Exosomal small RNAs as biomarker of canine oral melanoma

（犬口腔内メラノーマのバイオマーカーとしてのエクソソーム内スモール RNA）

The United Graduate School of Veterinary Science

YAMAGUCHI UNIVERSITY

HUSNA AL ASMAUL

September, 2022

Exosomal small RNAs as biomarker of canine oral melanoma

Academic Dissertation

Submitted to

The United Graduate School of Veterinary Science

Yamaguchi University, Japan

By

HUSNA AL ASMAUL

Department of Veterinary Medicine

Joint Faculty of Veterinary medicine

Kagoshima University, Japan

In partial fulfillment of requirements of the degree of

Doctor of Philosophy

In

Veterinary Medicine

September, 2022

Major Supervisor

Professor Dr. Naoki Miura

Department of Veterinary Medicine

Joint Faculty of Veterinary medicine

Kagoshima University, Japan

Co-Supervisors

Professor Dr. Takuya Mizuno

Department of Veterinary Medicine

Joint Faculty of Veterinary medicine

Yamaguchi University

Professor Dr. Yoshiharu Okamoto

Joint Department of Veterinary Medicine

Faculty of Agriculture

Tottori University

Professor Dr. Mitsuya Shiraishi

Department of Veterinary Medicine

Joint Faculty of Veterinary medicine

Kagoshima University

Associate Professor Dr. Masashi Takahashi

Department of Veterinary Medicine

Joint Faculty of Veterinary medicine

Kagoshima University

DEDICATION

The thesis is dedicated to my beloved husband Dr. Mahfuzur Rahman, my parents and to my little children Mehroosa Muntaha and Md Munzir Rahman who are the inspiration of my study.

Table of Contents

List of Tables

List of Figures

Overview

Considering the importance of the canine cancer model of human disease, as well as the need for strategies for canine cancer management, the properties of exosomes are an emerging topic in canine oncology.

In my graduation study, exosomal RNA was isolated and investigated by next-generation sequencing. I identified several differentially expressed microRNAs (miRNAs/miRs) and novel small non-coding RNAs (sRNAs) rather than miRNAs in the exosomes of two melanoma cell lines (KMeC and LMeC) compared with non-tumor reference exosomes. Further I explored these potential melanoma-specific exosomal small RNAs.

In the first chapter, I focus on the miRNAs in the exosome of melanoma. Among the miRNAs, I found that miR-143 and let-7b increased in primary, whereas miR-210, 708, 221, and 222 increased in metastatic site originated melanoma cells. Further analysis showed miR-143 and 221 significantly increased in plasma exosomes of metastatic melanoma patients. Moreover, the sensitivity and specificity are >85% for differentiating the non-metastatic and metastatic patients. Therefore, these miRNAs can be an incredible biomarker candidate to identify metastatic melanoma and facilitate a better prognosis.

In the second chapter, I focus on the sRNA other than miRNAs in the exosome of melanoma. Among the novel sRNAs, long noncoding RNA fragments, tRNA-derived fragments, snoRNAs and snRNAs were abundantly expressed. I selected four novel sRNAs upregulated in each cell line, and validated their aberrant expression with qPCR. In analysis using plasma-derived exosomes from melanoma patients, six out of the eight selected novel sRNAs showed significantly elevated expression. Receiver operating curve (ROC) analysis showed that one long non-coding RNA-derived small fragment (ENSCAFT00000069599.1) and one transfer RNA-derived small fragment (tRNA-Ala-TGC-5-1) have more than 85% sensitivity and specificity for differentiating melanoma patients from tumor-free dogs.

In conclusion, the significance of my study is to show the suitability of melanoma exosomal sRNAs (miRNAs and other novel sRNAs) as biomarker. I found plasma exosomal miRNAs and novel sRNAs are potential biomarker for detecting melanoma patients. Furthermore, miRNAs are also good candidates to differentiate the metastatic and non-metastatic melanoma patients. Therefore, I consider that exosomal miRNAs and novel sRNAs may serve as candidate biomarkers to facilitate more accurate diagnosis of canine oral melanoma in clinical settings. For sure, my study is a valuable resource for establishing a blood-based biomarker for canine melanoma.

General Introduction

Exosomes are the functional nano-sized (30-150 nm) vesicles originating from the endocytic pathway in almost all types of cells and are secreted into the extracellular spaces under both physiological and pathological conditions (1–3). They are released into biofluids such as blood, saliva, breast milk, cerebrospinal fluid, urine as well as into cell culture medium and play an important role in cell-to-cell communication (4,5) through delivering distinct cargo of various biologically active molecules such as DNA, coding and non-coding RNA (miRNAs, mRNAs), lipid and proteins (6,7). Interestingly, exosomal cargo is already known to be dysregulated in human and other cancers(8–10). The exosomal transcriptome is thus a potentially rich source for biomarkers (9,11,12).

miRNAs are small non-coding RNAs that can play important roles in cancer pathogenesis. Aberrant miRNA expression is reported in tissue, plasma, exosomes and other biofluids of several human diseases including cancer (13,14). Human studies show that miRNA has the potential to be a blood-based biomarker in various diseases including cancer. In recent years extracellular exosomal miRNAs (exomiRs) are attracting much attention because of their specific disease or cancerous origin. Several human and one canine study

shows that exosomal miRNAs have the potential as predictive biomarker to diagnose and predict cancer stages (15,16).

On the other hand, research on human cancer has implicated a number of exosomal transcripts, other than miRNAs, in cancer development and progression (12,17). The transcripts receiving this attention are small non-coding RNAs (sRNAs), and they have been described as “rare sRNAs” in human medicine or more widely as novel sRNAs (18). Such novel sRNAs appear to be promising biomarkers in canine oncology. They encompass a diverse range of sRNA species, including small nucleolar RNAs (snoRNAs), small nuclear RNAs (snRNAs), small cajal body-specific RNA (scaRNA), transfer RNA-derived small fragments (tRFs), and PIWI-interacting RNAs (piRNAs) (19,20). In general, novel sRNAs possess high stability and low variability, and are conserved across a range of species. Accordingly, they can be regarded as a more reliable source of non-invasive biomarkers for the investigation of cancer than commonly used miRNAs or lncRNAs (18). Identifying novel sRNAs aberrantly expressed in exosomes from malignant cells is thus a promising line of research on new diagnostic and therapeutic approaches in canine oncology in general, and for melanomas in particular (18).

Melanoma is an aggressive tumor occurring in both human and dog and its incidence is increasing worldwide (21–23). They can progress to a metastatic phase dramatically, with

a concomitantly poor prognosis (18,23–25). Canine oral melanoma (COM) is one of the most common malignancies in dogs and accounting for 7% of all malignant tumors in dogs (25). Patients having COM have poor prognosis and the survival rate dramatically decreases once the tumor has metastasized (26,27). COM has been reported as spontaneous natural model for human melanoma.

Aberrant miRNAs expression is reported in COM tissues (28). On the other hand tissue and exosomal miRNAs profile of human melanoma have been studied (29). For example, human melanoma exosome study showed miR-155, 210 and/or miR-221, 222 play vital role in pre-metastatic microenvironment or define malignancy (30-34). Moreover, novel sRNAs have been linked with a range of cancers including gastric cancer, breast cancer, oral squamous cell carcinoma, head and neck cancer in human and/or canine populations, especially where they show a high level of exosomal loading (23,25,35,36). A further four snoRNAs (ACA17, ACA45, HBII-276, and SNORD12) have been proposed as prognostic markers of human uveal melanoma (20). My laboratory's previous study has also reported aberrantly expressed snoRNA, snRNA, tRFs, and piRNAs in canine melanoma tissues, cell lines, and plasma (Rahman MM, Lai YC, Husna AA, et al., 2019). However, no study investigates the COM exosome profile till now. To the best of my knowledge, this was the first study which represents the status of exosomal miRNAs and novel sRNAs of COM.

A few studies reveal exosomal miRNAs indicative for early stage of cancer (15,16). In my present study I aimed to develop exosome specific miRNAs biomarkers which may define COM stages. Another part of my project aimed to identify novel sRNAs with potential as biomarkers of COM and to elucidate their diagnostic efficacy. To attain my goal, I collect the COM exosome from cell lines and analyze their sRNAs (miRNAs and others) profile by next generation sequencing. I found exosomal miRNAs are differentially expressed comparing to the control. Expression pattern of exosomal miRNAs is also different from their parental cells of origin. Moreover, I also confirmed a unique expression pattern of miR-210, 221, 222, 708 and 143 that reflect the primary and metastatic status of COM. I also investigated sRNA species by comparing their exosomal expression between COM cell lines and healthy dogs and subjected aberrantly expressed sRNAs for a validation of expression with qPCR and investigations of expression, and specificity and sensitivity as biomarkers, in live patients.

Chapter 1

Identification of melanoma-specific exosomal miRNAs as the potential biomarker for canine oral melanoma

(Husna, AA. *et al*., 2021 Pigm*ent Cell Melanoma Res . 2021 Nov;34(6):1062-1073*)

Chapter 1

1.1 Abstract

Considering the importance of the canine cancer model of human disease, as well as the need for strategies for canine cancer management, the properties of exosomes are an emerging topic in canine oncology. In my first study, exosomal RNA was isolated and investigated by next-generation sequencing. I identified several differentially expressed microRNAs (miRNAs/miRs) in the exosomes of two melanoma cell lines compared with non-tumor reference exosomes. I explored these potential melanoma-specific exosomal miRNAs further and found that miR-143 and let-7b increased in primary, whereas miR-210, 708, 221, and 222 increased in metastatic site originated melanoma cells. Further analysis showed miR-143 and 221 significantly increased in plasma exosomes of metastatic melanoma patients. Moreover, the sensitivity and specificity are greater than 85% for differentiating the non-metastatic and metastatic patients. Therefore, these miRNAs can be an incredible biomarker candidate to identify metastatic melanoma and facilitate a better prognosis.

1.2 Introduction

Exosomes are the nano-sized (30-150 nm) vesicles produced by almost all types of cells and secreted into the extracellular spaces under both physiological and pathological conditions (1–3). They are present in bio-fluids such as blood, saliva, breast milk, cerebrospinal fluid, urine as well as into cell culture medium (4,5). Exosomes contain various biologically active molecules such as miRNAs, mRNAs, DNA and proteins (6). miRNAs are small non-coding RNAs that can play important roles in cancer pathogenesis. Aberrant miRNA expression is reported in tissue, plasma, exosomes and other biofluid of several human diseases including cancer (13,14). Human studies showed that miRNA have the potential to be a blood-based biomarker in various diseases including cancer. In recent years extracellular exosomal miRNAs (exomiRs) are attracting much attention because of their specific disease or cancerous origin. Several human studies and one canine study showed that exosomal miRNAs have the potential as predictive biomarker to diagnose and predict cancer stages (15,16).

Melanoma is an aggressive tumor occurring in both human and dog and its incidence is increasing worldwide (21–23). COM is one of the most common malignancies in dog with poor prognosis and the survival rate dramatically decreases once the tumor has metastasized

(26,27). COM reported as spontaneous natural model for human melanoma. Aberrant miRNAs expression is reported in COM tissues. On the other hand tissue and exosomal miRNAs profile of human melanoma have been studied (29). For example, human melanoma exosome study showed miR-155, 210 and/or miR-221, 222 play vital role in pre-metastatic microenvironment or define malignancy (30-34). However, no study investigates the COM exosome profile till now. To the best of my knowledge, this is the first study which represents the status of exosomal miRNAs of COM.

A few studies revealed exosomal miRNAs indicative for early stage of cancer (15,16). In my present study I aimed to develop exosome specific miRNAs biomarker which may define COM stages. To attain my goal, I collect the COM exosome from cell lines and analyze their miRNA profile by next generation sequencing. I found exosomal miRNAs are differentially expressed comparing to the control. Expression pattern of exosomal miRNAs is also different from their parental cells of origin. Moreover, I also confirmed a unique expression pattern of miR-210, 221, 222, 708 and 143 that reflect the primary and metastatic status of COM.

1.3 MATERIALS AND METHODS

1.3.1 Exosome preparation

Melanoma exosome (Cell culture)

Two COM cell lines, KMeC (originated from primary oral melanoma) and LMeC (originated from a metastatic mandibular lymph node of oral melanoma) used in this study were received from the University of Tokyo. The cell lines mentioned above were cultured in RPMI-1640 medium (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) containing exosome-depleted FBS; EFBS (System Biosciences, Mountain View, CA, USA), as previously described, until confluent (Inoue et al., 2004). The culture media were collected and then processed for exosome isolation. These exosomes will be referred to as melanoma exosomes through the text. Cells were lysed with the lysis buffer for RNA extraction referred to as cellular RNAs in the text.

Reference exosome (non-tumor) and Melanoma patients' plasma exosomes

As the primary melanocyte cell line from dogs is not established yet, reference exosomes were collected from non-tumor dog plasma. Blood samples were collected from non-tumor dogs (n=6) housed at Kagoshima University for use in another project. Whole

blood was also collected (during surgery) from melanoma patients (n=9) treated in the Veterinary teaching hospital, Kagoshima University, with informed consent from the patient's owner. Patient's signalment are presented in Appendix 1. Then they were immediately centrifuged at 3000 xg for 10 minutes and the supernatants (plasma) were aliquoted into tubes and processed for exosome isolation. The non-tumor exosomes will be referred to as reference exosomes throughout the text. The experimental design and protocols were approved by the Kagoshima university and the teaching hospital ethics committee (KV0004).

Isolation and identification of exosomes

Exosomes were collected from the culture supernatant using the Total Exosome Isolation (from cell culture) kit (Invitrogen, Thermo Fisher Scientific, Carlsbad, CA, USA) according to the manufacturer's protocol. Ten milliliter of culture media was used to isolate the exosomes. Total exosome precipitation reagent (from plasma) (Invitrogen) was used to isolate reference exosomes from the non-tumor dog plasma. Two different volume of plasma was used to isolate the exosomes. For next generation sequencing study, I need more RNA therefore I used one milliliter of plasma to isolate the exosomes. Besides, for validation in the clinical patients, exosome was isolated from 300 microliter of plasma both from control and melanoma patients. The size of both the cell lines and reference exosomes were

determined by nano tracking analysis (NTA) (Quantum Design, Tokyo, Japan). Exosomal marker protein TSG101 (Tumor Susceptibility Gene 101) and CD9 were identified by western blot analysis.

1.3.2 Western blot analysis

The yield of exosome protein was quantified using the Micro BCA™ Protein Assay Kit (Thermo Fisher Scientific). Next, 10 μg of protein were separated using 15% gradient polyacrylamide gels. The protein was then transferred onto PVDF membranes (Millipore, Croxley Green, Watford, UK). Membranes were blocked with Pierce™ Protein-Free (TBS) Blocking Buffer (Thermo Fisher Scientific) at room temperature for 2 hours. Primary antibodies were incubated overnight at 4°C as follows: TSG101 (1:1000 dilution, ARP37310-T100, Aviva System Biology, San Diego, CA, USA), CD9 (1:1000 dilution, SM1065PS, Acris-an Origene Company, Rockville, MD, USA) and bet -actin (1:10000 dilution, GTX109639; GeneTex, San Antonio, Texas). Membranes were incubated with secondary antibody at room temperature for 1 or 2 hours as follows: anti-rabbit IgG (1:50000 and 1:80000, 55689, Jackson Immuno Research, Baltimore, PA, USA) for TSG101, beta-actin and anti-mouse IgG (1:50000, 115-035-062, Jackson Immuno Research) for CD9.

