284w
KCNMB2	miR-1246, miR-338
BDNF	miR-10a, miR-15
TFRC	miR-10a, miR-23b-3p
LPP	miR-142-3p, miR-2284aa
GADD45A	miR-142-3p, miR-2285p
GNAQ	miR-142-3p, miR-338
FKBP1A	miR-2285p, miR-338
ECT2	miR-21-3p, miR-223
PMAIP1	miR-21-3p, miR-23b-3p
TRADD	miR-21-3p, miR-15a

Table 3-12 Gene ontology (GO) and KEGG Pathway analysis. The fifteen most significantly (FDR < 0.05) enriched GO terms in biological process category were listed; all of the significantly enriched GO terms in molecular function category and KEGG Pathway were listed.

Term	Count	%	FDR
Gene Ontology (Biological Process)			
Regulation of Nitrogen Compound Metabolic Process	206	22.8	4E-06
Regulation of Gene Expression	201	22.3	3E-05
Regulation of Cellular Macromolecule Biosynthetic Process	186	20.6	4E-05
Regulation of RNA Metabolic Process	176	19.5	4E-05
Positive Regulation of Nitrogen Compound Metabolic Process	103	11.4	7E-05
Positive Regulation of Macromolecule Metabolic Process	153	17	7E-05
Regulation of Nucleobase-containing Compound Metabolic Process	190	21.1	7E-05
Protein Modification Process	186	20.6	0.0001
Cellular Protein Modification Process	186	20.6	0.0001
Positive Regulation of RNA Metabolic Process	87	9.6	0.0001
Regulation of RNA Biosynthetic Process	168	18.6	0.0001
Positive Regulation of Metabolic Process	159	17.6	0.0001
Regulation of Macromolecule Biosynthetic Process	188	20.8	0.0002
Regulation of Nucleic Acid-templated Transcription	167	18.5	0.0002
Positive Regulation of Gene Expression	98	10.9	0.0002
Gene Ontology (Molecular Function)			
Heterocyclic Compound Binding	303	33.6	0.0003
Organic Cyclic Compound Binding	304	33.7	0.0008
Regulatory Region DNA Binding	65	7.2	0.0011
Regulatory Region Nucleic Acid Binding	65	7.2	0.0011
Nucleic Acid Binding Transcription Factor Activity	78	8.6	0.0018
Transcription Factor Activity, Sequence-specific DNA Binding	78	8.6	0.0018
Transcription Regulatory Region DNA Binding	63	7	0.0039
Sequence-specific DNA Binding	76	8.4	0.0054
Kinase Activity	62	6.9	0.044
KEGG Pathway			
FoxO Signaling Pathway	26	2.9	7E-07
Progesterone-mediated Oocyte Maturation	17	1.9	0.0018

Term	Count	%	FDR
Oocyte Meiosis	19	2.1	0.0026
Proteoglycans in Cancer	26	2.9	0.0045
KEGG Pathway			
PI3K-Akt Signaling Pathway	36	4	0.0057
ErbB Signaling Pathway	16	1.8	0.0066
MicroRNAs in Cancer	28	3.1	0.019
Prolactin Signaling Pathway	14	1.6	0.026
MAPK Signaling Pathway	28	3.1	0.036
Cell Cycle	18	2	0.048

Table 3-13 Novel miRNA with mean read counts in normal, CMT− or CMT+ groups > 50. Read counts were computed using CLC Genomics Workbench 10. N: Normal; Ne: CMT−; P: CMT+; Avg: Mean read counts.

Name	sequence	N1	N2	N3	N4	N5	Avg	Ne1	Ne2	Ne3	Ne4	Ne5	Avg	P1	P2	P3	P4	P5	Avg
chr17_7049	gcggcggcggcgccggggc	22	57	31	9	31	30	107	85	46	31	51	64	58	116	39	27	143	77
chr1_306	ggccgcgccgggccgcgcc	42	186	73	26	53	76	183	118	102	81	22	101	85	216	102	87	169	132
chr20_10561	uuuuccaaugaguuaguccuuu	114	1243	1278	168	29	566	8	338	266	665	0	255	56	141	114	319	75	141
chr26_14041	ccaaaccaguugugccuguaga	437	1216	1457	184	137	686	114	737	775	898	0	505	261	153	374	401	114	261
chr26_14097	ccgagccugacagaucacaca	1	17	2	1	1	4	0	5	3	4	0	2	5	868	15	547	307	348
chr2_9851	acucuucccucccucggccggc	51	97	66	33	43	58	27	42	59	109	5	48	33	6	58	41	10	30
chr4_17024	aaggaggagaagggggggu	472	799	1549	190	1043	811	1705	2414	483	3446	2296	2069	1907	4941	889	2886	9622	4049
chr4_17403	cucuccaaucgcgacggguaucucu	6151	2490	479	354	939	2083	22560	2126	2085	2659	2376	6361	35839	4199	9788	5393	12820	13608
chr8_21934	ccggcggcggcggcgacu	27	134	46	29	44	56	163	144	100	56	73	107	70	138	68	51	179	101

Table 3-14 The significantly different expressed (FDR < 0.05) novel miRNA. Three of them had the same sequences. FDR analysis by Empirical analysis of DGE based on tagwise dispersions were computed using CLC Genomics Workbench 10. FDR of novel miRNA was analyzed with ncRNA (did not include known miRNA). N: Normal; P: CMT+; Avg: Mean read counts.

Name	FDR	sequence	N1	N2	N3	N4	N5	Avg	P1	P2	P3	P4	P5	Avg
chr15_5392	0.01255	aacuuugaagacugaagu	5	21	15	3	22	13	11	2	2	5	1	4
chr15_5413	0.00213	aaguuuaaagaagucugccuug	0	0	0	0	0	0	1	31	1	23	20	15
chr15_5866	0.00711	aaguuuaaagaagucugccuug	0	1	0	0	0	0	1	28	1	20	18	14
chr15_5890	0.00239	ucacggagaggagaaacugcgaa	0	0	0	0	0	0	6	19	2	5	11	9
chr17_7169	0.01601	cggcuccgugacucguccgugg	2	5	4	4	4	4	2	1	0	1	2	1
chr18_7790	0.02743	aggagaggcacugucagagcug	0	0	0	0	0	0	2	11	0	6	3	4
chr19_8664	0.02775	ugcagggugguaggcugugggcu	33	57	50	8	25	35	17	2	23	6	3	10
chr19_8860	0.03187	gucuggaauccugaagagu	1	4	6	6	5	4	1	0	0	5	1	1
chr19_8907	0.04169	uuggcuagggaggaaugcugaacu	4	6	10	1	8	6	0	3	3	4	0	2
chr20_10561	0.01177	uuuuccaaugaguuaguccuuu	114	1243	1278	168	29	566	56	141	114	319	75	141
chr21_11091	0.02381	cccguuucucucgcgcgccggcu	23	18	18	13	19	18	5	10	15	10	3	9
chr22_11977	0.04545	gaacgaaauccaagcgcagcug	11	25	37	5	11	18	4	10	13	11	7	9
chr26_14041	0.02726	ccaaaccaguugugccuguaga	437	1216	1457	184	137	686	261	153	374	401	114	261

Name	FDR	sequence	N1	N2	N3	N4	N5	Avg	P1	P2	P3	P4	P5	Avg
chr26_14095	0.03653	ucgagccugacagaucacaca	1	0	0	0	0	0	1	18	1	12	6	8
chr26_14097	7.9E-05	ccgagccugacagaucacaca	1	17	2	1	1	4	5	868	15	547	307	348
chr26_14099	0.02031	ucgagccugacagaucacaca	0	0	0	0	0	0	0	22	1	11	6	8
chr26_14101	0.01553	ucgagccugacagaucacaca	0	0	0	0	0	0	0	18	0	10	7	7
chr29_15404	0.03117	aggaaggggcuucugagc	0	0	0	0	0	0	2	13	0	7	8	6
chr3_16229	0.00046	aaaaaccugaaugacccuuuugg	0	3	3	0	0	1	8	106	2	54	43	43
chr7_19975	0.0282	uuagauaacuaauaaaaaccug	0	3	0	0	0	1	3	48	0	8	17	15
chr7_20570	0.01275	acaccaggacuugucuccccaga	4	8	6	4	1	5	1	0	2	1	0	1

Table 3-15 Analysis results of the miRWalk 2.0.

Subcategory	p-value	miRNA
Carcinoma Non-Small-Cell Lung	0.040	hsa-miR-221-3p; hsa-miR-222-3p; hsa-miR-15a-5p
Infection	0.040	hsa-miR-221-3p; hsa-miR-146a-5p; hsa-miR-222-3p; hsa-miR-15a-5p
Inflammation	0.040	hsa-miR-221-3p; hsa-miR-146a-5p; hsa-miR-10a-5p; hsa-miR-146b-5p; hsa-miR-223-3p
Leukemia	0.040	hsa-miR-221-3p; hsa-miR-10a-5p; hsa-miR-222-3p; hsa-miR-130b-3p; hsa-miR-223-3p; hsa-miR-15a-5p
Leukemia Myelogenous Chronic BCR-ABL Positive	0.040	hsa-miR-221-3p; hsa-miR-10a-5p; hsa-miR-222-3p

Table 3-16 Analysis results of the HMDD 2.0.

Subcategory	p-value	miRNA precursor
Arthritis, Rheumatoid	0.013	hsa-mir-146a; hsa-mir-223; hsa-mir-15a; hsa-mir-132
Atherosclerosis	0.002	hsa-mir-221; hsa-mir-146a; hsa-mir-10a; hsa-mir-222; hsa-mir-146b; hsa-mir-21
Autistic Disorder	0.006	hsa-mir-146a; hsa-mir-146b; hsa-mir-21; hsa-mir-15a; hsa-mir-23b; hsa-mir-132
Breast Neoplasms	0.005	hsa-mir-221; hsa-mir-146a; hsa-mir-10a; hsa-mir-222; hsa-mir-146b; hsa-mir-223; hsa-mir-147a; hsa-mir-21; hsa-mir-301a; hsa-mir-874; hsa-mir-338; hsa-mir-15a; hsa-mir-23b; hsa-mir-132; hsa-mir-505
Carcinoma	0.011	hsa-mir-221; hsa-mir-10a; hsa-mir-142; hsa-mir-222; hsa-mir-146b; hsa-mir-21
Carcinoma, Hepatocellular	0.043	hsa-mir-221; hsa-mir-146a; hsa-mir-10a; hsa-mir-142; hsa-mir-222; hsa-mir-146b; hsa-mir-130b; hsa-mir-223; hsa-mir-147a; hsa-mir-21; hsa-mir-301a; hsa-mir-338; hsa-mir-15a
Carcinoma, Non-Small-Cell Lung	0.048	hsa-mir-221; hsa-mir-146a; hsa-mir-10a; hsa-mir-222; hsa-mir-146b; hsa-mir-223; hsa-mir-21; hsa-mir-15a
Carcinoma, Squamous Cell	0.018	hsa-mir-146a; hsa-mir-10a; hsa-mir-142; hsa-mir-222; hsa-mir-223; hsa-mir-21; hsa-mir-874; hsa-mir-15a
Carotid Artery Diseases	0.007	hsa-mir-221; hsa-mir-222
Cholesteatoma	0.035	hsa-mir-221; hsa-mir-21
Colonic Neoplasms	0.016	hsa-mir-221; hsa-mir-146a; hsa-mir-142; hsa-mir-223; hsa-mir-21; hsa-mir-15a; hsa-mir-23b; hsa-mir-132
Cryptosporidium	0.024	hsa-mir-21; hsa-mir-23b
Demyelinating Diseases	0.015	hsa-mir-21; hsa-mir-301a
Diabetes Mellitus	0.011	hsa-mir-146a; hsa-mir-21; hsa-mir-301a
Diabetes Mellitus, Type 2	0.011	hsa-mir-146a; hsa-mir-223; hsa-mir-21; hsa-mir-15a
Eclampsia	0.035	hsa-mir-222; hsa-mir-21
Eosinophilic Esophagitis	0.002	hsa-mir-146a; hsa-mir-142; hsa-mir-222; hsa-mir-146b; hsa-mir-223; hsa-mir-21; hsa-mir-132