Bands were visualized using ECL reagents (Roche, Nutley, NJ, USA) and imaged by myECL (Thermo Fisher Scientific).

1.3.3 RNA extraction and next-generation sequencing

Following the isolation and confirmation of exosomes, total RNA was isolated from the exosomes using a Total Exosome RNA and Protein Isolation kit (Invitrogen, Thermo Fisher Scientific). During the exosomal RNA isolation, 25 fmol cel-39 was spiked. The mirVana RNA Isolation kit (Invitrogen, Thermo Fisher Scientific) was used to isolate the total RNA from cells. The concentration of the exosomal RNA was measured by a Qubit™ microRNA Assay Kit (Thermo Fisher Scientific) according to the manufacturer's guidelines. The amount of total RNA from cells was measured using a NanoDrop 2000c spectrophotometer (ND2000C, Thermo Fisher Scientific). The quality and integrity of the RNAs were assessed on an Agilent 2100 Bioanalyzer (G2939BA, Agilent Technologies, Santa Clara, CA, USA). The RNA integrity number (RIN) mean value was > 9.0 (range 9.6–10) for the RNA from the KMeC and LMeC cell lines. Then, RNA was sent to a sequencing facility (Hokkaido System Science Co., Ltd., Sapporo, Hokkaido, Japan), for analysis. Briefly, libraries for small RNA were constructed using 1 μg of total RNA with the TruSeq Small RNA Library Preparation kit (Illumina, San Diego, CA, USA), according to the

manufacturer's protocol. Small RNAs were ligated to 5′ and 3′ adaptors. To create cDNA constructs, reverse transcription followed by amplification was performed. A gel purification step was followed to purify the amplified cDNA constructs for cluster generation. Sequencing was performed on an Illumina/HiSeq2500 instrument (Hokkaido System Science Co., Ltd.). I obtained high-quality clean reads (Phred score > 34) from the NGS assay. Raw sequences have been submitted to the NCBI sequence read archive database under accession number PRJNA656859.

1.3.4 Analysis of the sequencing reads

Sequencing reads were analyzed by the CLC Genomics Workbench (CLC Bio, Qiagen, Hilden, NRW, Germany) as recommended in the manufacturer's manual[1]. First, reads were imported into CLC Genomics Workbench (versions 10 and 12), then normalization, ambiguity, and adapter trimming, as well as quality control, were performed. Low quality, ambiguous nucleotides, 3′ adapters, and short (>15 nt) and long reads (>55 nt) were removed during the trimming process. Clean reads were analyzed according to the small RNA analysis guidelines within the Genomics Workbench. Workbench extracted and counted the small

[1]http://resources.qiagenbioinformatics.com/

RNAs from the clean reads. Then, the small RNAs were compared with the databases for annotation. MiRBase and non-coding RNA databases from the ensemble (Canis_familiris-canfam3.1. ncRNA and Homo_sapiens-GRCh37.ncRNA) were used as references. Expression values for the miRNAs/small RNAs were measured by the sequence/fragment counts. Differential expression between the groups was detected by an EDGE (empirical analysis of differential gene expression) test in the CLC Genomics Workbench. Default parameters were used throughout the analysis. The estimated average count per million (cpm) for each group was calculated to determine the fold change. This indicated the difference in the average cpm values between the groups. The false discovery rate (FDR) refers to the exact test of the p-value. For differential expression analysis between two groups, the following stringent filtering criteria were used: a fold change absolute value >2 and FDR ≤0.05, and an expression value per sample >10.

1.3.5 Quantitative real-time PCR (qRT-PCR)

The expression levels of exomiRs were determined by TaqMan microRNA assays (Thermo Fisher Scientific). Exosomal miRNA (0.205 ng/µl) was reverse transcribed into cDNA using the TaqMan MicroRNA Reverse Transcription Kit (Thermo Fisher Scientific) according to the manufacturer's instructions. The TaqMan First Advanced Master Mix Kit

and a one-step plus real-time PCR system (Thermo Fisher Scientific) were used for qRT-PCR analysis. Optimum PCR reaction conditions were employed according to the manufacturer's guidelines. The experiments were performed in duplicate. The $2^{-\Delta\Delta CT}$ method was used to measure miRNA expression. Mature miRNAs were quantified using TaqMan MicroRNA assays. The primer IDs were miR-186 (ID: 002285), miR-143 (ID: 002249), Let-7b (ID: 002619), miR-708 (ID: 002342), miR-210 (ID: 000512), miR-221 (ID: 001134) and miR-222 (ID: 002276). Primer information can be found at https://www.thermofisher.com/order/genome-database/. According to the NGS results, the expression values of miR-186 were consistent in both the cell lines exosomes and reference exosomes. The qRT-PCR results for miR-186 revealed Ct values that were also consistent in both the exosomes. I, therefore, considered this as an internal control to measure relative expression. I also calculated the relative expression using cel-39 as an internal control, which was spiked during RNA isolation. The relative expression levels of target miRNAs were consistent with both internal controls (miR-186 and cel-39) and I, therefore, used miR-186 as an internal control in subsequent experiments.

1.3.6 Statistical analysis

To analyze my data statistically, GraphPad Prism 7 software (www.graphpad.com) was used. To compare the qRT-PCR data, one-way ordinary ANOVA followed by Tukey's multiple comparison test was used. A p-value <0.05 was considered to indicate a statistically significant difference.

1.4 Results

1.4.1 Characterization of canine oral melanoma exosomes and their miRNAs

Melanoma exosomes were isolated from the culture supernatant of two COM cell lines, KMeC and LMeC. Reference exosomes were collected from the non-tumor dog plasma. The size of the isolated exosomes was confirmed by nano tracking analysis (~100 nm) (Figure 1-1a) and the presence of exosome marker proteins TSG101 and CD9 was confirmed by western blot analysis (Figure 1-1b). Thus, the exosomes were confirmed by size and exosomal protein analysis from cell lines, non-tumor dog plasma and melanoma dog plasma. Besides, absence of beta-actin in the exosomes comparing to the total plasma confirmed the presence of pure exosomes without cytoplasmic contaminants (Figure 1-1c). Total RNA was then isolated from the exosomes. The size and quantitation of the small RNA fragments were confirmed by an Agilent 2100 small RNA bioanalyzer and Qubit® 4 microRNA assay. The data showed that exosomes contain small RNAs of around 22 nt in length (Figure 1-1d).

1.4.2 Differential expression of exomiRs in COM

Differential miRNA expression observed among reference exosomes, cellular RNAs, and melanoma exosomes

To observe the overall expression patterns of miRNAs among reference exosomes, cellular RNAs, and melanoma exosomes, I performed principal component analysis (PCA). Based on miRNA expression, samples from each group are clustered together (Figure 1-2a). Even the miRNA expression profiles from exosomes and cellular RNAs (their cells of origin) clustered separately. This indicated miRNA expression differs among cellular RNAs, melanoma, and reference exosomes, leading us to further investigate the differentially expressed miRNAs between the groups.

Expression of miRNAs differs between the exosomes and their cells of origin

Based on the PCA results, I investigated the expression of miRNAs between melanoma exosomal RNAs and cellular RNAs (their cells of origin). Volcano plot analysis revealed 275 significantly differentially expressed miRNAs in the KMeC exosomes compared with cellular RNAs (Figure 1-2b). Among these miRNAs, 146 were upregulated and 129 were downregulated in the KMeC exosomes (Appendix 2).

In the case of the LMeC cell line, I found 279 significantly differentially expressed miRNAs between the exosomes and cellular RNAs (Figure 1-2c). Among these miRNAs, 140 were upregulated and 139 were downregulated in the LMeC exosomes (Appendix 3).

Expression of miRNAs differs between the reference and melanoma exosomes, as well as in between the cell line exosomes

I investigated the differentially expressed miRNAs between the reference and the melanoma exosomes. To determine the miRNAs that were differentially expressed between the groups, I applied the same stringent filtering criteria (fold change absolute value >2 and FDR ≤0.05, expression >10). A total 189 miRNAs (129 upregulated and 60 downregulated) were found to be significantly differentially expressed between reference and the KMeC melanoma exosomes (Appendix 4 and Appendix 6a), 205 miRNAs (136 upregulated and 69 downregulated) were significantly differentially expressed between reference and the LMeC melanoma exosomes (Appendix 5 and Appendix 6b), and 79 miRNAs (36 upregulated and 43 downregulated) were differentially expressed in between the KMeC and LMeC exosomes (Figure 1-2d and Appendix 7).

To observe the commonly expressed up-and downregulated exomiRs among the groups, I compared the following groups: Reference vs KMeC, Reference vs LMeC and KMeC vs LMeC (Figure 1-2e, 2f). Among the upregulated miRNAs, nine were commonly increased in all of the groups (Reference vs KMeC, Reference vs LMeC and KMeC vs LMeC) (Figure 1-2e and Table 1). These miRNAs were more significantly increased in the LMeC exosomes than in the KMeC and reference exosomes, indicating that they may play a role in metastasis.

Similarly, among the downregulated miRNAs, one exomiR was commonly decreased in all three groups (Reference vs KMeC, Reference vs LMeC and KMeC vs LMeC) (Figure 1-2f and Appendix 8). This miRNA was more significantly decreased in the LMeC exosomes than in the KMeC and reference exosomes, indicating that it may have the potential to be a prognostic marker of melanoma.

1.4.3 COM cell lines specific ExomiRs

To identify the exomiRs in COM cell lines, I investigated the KMeC and LMeC cell line-derived upregulated exomiRs, as shown in Figure 1-3a and 3b.

Primary melanoma cell line (KMEC)-derived exomiRs

To identify KMeC cell line-derived exomiRs, I first determined that 101 miRNAs were upregulated in KMeC cells compared with LMeC cells. Besides, I found that 146 miRNAs were upregulated in the KMeC exosomes compared with KMeC cellular RNAs in which, 134 miRNAs were upregulated only in KMeC exosomes (Figure 1-3a), the remaining 12 miRNAs were commonly increased in both KMeC cells and exosomes comparing to the LMeC cellular RNAs.

Metastatic melanoma cell line (LMeC)-derived exomiRs

Similar to my previous approach, first I identified 143 miRNAs that were upregulated in LmeC cells compared to KmeC cells. Besides, 140 miRNAs were upregulated in LmeC exosomes compared to LmeC cellular RNAs in which, 107 miRNAs were upregulated only in LmeC exosomes (Figure 1-3b), the remaining 33 miRNAs were commonly increased in both LmeC cells and exosomes comparing to the KmeC cellular RNAs.

Melanoma cell line-specific exomiRs

To reveal the melanoma-specific exomiRs, I further analyzed the upregulated miRNAs derived from only KMeC and LMeC melanoma exosomes. A Venn diagram revealed that, out of 134 upregulated miRNAs in KMeC exosomes and 107 upregulated miRNAs in LMeC exosomes, 55 were common between both types of melanoma exosomes (Figure 1-3c, Appendix 9). I also found that 79 miRNAs were specifically increased in the exosomes of KMeC, whereas 52 were specifically increased in LMeC exosomes (Figure 1-3c and Appendix 10, 11). The expression of these exomiRs may therefore be an indicator of the existence of melanoma. Moreover, these analyses also shaded lights that exomiRs expression can distinguish primary and metastatic cell lines.

ExomiRs define primary and metastatic sites originated melanoma cells

The findings above indicated that the expression of exomiRs is specific to cell lines. I, therefore, predicted that KMeC and LMeC exomiRs may have a distinct expression trend. To investigate further, I need a reference control. But, the primary melanocyte cell line was not available in dogs. So, the ideal exosome control from melanocytes was absent. To minimize the issue, I considered non-tumor exosomes as a reference control. From my prediction, I proposed three hypothetical exomiRs expression patterns depending on the primary and metastatic site of melanoma from where KMeC and LMeC cell line originates (Appendix 12a–c). Therefore, in this study, I addressed that KMeC and LMeC related exomiR expressions were specific to primary and metastatic sites, respectively. The exomiRs that were upregulated in the COM cell lines were validated by qRT-PCR.

ExomiRs expression increased only at primary site cells

miRNAs that were only increased in KMeC exosomes and remained unchanged or were not increased in LMeC exosomes were considered to be exomiRs upregulated at the primary site of melanoma (Appendix 2a). The specific stringent filtering criteria were applied to the NGS data. A total of 16 exomiRs were found to be increased in the primary melanoma

cell line exosomes, as shown in Table 2, and the expression levels of miR-143 and let-7b were validated by qRT-PCR (Figure 1-4a1, 4a2).

ExomiRs increased only at metastatic site cells

To determine any metastatic site-related exomiRs, I considered those miRNAs that were not changed in the reference exosomes or in KMeC exosomes but were considerably upregulated in LMeC exosomes (Appendix 12b). To obtain such exomiRs from my NGS analysis, I applied specific filtering criteria. A total of nine metastatic melanoma cell line-derived exomiRs were identified from my NGS data (Table 3), of which miR-210 and miR-708 were validated by qRT-PCR (Figure 1-4b1, 4b2).

ExomiRs gradually increased in expression at primary and metastatic site cells

To determine which exomiRs were gradually increased at the metastatic melanoma cells, I identified the miRNAs that were significantly successively increased in the KMeC and LMeC exosomes (Appendix 12c) compared to reference. I applied the specific stringent filtering criteria. In total, nine exomiRs were found that were gradually increased in the exosomes of primary (KMeC) and metastatic (LMeC) melanoma cells, as shown in Table 1. Among them, miR-221 and miR-222 were validated by qRT-PCR (Figure 1-4c1, 4c2).

1.4.4 ExomiRs- miR-143 and miR-221 are biomarkers for metastatic melanoma

Based on the cell culture experiment, I choose the topmost three expressed miRNAs (miR-143, 221, and 210) to investigate their suitability as biomarkers for melanoma patients. ExomiRs isolated from plasma of non-tumor dog and melanoma patients were compared. I measured the expression of these miRNAs by qRT-PCR and digital PCR (dPCR). Among these three candidates, miR-143 and 221 were significantly increased in the metastatic patients' exosomes. On the other hand, the expression of miR-210 remains unchanged. (Figure 1-5a1-3). Results from dPCR and qRT-PCR of these two miRs were highly correlated (Figure 1-5b1-3). To evaluate the suitability as a biomarker for melanoma, I did receiver operating characteristic (ROC) curve analysis for these two miRNAs. The combined use of miR-143 and 221 showed significantly higher diagnostic efficacy than the individual ones to detect the melanoma patients (AUC= 0.80; p= 0.059) (Figure 1-5c1). Besides, miR-143 and 221 could serve as biomarkers to distinguish metastatic from non-metastatic melanoma patients (Figure 1-5c2-3).

1.5 DISCUSSION

MiRNA is a promising candidate as blood or other biofluid-based biomarkers (37). In this study, I revealed the exomiR profile of COM, which is a natural spontaneous model of human melanoma (35,38). To the best of my knowledge, this is the first study to report the exomiR profile of COM. My findings indicated that specific cell-secreted exosomes bear distinct miRNA cargos, which is consistent with several previous human studies (39). Moreover, I also reported exosome biomarker candidates for primary melanoma as well as metastatic melanoma. Furthermore, my results revealed several exomiRs with distinct expression patterns compared with their parental COM cell lines consistent with previous human melanoma studies (30,40).

Exploring the exosome profile, I hypothesized that exomiRs reflect primary and metastatic sites of melanoma. Accordingly, I identified exomiRs that showed increased expression in primary (KMeC) and metastatic (LMeC) site originated melanoma cells. In KMeC exosomes, among these, increased exomiRs, miR-143 and let-7b was validated by qRT-PCR. In my previous study, miR-143 is reported to be downregulated in COM tissue (41). A similar pattern of expression is downregulation of miR-143 in tissue, but upregulation in the exosome has also been reported in human ovarian cancer (42). The discrepancy in the expression of miR-143 in tissue, plasma, and exosomes can be explained by the efficient

loading of miRNA into exosomes, which reduces its existence in cells or tissue. Previous studies on breast cancer reported that miR-143 is upregulated in cancer-associated fibroblast exosomes, while downregulated in cells (43, 44). The expression of another miRNA in this group, let-7b, is controversial among human studies (45–48). In my study, let-7b was one of the most highly upregulated miRNAs in KMeC exosomes, which was consistent with the findings of Ohshima *et al*.

Besides, among the nine exomiRs that were subsequently increased in metastatic site originated melanoma cells, miR-221 and miR-222 were the most highly expressed and confirmed by qPCR. A previous study identifies miR-221 and miR-222, along with seven other miRNAs, as diagnostic and therapeutic markers of human melanoma (49). The upregulated expression of miR-221 and miR-222 has also been reported in plasma exosomes during human malignant melanoma (30). Another study confirmed the role of exosomal miR-222 in melanoma progression and malignancy (50).

Nine miRNAs were only increased in aggressive LMeC exosomes; among these, miR-210 and miR-708 were the most highly expressed in LMeC exosomes. This result may indicate their relation to metastasis. It has previously been reported that miR-210 is upregulated during metastasis in melanoma (31). Moreover, miR-210 has also been considered as a potential cell or exosomal biomarker of several human cancers including lung

adenocarcinoma and renal cell carcinoma (51,52). Whereas for miR-708, a role has been reported in head-neck, lung, and colorectal cancer as an oncogenic, pharmacodynamic or diagnostic marker (53–55), but its role in melanoma has not been studied. Of note, in my analysis, miR-210, miR-708, and miR-212 were found to be both suddenly and subsequently increased at metastatic sites. This was particularly pronounced in LMeC (the metastatic cell line) because of the higher fold change observed (Tables 1 and 3). Besides, in my exosome NGS profile, several miRNAs (miR-212, miR-335, miR-551a, miR-574-3p, miR-935, and miR-214) upregulated in the COM cell line exosomes that were not validated by qPCR, which were also consistent with a previous human melanoma study (30). The results represent the reliability of exosomal miRNAs screening by NGS.

I further analyzed the selectively targeted exomiRs in melanoma-bearing dog plasma samples to evaluate their suitability as a biomarker of melanoma. MiR-143 and 221 were significantly highly expressed in the exosomes from metastatic patients' plasma than non-tumor individuals. However, the expression pattern of miR-143 in cell culture and clinical samples showed some discrepancy. In the cell culture, experiment miR-143 increased in primary originated cell's exosomes, whereas in clinical samples, increased expression was found in the metastatic cases. One logical explanation can be, in a cell culture experimental setting I analyzed only exomiRs from primary or metastatic site originated cell lines

individually. However, in the case of metastatic melanoma patients, plasma bears the exosomes from both the primary and metastatic tumor cells simultaneously, and also primary melanoma tends to be large in these cases, which may result in higher expression of miR-143.

Another exomiR (miR-210) expression remains unchanged in melanoma patients which is also inconsistent with the cell culture study. The expression magnitude of miR-210 was less than the other two candidates. Therefore, it may dilute or reduce the concentration of miR-210 in plasma, resulting in the mask of expression difference between reference and melanoma exosomes.

There are limitations to my study. The first, exomiRs profile was only investigated for melanoma cell lines; circulating melanoma exosomes from dogs were not included. It is not currently possible to separate circulating melanoma exosomes from other cell-derived exosomes in blood. Therefore, profiling the circulating exosomal RNAs from dogs is not representative of melanoma exosomes as exosomes from other cell sources will be included. Furthermore, a strategy for the identification of melanoma exosomes from plasma could facilitate the definition of a patient's disease state more precisely by these candidate miRNAs. Second, I compare the COM cell lines exomiRs with the exomiRs from reference exosomes that were collected from non-tumor dog plasma. Till now the primary melanocyte cell line

from dogs is not established. Therefore, I chose the non-tumor plasma exosome as a reference which is an ideal control for the blood-based exosomal study. Third, reference exosomes were isolated from plasma of beagle dogs.

I successfully present a comprehensive exomiRs profile of two COM cell lines. My profiling studies revealed that unique exomiRs expression profiles exist between exosomes and their cells of origin. I further reported that miR-143 and 221 are suitable biomarker candidates for melanoma.

Table 1-1 Common and gradually increased exosomal miRNAs in all groups (Reference vs KMeC, Reference vs LMeC and KMeC vs LMeC)

[†]Reference exosomes, [‡]KMeC cell line exosomes, [§]LMeC cell line exosomes, [¶]False discovery rates

miRNAs	Fold Change			FDR[¶]		
	R[†] vs K[‡]	R vs L[§]	K vs L	R vs K	R vs L	K vs L
mir-221	9.02	42.18	4.68	3.02E-07	8.12E-16	9.44E-04
mir-222	18.93	93.37	4.93	4.62E-09	1.46E-16	3.04E-03
mir-574	25.85	91.25	3.53	2.4E-13	1.7E-21	6.20E-03
mir-146b	3.67	23.95	6.53	1.28E-02	2.41E-09	7.08E-04
mir-210	3.97	23.29	5.87	7.73E-04	2.03E-13	3.94E-05
mir-335	7.76	25.39	3.27	4.88E-05	4.64E-10	4.45E-02
mir-708 (hsa/cfa-5p)	11.15	241.88	21.69	6.81E-05	3.35E-21	6.83E-11
mir-1224	28.90	173.85	6.01	8.97E-09	2.54E-19	5.82E-05
mir-212	3.86	23.49	6.09	1.86E-02	5.23E-09	6.24E-04

Table 1-2 ExomiRs expression increased only at primary site cells

miRNAs	Fold Change		FDR¶	
	R† vs K‡	K vs L§	R vs K	K vs L
mir-143	21.55	-595.62	1.22E-10	0
let-7b	3.15	-3.70	1.39E-02	1.37E-02
mir-181b-1//mir-181b-2	4.65	-4.92	3.13E-04	6.50E-04
mir-371	5.59	-5145.05	4.79E-04	1.7E-30
mir-92b	2.94	-2.13	3.15E-02	3.21E-01
let-7c	4.88	-47.38	1.75E-02	3.59E-07
mir-125b-2	25.16	-27.96	2.43E-13	1.78E-13
mir-1307	5.03	-3.15	1.31E-03	6.34E-02
mir-145	6.51	-44.31	8.98E-04	3.2E-09
mir-455	46.01	-594.05	4.81E-10	9.17E-19
mir-1296	3.30	-3.93	1.60E-02	1.37E-02
mir-99a	5.25	-9.66	2.81E-04	2.05E-06
mir-4286	5.95	-4.68	3.01E-04	1.23E-03
mir-197	8.77	-4.71	8.65E-04	1.78E-02
mir-1249	4.57	-7.10	9.60E-03	4.57E-04
mir-551a	7.56	-191.72	2.26E-03	2.05E-06

†Reference exosomes, ‡KMeC cell line exosomes, §LMeC cell line exosomes, ¶False discovery rates

Table 1-3 ExomiRs increased only at metastatic site cells

miRNAs	Fold Change		FDR[¶]	
	R[†] vs L	K[‡] vs L[§]	R vs L	K vs L
mir-210	16.64	8.97	9.684E-11	9.0583E-07
mir-708	26.97	14.26	4.597E-14	9.8203E-10
mir-8826	214.85	478.96	2.504E-21	2.1231E-24
mir-8884	3.15	4.62	2.86E-02	1.01E-02
mir-1298	9.84	45.31	2.56E-03	6.7783E-06
mir-504	19.48	35.43	5.333E-06	4.5635E-08
mir-1911	474.54	474.54	7.451E-09	1.8588E-10
mir-491	8.59	9.35	2.71E-03	4.86E-03
mir-212	10.11	14.08	3.07E-03	3.45E-03

[†]Reference exosomes, [‡]KMeC cell line exosomes, [§]LMeC cell line exosomes, [¶]False discovery rates

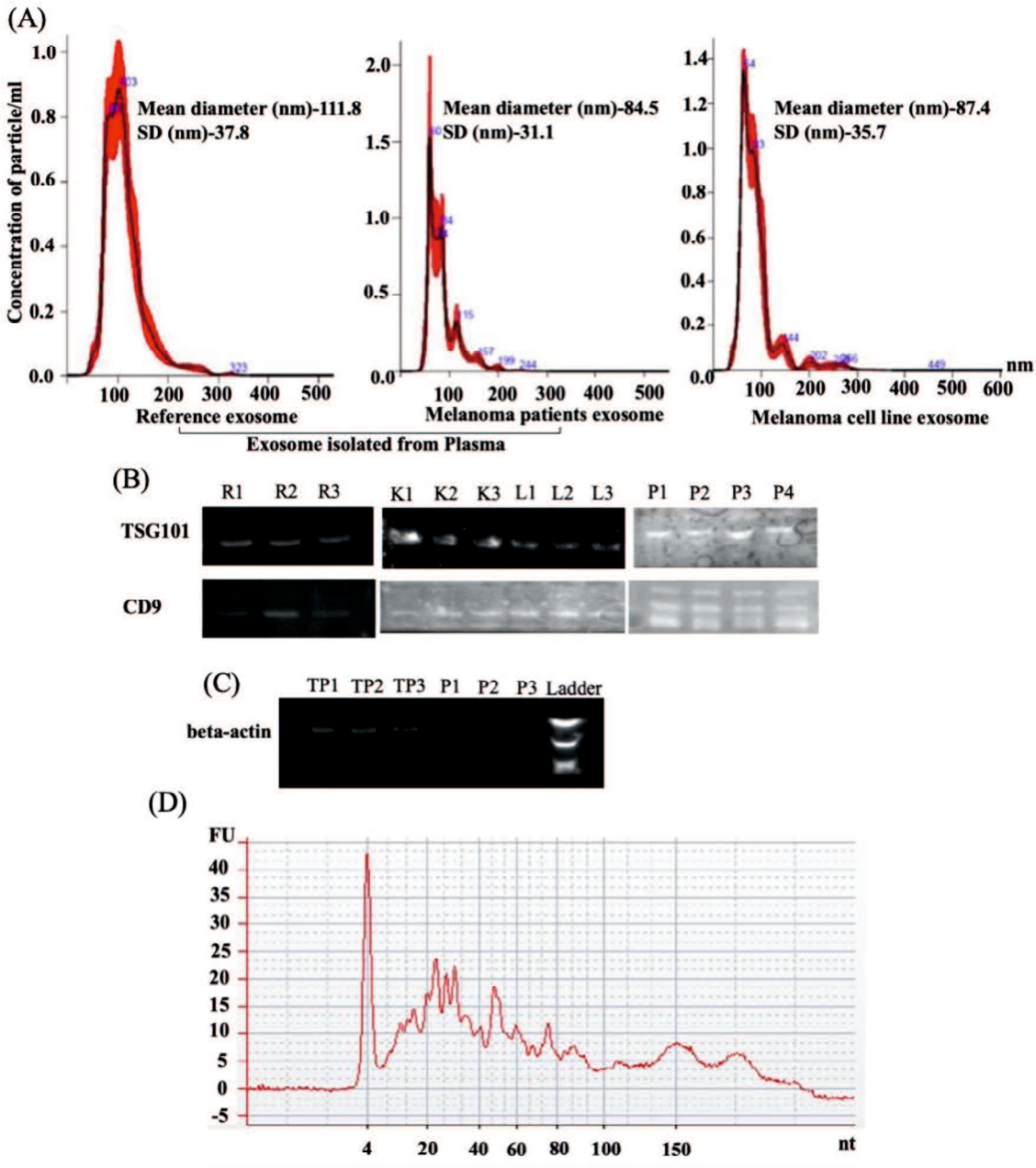

(A)
Concentration of particle/ml
Mean diameter (nm)-111.8
SD (nm)-37.8
Reference exosome
Mean diameter (nm)-84.5
SD (nm)-31.1
Melanoma patients exosome
Mean diameter (nm)-87.4
SD (nm)-35.7
Melanoma cell line exosome
nm
Exosome isolated from Plasma
(B)
R1 R2 R3 K1 K2 K3 L1 L2 L3 P1 P2 P3 P4
TSG101
CD9
(C)
TP1 TP2 TP3 P1 P2 P3 Ladder
beta-actin
(D)
FU
nt

Figure 1-1 Characterization of COM exosomes and their miRNAs. (A) Determination of particle size (~100 nm) by the Nano Sight. (B) Confirmation of the exosome marker proteins TSG101 and CD9 by western blot. (C) Absence of beta-actin in exosomes compared to total plasma confirmed the presence of pure exosomes with minimal contaminants. (D) Analysis of exosomal small RNA fragments by the Agilent 2100 bioanalyzer. R, Reference; K, KMeC (primary melanoma cell line); L, LMeC (metastatic melanoma cell line); P, Plasma exosomes from Melanoma Patients; TP, Total Plasma from Melanoma Patients.