Subcategory	p-value	miRNA precursor
Gastrointestinal Neoplasms	0.011	hsa-mir-221; hsa-mir-146a; hsa-mir-142; hsa-mir-222; hsa-mir-338
Glioblastoma	0.021	hsa-mir-221; hsa-mir-146a; hsa-mir-10a; hsa-mir-142; hsa-mir-222; hsa-mir-146b; hsa-mir-21; hsa-mir-15a; hsa-mir-23b
HEV	0.007	hsa-mir-221; hsa-mir-222
HIV	0.004	hsa-mir-221; hsa-mir-222; hsa-mir-223; hsa-mir-21
Head and Neck Neoplasms	0.026	hsa-mir-221; hsa-mir-142; hsa-mir-146b; hsa-mir-130b; hsa-mir-223; hsa-mir-21; hsa-mir-15a
Hepatitis B	0.005	hsa-mir-221; hsa-mir-146a; hsa-mir-338; hsa-mir-15a; hsa-mir-23b; hsa-mir-132
Inflammation	0.004	hsa-mir-221; hsa-mir-146a; hsa-mir-222; hsa-mir-21; hsa-mir-23b; hsa-mir-132
Kidney Neoplasms	0.007	hsa-mir-21; hsa-mir-15a; hsa-mir-23b
Leukemia, Lymphocytic, Chronic, B-Cell	0.002	hsa-mir-221; hsa-mir-146a; hsa-mir-222; hsa-mir-146b; hsa-mir-223; hsa-mir-21; hsa-mir-15a; hsa-mir-23b
Leukemia, Myeloid, Acute	0.024	hsa-mir-221; hsa-mir-146a; hsa-mir-10a; hsa-mir-142; hsa-mir-222; hsa-mir-223; hsa-mir-15a
Lung Neoplasms	0.036	hsa-mir-221; hsa-mir-146a; hsa-mir-142; hsa-mir-222; hsa-mir-146b; hsa-mir-223; hsa-mir-21; hsa-mir-301a; hsa-mir-338; hsa-mir-132
Lymphoma, Large B-Cell, Diffuse	0.009	hsa-mir-221; hsa-mir-223; hsa-mir-21
Marek Disease	0.003	hsa-mir-221; hsa-mir-222; hsa-mir-223
Multiple Myeloma	0.005	hsa-mir-221; hsa-mir-222; hsa-mir-130b; hsa-mir-223; hsa-mir-21; hsa-mir-15a
Multiple Sclerosis	0.007	hsa-mir-146a; hsa-mir-146b; hsa-mir-21; hsa-mir-338; hsa-mir-15a
Muscular Disorders, Atrophic	0.006	hsa-mir-221; hsa-mir-146a; hsa-mir-222; hsa-mir-146b; hsa-mir-223; hsa-mir-21; hsa-mir-132
Myelodysplastic Syndromes	0.029	hsa-mir-221; hsa-mir-146a; hsa-mir-222; hsa-mir-21; hsa-mir-15a

Subcategory	p-value	miRNA precursor
Myocardial Reperfusion Injury	0.007	hsa-mir-146a; hsa-mir-21
Neoplasms, Squamous Cell	0.002	hsa-mir-221; hsa-mir-222; hsa-mir-146b; hsa-mir-21; hsa-mir-874; hsa-mir-338; hsa-mir-23b
Obesity	0.002	hsa-mir-221; hsa-mir-146b; hsa-mir-21; hsa-mir-132
Pancreatic Neoplasms	0.003	hsa-mir-221; hsa-mir-146a; hsa-mir-10a; hsa-mir-142; hsa-mir-222; hsa-mir-146b; hsa-mir-223; hsa-mir-21; hsa-mir-338; hsa-mir-15a; hsa-mir-132
Papilary thyroid carcinoma	0.007	hsa-mir-221; hsa-mir-222
Periodontal Diseases	0.035	hsa-mir-146a; hsa-mir-146b
Polycythemia Vera	0.018	hsa-mir-221; hsa-mir-222; hsa-mir-21
Prostatic Neoplasms	0.006	hsa-mir-221; hsa-mir-146a; hsa-mir-222; hsa-mir-146b; hsa-mir-130b; hsa-mir-223; hsa-mir-21; hsa-mir-301a; hsa-mir-15a; hsa-mir-23b; hsa-mir-132
Psoriasis	0.002	hsa-mir-146a; hsa-mir-142; hsa-mir-146b; hsa-mir-223; hsa-mir-21
Reperfusion Injury	0.006	hsa-mir-146a; hsa-mir-223; hsa-mir-21; hsa-mir-15a
Scleroderma, Systemic	0.007	hsa-mir-146a; hsa-mir-142; hsa-mir-146b; hsa-mir-21
Sepsis	0.043	hsa-mir-146a; hsa-mir-223; hsa-mir-15a
Sjogrens Syndrome	0.007	hsa-mir-146a; hsa-mir-146b
Tuberculosis, Pulmonary	0.011	hsa-mir-146a; hsa-mir-130b; hsa-mir-223; hsa-mir-147a; hsa-mir-21
Uterine Cervical Neoplasms	0.029	hsa-mir-221; hsa-mir-146a; hsa-mir-10a; hsa-mir-21; hsa-mir-338; hsa-mir-23b

Conclusion

Comparing miR-92a, miR-375, and let-7g, we find that miR-92a is suitable as a housekeeping gene for bovine mastitis milk miRNA analysis. The expression level miR-21, miR-146a, miR-155, miR-222 and miR-383 was significantly upregulated in CMT+ milk. These miRNA have a potential to be used as biomarkers for bovine mastitis. These miRNA are also increased in digital PCR analysis, suggesting that digital PCR can be a tool for mastitis milk miRNA analysis. miR-21 expression is also increased in mastitis cow serum, suggesting that focal mastitis can cause molecular biological mechanisms changed systemically. Milk samples are superior to serum samples as biomarker and diagnostic tools, because inflammation related miRNA are changed much clearly in mastitis affected milk samples and milk samples have better accessibility. In genome-wide miRNA study by Illumina small RNA sequencing technic, we find 23 miRNA being upregulated and 2 downregulated in mastitis milk. U2 small nuclear RNA is upregulated in mastitis milk, and the significantly increased miR-1246 is likely derived from U2 small nuclear RNA. Bovine chromosome 19, which is highly conserved with human chromosome 17, is a hotspot of upregulated small RNA source. Human breast cancer related oncogenes and tumor suppressor genes such as HER2, p53 and BRCA1 are located on chromosome 17, suggesting bovine chromosome 19 might play a role in

breast related diseases. Gene ontology analysis of significantly upregulated miRNA putative mRNA targets show that the upregulated miRNA are involved in regulate target gene expression, while KEGG pathway analysis shows that upregulated miRNA are mainly related to cancer and immune system pathways. Three of novel miRNA are related with bovine mastitis. One of the highly expressed mastitis related novel miRNA is significantly upregulated in milk using a digital PCR system. The genome-wide study provides insights into bovine mastitis and inflammatory diseases. We have shown that the miRNA play a role in bovine mastitis. The functions of individual miRNA or it caused by released from dead somatic cells into cow milk needs further study. Other RNA derived small RNA might also play a role in inflammation and disease conditions, especially RNU2 (miR-1246), their functions and mechanisms are also an interesting research field in the future.

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Appendix

Appendix 1-1 Sample name, sample type and CT values of housekeeping gene candidates. CT values shown as the mean values of duplicated experiment.

Name	Type	miR-375	miR-92a	let-7g
528 右前	Normal	25.57	27.41	27.35
1	Normal	23.09	25.33	25.10
3	Normal	22.43	25.33	25.78
4	Normal	24.19	26.28	26.63
5	Normal	24.07	25.83	27.18
550M 右後	CMT 4+	23.65	25.12	25.17
585M 右後	CMT 3+	23.56	26.08	26.19
593M	Normal	23.55	26.14	27.54
601M 左後	CMT 2+	23.65	26.06	27.41
603M 左後	CMT 2+	24.68	26.20	28.21
604M 左前	CMT 4+	23.57	25.73	28.11
605M	Normal	23.58	25.67	27.95
617M	Normal	23.57	25.55	27.70
620M	Normal	23.94	25.92	29.17
622M	Normal	24.01	26.15	28.58
420 左後	CMT 3+	23.56	26.51	27.34
458 左後	CMT +	24.51	26.26	28.34
458 右後	CMT 3+	26.04	27.02	27.31
473 左前	CMT +	24.17	26.80	28.09
505 左後	CMT 3+	24.60	27.92	27.44
511 右後	CMT 2+	24.02	26.86	29.15

Appendix 1-2 Sample name, sample type, miR-146a CT values and their relative expression values normalized to housekeeping gene candidates. CT values shown as the mean values of duplicated experiment.

Sample		CT value	Housekeeping gene normalized expression					
Name	**Type**	**miR-146a**	**miR-92a**	**miR-375**	**let-7g**	**miR-92a+ miR-375**	**miR-92a+ let-7g**	**let-7g+ miR-375**
170A	Normal	32.98	0.53	0.45	0.47	0.49	0.50	0.46
170B	Normal	34.50	0.18	0.17	0.18	0.18	0.18	0.18
170C	Normal	36.45	0.04	0.04	0.05	0.04	0.05	0.05
170D	Normal	33.11	0.40	0.49	0.50	0.44	0.45	0.49
534A	Normal	30.13	3.39	2.92	2.14	3.15	2.69	2.50
534B	Normal	31.87	1.43	0.96	0.81	1.17	1.07	0.88
534C	Normal	32.84	0.82	0.49	0.42	0.64	0.59	0.45
534D	Normal	31.59	2.07	1.21	1.08	1.59	1.49	1.14
566A	Normal	33.00	0.54	0.38	0.39	0.45	0.46	0.38
566B	Normal	31.25	1.70	2.34	1.43	1.99	1.56	1.83
566C	Normal	31.96	0.75	0.65	0.91	0.70	0.82	0.77
566D	Normal	32.55	0.65	0.41	0.63	0.52	0.64	0.51
574D	Normal	33.19	0.49	1.09	2.95	0.73	1.21	1.79
594A	Normal	32.18	0.90	0.62	1.19	0.75	1.04	0.86
594B	Normal	32.40	0.62	0.41	0.87	0.50	0.73	0.60
594C	Normal	32.06	0.79	0.62	0.79	0.70	0.79	0.70
594D	Normal	34.78	0.17	0.12	0.18	0.14	0.18	0.14
609A	Normal	33.21	0.52	1.51	1.00	0.88	0.72	1.23
609B	Normal	30.99	2.72	2.13	1.64	2.41	2.11	1.87
609C	Normal	31.46	1.33	1.21	1.25	1.27	1.29	1.23
609D	Normal	32.71	0.81	0.61	0.49	0.70	0.63	0.55
後 12/26D	CMT 1+	30.55	6.42	5.36	2.04	5.87	3.62	3.31
305 右前	CMT 3+	25.54	29.13	25.62	28.48	27.32	28.80	27.02
305 右後	CMT 4+	23.46	53.70	220.10	193.53	108.72	101.95	206.39
513 右後	CMT 3+	28.00	9.59	4.23	4.99	6.37	6.92	4.60
514 左前	CMT 3+	26.70	12.19	29.49	6.96	18.96	9.21	14.33
514 右前	CMT 3+	26.03	19.09	62.83	10.70	34.63	14.29	25.93
528 左前	Normal	40.00	0.00	0.01	0.01	0.00	0.01	0.01
528 右前	Normal	38.51	0.02	0.02	0.01	0.02	0.01	0.01

Sample		CT value	Housekeeping gene normalized expression					
Name	**Type**	**miR-146a**	**miR-92a**	**miR-375**	**let-7g**	**miR-92a+ miR-375**	**miR-92a+ let-7g**	**let-7g+ miR-375**
1	Normal	32.07	0.33	0.32	0.12	0.32	0.20	0.20
3	Normal	33.56	0.12	0.07	0.07	0.09	0.09	0.07
4	Normal	34.16	0.15	0.16	0.08	0.16	0.11	0.11
5	Normal	32.92	0.26	0.35	0.28	0.30	0.27	0.31
550M 右後	CMT 4+	27.39	7.29	12.10	3.25	9.39	4.87	6.28
585M 右後	CMT 3+	28.89	5.00	4.01	2.32	4.48	3.41	3.05
593M	Normal	33.83	0.17	0.13	0.19	0.15	0.18	0.16
601M 左後	CMT 2+	29.25	3.85	3.33	4.21	3.58	4.02	3.75
603M 左後	CMT 2+	29.89	2.73	4.38	4.70	3.45	3.58	4.54
604M 左前	CMT 4+	29.43	2.70	2.78	6.04	2.74	4.04	4.10
605M	Normal	34.26	0.09	0.10	0.19	0.09	0.13	0.14
617M	Normal	34.31	0.08	0.10	0.15	0.09	0.11	0.12
620M	Normal	33.54	0.18	0.21	0.73	0.19	0.36	0.39
622M	Normal	33.76	0.18	0.19	0.42	0.18	0.27	0.28
420 左後	CMT 3+	26.75	29.79	17.75	22.75	22.99	26.03	20.10
458 左後	CMT +	30.84	1.48	2.01	2.68	1.73	1.99	2.33
458 右後	CMT 3+	27.92	18.83	44.06	9.91	28.80	13.66	20.90
473 左前	CMT +	30.09	3.59	2.68	3.78	3.10	3.68	3.18
505 左後	CMT 3+	26.82	75.47	34.82	23.32	51.26	41.95	28.50
511 右後	CMT 2+	29.86	4.41	2.83	9.24	3.53	6.38	5.11

Appendix 2-1 CT values of miRNA in normal and mastitis cows. CT values shown as the mean values of duplicated experiment.