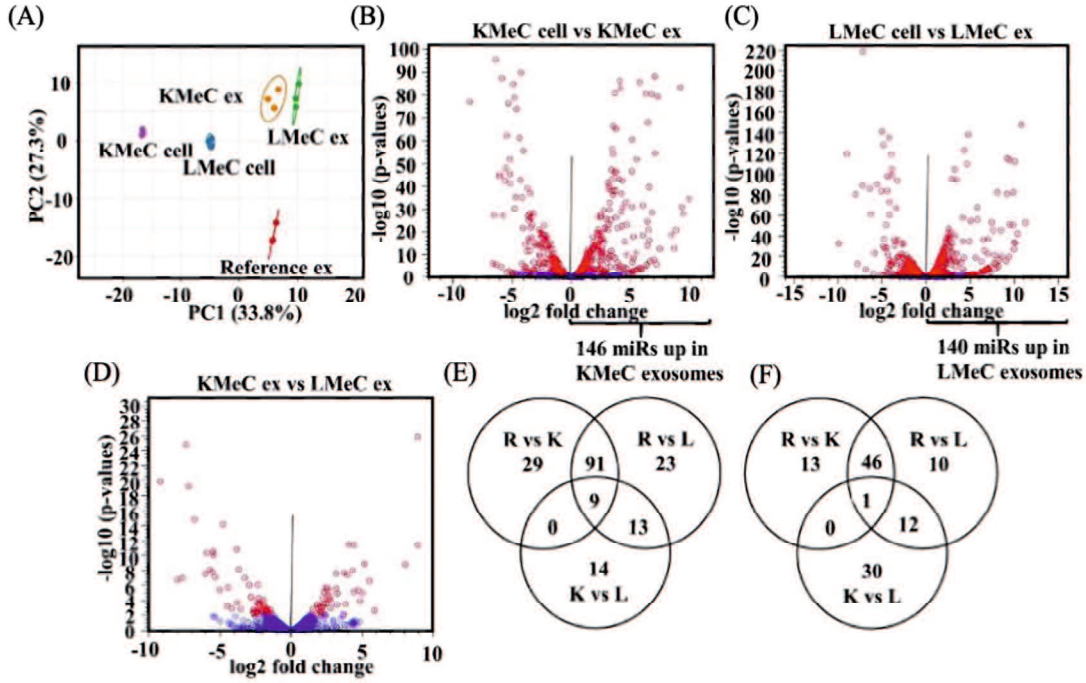

(A)
KMeC ex
LMeC ex
KMeC cell
LMeC cell
Reference ex
PC2 (27.3%)
PC1 (33.8%)
(B)
KMeC cell vs KMeC ex
-log10 (p-values)
log2 fold change
146 miRs up in
KMeC exosomes
(C)
LMeC cell vs LMeC ex
-log10 (p-values)
log2 fold change
140 miRs up in
LMeC exosomes
(D)
KMeC ex vs LMeC ex
-log10 (p-values)
log2 fold change
(E)
R vs K
29
91
R vs L
23
9
0
13
14
K vs L
(F)
R vs K
13
46
R vs L
10
1
0
12
30
K vs L

Figure 1-2 Differential expression of exomiRs in COM. (A) Principle component analysis (PCA) shows the overall expression pattern of miRNAs among reference exosomes, cellular RNAs and melanoma exosomes. (B, C) Volcano plot analysis represents the differentially expressed miRNAs between the exosomal and cellular RNAs; (B) K ex and cellular RNAs and (C) L ex and cellular RNAs. (D) Volcano plot stands for the differentially expressed exosomal miRNAs between the two melanoma cell lines. (E, F) Venn diagram represents commonly upregulated and downregulated differentially expressed exosomal miRNAs among the groups (R vs K; R vs L and K vs L). N=3 in each group. In the Volcano plot analysis, the X-axis represents the fold change, whereas the Y-axis shows the FDR p-values. Each circle indicates a single miRNA. Red circles indicate significantly differentially expressed miRNAs (fold change, absolute value >2 and FDR ≤0.05, expression >10). FDR, false discovery rate; R, Reference; K, KMeC (primary melanoma cell line); L, LMeC (metastatic melanoma cell line); ex, exosome.

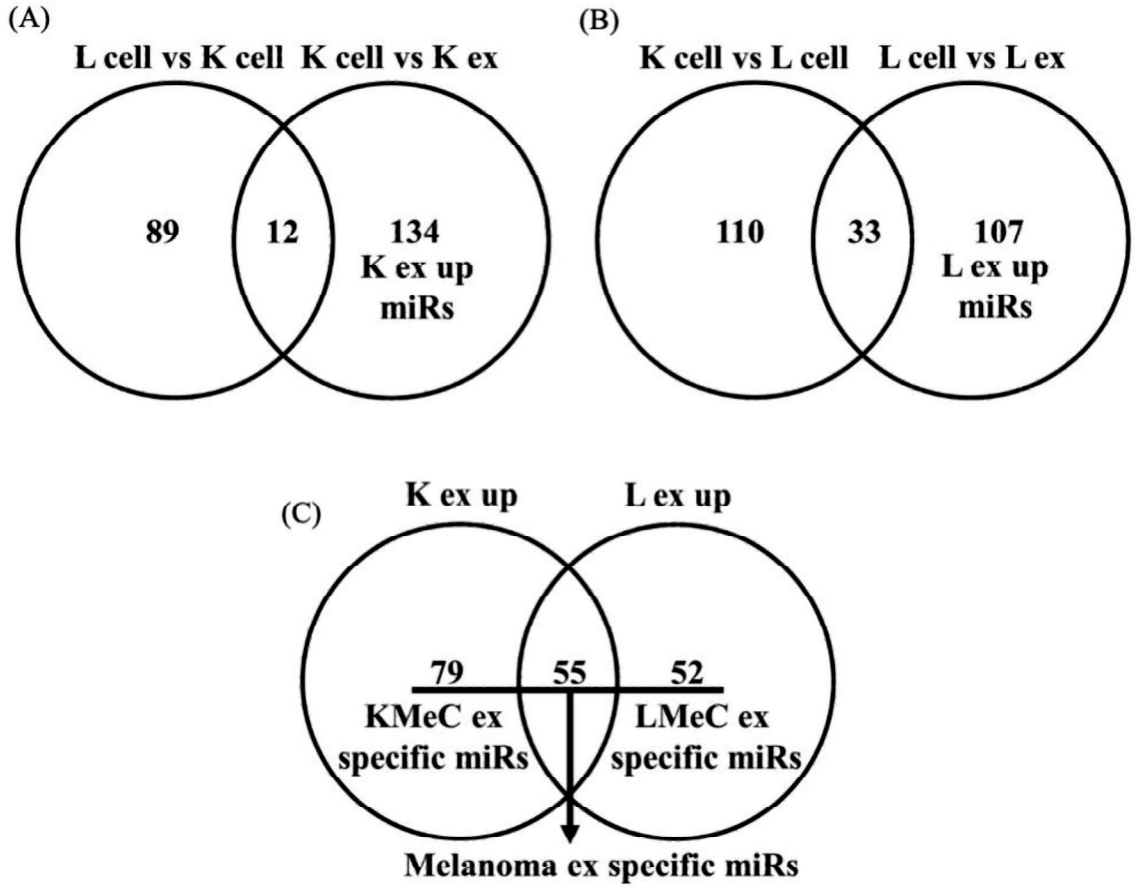

(A)
L cell vs K cell
K cell vs K ex
89
12
134
K ex up
miRs
(B)
K cell vs L cell
L cell vs L ex
110
33
107
L ex up
miRs
(C)
K ex up
L ex up
79
55
52
KMeC ex
specific miRs
LMeC ex
specific miRs
Melanoma ex specific miRs

Figure 1-3 Exosomal miRNAs in COM. (A-B) Venn diagram represents the miRNAs increased in the (A) KMeC and (B) LMeC exosomes. (C) Venn diagram shows the melanoma exosome-specific miRNAs, as well as the K and L exosome-specific miRNAs. K, KMeC (primary melanoma cell line); L, LMeC (metastatic melanoma cell line); ex, exosome.

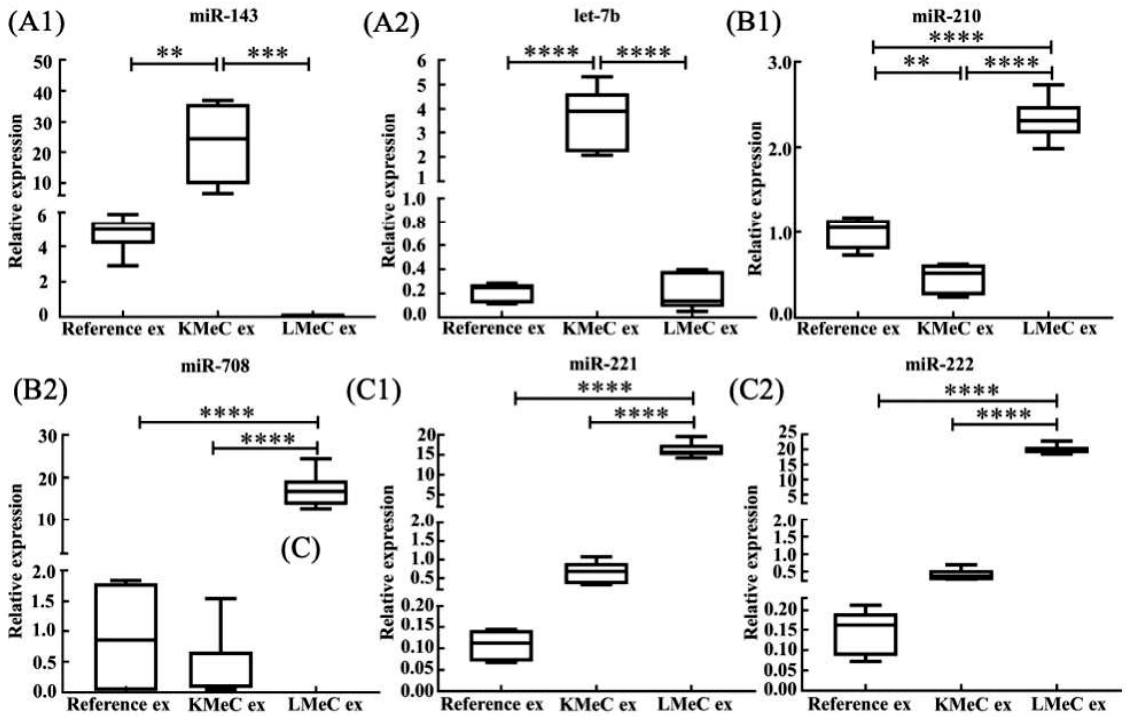
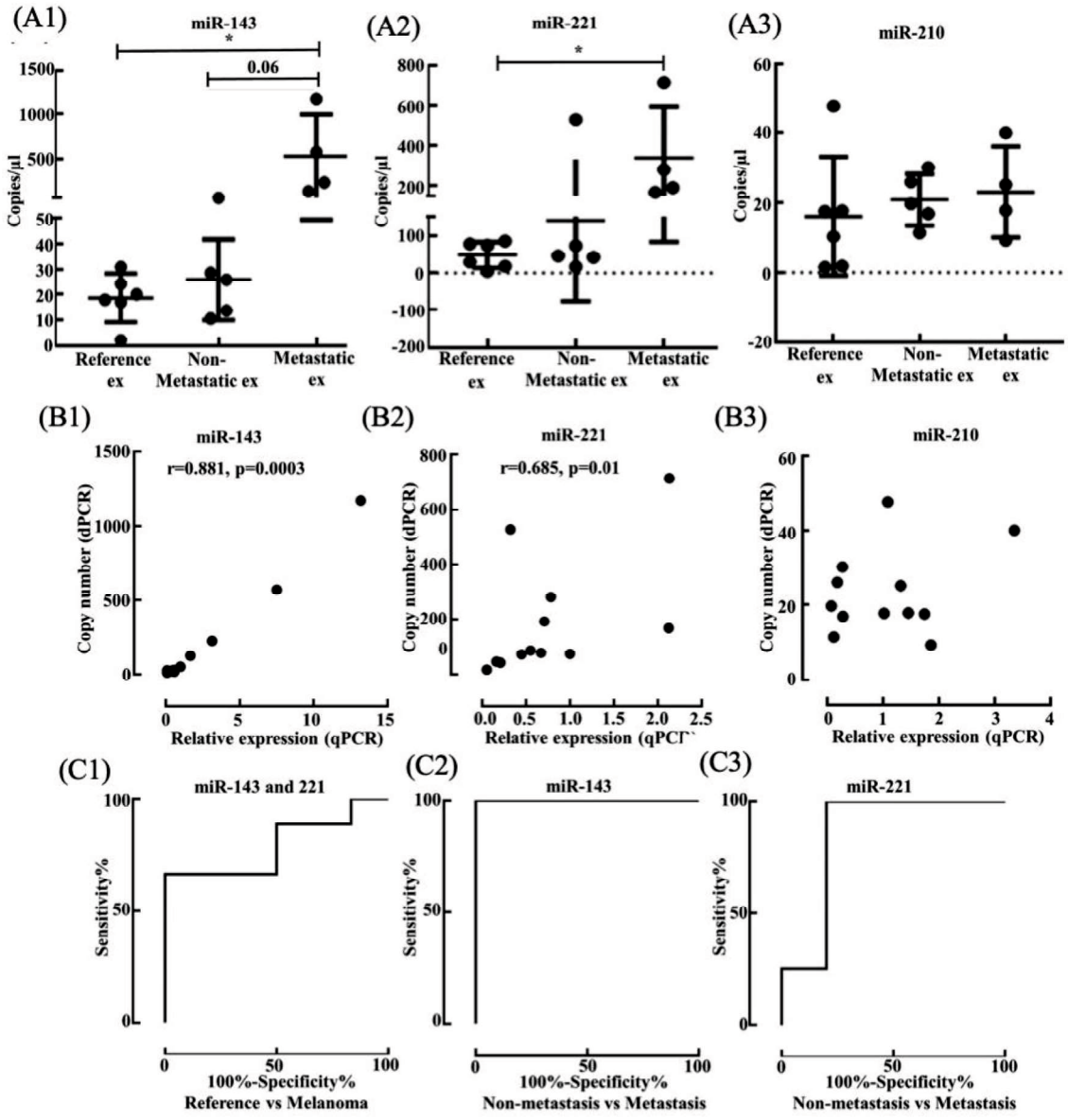

(A1)
miR-143
**
***
Relative expression
Reference ex
KMeC ex
LMeC ex
(A2)
let-7b
****
****
(B1)
miR-210
****
**
****
(B2)
miR-708
****
****
(C)
(C1)
miR-221
****
****
(C2)
miR-222
****
****

Figure 1-4 qRT-PCR validation of the exomiRs that define the primary and metastatic site cells of melanoma. (A1-2) miR-143 and let-7b were increased in KMeC cell line exosomes; (B1-2) miR-210 and miR-708 were increased in LMeC cell line exosomes; (C1-2) miR-221 and miR-222 were gradually increased in KMeC and LMeC cell line exosomes. N=6 in each group. The Y-axes indicate the relative miRNA expression levels normalized against miR-186 (Tukey's multiple comparisons test; **P <0.01; ***P <0.001; ****P <0.0001).



miRNA	Reference vs Melanoma	Reference Vs Non-metastasis	Reference vs Metastasis	Non-metastasis Vs Metastasis
	AUC (p-value)	AUC (p-value)	AUC (p-value)	AUC (p-value)
miR-143	0.78 (0.077)	0.60 (0.583)	1.00 (0.011)	1.00 (0.014)
miR-221	0.74 (0.125)	0.53 (0.855)	1.00 (0.011)	0.85 (0.086)
miR-143 and 221	0.80 (0.059)	0.63 (0.465)	1.00 (0.011)	0.90 (0.050)

Figure 1-5 Analysis of ExomiRs (miR-143 and miR-221) in clinical patients' plasma as a biomarker for metastatic melanoma. (A1-3) Expression of exomiRs- (A1) miR-143; (A2) miR-221 and (A3) miR-210 in patients' plasma was determined by dPCR. (B1-3) Correlation between the qRT-PCR and dPCR results regarding (B1) miR-143; (B2) miR-221 and (B3) miR-210. (C1-3) ROC curve to determine the diagnostic efficacy of (C1) miR-143 and 221 together; (C2) miR-143 and (C3) miR-221 as biomarker for melanoma.

Chapter 2

Long non-coding RNA and transfer RNA-derived small fragments in exosomes are potential biomarkers for canine oral melanoma

(Husna, AA *et al*., 2022 *Vet. Comp. Oncol).*

Chapter 2

2.1 Abstract

Novel small non-coding RNAs (sRNAs) represent an emerging line of research in both human and canine oncology, due to their diverse regulatory and functional roles. Novel sRNAs are regarded as distinct from microRNAs, although both are part of the exosomal cargo. Recently, I reported on exosomal miRNAs as biomarkers for canine melanoma; however, it is unknown if novel sRNAs hold similar potential. Accordingly, I aimed to identify and validate novel sRNAs as potential biomarkers of canine oral melanoma, as part of my larger project on sequencing small exosomal RNA for this disease. Next generation sequencing revealed several differentially expressed novel sRNAs in exosomes from two melanoma cell lines (KMeC and LMeC) when compared with reference exosomes (from tumor-free dogs). Among these novel sRNAs, long noncoding RNA fragments, tRNA-derived fragments, snoRNAs, and snRNAs were abundantly expressed. I selected four novel sRNAs upregulated in each cell line, and validated their aberrant expression with qPCR. In analysis using plasma-derived exosomes from melanoma patients, six out of the eight selected novel sRNAs showed significantly elevated expression. Receiver operating curve (ROC) analysis showed that one long non-coding RNA-derived small fragment (ENSCAFT00000069599.1) and one transfer RNA-derived small fragment (tRNA-Ala-

TGC-5-1) have more than 85% sensitivity and specificity for differentiating melanoma patients from tumor-free dogs. Therefore, I consider that novel sRNAs may serve as candidate biomarkers to facilitate more accurate diagnosis of canine oral melanoma in clinical settings.

2.2 Introduction

Exosomes are functional nanosized vesicles (30–150 nm) originating from the endocytic pathway in a wide range of cells, in both healthy and diseased individuals. They play a role in cell-to-cell communication after being released into biofluids such as blood, urine, saliva, and cerebrospinal fluid (7), with a cargo of DNA, coding and non-coding RNA, lipids, and proteins. Interestingly, exosomal cargo is already known to be dysregulated in human and other cancers (8–10). The exosomal transcriptome is thus a potentially rich source for biomarkers (9,11,12).

One type of molecule within the exosomal cargo—microRNAs (miRNAs)—has already been the focus of canine oral melanoma-related research. I previously described a potential role for exosomal miRNAs as biomarker of this disease (17). However, research on human cancer has implicated a number of exosomal transcripts, not just miRNAs, in cancer development and progression (6,7). The transcripts receiving this attention are small non-coding RNAs (sRNAs), and they have been described as “rare sRNAs” in human medicine (18), or more widely as novel sRNAs; in this paper I will adopt the latter term.

Such novel sRNAs appear to be promising biomarkers in canine oncology. They encompass a diverse range of sRNA species, including small nucleolar RNAs (snoRNAs),

small nuclear RNAs (snRNAs), small cajal body-specific RNA (scaRNA), transfer RNA-derived small fragments (tRFs), and PIWI-interacting RNAs (piRNAs)(9,10). I previously reported on aberrantly expressed snoRNA, snRNA, tRFs, and piRNAs in canine melanoma tissues, cell lines, and plasma (19). Moreover, novel sRNAs have been linked with a range of cancers including gastric cancer, breast cancer, oral squamous cell carcinoma, head and neck cancer in human and/or canine populations, especially where they show a high level of exosomal loading (11,16). A further four snoRNAs (ACA17, ACA45, HBII-276, and SNORD12) have been proposed as prognostic markers of human uveal melanoma (20). In general, novel sRNAs possess high stability and low variability, and are conserved across a range of species. Accordingly, they can be regarded as a more reliable source of non-invasive biomarkers for the investigation of cancer than commonly used miRNAs or lncRNAs (18).

Identifying novel sRNAs aberrantly expressed in exosomes from malignant cells is thus a promising line of research on new diagnostic and therapeutic approaches in canine oncology in general, and for melanomas in particular. Melanomas are highly aggressive skin cancers in canine as well as human medicine. They can progress to a metastatic phase dramatically, with a concomitantly poor prognosis (18,23–25). Canine oral melanoma is the most common oral tumor in dogs, accounting for 7% of all malignant tumors in dogs (25),

and has been proposed as a spontaneous model for human melanoma (35). Both clinicians and researchers stand to benefit from the identification of new biomarkers for this disease, and novel sRNAs hold great promise in that role, as discussed above. Clearly, their potential as biomarkers in canine oncology could be overlooked through an over-narrow focus on miRNAs alone.

Accordingly, in this study, I aimed to identify novel sRNAs with potential as biomarkers of canine oral melanoma, and then elucidate their diagnostic efficacy. I investigated sRNA species by comparing their exosomal expression between canine oral melanoma cell lines and healthy dogs with next generation sequencing (NGS), and subjected aberrantly expressed sRNAs for a validation of expression with qPCR and investigations of expression, and specificity and sensitivity as biomarkers, in live patients.

2.3 Materials and Methods

2.3.1 Preparation and identification of exosomes

Cell culture and exosome preparation were performed as described in my previous study (17). Briefly, exosomes were prepared from two canine oral melanoma cell lines: KMeC (derived from primary oral melanoma) and LMeC (derived from a metastatic mandibular lymph node of oral melanoma) (56). These preparations were regarded as melanoma exosomes in this study. The cell lines were stored in a frozen liquid nitrogen medium (CultureSure; Fujifilm Wako Pure Chemical Corporation, Osaka, Japan). Cells were cultured until confluence as previously described (Inoue et al., 2004). Exosome-depleted fetal bovine serum (FBS; System Bioscience, Mountain View, CA, USA) was used. Culture media were harvested for exosome preparation. Exosomes were also isolated from plasma from tumor-free dogs (housed at Kagoshima University, Japan) and dogs with melanoma (undergoing treatment at the Veterinary Teaching Hospital, Kagoshima University, Japan). In each case, the condition had been diagnosed by qualified veterinarians through clinical examinations and pathological examination. The exosomes obtained from tumor-free dogs were adopted as reference exosomes, in the absence of any established primary canine melanocyte cell line. Whole blood was collected from both the tumor-free and melanoma-bearing dogs to obtain plasma. Informed consent was obtained from each patient's owner before sample collection. Plasma was isolated by centrifugation at 3000 x g for 10 minutes immediately after blood collection.

The obtained plasma was then aliquoted into eppendorf tubes and processed for exosome preparation. The demographic (age, sex, and breed) and clinical characteristics of the canine melanoma patients are presented in the supplementary data (Table S1). The study design and experimental conditions were approved by the Kagoshima University Veterinary Teaching Hospital Ethics Committee (Approval No. KV0004).

Exosomes were isolated from ten-milliliter aliquots of culture media containing cells from the relevant cell line. Exosomes were isolated from the plasma of tumor-free or melanoma-bearing dogs, using one-milliliter amounts of plasma for NGS, and 300-microliter amounts for qPCR validation. The Total Exosome Isolation Kit (Invitrogen, Thermo Fisher Scientific, Carlsbad, CA, USA) was used for cell culture and plasma. Exosomes were characterized by nanoparticle tracking analysis (NTA) and Western blot analysis as described in my previous work (17). Cell culture and exosome preparation were performed as described in my previous study (17). Briefly, exosomes were prepared from two canine oral melanoma cell lines: KMeC (derived from primary oral melanoma) and LMeC (derived from a metastatic mandibular lymph node of oral melanoma) (56). These preparations were regarded as melanoma exosomes in this study. The cell lines were stored in a frozen liquid nitrogen medium (CultureSure; Fujifilm Wako Pure Chemical Corporation, Osaka, Japan). Cells were cultured until confluence as previously described (Inoue et al., 2004). Exosome-depleted fetal bovine serum (FBS; System Bioscience, Mountain View, CA, USA) was used.

Culture media were harvested for exosome preparation. Exosomes were also isolated from plasma from tumor-free dogs (housed at Kagoshima University, Japan) and dogs with melanoma (undergoing treatment at the Veterinary Teaching Hospital, Kagoshima University, Japan). In each case, the condition had been diagnosed by qualified veterinarians through clinical examinations and pathological examination. The exosomes obtained from tumor-free dogs were adopted as reference exosomes, in the absence of any established primary canine melanocyte cell line. Whole blood was collected from both the tumor-free and melanoma-bearing dogs to obtain plasma. Informed consent was obtained from each patient's owner before sample collection. Plasma was isolated by centrifugation at 3000 x g for 10 minutes immediately after blood collection. The obtained plasma was then aliquoted into eppendorf tubes and processed for exosome preparation. The demographic (age, sex, and breed) and clinical characteristics of the canine melanoma patients are presented in the supplementary data (Table S1). The study design and experimental conditions were approved by the Kagoshima University Veterinary Teaching Hospital Ethics Committee (Approval No. KV0004).

Exosomes were isolated from ten-milliliter aliquots of culture media containing cells from the relevant cell line. Exosomes were isolated from the plasma of tumor-free or melanoma-bearing dogs, using one-milliliter amounts of plasma for NGS, and 300-microliter amounts for qPCR validation. The Total Exosome Isolation Kit (Invitrogen, Thermo Fisher Scientific, Carlsbad, CA, USA) was used for cell culture and plasma. Exosomes were characterized by nanoparticle tracking analysis (NTA) and Western blot analysis as described in my previous work (17).

2.3.2 Exosomal RNA extraction and NGS

Total RNA was isolated from both the cell lines and plasma-derived exosomes using the Total Exosome RNA and Protein Isolation Kit (Invitrogen, Thermo Fisher Scientific). The relevant concentration was measured using a Qubit® microRNA Assay Kit (Life Technologies, Thermo Fisher Scientific). Cel-39 was spiked during exosome RNA isolation to confirm the isolation efficiency (Appendix 13a-b). The quality and integrity of the RNA were assessed using an Agilent 2100 Bioanalyzer (G2939BA, Agilent Technologies, Santa Clara, CA, USA). sRNA libraries were prepared and sequenced by the Hokkaido System Sciences Company (Sapporo, Hokkaido, Japan). In brief, one microgram of total RNA was analyzed using the TruSeq Small RNA Library Preparation Kit (Illumina, San Diego, California) for sRNA-library constructions, in accordance with the manufacturer's guidelines. 5′ and 3′ adaptors were ligated to the sRNAs (18-55 nt). cDNA was constructed by reverse transcription followed by amplification. For cluster generation, the amplified cDNA constructs were purified with a gel purification step. An Illumina/HiSeq2500 instrument (Hokkaido System Science Co., Ltd.) was used for sequencing.

2.3.3 Analysis of exosomal sRNA sequencing reads

CLC Genomics Workbench (versions 10 and 12; CLC Bio, Qiagen, Hilden, NRW, Germany) was used to import and analyze the sequencing reads in accordance with the manufacturer’s sRNA analysis protocol. Default parameters were followed.

Normalization, ambiguity, adapter and trimming were performed together with quality control. ENSEMBL (Release 104) human and dog and RNA central databases were used to annotate the reads. However, interested readers can find out the individual candidate by searching manually in the database websites. In the Biomart section of Ensembl; genes 104 databases should be selected. In the dataset section dog/human genes (CanFam 3.1 or human genes GRCH 38. p13) should be chosen. For RNAcentral following weblink-https://rnacentral.org/search?q=URS000194BF29_9823 can be used to find the individual candidate. The EDGE (empirical analysis of differential gene expression) program was used to determine differential expression. The statistical filtering criteria consisted of an absolute fold change >2, a false discovery rate (FDR) ≤0.05, and an expression value per sample >10; were used in differential expression analysis.

2.3.4 Quantitative real-time PCR (qRT-PCR)

Exosomal sRNA expression levels were determined using custom TaqMan gene expression assays (Thermo Fisher Scientific). 1.25µl (0.205 ng/µl) of total exosomal RNA was reverse transcribed into cDNA by using the TaqMan MicroRNA Reverse Transcription Kit (Thermo Fisher Scientific) in accordance with the manufacturer's protocol. qRT-PCR was performed using the TaqMan® Fast Advanced Master Mix Kit (Thermo Fisher Scientific) and the StepOne Plus™ Real-Time PCR System (Applied Biosystems; Thermo Fisher Scientific). Thermal cycling was maintained following the manufacturer's instructions.

All experiments were performed in duplicate and sRNAs expression was measured using the $2^{-\Delta\Delta CT}$ method. $2^{-\Delta\Delta CT}$ was plotted as relative expression. MiR-186 was used as an internal control as described in my previous study.(17) The ct value of miR-186 among the all groups are shown in Appendix 13c-d. Custom-made primer sequences were as follows: lncRNA fragments 5′-GGCCGUGAUCGUAUAGUGGUUAGUACUCUGCG-3′ (ENSCAFT00000069599.1) and 5′-CCUGGACAUGAUGACUC-3′ (ENSCAFT00000090032.1), tRNA fragments 5′-TCGTTTCCCGGCCAACGCACCA-3′ (tRNA-Gly); 5′- GTTTCCGTAGTGTAGCGGTCATCACACTCG-3′ (tRNA-Val-AAC-3-1) and 5′-GGGGATGTAGCTCAGCGGTAGAGCATATG-3′ (tRNA-Ala-TGC-5-1), SCARNA16 fragment 5′-AUGGUCUGCGGAAAGGCUCCUGUGUGGCCGCACC-3′, SNORD20 fragment 5′-UAGUCAAGUUCUGAUCCAG-3′, and miRNA 5′-CAAGGTCCGCTGTGAACACGGA-3′.

2.3.5 Statistical analysis

Statistical analysis was carried out using GraphPad Prism 9 (www.graphpad.com). Mann-Whitney t-test and one-way ordinary ANOVA were used to compare the qRT-PCR data. A p-value <0.05 was considered statistically significant.

2.4 Results

2.4.1 Aberrantly expressed sRNAs in canine oral melanoma exosomes

To investigate novel sRNA species aberrantly expressed in canine oral melanoma, I set out to identify non-miRNA reads in melanoma exosomes (from the KMeC and LMeC cell lines) and reference exosomes as part of my exosomal sRNA sequencing project (PRJNA656859). Reads are annotated based on the Ensemble human and dog and RNAcentral databases. Non-miRNA sRNAs accounted for 14% of annotated reads for the reference exosomes, as opposed to 42% for KMeC exosomes and 18% for LMeC exosomes (Figure 2-1a-c). I then set out to identify specific novel sRNAs differentially expressed in the melanoma exosomes versus reference exosomes, by applying stringent filtering criteria (absolute fold change >2 and FDR ≤0.05).

For the KMeC cell line, I initially identified 1849 sRNAs as significantly differentially expressed in melanoma exosomes versus reference exosomes (Figure 2-2a). This total was winnowed down to only the most markedly differentially expressed sRNAs, with application of an additional filtering criterion (mean expression ≥1000). A total of 108 novel sRNAs spanning 11 types met all three criteria (Figure 2-2b; Table 1), among which lncRFs, snRNAs, tRFs, and snoRNAs were found to be the most abundant.

For the LMeC cell line, I initially identified 3185 sRNAs (Figure 2-2c) as significantly differentially expressed in melanoma exosomes versus reference exosomes. This total was winnowed down by applying the additional criterion described above. A total of 100 sRNAs spanning 10 sRNA types met all three criteria (Figure 2-2d; Table 2), among which lncRFs, snRNAs, and snoRNAs were found to be the most abundant.