Sample	Type	miR-26b	miR-29b	miR-92a	miR-122	miR-125b	miR-222	miR-204	miR-205	miR-383
1	Normal	25.60	30.85	25.24	30.24	27.65	33.02	35.61	33.69	36.07
2	Normal	26.39	31.57	25.97	31.02	27.94	33.46	36.14	34.35	35.66
3	Normal	26.18	31.61	25.45	30.26	28.05	32.49	35.26	34.73	36.47
4	Normal	26.28	32.63	25.92	30.21	28.50	33.42	36.05	34.38	35.79
5	Normal	25.27	30.02	25.64	35.89	27.07	28.09	35.32	34.66	36.23
6	Normal	25.37	29.33	25.68	40.00	27.30	27.42	35.00	34.15	35.81
7	Normal	25.39	29.31	25.63	40.00	27.29	32.64	35.79	34.34	35.12
8	Normal	25.39	29.87	25.43	40.00	27.17	30.11	35.18	34.52	34.29
9	Normal	24.58	29.60	24.99	40.00	27.01	33.52	38.29	33.96	34.20
10	Normal	25.13	29.69	24.81	40.00	26.76	30.25	35.55	34.17	35.24
11	Normal	24.87	29.48	25.30	35.24	27.34	34.17	36.99	34.89	35.44
12	Normal	24.99	29.76	25.38	40.00	27.78	35.42	36.71	35.42	36.78
13	Normal	25.26	30.41	25.43	36.64	27.58	35.54	38.63	35.00	36.86
14	Normal	25.74	30.18	25.83	40.00	27.93	34.96	36.66	34.55	35.77
15	Normal	25.17	29.33	25.20	40.00	27.59	31.66	36.19	33.40	36.15
16	Normal	25.09	29.58	25.31	35.75	27.60	31.66	35.29	32.30	36.10
17	Normal	25.01	29.18	25.13	34.45	27.46	32.04	38.87	32.27	37.03
18	Normal	25.66	30.15	25.56	38.67	28.15	32.98	36.14	33.81	35.66
19	Normal	25.18	29.85	25.06	40.00	27.34	33.35	35.56	34.30	35.73
20	Normal	25.63	30.46	25.57	34.66	27.76	29.58	36.78	34.30	36.50
21	Normal	25.02	29.57	25.47	40.00	27.11	29.28	35.85	34.74	35.89
22	Normal	25.24	31.36	25.08	34.49	27.52	31.73	35.51	33.87	34.18
23	Mastitis	27.68	33.77	25.58	30.12	26.45	30.95	36.62	34.13	33.98
24	Mastitis	25.87	32.04	25.78	30.32	25.89	27.73	34.75	32.25	30.82
25	Mastitis	25.35	32.43	25.66	29.70	25.74	27.98	34.89	32.95	32.80
26	Mastitis	27.69	31.62	25.21	29.16	26.18	32.22	36.58	34.36	33.58
27	Mastitis	27.58	31.52	25.94	29.03	25.99	27.97	34.90	34.37	34.01
28	Mastitis	26.43	31.29	25.94	28.95	26.20	27.75	36.70	33.43	32.11
29	Mastitis	29.12	32.34	26.60	29.56	26.25	28.01	37.62	34.58	33.63
30	Mastitis	26.14	29.52	25.16	29.92	27.31	32.28	35.81	32.76	35.78
31	Mastitis	24.04	29.78	25.06	28.48	26.85	24.28	36.13	31.19	31.00

Appendix 2-2 miRNA relative expression normalized to miR-92a in normal and mastitis cows.

Sample	Type	miR-26b	miR-29b	miR-122	miR-125b	miR-222	miR-204	miR-205	miR-383
1	Normal	0.94	0.70	17.44	0.61	0.23	1.25	1.00	0.40
2	Normal	0.90	0.71	16.79	0.83	0.29	1.44	1.05	0.88
3	Normal	0.73	0.48	19.80	0.53	0.39	1.84	0.56	0.35
4	Normal	0.94	0.33	28.54	0.55	0.28	1.48	0.99	0.78
5	Normal	1.56	1.64	0.45	1.20	9.36	2.01	0.67	0.47
6	Normal	1.50	2.75	0.03	1.06	15.43	2.59	0.98	0.65
7	Normal	1.42	2.67	0.03	1.03	0.40	1.44	0.83	1.01
8	Normal	1.24	1.58	0.02	0.97	1.99	1.92	0.64	1.55
9	Normal	1.60	1.41	0.02	0.80	0.14	0.16	0.69	1.22
10	Normal	0.97	1.17	0.01	0.84	1.18	0.97	0.53	0.53
11	Normal	1.63	1.90	0.57	0.79	0.11	0.50	0.45	0.64
12	Normal	1.58	1.64	0.02	0.61	0.05	0.64	0.33	0.27
13	Normal	1.36	1.09	0.24	0.74	0.05	0.18	0.46	0.26
14	Normal	1.28	1.69	0.03	0.76	0.09	0.91	0.82	0.74
15	Normal	1.23	1.96	0.02	0.62	0.59	0.81	1.19	0.37
16	Normal	1.41	1.78	0.40	0.66	0.63	1.63	2.74	0.41
17	Normal	1.31	2.07	0.87	0.65	0.43	0.12	2.46	0.19
18	Normal	1.13	1.42	0.06	0.54	0.30	1.08	1.15	0.66
19	Normal	1.11	1.24	0.02	0.67	0.16	1.14	0.58	0.45
20	Normal	1.15	1.16	1.02	0.71	3.17	0.70	0.82	0.37
21	Normal	1.64	2.00	0.02	1.04	3.65	1.24	0.56	0.53
22	Normal	1.08	0.44	0.82	0.60	0.51	1.20	0.78	1.32
23	Mastitis	0.28	0.12	23.83	1.78	1.24	0.79	0.92	2.14
24	Mastitis	1.14	0.45	23.94	3.01	13.26	3.30	3.94	22.13
25	Mastitis	1.50	0.31	33.69	3.07	10.23	2.75	2.21	5.12
26	Mastitis	0.22	0.40	35.90	1.66	0.40	0.62	0.61	2.18
27	Mastitis	0.39	0.72	65.40	3.13	12.55	3.32	1.01	2.70
28	Mastitis	0.86	0.84	69.17	2.71	14.64	0.96	1.93	10.06
29	Mastitis	0.21	0.64	71.65	4.17	19.40	0.80	1.37	5.55
30	Mastitis	0.61	1.67	20.50	0.73	0.37	1.03	1.79	0.46
31	Mastitis	2.45	1.30	51.82	0.94	88.12	0.77	4.96	11.81

Appendix 2-3 CT values of miR-21-5p and miR-92a in normal and mastitis cows, and miRNA relative expression normalized to miR-92a in normal and mastitis cows. CT values shown as the mean values of duplicated experiment.

		CT		Expression
Sample	**Type**	**miR-92a**	**miR-21-5p**	**miR-21-5p**
1	Normal	25.80	23.51	0.77
2	Normal	25.75	23.70	0.65
3	Normal	25.70	23.98	0.52
4	Normal	25.53	24.37	0.35
5	Normal	26.30	23.21	1.34
6	Normal	26.38	23.07	1.56
7	Normal	25.85	23.15	1.02
8	Normal	25.75	22.86	1.17
9	Normal	25.28	22.71	0.93
10	Normal	25.66	22.58	1.33
11	Normal	26.48	24.61	0.57
12	Normal	26.14	24.14	0.63
13	Normal	26.36	24.13	0.74
14	Normal	25.91	23.72	0.72
15	Normal	25.77	23.05	1.03
16	Normal	25.44	23.09	0.80
17	Normal	25.46	23.03	0.85
18	Normal	25.98	23.62	0.81
19	Normal	25.99	23.30	1.02
20	Normal	26.17	22.86	1.56
21	Normal	25.61	22.84	1.08
22	Normal	26.14	23.00	1.39
23	Mastitis	29.54	26.53	1.27
24	Mastitis	30.33	24.20	10.94
25	Mastitis	26.97	23.98	1.25
26	Mastitis	27.10	26.03	0.33
27	Mastitis	28.64	25.41	1.48
28	Mastitis	28.48	24.09	3.28
29	Mastitis	28.68	26.94	0.53

Appendix 2-4 CT values and relative expression normalized to miR-92a of miRNA in normal, CMT− and CMT+ quarters. CT: CT value; Exp: relative expression. CT values shown as the mean values of duplicated experiment.

Sample	Type	miR-92a	miR-21-5p		miR-122		miR-146a		miR-155		miR-222		miR-383	
		CT	CT	Exp	CT	Exp	CT	Exp	CT	Exp	CT	Exp	CT	Exp
1	normal	25.80	23.51	0.80	29.53	131.25	32.98	0.84	32.76	0.57	30.83	0.45	35.20	0.53
2	normal	25.75	23.70	0.68	30.31	74.14	34.50	0.28	32.96	0.48	30.95	0.40	34.47	0.85
3	normal	25.70	23.98	0.54	30.83	50.08	36.45	0.07	32.55	0.62	30.68	0.47	35.21	0.49
4	normal	25.53	24.37	0.37	31.09	37.14	33.11	0.64	33.34	0.32	30.87	0.37	34.64	0.65
5	normal	26.30	23.21	1.46	37.88	0.54	32.84	5.40	31.96	2.28	28.90	5.15	34.09	0.43
6	normal	26.38	23.07	1.33	36.84	0.28	31.59	2.28	32.07	1.73	28.65	2.78	34.39	1.09
7	normal	25.85	23.15	1.39	34.09	0.57	33.00	1.31	32.69	1.41	30.82	2.44	34.47	1.61
8	normal	25.75	22.86	1.62	40.00	1.24	31.25	3.29	31.99	1.39	30.03	3.06	35.28	1.38
9	normal	25.28	22.71	1.06	40.00	5.75	31.96	0.86	32.18	0.62	30.58	0.47	34.57	0.91
10	normal	25.66	22.58	1.22	40.00	0.09	32.55	2.70	32.39	0.95	30.59	0.77	34.56	0.48
11	normal	26.48	24.61	0.97	40.00	0.06	33.45	1.19	33.09	0.60	30.81	0.38	35.18	0.57
12	normal	26.14	24.14	1.38	40.00	0.08	35.32	1.03	32.84	0.67	30.92	0.49	34.59	0.75
13	normal	26.36	24.13	0.60	40.00	0.15	33.97	0.98	33.46	0.73	30.85	0.74	34.75	0.86
14	normal	25.91	23.72	0.65	38.04	0.12	33.19	0.21	33.43	0.69	31.14	0.54	34.96	1.03
15	normal	25.77	23.05	0.77	36.72	0.14	32.18	0.62	31.53	0.52	30.56	0.66	34.10	1.06
16	normal	25.44	23.09	0.75	37.84	0.39	32.40	0.79	32.31	0.39	30.73	0.40	33.55	0.68

Sample	Type	miR-92a	miR-21-5p		miR-122		miR-146a		miR-155		miR-222		miR-383	
		CT	CT	Exp	CT	Exp	CT	Exp	CT	Exp	CT	Exp	CT	Exp
17	normal	25.46	23.03	1.08	40.00	0.88	32.06	1.43	31.81	1.32	30.57	0.53	35.31	1.11
18	normal	25.98	23.62	0.84	37.65	0.32	34.78	0.98	32.54	0.61	30.89	0.38	35.76	1.30
19	normal	25.99	23.30	0.88	37.46	0.07	33.21	1.26	32.98	0.88	30.72	0.43	34.40	0.39
20	normal	26.17	22.86	0.84	37.31	0.54	30.99	0.27	32.12	0.76	29.70	0.49	33.09	0.41
21	normal	25.61	22.84	1.06	37.76	0.62	31.46	0.82	32.31	0.56	29.74	0.56	35.18	1.05
22	normal	26.14	23.00	1.63	37.99	0.77	32.71	4.33	33.07	1.16	30.67	1.28	35.50	2.96
32	normal	25.64	22.48	1.12	37.28	0.39	30.13	2.12	30.61	0.69	27.16	0.85	35.34	0.47
33	normal	26.13	23.11	1.44	38.74	0.47	31.87	1.28	31.50	0.59	28.55	0.64	34.49	0.54
34	CMT−	29.42	28.20	0.89	40.00	7.08	40.00	1.47	30.18	1.24	30.71	0.43	34.87	1.31
35	CMT−	26.40	24.11	0.40	40.00	143.72	36.23	0.94	34.75	0.95	30.82	0.36	35.08	0.89
36	CMT3+	26.61	24.16	2.36	40.00	5.74	35.55	46.34	34.55	6.16	31.25	12.67	34.96	7.44
37	CMT3+	26.76	24.41	3.65	40.00	14.04	40.00	85.44	35.80	10.43	31.89	22.85	35.02	5.86
38	CMT−	25.97	24.55	1.27	40.00	0.28	40.00	1.56	31.32	0.48	30.88	0.54	35.86	0.96
39	CMT−	28.47	22.62	0.85	40.00	29.56	40.00	1.56	35.24	0.67	33.84	0.49	34.75	0.54
40	CMT−	27.40	21.48	0.77	40.00	0.63	38.51	0.86	34.41	0.37	30.53	0.40	35.40	0.49
41	CMT3+	28.88	23.53	1.55	40.00	12.56	40.00	15.25	35.19	2.32	32.25	3.61	34.77	1.53
42	CMT3+	25.31	18.80	2.96	32.46	3.97	32.07	19.40	30.84	10.17	29.19	15.29	35.70	3.36
43	CMT−	28.58	23.78	1.17	35.46	0.08	40.00	0.59	33.84	0.91	31.78	1.29	33.85	0.94
44	CMT3+	25.32	19.91	2.69	30.10	2.23	33.56	30.37	31.24	14.43	33.82	15.92	33.98	2.02
45	CMT−	26.28	19.80	0.78	36.26	0.09	34.16	0.09	31.29	0.35	28.79	0.93	33.18	0.79
46	normal	25.83	20.00	0.38	40.00	1.14	32.92	0.08	32.38	42.27	31.67	6.09	33.86	8.22
47	normal	26.14	20.87	0.80	33.75	0.14	33.83	0.13	32.62	0.22	35.90	0.69	32.81	0.88

Sample	Type	miR-92a	miR-21-5p		miR-122		miR-146a		miR-155		miR-222		miR-383	
		CT	CT	Exp	CT	Exp	CT	Exp	CT	Exp	CT	Exp	CT	Exp
48	normal	25.67	19.48	0.90	36.77	0.16	34.26	0.25	33.28	0.29	34.29	0.60	33.12	1.10
49	normal	25.54	19.50	0.84	40.00	0.18	34.31	0.01	32.96	0.14	34.64	0.42	33.10	1.17
50	normal	25.92	19.84	0.44	36.61	0.10	33.54	0.01	32.65	1.76	33.10	0.49	33.72	0.38
51	normal	26.14	20.50	0.72	39.32	0.15	33.76	0.03	33.26	0.32	34.60	0.58	33.00	1.18
52	normal	25.23	22.79	0.75	33.18	0.07	31.61	0.03	31.09	0.27	30.34	2.75	33.33	0.36
53	normal	25.34	24.05	0.51	28.94	0.19	32.36	0.03	31.58	0.45	30.70	2.31	34.00	1.55
54	normal	25.85	22.89	1.13	38.47	3.01	32.13	0.70	33.05	0.76	30.62	1.63	34.39	0.07
55	normal	25.59	23.21	0.35	31.48	3.63	31.88	0.03	32.33	0.93	30.52	2.60	34.95	2.39
56	normal	25.40	23.16	0.53	36.84	15.63	32.56	0.25	33.00	0.58	30.62	0.07	34.92	0.23
57	normal	25.63	22.79	1.11	40.00	0.42	33.31	0.32	31.93	1.10	29.15	4.19	34.19	0.77
58	normal	25.76	23.51	0.71	40.00	0.02	36.16	0.56	33.44	0.37	29.76	0.42	34.57	0.35
59	CMT−	25.22	19.84	0.52	35.86	0.27	32.31	0.56	31.23	0.55	32.45	0.16	32.28	0.69
60	CMT4+	25.76	19.65	1.86	31.07	3.73	33.50	15.72	31.95	2.69	30.12	13.66	32.11	7.21
61	CMT−	25.98	20.22	0.86	34.72	10.75	32.72	0.36	32.11	0.48	33.54	1.17	32.74	1.12
62	CMT3+	25.52	20.24	1.80	33.97	1.48	33.90	10.80	32.64	2.89	35.42	11.77	33.43	6.36
63	Normal	25.84	19.72	0.48	34.75	2.18	32.17	0.37	31.94	0.39	32.87	0.03	32.82	0.90
64	CMT−	25.68	19.41	0.67	34.76	0.99	32.54	0.71	29.41	0.50	31.41	0.13	33.27	0.84
65	CMT2+	25.86	20.54	1.81	33.15	1.79	32.24	8.30	31.12	1.99	30.61	3.99	32.70	3.90
66	CMT2+	25.87	19.88	1.20	37.39	4.88	33.37	5.88	31.83	2.22	32.26	3.21	33.04	3.72
67	CMT−	27.22	19.08	0.48	33.26	1.22	29.62	0.23	29.61	0.25	27.22	0.02	32.72	0.38
68	CMT−	24.78	19.93	0.87	34.21	0.89	34.32	0.95	31.63	0.51	36.59	0.18	35.51	0.73
69	CMT4+	26.03	20.52	1.71	37.48	3.20	32.22	5.84	31.88	1.64	31.84	8.93	33.81	5.21

Sample	Type	miR-92a	miR-21-5p		miR-122		miR-146a		miR-155		miR-222		miR-383	
		CT	**CT**	**Exp**	**CT**	**Exp**	**CT**	**Exp**	**CT**	**Exp**	**CT**	**Exp**	**CT**	**Exp**
70	Normal	24.14	20.29	0.91	32.39	0.19	25.54	0.20	27.68	0.18	24.37	0.06	29.73	0.52
71	Normal	22.95	18.47	0.82	29.91	0.02	23.46	0.18	25.73	0.21	22.33	0.04	28.88	0.49
72	Normal	25.00	21.75	0.84	32.12	0.26	28.00	0.39	29.94	0.33	27.04	0.16	32.87	0.41
73	Normal	24.04	19.87	0.62	32.82	0.05	26.70	0.39	26.86	0.25	24.00	0.07	30.78	0.79
74	CMT−	24.02	19.98	0.96	33.64	0.79	26.03	0.66	26.33	2.65	23.92	0.45	31.49	0.48
75	CMT3+	25.11	17.89	3.04	31.96	10.41	27.39	64.35	28.83	7.54	25.92	20.18	28.78	6.41
76	CMT−	26.08	18.90	0.49	34.25	2.73	28.89	0.91	29.68	0.92	27.10	0.89	29.93	0.80
77	CMT+	26.05	18.87	0.83	33.95	4.91	29.25	3.19	30.20	1.17	28.64	7.04	30.61	1.84
78	CMT−	26.20	19.61	0.79	32.65	0.15	29.89	0.42	30.19	0.57	29.10	0.29	30.82	0.64
79	CMT3+	25.73	18.63	8.67	32.79	4.24	29.43	40.62	30.16	23.67	27.15	203.17	29.87	8.12
80	CMT+	26.51	18.58	2.43	31.87	9.69	26.75	7.75	28.74	1.95	26.76	7.22	30.35	1.19
81	CMT−	26.26	20.20	3.51	32.70	6.49	30.84	14.43	31.17	6.75	28.02	23.95	31.90	2.02
82	CMT3+	27.01	17.57	10.95	33.67	10.53	27.92	162.45	27.59	25.80	23.93	184.99	30.51	9.00
83	CMT−	26.79	19.18	0.36	32.26	0.62	30.09	0.10	30.97	0.31	28.52	0.01	33.06	0.05
84	CMT2+	27.91	18.13	2.55	33.25	5.09	26.82	9.49	28.36	2.90	24.96	5.74	31.26	0.81
85	CMT−	26.86	19.17	0.56	33.25	0.15	29.86	1.04	30.46	0.61	28.92	0.43	33.69	0.42
86	CMT2+	25.68	21.54	0.22	36.02	0.33	33.50	0.34	29.58	2.36	32.68	0.19	33.68	0.36

Appendix 2-5 CT values of miRNA in bovine mastitis serum. CT values shown as the mean values of duplicated experiment.

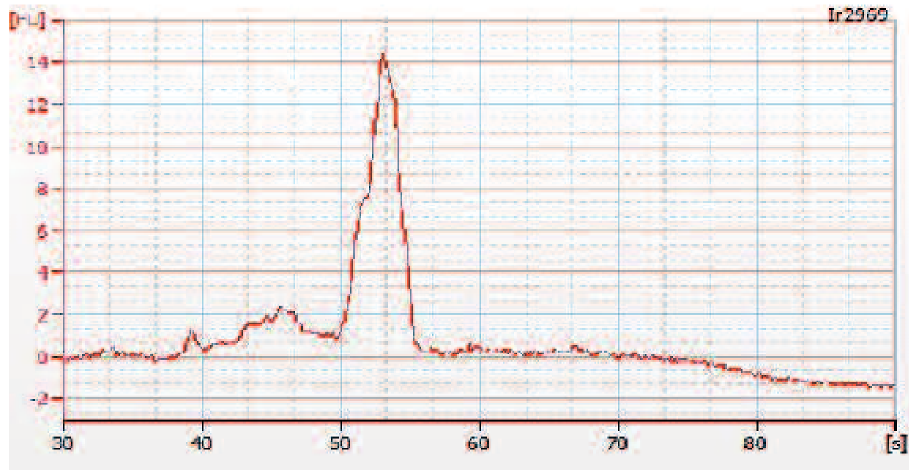
Sample	Type	cel-miR-39	miR-16	miR-146a	miR-155	miR-21	miR-222	miR-383
1	Normal	16.75	23.45	31.13	33.06	30.58	29.48	34.50
2	Normal	17.09	22.84	30.54	33.02	30.59	29.25	34.66
3	Normal	17.03	27.49	32.84	33.69	31.00	30.61	35.39
4	Normal	17.72	27.50	35.62	33.52	30.49	30.45	34.25
5	Normal	17.36	23.86	31.46	33.40	31.20	28.96	34.58
6	Normal	15.91	24.20	31.99	33.59	31.49	29.43	35.49
7	Normal	16.70	25.60	37.23	33.98	30.70	30.29	35.70
8	Normal	16.33	25.51	33.66	33.27	30.81	29.96	34.04
9	Normal	16.92	24.45	31.82	32.17	30.18	28.73	32.69
10	Normal	17.30	26.22	33.21	33.07	30.39	29.97	33.59
11	Normal	17.43	24.03	31.78	32.36	30.05	28.61	33.66
12	Mastitis	16.94	24.00	32.09	33.82	30.01	29.17	35.28
13	Mastitis	16.30	25.92	31.70	33.91	32.00	30.61	33.59
14	Mastitis	17.61	26.04	33.15	33.36	30.26	29.15	33.70
15	Mastitis	17.29	26.69	33.17	32.42	29.05	29.48	35.47
16	Mastitis	17.00	24.85	32.77	32.03	29.47	28.97	35.94
17	Mastitis	17.44	26.16	38.18	33.62	30.65	30.01	35.22
18	Mastitis	17.42	24.31	32.54	32.99	29.83	29.14	34.64
19	Mastitis	16.87	25.11	32.02	33.43	30.39	29.55	34.65
20	Mastitis	17.48	25.08	31.33	34.18	30.50	29.74	35.10
21	Mastitis	18.08	25.78	33.26	33.29	29.89	28.64	34.09
22	Mastitis	16.26	26.64	32.59	33.02	29.94	29.80	33.62
23	Mastitis	16.98	27.26	35.51	33.68	30.05	30.29	34.31
24	Mastitis	16.80	23.64	34.24	32.68	29.49	29.03	34.53
25	Mastitis	17.32	26.42	33.61	34.12	31.31	30.72	34.80
26	Mastitis	17.25	26.08	33.43	32.93	30.12	29.61	33.90

Appendix 2-6 Relative expression values normalized to cel-miR-39 and miR-16. CT values shown as the mean values of duplicated experiment. cel-39: cel-miR-39.