2.4.2 qRT-PCR validation of sRNAs

To validate the findings in exosomal NGS, I selected eight representative novel sRNAs for qPCR validation of their expression levels in melanoma exosomes (from the KMeC and LMeC cell lines) relative to reference exosomes. For each cell line, I selected sRNAs from higher and lower expression levels (within the acceptable range). The sequencing results for KMeC exosomes were confirmed by the upregulation of the relevant lncRF (transcript ID: ENSCAFT00000069599.1), SCARNA16 (ENSCAFT00000042192.1), and two tRFs (URS00004C0DBA_7091; tRNA-Gly and URS0000EE95ED_9615; tRNA-Val-AAC-3-1) relative to the reference exosomes (Figure 2-3a-d). Likewise, the sequencing results for LMeC exosomes were confirmed by upregulation of the relevant lncRF (ENSCAFT00000090032.1), SNORD20 (ENSCAFT00000033483.1), gga-miR-124c

(URS000075EF3B_9031), and tRNA-Ala-TGC-5-1 (URS00006CDE6B_9615) relative to reference exosomes (Figure 2-3e-h).

2.4.3 Expression of sRNA fragments in plasma-derived exosomes from canine oral melanoma patients

To establish whether differential expression patterns in validated melanoma cell lines could be replicated in dogs actually diagnosed with oral melanoma, I measured the relative expression in plasma-derived exosomes from canine oral melanoma patients versus reference exosomes, for each of the eight novel sRNAs that yielded positive results in exosomal NGS and qRT-PCR validation.

Five of the eight representative novel sRNAs showed significantly elevated relative expression in canine oral melanoma patients: the two lncRFs, ENSCAFT00000069599.1 and ENSCAFT00000090032.1 (82 and 24-fold elevations, respectively; Figure 2-4a-b), the two tRNA-derived fragments, tRNA-Gly and tRNA-Ala-TGC-5-1 (32 and 45-fold, respectively; Figure 2-4c-d), and the snoRD20-derived fragment (six-fold; Figure 2-4e).

One of the eight representative novel sRNAs showed a tendency toward elevated relative expression in canine oral melanoma patients: tRNA-Val-AAC-3-1 (p=0.087; Figure 2-4f; validated as differentially expressed in the KMeC cell line).

The other two representative novel sRNAs showed no significant elevation in relative expression in canine oral melanoma patients: SCARNA16 (two-fold elevation; p=0.23; Figure 2-4g; validated as differentially expressed in the KMeC cell line), and gga-miR-124c (three-fold elevation; Figure 2-4h; validated as differentially expressed in the LMeC cell line).

2.4.4 Receiver operating characteristic curve analysis of the candidates for biomarker

The representative novel sRNAs showing or tending to show elevated relative expression in clinical patients were regarded as candidate biomarkers. To evaluate the suitability of the candidate sRNAs as biomarkers for canine melanoma, I analyzed their sensitivity and specificity in receiver operating characteristic (ROC) curve analysis, regarding an area under the ROC curve (AUC) $\geq$0.80 as confirming diagnostic efficacy. One of the lncRFs (ENSCAFT00000069599.1) showed the greatest diagnostic efficacy (AUC = 0.91, p-value $\leq$ 0.01; Figure 2-5a), and four other candidate sRNAs—tRNA-Ala-TGC-5-1, tRNA-Gly, SNORD20, and lncRF-ENSCAFT00000090032.1—also yielded values above

the threshold for potential diagnostic efficacy (AUC ≥0.80; p-value ≤0.05; Figure 2-5b-e).

The only exception was tRNA-Val-AAC-3-1(AUC =0.78; p-value 0.07) Figure 2-5f.

2.5 Discussion

To the author's knowledge, this is the first report on novel exosomal sRNAs (non-miRNA sRNAs) as potential biomarkers of canine oral melanoma, and the first report on a lncRF as a cancer biomarker.

As major novel findings in this study, I identified 108 and 100 novel sRNAs as aberrantly expressed in the KMeC and LMeC canine oral melanoma cell lines, respectively, and established that five of these novel sRNAs (from a representative subset of eight) were also aberrantly expressed in clinical cases of canine oral melanoma, and met criteria for diagnostic efficacy as biomarkers for this disease. Taking novel exosomal sRNAs from molecules identified in canine oral melanoma cell lines with NGS, through valuation with PCR and evaluation in real patients, to successful evaluations for diagnostic efficacy serves as a proof of concept for such research on these sRNAs.

The second stage of my research (that in live animals) only involved a small, representative subset of the sRNAs identified and validated through NGS and PCR analyses with cell lines. From these novel sRNAs, I selected eight sRNAs (comprising two lncRFs, one scaRNA, three tRFs, one snoRNA and one miRNA) at the higher and lower ends of the spectrum of expression levels allowed by the stringent filtering criteria, and the expression results were successfully validated for each of them in PCR analysis. Six of these novel

sRNAs were overexpressed in canine oral melanoma patients, and five of them showed sufficient sensitivity and specificity to be regarded as candidate biomarkers. It is noteworthy that just this small subset yielded some promising candidate biomarkers, and suggests that the wider pool of aberrantly expressed sRNAs I identified also represent promising lines of future research.

The results of my diagnostic efficacy evaluation are particularly interesting. I targeted six novel sRNAs (that had been identified in NGS and validated in PCR) showing high expression levels (significantly high or tending to significance) in canine oral melanoma patients for ROC curve analysis of sensitivity and specificity. Five of the six novel sRNAs met the criterion for diagnostic efficacy, and two of these sRNAs appear particularly promising (based on their AUC and p values). These three novel sRNAs represent two derived-fragment RNA types; specifically, one was a lncRF (transcript ID: ENSCAFT00000069599.1) and the other two were tRFs (tRNA-Gly and tRNA-Ala-TGC-5-1). This is the first time such novel derived-fragment type sRNAs have been identified as candidate biomarkers for melanoma. I consider that the plasma levels of these exosomal sRNAs could serve as potential diagnostic biomarkers for canine oral melanoma.

My results are consistent with a number of previous indications that these novel sRNAs may play specific roles in a range of cancers (3,16). Other researchers are already looking at

these novel sRNA types as cancer biomarkers both generally and in specific instances (36,57). Studies in humans and dogs reveal that melanoma is one of many cancers in which tRFs may be differentially expressed, indicating their diagnostic and prognostic significance (19,36,58–62). Two scaRNAs (SCARNA2 and SCARNA22) have been found to play an oncogenic role in colorectal cancer and multiple myeloma (63–65). SNORD20 is reported as one of the most highly expressed RNAs in glioblastoma cell lines (18). In another study, melanoma exosomes were found to be enriched with SNORD83A and SNORD89 (66). Moreover, SNORD126, SNORA23, SNORA42, and SNORD46 are reportedly similarly overexpressed in multiple cancer types (67). My findings extend this evidence, and through a particular focus on sRNAs in the field of canine oral melanoma, bring to light the role of an lncRF (ENSCAFT00000090032.1), a newly detected transcript derived from the ENSCAFG00000047740 gene (68).

My study possesses two limitations. First, I initially identified sRNAs with exosomal profiling in two melanoma cell lines; I did not investigate circulating exosomes in melanoma-bearing dogs at this stage. This is due to the absence of established techniques for separating circulating exosomes derived from melanoma cells and other cells in the blood. Since exosomes from all cells throughout the body are present in plasma, the true circulating exosomal RNA profiles of melanoma-bearing dog plasma would encompass both melanoma

exosomes and exosomes from normal cells. Second, I compared novel sRNA expression between exosomes from canine oral melanoma cell lines and reference exosomes (plasma-derived exosomes from tumor-free dogs) because no primary canine melanocyte cell line is currently established. Accordingly, I consider that tumor-free dog plasma was the optimum source of reference data for this blood-based exosomal study.

In conclusion, I successfully demonstrated the aberrant expression of a number of novel sRNAs in exosomes from canine oral melanoma cell lines and canine melanoma patient plasma. These findings also included several newly detected transcripts. In further analysis, I identified five of these differentially expressed sRNAs as candidate liquid biopsy biomarkers for diagnosing canine oral melanoma. Taking the results together, my study represents an expansion to existing knowledge of novel exosomal sRNAs, and sheds light on their diagnostic utility of these sRNA species as promising biomarkers for canine oral melanoma.

Table 2-1 Differentially expressed exosomal RNAs (KMeC cell line versus reference exosomes)

exosomal RNA	RNA type
URS0000811DC6_10090	lncRNA†
URS00009BD94D_10090	
URS00009C1FD1_10090	
URS0000A89D34_10090	
URS0000A9325C_10090	
URS0000A9F5C6_10090	
URS0000AA63A7_10090	
URS0000E6FDBF_10090	
URS000194BF29_9823	
ENSCAFT00000052854.2	
ENSCAFT00000054104.2	
ENSCAFT00000067106.1	
ENSCAFT00000069002.1	
ENSCAFT00000069599.1	
ENSCAFT00000076690.1	
ENSCAFT00000078237.1	
ENSCAFT00000083630.1	
ENSCAFT00000086830.1	
ENSCAFT00000090032.1	
ENSCAFT00000091135.1	
ENSCAFT00000091455.1	
ENST00000429798.1	
ENST00000548322.1	
ENST00000548819.5	
ENST00000565336.1	
ENST00000566930.6	

ENST00000569473.1	
ENST00000602361.1	
ENST00000630360.1	
ENST00000655322.1	
ENST00000658801.1	
ENST00000660868.1	
ENST00000668190.1	
URS000075A277_9601	miRNA‡
URS0000A7D9CF_9365	
URS0000AB095F_59463	
URS0000CCAC0F_9568	
URS00002B5CD1_68728	miscRNA§
URS00003A1C55_32644	
URS0000582A62_55040	
ENSCAFT00000046542.2	
ENSCAFT00000049090.2	
ENSCAFT00000050083.2	
ENSCAFT00000040266.1	ribozyme
URS000195D6D0_9685	rRNA¶
ENSCAFT00000033466.1	
ENSCAFT00000042192.1	scaRNA††
ENSCAFT00000033483.1	snoRNA‡‡
ENSCAFT00000034285.1	
ENSCAFT00000034404.2	
ENSCAFT00000040097.1	
ENSCAFT00000040113.2	
ENSCAFT00000040962.1	
ENSCAFT00000071727.1	
ENST00000365223.1	
ENSCAFT00000032824.1	snRNA§§
ENSCAFT00000032993.1	

ENSCAFT00000033229.1	
ENSCAFT00000033276.1	
ENSCAFT00000033315.2	
ENSCAFT00000033317.1	
ENSCAFT00000033408.1	
ENSCAFT00000033543.1	
ENSCAFT00000033579.1	
ENSCAFT00000034089.1	
ENSCAFT00000034121.1	
ENSCAFT00000034417.2	
ENSCAFT00000034428.1	
ENSCAFT00000034434.1	
ENSCAFT00000035003.1	
ENSCAFT00000040041.1	
ENSCAFT00000040145.1	
ENSCAFT00000040312.1	
ENSCAFT00000040756.1	
ENSCAFT00000043401.1	
ENSCAFT00000043565.1	
ENSCAFT00000043978.1	
ENSCAFT00000044351.1	
ENSCAFT00000044781.1	
ENSCAFT00000045740.1	
ENSCAFT00000046881.1	
ENSCAFT00000047494.1	
ENSCAFT00000048459.1	
ENSCAFT00000048898.1	
ENSCAFT00000049695.1	
ENSCAFT00000049813.1	
URS00003B4E0F_9986	tRNA[¶¶]
URS00004C0DBA_7091	

URS0000633F72_7955	
URS0000662B0F_31033	
URS0000682E19_400682	
URS0000687872_9615	
URS0000691C1E_9785	
URS000069EF13_9785	
URS00006AE498_7955	
URS00006C9C28_7955	
URS0000868532_9986	
URS0000BE33FF_8969	
URS0000BE7A21_246437	
URS0000C0799D_37040	
URS0000EE95ED_9615	
URS0000EEABAF_9940	
URS0001894629_623744	
URS0000A9948C_9615	Unknown
URS0000AAFC4C_9615	
ENSCAFT00000040573.1	vaultRNA
ENSCAFT00000034677.1	Y_RNA
ENSCAFT00000034682.1	

† Long non-coding RNAs, ‡ microRNA, § miscellaneous RNA, ¶ Ribosomal RNA, †† Small Cajal body-specific RNAs, ‡‡ Small nucleolar RNAs, §§ Small nuclear RNA, ¶¶ Transfer RNA

Table 2-2 Differentially expressed exosomal RNAs (LMeC cell line versus reference exosomes)

Exosomal RNA	RNA Type
URS00009BD94D_10090	lncRNA†
URS00009C1FD1_10090	
URS0000A89D34_10090	
URS0000A9325C_10090	
URS0000A9F5C6_10090	
URS0000B28FA1_10116	
URS0000DFA586_9627	
URS0000DFCBA1_10090	
URS0000E6FDBF_10090	
URS000194BF29_9823	
ENSCAFT00000043311.2	
ENSCAFT00000044771.2	
ENSCAFT00000049587.2	
ENSCAFT00000052854.2	
ENSCAFT00000054104.2	
ENSCAFT00000055431.2	
ENSCAFT00000057426.2	
ENSCAFT00000064971.1	
ENSCAFT00000069002.1	
ENSCAFT00000069152.1	
ENSCAFT00000069208.1	
ENSCAFT00000070429.1	
ENSCAFT00000072611.1	
ENSCAFT00000076075.1	
ENSCAFT00000076690.1	
ENSCAFT00000078237.1	

ENSCAFT00000080360.1	
ENSCAFT00000081917.1	
ENSCAFT00000086830.1	
ENSCAFT00000087428.1	
ENSCAFT00000089723.1	
ENSCAFT00000090032.1	
ENSCAFT00000091067.1	
ENSCAFT00000091135.1	
ENSCAFT00000092146.1	
ENST00000566930.6	
ENST00000570843.1	
ENST00000630360.1	
ENST00000648228.1	
ENST00000650189.1	
URS0000A7D9CF_9365	miRNA[‡]
URS0000AB095F_59463	
URS0000CCAC0F_9568	
URS000075A277_9601	
ENST00000580344.1	
ENSCAFT00000032578.1	
URS00002B5CD1_68728	miscRNA[§]
URS00003A1C55_32644	
URS0000582A62_55040	
ENSCAFT00000034816.1	Mt_rRNA[¶]
ENSCAFT00000043634.2	rRNA[††]
ENSCAFT00000076881.1	
ENSCAFT00000042192.1	scaRNA[‡‡]
ENST00000365223.1	snoRNA[§§]
ENSCAFT00000032979.1	
ENSCAFT00000033483.1	
ENSCAFT00000033754.1	

ENSCAFT00000033831.1	
ENSCAFT00000034285.1	
ENSCAFT00000040031.1	
ENSCAFT00000040069.1	
ENSCAFT00000040097.1	
ENSCAFT00000040113.2	
ENSCAFT00000032824.1	snRNA¶¶
ENSCAFT00000032993.1	
ENSCAFT00000033229.1	
ENSCAFT00000033276.1	
ENSCAFT00000033315.2	
ENSCAFT00000033317.1	
ENSCAFT00000033408.1	
ENSCAFT00000033543.1	
ENSCAFT00000033579.1	
ENSCAFT00000034121.1	
ENSCAFT00000035003.1	
ENSCAFT00000040041.1	
ENSCAFT00000040145.1	
ENSCAFT00000040156.1	
ENSCAFT00000040312.1	
ENSCAFT00000040756.1	
ENSCAFT00000042047.1	
ENSCAFT00000042310.1	
ENSCAFT00000043401.1	
ENSCAFT00000043565.1	
ENSCAFT00000043978.1	
ENSCAFT00000044781.1	
ENSCAFT00000045740.1	
ENSCAFT00000046881.1	
ENSCAFT00000047494.1	

ENSCAFT00000048459.1	
ENSCAFT00000049813.1	
URS00000FA8B2_9606	tRNA[†††]
URS0000633F72_7955	
URS0000682E19_400682	
URS0000687872_9615	
URS0000EEABAF_9940	
URS0000A86CA7_9615	Unknown
URS0000A9948C_9615	
URS0000A9A080_9615	
URS0000AAA8B1_9615	
ENSCAFT00000040573.1	vaultRNA

[†] Long non-coding RNAs, [‡] microRNA, [§] miscellaneous RNA, [¶] Mitochondrial Ribosomal RNA, [††]Ribosomal RNA, [‡‡] Small Cajal body-specific RNAs, [§§] Small nucleolar RNAs, [¶¶] Small nuclear RNA, [†††] Transfer RNA

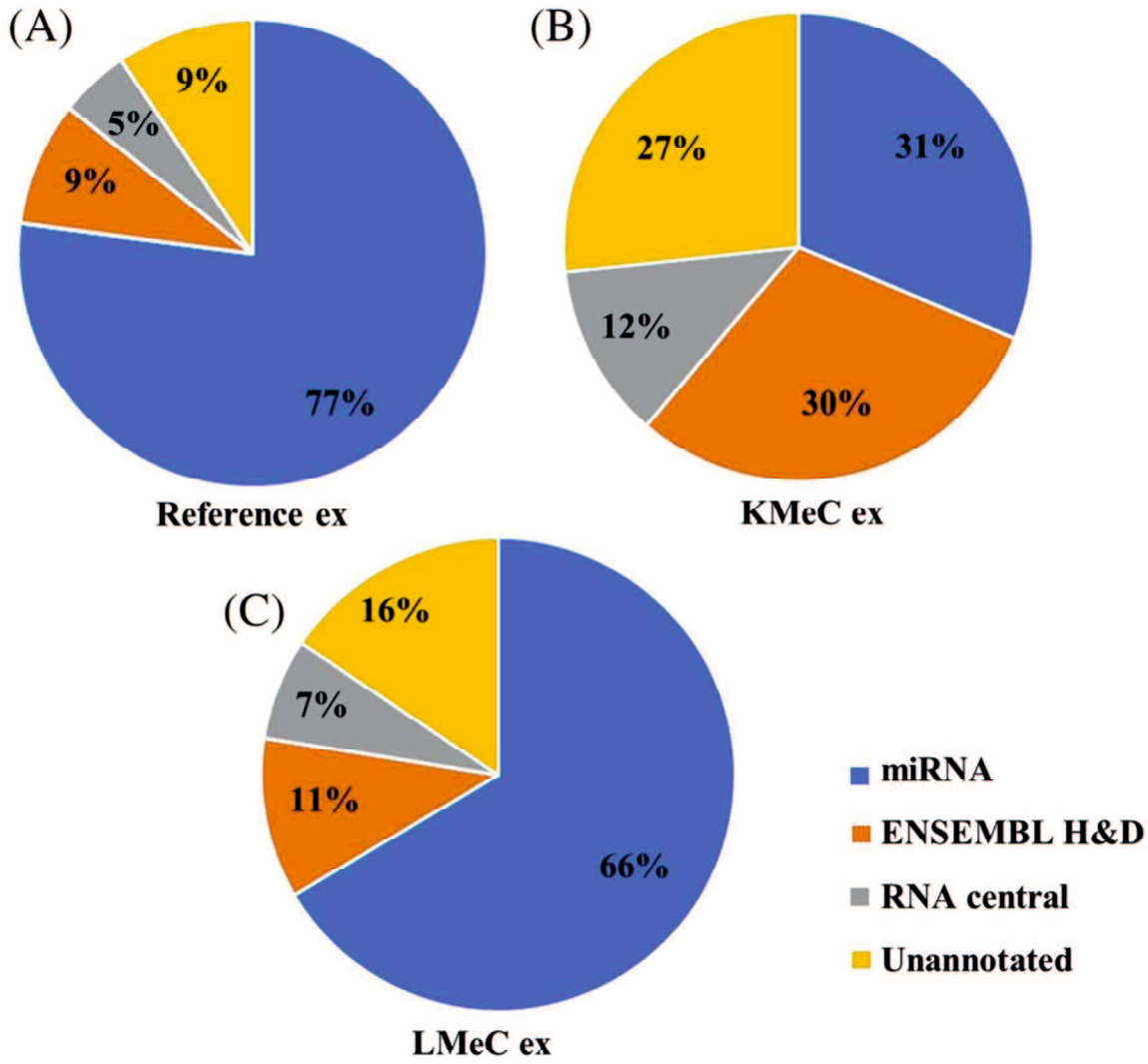

(A)
9%
5%
9%
77%
Reference ex
(B)
27%
31%
12%
30%
KMeC ex
(C)
16%
7%
11%
66%
LMeC ex
miRNA
ENSEMBL H&D
RNA central
Unannotated

Figure 2-1 Annotation of small RNA reads in reference and melanoma exosomes. (a-c) Small RNA reads are annotated with the Ensembl H&D (human and dog) and RNAcentral databases with reference exosomes (a); KMeC cell line exosomes (b) and LMeC cell line exosomes (c). Ex, exosome; KMeC, primary melanoma cell line; LMeC, metastatic melanoma cell line.

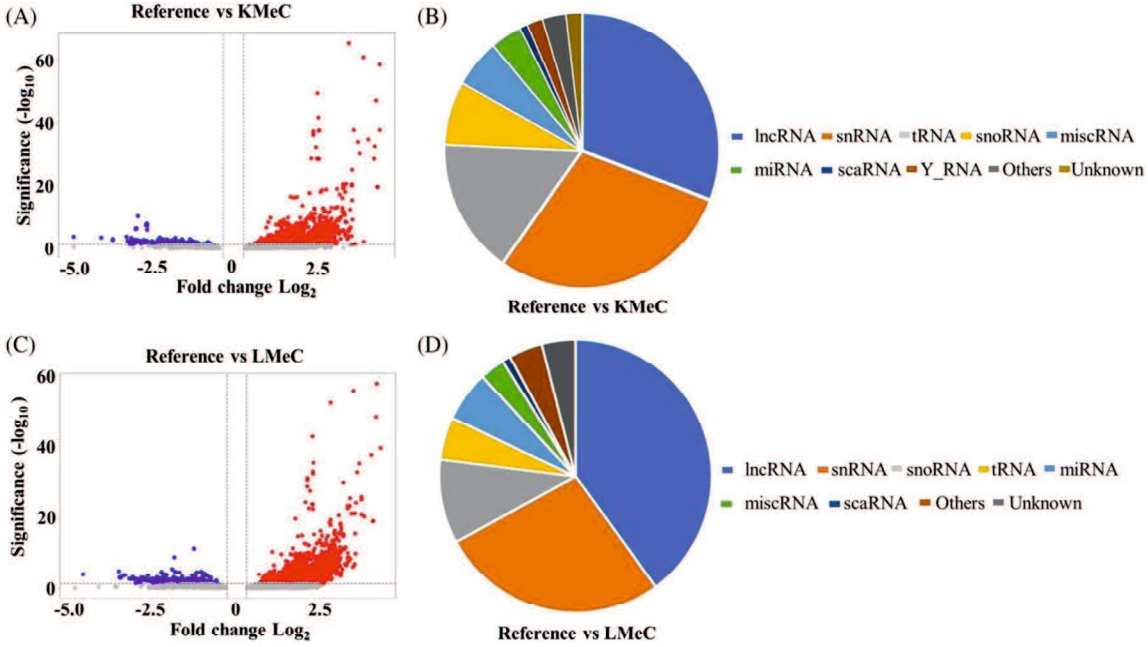

(A)
Reference vs KMeC
Significance (-log10)
60
40
20
0
-5.0
-2.5
0
2.5
Fold change Log2
(B)
lncRNA
snRNA
tRNA
snoRNA
miscRNA
miRNA
scaRNA
Y_RNA
Others
Unknown
Reference vs KMeC
(C)
Reference vs LMeC
Significance (-log10)
60
40
20
0
-5.0
-2.5
0
2.5
Fold change Log2
(D)
lncRNA
snRNA
snoRNA
tRNA
miRNA
miscRNA
scaRNA
Others
Unknown
Reference vs LMeC

Figure 2-2 Differentially expressed small non-coding RNAs (sRNAs) in melanoma cell lines versus reference exosomes. (a) Volcano plots represent differentially expressed sRNAs in KMeC cell line exosomes versus reference exosomes. Each circle signifies a single sRNA. Red and blue circles indicate significantly differentially expressed sRNAs (Fold change absolute value >2, FDR ≤0.05). (b) Pie chart illustrating abundantly expressed rare RNA species with mean expression ≥1000. (c) Volcano plots represent differentially expressed sRNAs in LMeC cell line exosomes versus reference exosomes. Each circle signifies a single sRNA. Red and blue circles indicate significantly differentially expressed sRNAs (fold change absolute value >2, FDR ≤0.05). (d) Pie chart analysis showed different rare species of sRNAs with mean expression ≥1000. KMeC, primary melanoma cell line; LMeC, metastatic melanoma cell line; ex, exosome; FDR, false discovery rate; Others, ; others include vault and other RNA types.

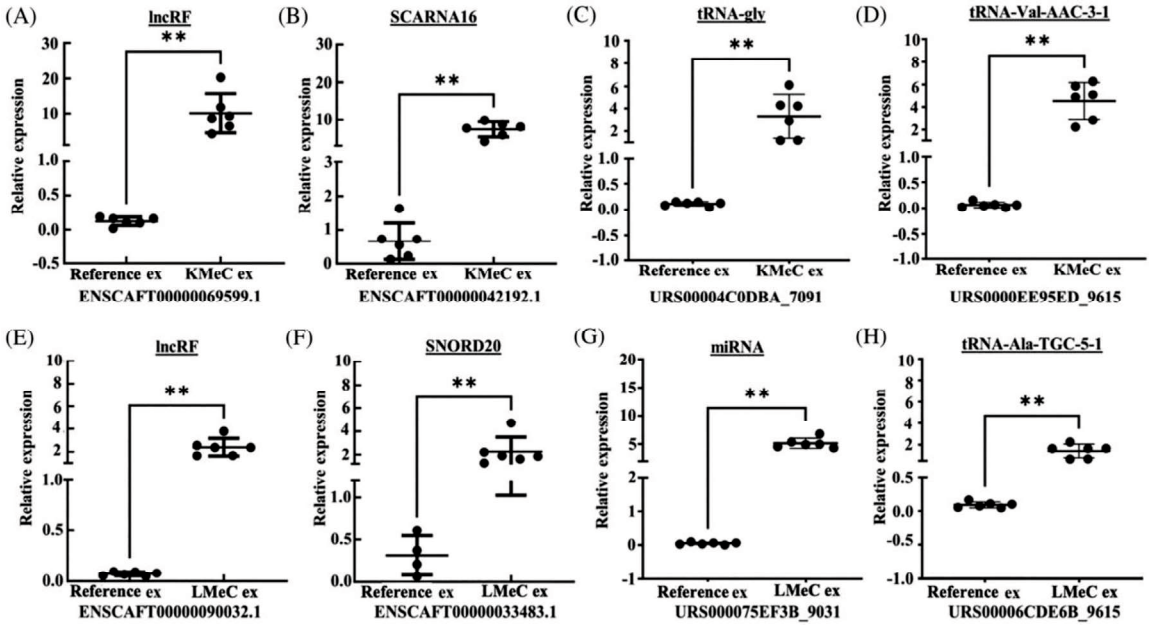

(A)
lncRF
**
Relative expression
Reference ex
KMeC ex
ENSCAFT00000069599.1
(B)
SCARNA16
**
Relative expression
Reference ex
KMeC ex
ENSCAFT00000042192.1
(C)
tRNA-glv
**
Relative expression
Reference ex
KMeC ex
URS00004C0DBA_7091
(D)
tRNA-Val-AAC-3-1
**
Relative expression
Reference ex
KMeC ex
URS0000EE95ED_9615
(E)
lncRF
**
Relative expression
Reference ex
LMeC ex
ENSCAFT00000090032.1
(F)
SNORD20
**
Relative expression
Reference ex
LMeC ex
ENSCAFT00000033483.1
(G)
miRNA
**
Relative expression
Reference ex
LMeC ex
URS000075EF3B_9031
(H)
tRNA-Ala-TGC-5-1
**
Relative expression
Reference ex
LMeC ex
URS00006CDE6B_9615

Figure 2-3 qRT-PCR validation of sRNAs in COM exosomes. (a-d) A lncRF-ENSCAFT00000069599.1 (a); SCARNA16 fragment- ENSCAFT42192.1 (b); two tRFs-URS00004C0DBA_7091/tRNA-gly (c) and URS0000EE95ED_9615/tRNA-Val-AAC-3-1 (d) were increased in KMeC cell line exosomes. (e-f) A lncRF- ENSCAFT00000090032.1 (e); SNORD20 fragment- ENSCAFT00000033483.1 (f); gga-miR-124c (g) and tRFs-URS00006CDE6B_9615/tRNA-Ala-TGC-5-1 (h) were highly expressed in LMeC cell line exosomes. n=6 in each group. LncRFs, long non-coding RNA derived small fragments; tRFs, transfer RNA-derived small fragments; and snoRNAs, small nucleolar RNAs; scaRNA, small cajal body specific RNA. The Y-axes represent the relative sRNA expression levels normalized against miR-186 (Mann Whitney test; **P <0.01; ***P <0.001).