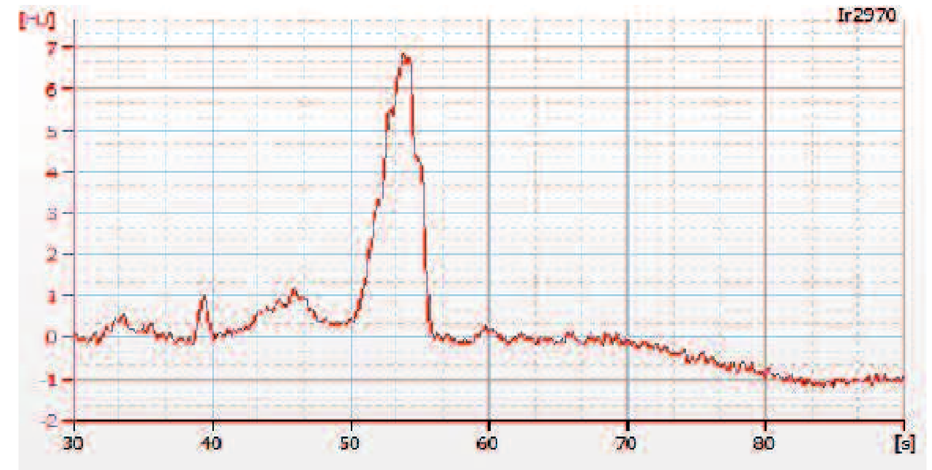
Sample	**Type**	**miR-146a**		**miR-155**		**miR-21**		**miR-222**		**miR-383**	
		cel-39	miR-16	cel-39	miR-16	cel-39	miR-16	cel-39	miR-16	cel-39	miR-16
1	Normal	3.46	1.03	0.88	0.26	0.67	0.20	0.83	0.25	0.77	0.23
2	Normal	6.57	1.01	1.15	0.18	0.84	0.13	1.24	0.19	0.88	0.13
3	Normal	1.29	5.18	0.69	2.81	0.61	2.46	0.47	1.87	0.51	2.05
4	Normal	0.30	0.76	1.25	3.17	1.39	3.52	0.84	2.10	1.80	4.53
5	Normal	4.22	1.09	1.07	0.28	0.66	0.17	1.83	0.47	1.12	0.29
6	Normal	1.07	0.95	0.34	0.31	0.20	0.18	0.49	0.43	0.22	0.19
7	Normal	0.05	0.07	0.45	0.62	0.59	0.82	0.46	0.63	0.32	0.44
8	Normal	0.45	0.74	0.57	0.96	0.43	0.72	0.45	0.74	0.80	1.32
9	Normal	2.42	1.28	1.85	0.98	1.00	0.53	1.58	0.84	3.05	1.61
10	Normal	1.20	1.67	1.28	1.80	1.12	1.56	0.87	1.21	2.12	2.95
11	Normal	3.54	0.98	2.30	0.64	1.55	0.43	2.45	0.68	2.22	0.61
12	Mastitis	2.02	0.77	0.60	0.23	1.14	0.44	1.18	0.45	0.51	0.20
13	Mastitis	1.70	3.83	0.36	0.81	0.18	0.42	0.28	0.63	1.07	2.40
14	Mastitis	1.55	1.53	1.31	1.30	1.51	1.50	1.91	1.89	2.45	2.42
15	Mastitis	1.22	2.36	2.00	3.90	2.81	5.48	1.21	2.35	0.57	1.11
16	Mastitis	1.32	0.87	2.15	1.43	1.72	1.14	1.42	0.94	0.34	0.22
17	Mastitis	0.04	0.05	0.97	1.17	1.03	1.25	0.94	1.12	0.76	0.91
18	Mastitis	2.07	0.70	1.48	0.50	1.79	0.61	1.68	0.57	1.12	0.38
19	Mastitis	2.02	1.75	0.74	0.65	0.83	0.72	0.86	0.75	0.76	0.66
20	Mastitis	4.97	2.76	0.67	0.38	1.17	0.65	1.16	0.64	0.84	0.47
21	Mastitis	1.98	1.18	1.88	1.13	2.70	1.63	3.74	2.23	2.58	1.54
22	Mastitis	0.89	3.41	0.64	2.48	0.74	2.86	0.48	1.82	1.01	3.88
23	Mastitis	0.19	0.69	0.67	2.41	1.14	4.06	0.56	1.99	1.04	3.69
24	Mastitis	0.41	0.14	1.19	0.39	1.48	0.48	1.19	0.39	0.78	0.26
25	Mastitis	0.92	1.45	0.63	0.99	0.60	0.95	0.53	0.83	0.93	1.47
26	Mastitis	0.99	1.30	1.37	1.80	1.30	1.71	1.08	1.41	1.66	2.17

Appendix 3-1 NanoDrop Spectrophotmeter and 2100 Bioanalyzer System results. Analysis and the report were provided by Hokkaido System Science Co., Ltd.

No.	Sample		Nanodrop		
			A260/A280	A260/A230	ng/µl
1	618 右後	Normal 1	1.39	0.29	134.6
2	626 左前	Normal 2	1.35	0.22	105.2
3	629 右前	Normal 3	1.43	0.26	129.9
4	632 右前	Normal 4	1.29	0.25	124.5
5	633 左前	Normal 5	1.35	0.33	155.7
6	601 右後	CMT− 1	1.07	0.34	133.9
7	603 右後	CMT− 2	1.22	0.23	110.4
8	608 左前	CMT− 3	1.33	0.2	96.2
9	620 右前	CMT− 4	1.13	0.4	128.6
10	634 左前	CMT− 5	1.23	0.27	137.2
11	601 左後	CMT+ 1	1.39	0.78	318.6
12	603 左後	CMT+ 2	1.54	0.5	252.6
13	608 右前	CMT+ 3	1.37	1.13	207.5
14	620 右後	CMT+ 4	1.45	0.61	266.4
15	634 左後	CMT+ 5	1.45	0.51	264.1



618 右後_Normal 1



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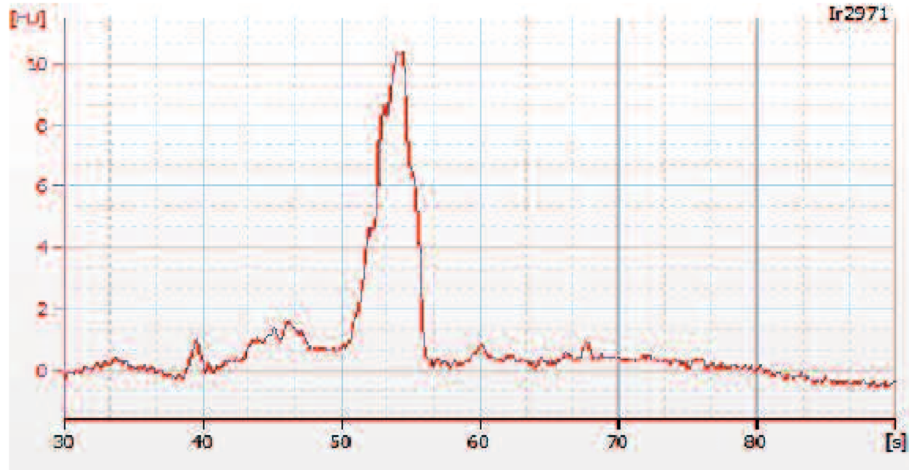
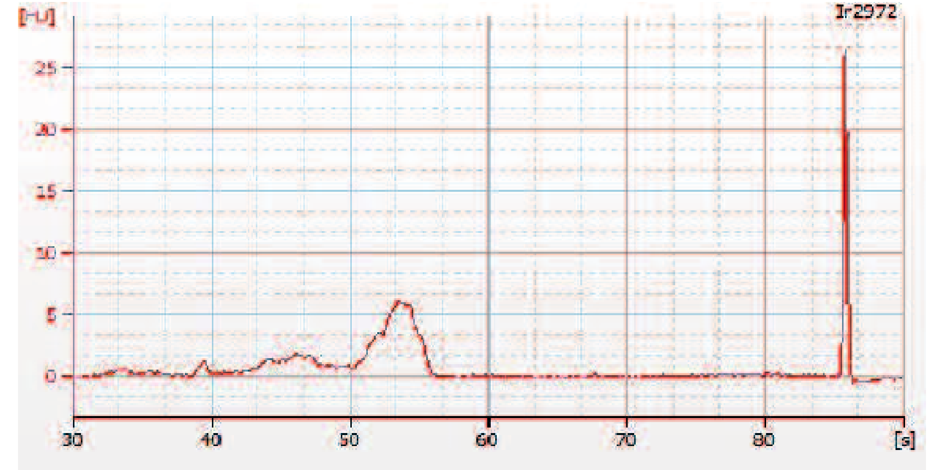


629 右前_Normal 3



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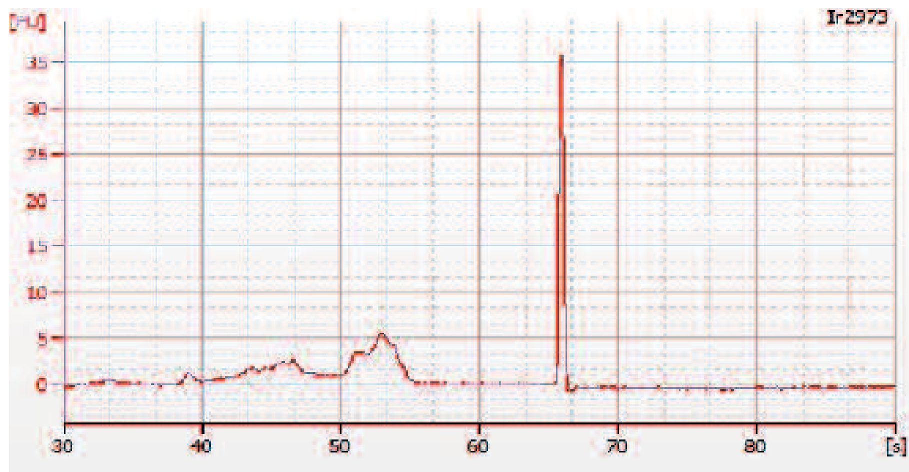
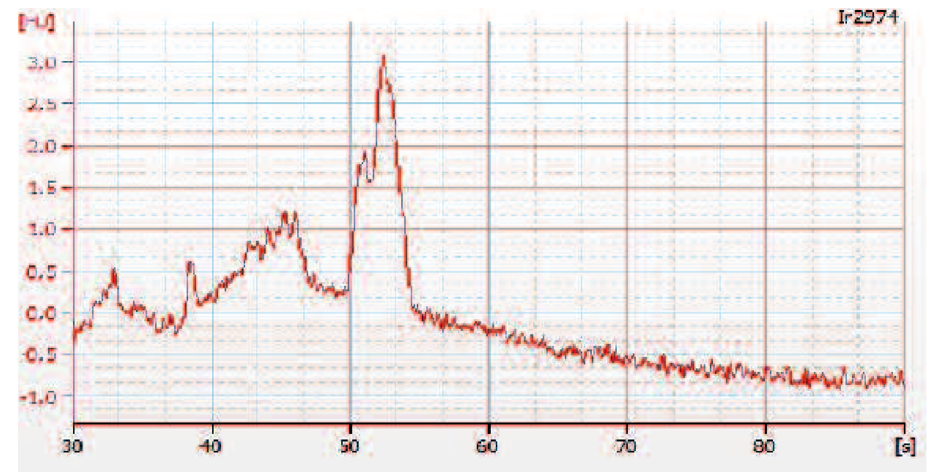
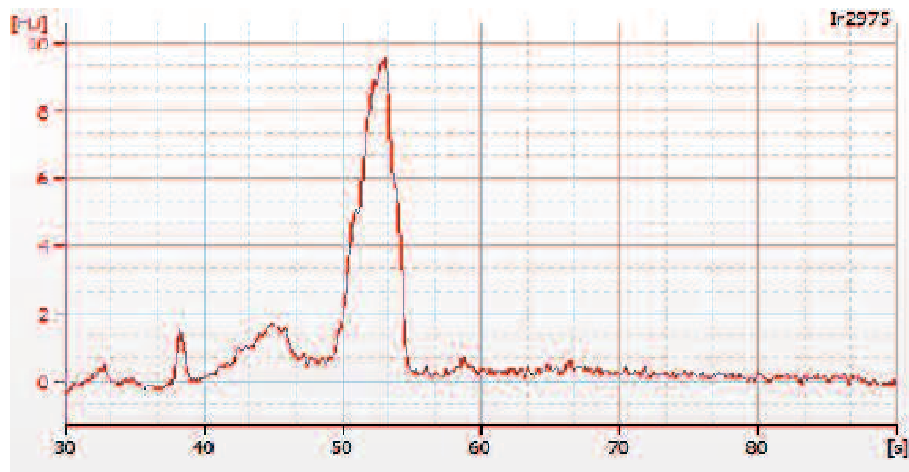


633 左前_Normal 5



601 右後_CMT−1



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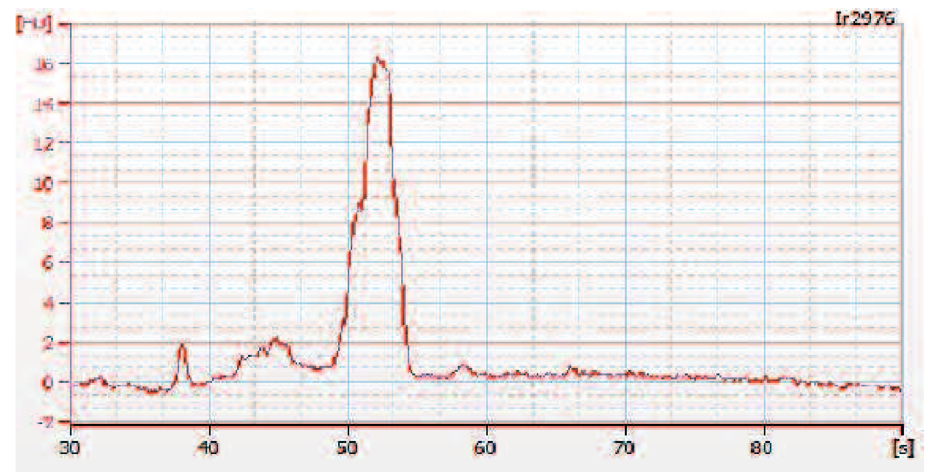

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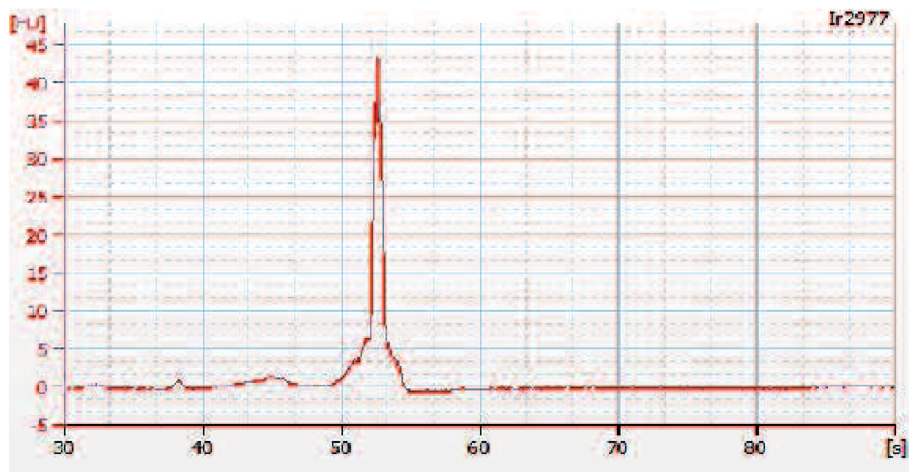

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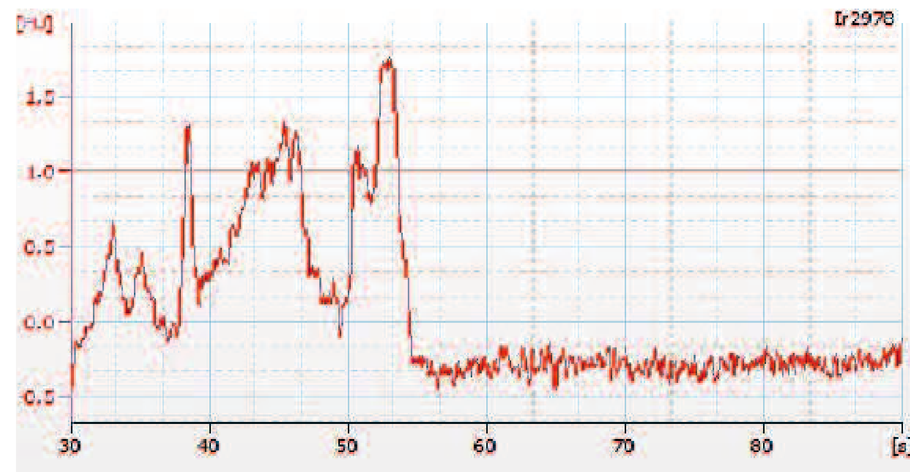

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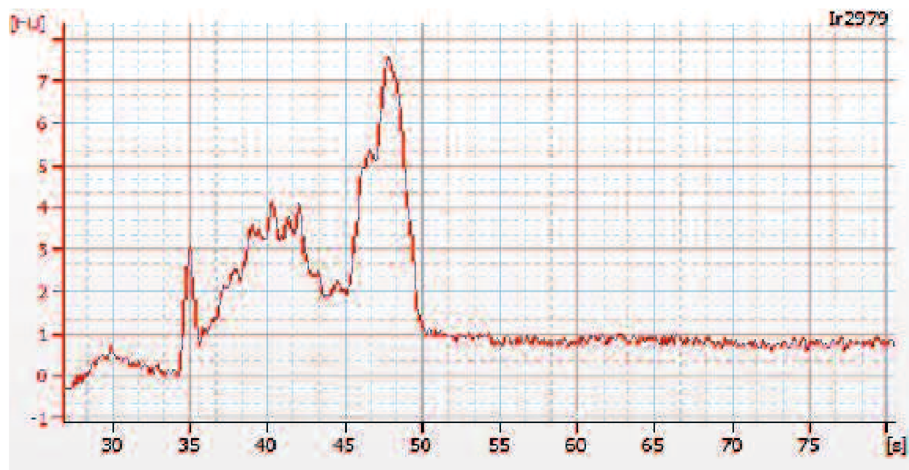

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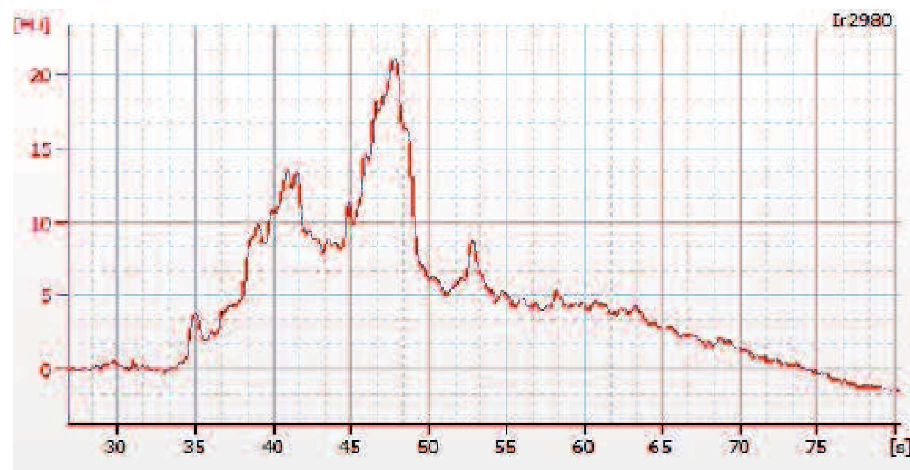

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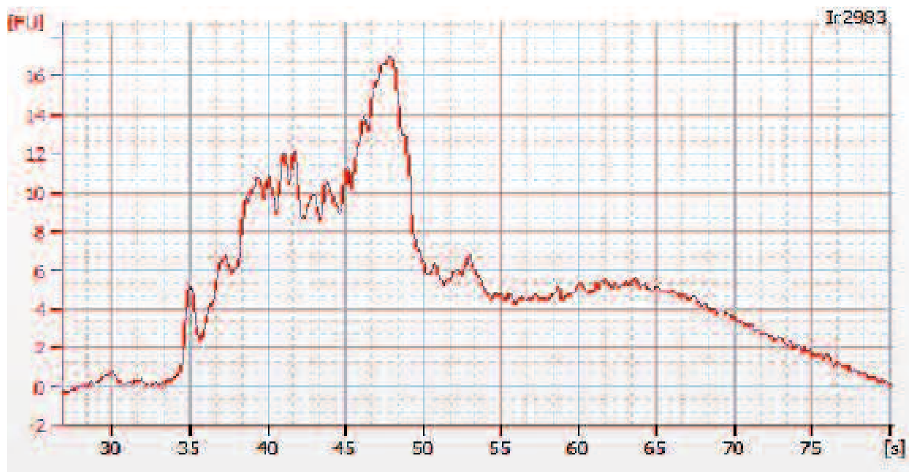

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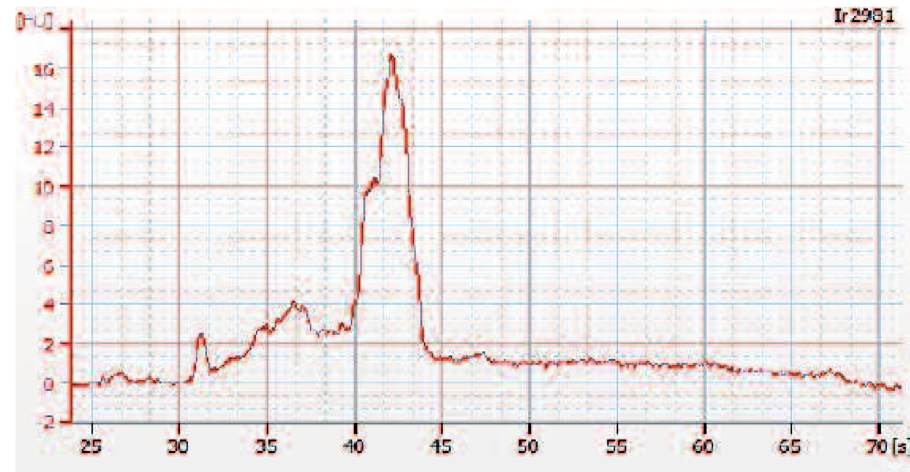
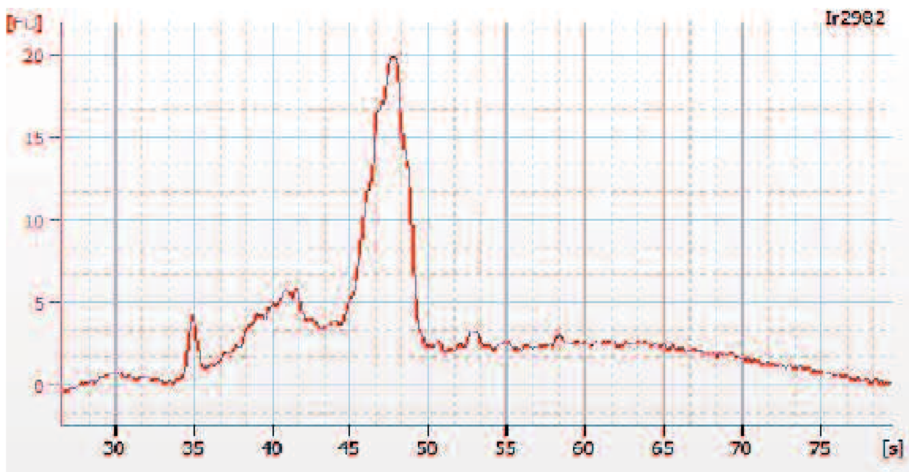


620 右後 CMT+ 4



634 左後 CMT+ 5

Appendix 3-2 CT values and relative expression normalized to miR-92a of miRNA in normal, CMT− and CMT+ quarters. CT: CT value; Exp: relative expression.

Sample	Type	miR-92a	miR-142-5p		miR-221		miR-15a		miR-23b-3p		miR-2284w		miR-6529a		U2	
		CT	CT	Exp	CT	Exp	CT	Exp	CT	Exp	CT	Exp	CT	Exp	CT	Exp
1	normal	25.80	33.73	0.10	31.39	0.43	28.10	0.87	26.40	4.50	40.00	0.24	33.35	0.27	23.45	0.35
2	normal	25.75	34.45	0.06	32.17	0.25	28.60	0.60	26.92	3.04	40.00	0.23	34.06	0.16	24.84	0.13
3	normal	25.70	33.60	0.10	31.45	0.39	28.34	0.69	27.22	2.38	40.00	0.22	33.38	0.25	23.54	0.30
4	normal	25.53	33.71	0.08	31.42	0.35	28.23	0.67	26.76	2.91	40.00	0.20	34.87	0.08	23.83	0.22
5	normal	25.85	33.93	0.09	30.94	0.61	28.15	0.87	26.38	4.72	40.00	0.25	32.71	0.43	22.07	0.93
6	normal	25.75	30.84	0.69	28.00	4.42	27.89	0.98	26.86	3.17	38.37	0.71	32.41	0.50	20.54	2.53
7	normal	25.28	33.72	0.07	30.88	0.43	27.59	0.87	25.85	4.58	40.00	0.17	31.62	0.62	21.34	1.04
8	normal	25.66	31.36	0.45	28.73	2.49	27.47	1.23	25.85	5.96	40.00	0.22	32.41	0.47	20.92	1.81
9	normal	26.48	34.79	0.07	32.37	0.35	28.15	1.35	26.20	8.29	40.00	0.38	32.54	0.75	23.21	0.65
10	normal	26.14	34.48	0.07	32.51	0.25	27.99	1.20	25.85	8.36	40.00	0.30	34.11	0.20	22.65	0.76
11	normal	26.36	33.61	0.15	32.96	0.22	27.77	1.62	25.87	9.59	40.00	0.35	33.06	0.48	23.56	0.47
12	normal	25.91	35.60	0.03	32.20	0.27	27.89	1.09	25.83	7.22	40.00	0.26	33.55	0.25	22.76	0.60
13	normal	25.46	32.65	0.16	30.23	0.77	27.76	0.88	25.88	5.11	40.00	0.19	32.42	0.40	22.18	0.66
14	normal	25.98	33.31	0.15	31.20	0.56	28.13	0.97	26.31	5.42	40.00	0.27	33.82	0.22	24.09	0.25
15	normal	25.99	33.68	0.11	31.58	0.44	27.32	1.72	25.61	8.89	40.00	0.27	32.39	0.60	23.72	0.33
16	normal	26.17	29.03	3.26	27.56	8.00	27.11	2.25	25.93	8.07	38.17	1.10	32.32	0.71	21.29	2.00
17	normal	25.61	28.79	2.61	27.70	4.95	27.37	1.28	26.37	4.05	38.48	0.60	32.61	0.39	21.38	1.28

Sample	Type	miR-92a	miR-142-5p		miR-221		miR-15a		miR-23b-3p		miR-2284w		miR-6529a		U2	
		CT	CT	Exp	CT	Exp	CT	Exp	CT	Exp	CT	Exp	CT	Exp	CT	Exp
18	normal	26.14	32.34	0.32	29.85	1.61	28.93	0.62	26.10	7.02	40.00	0.30	33.40	0.33	23.03	0.59
19	CMT−	25.23	32.75	0.13	29.47	1.11	27.01	1.26	25.53	5.57	40.00	0.16	31.22	0.79	20.57	1.72
20	CMT−	25.34	35.39	0.02	31.29	0.34	27.88	0.74	25.66	5.47	40.00	0.17	32.50	0.35	21.72	0.83
21	CMT3+	24.14	23.46	37.67	19.97	376.79	22.65	12.09	26.48	1.35	30.92	40.76	25.17	24.58	13.84	85.70
22	CMT4+	22.95	20.71	111.48	18.54	445.38	20.81	19.08	26.99	0.41	28.75	80.62	23.68	30.29	13.02	66.13
23	CMT−	25.85	35.49	0.03	31.44	0.44	28.56	0.66	27.12	2.82	40.00	0.25	32.96	0.36	22.86	0.54
24	CMT−	25.59	33.61	0.09	30.60	0.65	28.78	0.47	26.68	3.22	40.00	0.21	33.20	0.26	21.92	0.87
25	CMT−	25.40	34.38	0.05	32.09	0.20	28.61	0.47	28.05	1.09	40.00	0.18	32.34	0.41	23.53	0.25
26	CMT3+	25.00	25.49	16.84	27.44	3.88	27.69	0.67	27.95	0.88	37.71	0.67	31.94	0.41	18.72	5.30
27	CMT3+	24.04	23.31	39.16	21.08	163.41	23.75	5.30	25.27	2.93	30.75	43.01	25.69	16.01	13.95	74.24
28	CMT−	25.63	27.83	5.13	29.53	1.40	29.94	0.22	27.84	1.47	39.46	0.31	32.24	0.51	21.82	0.95
29	CMT3+	24.02	22.27	79.29	20.86	187.49	23.27	7.26	24.99	3.48	30.61	46.63	25.63	16.53	14.99	35.68
30	CMT−	25.76	29.71	1.52	31.08	0.53	29.07	0.44	26.88	3.14	40.00	0.23	33.17	0.29	22.17	0.82
31	normal	29.42	36.86	0.14	35.76	0.26	35.46	0.07	35.80	0.08	40.00	2.94	35.23	0.90	26.46	0.53
32	normal	26.40	33.39	0.19	32.43	0.32	28.34	1.13	28.09	2.13	40.00	0.36	33.80	0.30	23.20	0.63
33	normal	26.61	32.15	0.51	33.52	0.17	30.37	0.32	31.27	0.27	40.00	0.42	36.33	0.06	23.91	0.44
34	normal	26.76	33.60	0.21	35.96	0.04	31.85	0.13	30.60	0.48	40.00	0.46	36.60	0.05	23.79	0.53
35	normal	25.97	35.27	0.04	33.89	0.09	36.23	0.00	33.05	0.05	40.00	0.27	36.11	0.04	27.02	0.03
36	normal	28.47	34.44	0.38	36.24	0.10	28.83	3.38	26.99	19.13	40.00	1.52	33.67	1.37	24.31	1.22
37	normal	27.40	32.84	0.54	32.13	0.79	26.05	11.01	25.94	18.81	40.00	0.72	33.62	0.67	24.21	0.62
38	normal	28.88	32.18	2.40	35.87	0.17	30.00	1.99	28.92	6.63	40.00	2.01	35.96	0.37	24.62	1.30
39	normal	25.31	29.45	1.34	28.62	2.12	24.94	5.59	24.51	11.91	35.31	4.39	29.02	3.86	20.04	2.64

Sample	Type	miR-92a	miR-142-5p		miR-221		miR-15a		miR-23b-3p		miR-2284w		miR-6529a		U2	
		CT	CT	Exp	CT	Exp	CT	Exp	CT	Exp	CT	Exp	CT	Exp	CT	Exp
40	normal	28.58	32.01	2.19	34.45	0.36	30.51	1.13	29.25	4.28	40.00	1.64	37.60	0.10	24.78	0.95
41	normal	25.32	33.54	0.08	32.42	0.15	26.74	1.61	28.91	0.57	40.00	0.17	32.98	0.25	24.11	0.16
42	normal	26.28	28.41	5.40	28.19	5.57	26.25	4.42	27.90	2.22	34.16	18.98	29.80	4.37	19.99	5.32
43	normal	25.83	31.02	0.64	31.08	0.55	25.96	3.93	26.16	5.40	40.00	0.24	31.74	0.83	22.76	0.57
44	CMT−	25.22	30.79	0.50	30.49	0.54	25.94	2.62	27.15	1.79	37.35	1.00	30.71	1.12	21.88	0.69
45	CMT4+	25.11	24.73	30.81	24.52	31.63	23.43	13.88	27.28	1.52	32.01	37.60	26.34	21.44	16.01	37.47
46	CMT−	25.76	29.98	1.26	29.43	1.65	26.06	3.51	27.05	2.78	36.95	1.92	30.26	2.21	20.95	1.90
47	CMT3+	26.08	26.95	12.87	25.63	28.53	24.46	13.21	26.43	5.35	36.62	3.00	27.44	19.54	16.02	72.18
48	Normal	26.14	35.63	0.03	34.57	0.06	26.42	3.56	27.89	2.03	40.00	0.30	31.77	1.01	24.94	0.16
49	CMT−	25.98	32.01	0.36	31.75	0.38	27.07	2.02	29.54	0.58	40.00	0.27	31.35	1.21	23.21	0.46
50	CMT2+	26.05	26.68	15.34	25.49	31.04	24.02	17.66	29.00	0.88	36.98	2.30	27.81	14.88	16.61	47.43
51	CMT2+	26.20	27.04	13.20	25.98	24.40	24.83	11.13	30.03	0.48	36.23	4.27	28.23	12.33	17.23	33.98
52	CMT−	25.52	34.79	0.04	34.44	0.04	26.99	1.56	28.17	1.08	40.00	0.20	32.00	0.56	23.71	0.24
53	CMT−	25.84	32.86	0.18	31.14	0.53	26.02	3.83	27.03	2.99	40.00	0.25	30.46	2.04	20.42	2.92
54	CMT4+	25.73	26.22	16.85	25.25	29.18	23.73	17.31	27.85	1.57	33.56	19.74	26.93	21.92	17.09	27.11
55	Normal	25.67	34.92	0.04	34.19	0.06	26.32	2.75	26.45	3.95	40.00	0.22	31.47	0.90	24.53	0.15
56	Normal	25.54	34.13	0.06	33.73	0.07	26.95	1.63	27.42	1.85	40.00	0.20	32.62	0.37	23.22	0.34
57	Normal	25.92	33.00	0.17	32.45	0.23	26.03	3.99	26.04	6.27	40.00	0.26	31.29	1.21	23.44	0.38
58	Normal	26.14	34.09	0.10	33.12	0.17	26.96	2.46	28.45	1.38	40.00	0.30	31.92	0.91	23.80	0.34
59	CMT−	25.68	32.35	0.23	30.17	0.93	26.88	1.87	28.37	1.06	40.00	0.22	31.53	0.87	22.13	0.80
60	CMT3+	26.51	25.94	35.10	25.00	59.72	23.62	31.98	26.98	4.92	34.77	14.66	27.98	18.16	14.90	213.00
61	CMT−	25.86	31.18	0.59	29.67	1.49	25.77	4.57	25.42	9.25	35.50	5.60	30.27	2.35	20.61	2.58

Sample	Type	miR-92a	miR-142-5p		miR-221		miR-15a		miR-23b-3p		miR-2284w		miR-6529a		U2	
		CT	CT	Exp	CT	Exp	CT	Exp	CT	Exp	CT	Exp	CT	Exp	CT	Exp
62	CMT+	26.26	28.49	5.02	27.04	12.19	25.68	6.44	26.44	6.01	34.64	13.49	28.74	8.99	18.56	14.07
63	CMT−	25.87	32.44	0.25	31.56	0.41	27.10	1.84	26.32	5.00	40.00	0.25	30.45	2.10	22.07	0.95
64	CMT3+	27.01	23.79	220.39	23.34	267.29	22.54	95.80	25.95	14.30	32.02	139.24	25.46	147.01	15.21	243.05
65	CMT+	26.79	26.73	24.69	26.64	23.41	25.43	11.08	28.21	2.56	40.00	0.47	28.89	11.73	17.90	32.30
66	CMT−	27.22	27.20	23.96	26.94	25.43	25.37	15.59	26.54	10.94	35.21	17.63	28.36	22.76	17.97	41.32
67	CMT3+	27.91	24.27	294.39	22.98	638.39	22.60	171.08	26.94	13.36	33.53	91.22	25.81	215.00	14.47	757.07
68	CMT−	24.78	36.38	0.01	33.72	0.04	27.50	0.66	30.19	0.16	40.00	0.12	32.96	0.17	22.78	0.27
69	CMT2+	26.86	28.36	8.34	27.24	16.02	26.00	7.82	29.20	1.34	40.00	0.50	29.65	7.26	17.72	38.35
70	CMT3+	24.60	28.05	2.16	26.67	4.98	24.39	4.99	25.11	4.77	37.37	0.64	27.77	5.57	16.90	14.07
71	CMT2+	24.86	27.98	2.70	26.92	5.00	24.07	7.42	25.94	3.21	34.69	4.91	28.76	3.35	16.74	18.83
72	CMT2+	24.91	26.71	6.79	25.96	10.14	23.76	9.61	26.60	2.12	36.16	1.85	27.98	5.98	16.80	18.81
73	CMT3+	25.03	29.18	1.34	28.60	1.77	25.70	2.72	26.52	2.43	35.71	2.75	29.56	2.18	19.88	2.42
74	CMT3+	24.19	27.24	2.86	26.12	5.52	23.70	6.05	26.09	1.83	35.49	1.78	26.98	7.28	16.59	13.23
75	CMT−	24.81	26.47	7.48	28.00	2.30	27.98	0.48	30.34	0.15	40.00	0.12	32.43	0.25	23.35	0.19
76	CMT−	24.55	30.11	0.50	30.64	0.31	27.73	0.48	30.05	0.15	38.13	0.37	33.59	0.10	24.08	0.09
77	CMT−	24.25	27.98	1.77	29.96	0.40	27.32	0.51	28.71	0.31	40.00	0.08	33.37	0.09	23.29	0.13
78	CMT−	24.23	31.27	0.18	30.69	0.24	27.91	0.34	30.37	0.10	40.00	0.08	34.25	0.05	22.77	0.19
79	CMT−	23.76	31.41	0.12	32.56	0.05	26.64	0.59	25.83	1.62	37.95	0.24	31.47	0.24	21.72	0.28
80	CMT−	24.41	27.63	2.53	29.22	0.74	26.35	1.12	27.69	0.70	35.52	2.02	31.15	0.47	21.95	0.37
81	CMT2+	26.14	33.05	0.20	31.48	0.52	38.50	0.00	35.04	0.01	38.18	1.06	33.19	0.38	29.53	0.01
82	CMT1+	25.56	31.37	0.42	30.89	0.52	35.42	0.00	36.20	0.00	37.78	0.94	33.68	0.18	29.08	0.01
83	CMT1+	24.76	29.25	1.05	29.20	0.97	30.66	0.07	33.42	0.02	37.60	0.61	32.20	0.29	23.95	0.12

Sample	Type	miR-92a	miR-142-5p		miR-221		miR-15a		miR-23b-3p		miR-2284w		miR-6529a		U2	
		CT	**CT**	**Exp**	**CT**	**Exp**	**CT**	**Exp**	**CT**	**Exp**	**CT**	**Exp**	**CT**	**Exp**	**CT**	**Exp**
84	CMT2+	24.04	26.09	5.70	28.22	1.15	25.75	1.32	25.68	2.19	33.99	4.53	28.83	1.82	18.82	2.54
85	CMT2+	24.20	25.84	7.55	27.51	2.12	26.93	0.65	29.85	0.14	34.76	2.97	28.81	2.05	21.84	0.35
86	CMT1+	24.63	25.21	15.78	27.89	2.19	25.98	1.70	27.85	0.73	33.85	7.56	29.07	2.32	21.04	0.82
87	CMT2+	24.24	26.75	4.14	28.75	0.92	27.43	0.47	30.51	0.09	35.53	1.79	30.52	0.65	23.09	0.15
88	Normal	23.51	27.55	1.44	28.99	0.47	24.68	1.91	24.41	3.66	33.58	4.18	28.85	1.24	17.89	3.34
89	Normal	23.96	29.21	0.62	30.16	0.29	25.25	1.77	25.00	3.32	33.74	5.12	30.02	0.75	19.01	2.10
90	Normal	25.03	31.55	0.26	33.04	0.08	28.55	0.38	30.03	0.21	40.00	0.14	32.67	0.25	23.38	0.21
91	Normal	25.05	31.36	0.30	33.13	0.08	28.15	0.50	30.31	0.18	40.00	0.14	32.27	0.34	24.47	0.10
92	Normal	25.58	32.52	0.19	34.30	0.05	27.67	1.01	28.35	1.00	37.57	1.10	33.26	0.25	24.83	0.11
93	Normal	25.24	32.82	0.12	33.56	0.07	28.18	0.56	30.39	0.19	38.20	0.56	35.70	0.04	26.42	0.03
94	Normal	25.50	32.85	0.14	33.97	0.06	28.95	0.39	31.52	0.10	40.00	0.19	35.07	0.07	26.82	0.03
95	Normal	24.96	32.87	0.10	34.13	0.04	27.26	0.88	28.67	0.52	40.00	0.13	34.85	0.05	25.23	0.06
96	Normal	25.19	34.38	0.04	34.94	0.02	27.86	0.68	29.83	0.27	40.00	0.16	33.72	0.14	26.50	0.03
97	CMT−	24.65	28.77	1.36	29.88	0.56	28.77	0.25	31.47	0.06	39.67	0.13	31.76	0.36	24.16	0.10
98	CMT−	24.37	26.65	4.87	28.85	0.94	26.30	1.13	26.47	1.59	35.75	1.68	29.53	1.40	20.05	1.36
99	CMT2+	26.30	27.54	9.99	28.10	6.00	28.85	0.74	31.82	0.15	33.75	25.58	30.17	3.42	21.64	1.72
100	CMT−	24.41	32.06	0.12	33.12	0.05	28.25	0.30	31.57	0.05	40.00	0.09	32.47	0.19	25.72	0.03
101	CMT2+	29.35	30.82	8.50	31.32	5.32	36.53	0.03	34.51	0.19	35.63	57.73	30.04	31.00	22.31	8.92
102	CMT−	23.98	30.64	0.23	32.26	0.07	26.40	0.81	27.83	0.47	38.10	0.25	30.54	0.53	23.27	0.11
103	CMT−	24.72	28.47	1.76	30.05	0.52	28.55	0.30	31.06	0.08	38.01	0.45	31.27	0.54	24.08	0.11
104	Normal	30.82	36.06	0.62	35.71	0.71	38.15	0.03	36.52	0.13	40.00	7.70	36.97	0.70	29.00	0.24
105	CMT−	24.87	30.26	0.56	31.49	0.21	26.28	1.62	26.55	2.13	40.00	0.13	30.62	0.93	20.68	1.25

Sample	Type	miR-92a	miR-142-5p		miR-221		miR-15a		miR-23b-3p		miR-2284w		miR-6529a		U2	
		CT	CT	Exp	CT	Exp	CT	Exp	CT	Exp	CT	Exp	CT	Exp	CT	Exp
106	CMT−	25.24	29.19	1.53	31.03	0.38	26.91	1.36	28.40	0.76	38.56	0.44	32.52	0.32	22.90	0.34
107	CMT−	25.46	31.62	0.33	31.58	0.30	28.41	0.56	31.53	0.10	40.00	0.19	33.30	0.22	24.39	0.14
108	CMT2+	27.84	29.89	5.70	32.32	0.94	33.71	0.07	33.49	0.14	34.90	33.60	31.01	5.56	22.96	1.99
109	CMT−	24.58	28.65	1.41	31.79	0.14	26.01	1.61	25.84	2.85	40.00	0.10	29.78	1.37	22.11	0.38
110	CMT2+	25.40	26.33	12.33	29.76	1.02	26.62	1.85	28.25	0.94	35.54	3.97	30.02	2.05	21.37	1.11
111	CMT−	25.31	23.70	72.44	26.38	9.99	25.59	3.56	29.26	0.44	34.40	8.25	28.30	6.34	20.04	2.63
112	CMT1+	24.77	26.76	5.96	29.22	0.96	26.59	1.23	26.68	1.82	38.38	0.36	29.66	1.70	21.00	0.93
113	CMT−	27.90	31.01	2.72	31.10	2.27	36.43	0.01	35.10	0.05	35.77	19.10	32.20	2.55	27.11	0.12
114	CMT−	26.55	30.62	1.40	30.02	1.89	31.25	0.17	33.84	0.04	36.41	4.82	31.86	1.27	25.78	0.12
115	CMT−	26.00	32.00	0.37	32.35	0.26	31.86	0.07	34.03	0.03	39.59	0.36	33.05	0.38	26.41	0.05
116	CMT2+	26.86	25.95	44.28	28.76	5.62	27.48	2.80	30.07	0.74	35.34	12.53	29.16	10.18	17.96	32.37
117	CMT+	26.63	27.02	17.99	28.16	7.25	28.15	1.50	28.84	1.48	38.03	1.65	29.92	5.14	18.71	16.41
118	CMT2+	26.48	25.61	43.02	28.72	4.43	28.60	0.99	30.17	0.53	37.31	2.46	28.76	10.31	19.60	8.01
119	CMT−	25.27	28.83	2.00	31.04	0.39	27.54	0.90	29.15	0.46	38.15	0.59	30.25	1.59	21.53	0.90
120	CMT4+	24.17	20.93	221.42	23.51	33.07	23.12	8.90	26.19	1.68	31.78	22.86	25.42	21.08	14.70	48.10
121	CMT−	23.58	26.93	2.31	29.78	0.29	25.23	1.38	24.64	3.28	37.24	0.35	28.57	1.58	20.05	0.79
122	CMT−	24.77	24.62	26.07	27.60	2.93	26.46	1.34	25.40	4.39	34.61	4.90	28.61	3.50	14.88	64.33
123	CMT+	25.15	26.27	10.88	26.33	9.28	26.55	1.64	26.92	2.01	36.98	1.23	28.83	3.93	18.27	8.01
124	CMT−	24.52	30.82	0.30	30.94	0.25	26.71	0.94	26.23	2.08	40.00	0.10	31.01	0.56	21.62	0.51
125	CMT2+	24.93	27.28	4.64	27.28	4.12	26.49	1.46	27.16	1.46	34.41	6.29	29.79	1.74	20.09	1.95
126	Normal	25.99	33.26	0.15	33.36	0.13	30.35	0.21	31.63	0.14	40.00	0.27	33.12	0.36	23.47	0.39
127	Normal	25.07	28.72	1.87	29.10	1.28	28.28	0.47	28.41	0.67	37.87	0.63	30.49	1.17	21.25	0.96

Sample	Type	miR-92a	miR-142-5p		miR-221		miR-15a		miR-23b-3p		miR-2284w		miR-6529a		U2	
		CT	CT	Exp	CT	Exp	CT	Exp	CT	Exp	CT	Exp	CT	Exp	CT	Exp
128	CMT−	24.92	30.17	0.62	29.85	0.69	26.93	1.07	26.41	2.43	38.76	0.31	30.35	1.17	20.98	1.05
129	CMT4+	25.51	24.94	35.02	24.77	35.04	26.35	2.42	28.48	0.87	33.76	14.78	29.08	4.23	18.18	10.93
130	Normal	26.91	31.38	1.07	31.74	0.74	31.81	0.14	31.67	0.25	40.00	0.52	35.47	0.13	26.05	0.12
131	Normal	26.02	31.59	0.50	32.16	0.30	29.71	0.33	29.15	0.78	40.00	0.28	32.19	0.69	24.68	0.17
132	Normal	26.80	34.82	0.09	33.65	0.18	30.81	0.27	30.74	0.44	40.00	0.48	33.91	0.36	25.13	0.22
133	Normal	26.40	32.26	0.40	32.35	0.34	32.44	0.07	32.16	0.13	37.74	1.73	31.67	1.30	23.63	0.46
134	Normal	25.98	33.11	0.17	32.80	0.19	30.26	0.22	32.05	0.10	40.00	0.27	34.89	0.10	25.87	0.07
135	Normal	27.20	35.21	0.09	35.76	0.06	35.45	0.01	35.46	0.02	40.00	0.63	35.25	0.19	27.55	0.05
136	CMT−	27.94	36.97	0.05	36.76	0.05	36.73	0.01	35.99	0.03	40.00	1.05	35.97	0.19	28.93	0.03
137	CMT4+	28.31	26.85	64.96	25.06	199.85	31.28	0.55	32.64	0.34	34.04	84.53	29.57	21.00	20.08	20.45
138	Normal	27.67	33.41	0.44	31.44	1.53	34.29	0.04	33.63	0.11	39.53	1.20	34.42	0.46	27.15	0.10

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