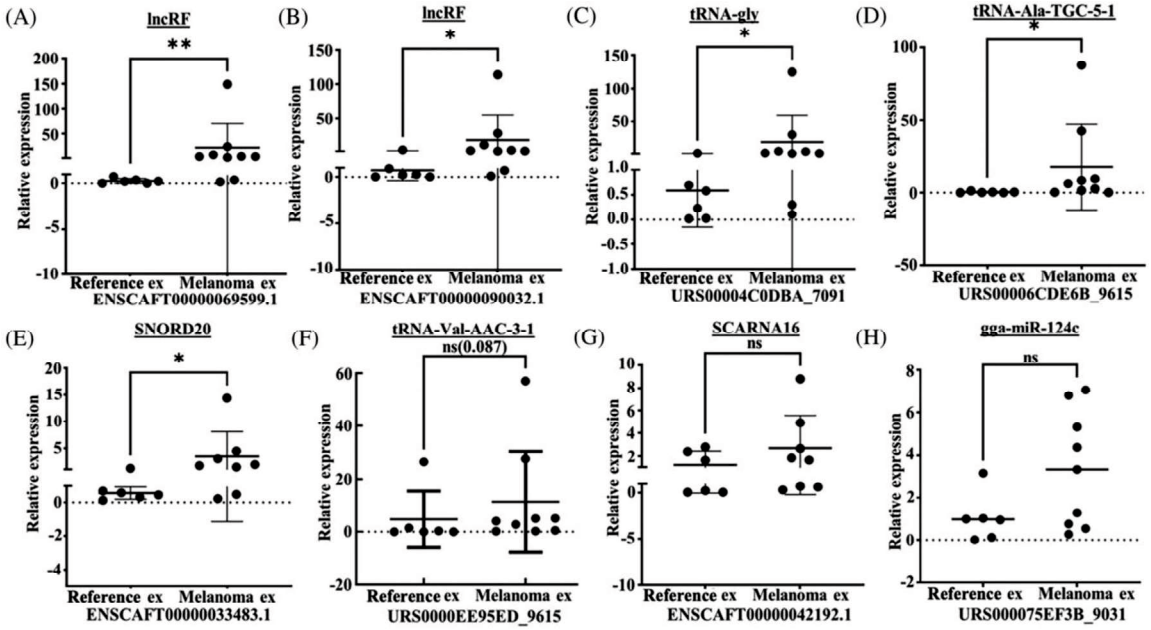
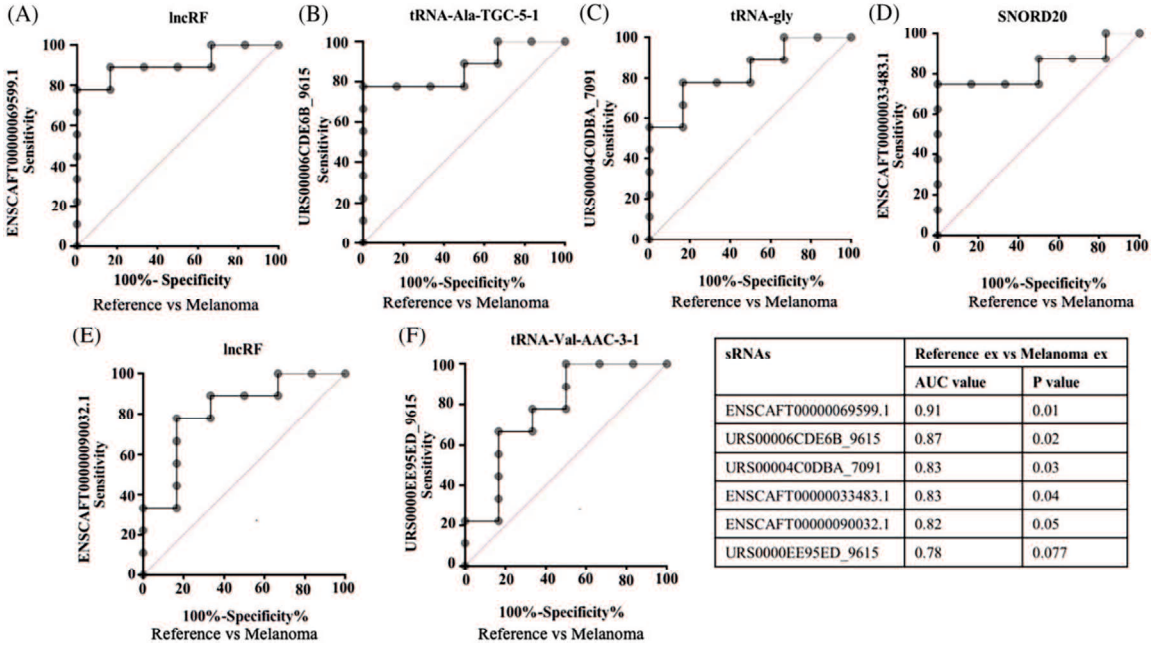

(A)
lncRF
**
Relative expression
Reference ex
Melanoma ex
ENSCAFT00000069599.1
(B)
lncRF
*
Relative expression
Reference ex
Melanoma ex
ENSCAFT00000090032.1
(C)
tRNA-gly
*
Relative expression
Reference ex
Melanoma ex
URS00004C0DBA_7091
(D)
tRNA-Ala-TGC-5-1
*
Relative expression
Reference ex
Melanoma ex
URS00006CDE6B_9615
(E)
SNORD20
*
Relative expression
Reference ex
Melanoma ex
ENSCAFT00000033483.1
(F)
tRNA-Val-AAC-3-1
ns(0.087)
Relative expression
Reference ex
Melanoma ex
URS0000EE95ED_9615
(G)
SCARNA16
ns
Relative expression
Reference ex
Melanoma ex
ENSCAFT00000042192.1
(H)
gga-miR-124c
ns
Relative expression
Reference ex
Melanoma ex
URS000075EF3B_9031

Figure 2-4 Expression of sRNA fragments in the plasma-derived exosomes of canine oral melanoma patients. Expression of LncRFs- ENSCAFT00000069599.1 (a) and ENSCAFT00000090032.1 (b); tRFs- URS00004C0DBA_7091/tRNA-gly (c) and URS00006CDE6B_9615-tRNA-Ala-TGC-5-1 (d); ENSCAFT00000033483.1/SNORD20 (e); tRFs-URS0000EE95ED_9615 /tRNA-Val-AAC-3-1 (f); SCARNA16 fragment-ENSCAFT42192.1 (g) and gga-miR-124c (h) in melanoma dog plasma-derived exosomes were determined by qRT-PCR analysis. n=6 in reference and n=9 in melanoma group except SNORD20 and SCARNA16 (n=8). LncRFs, long non-coding RNA derived small fragments; tRFs, transfer RNA-derived small fragments; and snoRNAs, small nucleolar RNAs; scaRNA, small cajal body specific RNA. The Y-axes represent the relative sRNA expression levels normalized against miR-186 (Mann Whitney test; *P <0.05; **P <0.01).



sRNAs	Reference ex vs Melanoma ex	
	AUC value	P value
ENSCAFT00000069599.1	0.91	0.01
URS00006CDE6B_9615	0.87	0.02
URS00004C0DBA_7091	0.83	0.03
ENSCAFT00000033483.1	0.83	0.04
ENSCAFT00000090032.1	0.82	0.05
URS0000EE95ED_9615	0.78	0.077

Figure 2-5 ROC curve analysis of exosomal sRNAs. (a-f) ROC curve represents the diagnostic efficacy of ENSCAFT00000069599.1/lncRFs (a); URS00006CDE6B_9615/tRNA-Ala-TGC-5-1) (b); URS00004C0DBA_7091/tRNA-gly (c); ENSCAFT00000033483.1/SNORD20 (d); ENSCAFT00000090032.1/lncRFs (e) and URS0000EE95ED_9615/tRNA-Val-AAC-3-1) (f). ROC, receiver operating characteristic curve; lncRFs, long non-coding RNA derived small fragments; tRFs, transfer RNA-derived small fragments; and snoRNAs, small nucleolar RNAs.

General Discussion

To the best of my knowledge, this is the first report on exosmiRs and other novel exosomal sRNAs (non-miRNA sRNAs) as potential biomarkers of canine oral melanoma, and also the first study on a lncRF as a cancer biomarker.

miRNAs and other novel sRNAs (rare sRNAs) appear to be promising candidate as blood or other biofluid-based biomarkers in cancer research (18). In this study, I revealed the exosomal sRNAs (miRNAs and non-miRNA sRNAs) profile of COM, which is a natural spontaneous model of human melanoma (24,28). My findings in the first part of my project indicated that specific cell-secreted exosomes bear distinct miRNA cargos, which is consistent with several previous human studies (39). Moreover, I also reported exosome biomarker candidates for primary melanoma as well as metastatic melanoma. Furthermore, my results revealed several exomiRs with distinct expression patterns compared with their parental COM cell lines consistent with previous human melanoma studies (30,40). In second part of this study, I identified aberrantly expressed novel sRNAs in the COM cell lines (KMeC and LMeC). I also established that, these novel sRNAs were also aberrantly expressed in clinical cases of COM, and met criteria for diagnostic efficacy as biomarkers for this disease.

Exploring the exosome profile, I hypothesized that exomiRs reflect primary and metastatic sites of melanoma. Accordingly, I identified exomiRs that showed increased expression in primary (KMeC) and metastatic (LMeC) site originated melanoma cells. In KMeC exosomes, among these increased exomiRs, miR-143, and let-7b was validated by qRT-PCR. In my previous study, miR-143 is reported to be downregulated in COM tissue (41). A similar pattern of expression is downregulation of miR-143 in tissue, but upregulation in the exosome has also been reported in human ovarian cancer (42). The discrepancy in the expression of miR-143 in tissue, plasma, and exosomes can be explained by the efficient loading of miRNA into exosomes, which reduces its existence in cells or tissue. Previous studies on breast cancer reported that miR-143 is upregulated in cancer-associated fibroblast exosomes, while downregulated in cells (43,44). The expression of another miRNA in this group, let-7b, is controversial among human studies (45–48). In my study, let-7b was one of the most highly upregulated miRNAs in KMeC exosomes, which was consistent with the findings of Ohshima et al.

Besides, among the nine exomiRs that were subsequently increased in metastatic site originated melanoma cells, miR-221 and miR-222 were the most highly expressed and confirmed by qPCR. A previous study identifies miR-221 and miR-222, along with seven

other miRNAs, as diagnostic and therapeutic markers of human melanoma (49). The upregulated expression of miR-221 and miR-222 has also been reported in plasma exosomes during human malignant melanoma (30). Another study confirmed the role of exosomal miR-222 in melanoma progression and malignancy (50).

Nine miRNAs were only increased in aggressive LMeC exosomes; among these, miR-210 and miR-708 were the most highly expressed in LMeC exosomes. This result may indicate their relation to metastasis. It has previously been reported that miR-210 is upregulated during metastasis in melanoma (31). Moreover, miR-210 has also been considered as a potential cell or exosomal biomarker of several human cancers including lung adenocarcinoma and renal cell carcinoma (52,67). Whereas for miR-708, a role has been reported in head-neck, lung, and colorectal cancer as an oncogenic, pharmacodynamic, or diagnostic marker (53–55), but its role in melanoma has not been studied. Of note, in my analysis, miR-210, miR-708, and miR-212 were found to be both suddenly and subsequently increased at metastatic sites. This was particularly pronounced in LMeC (the metastatic cell line) because of the higher fold change observed (Tables 1 and 3). Besides, in my exosome NGS profile, several miRNAs (miR-212, miR-335, miR-551a, miR-574-3p, miR-935, and miR-214) upregulated in the COM cell line exosomes that were not validated by qPCR,

which were also consistent with a previous human melanoma study (30). The results represent the reliability of exosomal miRNAs screening by NGS.

I further analyzed the selectively targeted exomiRs in melanoma-bearing dog plasma samples to evaluate their suitability as a biomarker of melanoma. miR-143 and 221 were significantly highly expressed in the exosomes from metastatic patients' plasma than non-tumor individuals. However, the expression pattern of miR-143 in cell culture and clinical samples showed some discrepancy. In the cell culture, experiment miR-143 increased in primary originated cell's exosomes, whereas in clinical samples, increased expression was found in the metastatic cases. One logical explanation can be, in a cell culture experimental setting, I analyzed only exomiRs from primary or metastatic site originated cell lines individually. However, in the case of metastatic melanoma patients, plasma bears the exosomes from both the primary and metastatic tumor cells simultaneously, and also primary melanoma tend to be large in these cases, which may result in higher expression of miR-143.

Another exomiR (miR-210) expression remains unchanged in melanoma patients, which is also inconsistent with the cell culture study. The expression magnitude of miR-210 was less than the other two candidates. Therefore, it may dilute or reduce the concentration of miR-210 in plasma, resulting in the mask of expression difference between reference and melanoma exosomes.

In second part of this study, I identified 108 and 100 aberrantly expressed novel sRNAs in the COM cell lines (KMeC and LMeC) respectively, as major novel findings. Moreover, I established that five of these novel sRNAs (from a representative subset of eight) were also aberrantly expressed in clinical cases of COM, and exhibit diagnostic efficacy as biomarkers for this disease. Successful evaluations for diagnostic efficacy were executed through identifying novel exosomal sRNAs in COM cell lines with NGS, their validation with PCR and evaluation in clinical patients serves as a proof of concept for such research on these sRNAs.

From these novel sRNAs, I selected eight sRNAs (comprising two lncRFs, one scaRNA, three tRFs, one snoRNA and one miRNA) at the higher and lower ends of the spectrum of expression levels allowed by the stringent filtering criteria, and the expression results were successfully validated for each of them in PCR analysis. Six of these novel sRNAs were overexpressed in COM patients, and five of them showed sufficient sensitivity and specificity to be regarded as candidate biomarkers. It is noteworthy that just this small subset yielded some promising candidate biomarkers, and suggests that the wider pool of aberrantly expressed sRNAs I identified also represent promising lines of future research.

The results of my diagnostic efficacy evaluation are particularly interesting. I targeted six novel sRNAs (that had been identified in NGS and validated in PCR) showing high

expression levels (significantly high or tending to significance) in COM patients for ROC curve analysis of sensitivity and specificity. Five of the six novel sRNAs met the criterion for diagnostic efficacy, and two of these sRNAs appear particularly promising (based on their AUC and p values). These three novel sRNAs represent two derived-fragment RNA types; specifically, one was a lncRF (transcript ID: ENSCAFT00000069599.1) and the other two were tRFs (tRNA-Gly and tRNA-Ala-TGC-5-1). This is the first time such novel derived-fragment type sRNAs have been identified as candidate biomarkers for COM. I consider that the plasma levels of these exosomal sRNAs could serve as potential diagnostic biomarkers for COM.

My results are consistent with a number of previous indications that these novel sRNAs may play specific roles in a range of cancers (9,36). Other researchers are already looking at these novel sRNA types as cancer biomarkers both generally and in specific instances (36,57). Studies in humans and dogs reveal that melanoma is one of many cancers in which tRFs may be differentially expressed, indicating their diagnostic and prognostic significance (19,36,58–62). Two scaRNAs (SCARNA2 and SCARNA22) have been found to play an oncogenic role in colorectal cancer and multiple myeloma (63–65). SNORD20 is reported as one of the most highly expressed RNAs in glioblastoma cell lines (18). In another study, melanoma exosomes were found to be enriched with SNORD83A and SNORD89 (66). Moreover, SNORD126,

SNORA23, SNORA42, and SNORD46 are reportedly similarly overexpressed in multiple cancer types (67). My findings extend this evidence, and through a particular focus on sRNAs in the field of canine oral melanoma, bring to light the role of an lncRF (ENSCAFT00000090032.1), a newly detected transcript derived from the ENSCAFG00000047740 gene (68).

My study posseses some limitations. The first, exosomal miRNAs and non-miRNA sRNAs profile was only investigated for melanoma cell lines; circulating melanoma exosomes from dogs were not included. It is not currently possible to separate circulating melanoma exosomes from other cell-derived exosomes in blood. Therefore, profiling the circulating exosomal RNAs from dogs is not representative of melanoma exosomes as exosomes from all cells throughout the body are present in plasma, the true circulating exosomal RNA profiles of melanoma-bearing dog plasma would encompass both melanoma exosomes and exosomes from normal cells. Furthermore, a strategy for the identification of melanoma exosomes from plasma could facilitate the definition of a patient's disease state more precisely by these candidate miRNAs. Second, I compare the COM cell lines exomiRs with the exomiRs from reference exosomes that were collected from non-tumor dog plasma. Until now, the primary melanocyte cell line from dogs is not established. Therefore, I chose

the non-tumor plasma exosome as a reference, which is an ideal control for the blood-based exosomal study. Third, reference exosomes were isolated from plasma of beagle dogs.

In conclusion, I successfully present a comprehensive exomiRs profile of two COM cell lines. My profiling studies revealed that unique exomiRs expression profiles exist between exosomes and their cells of origin. I further reported that miR-143 and 221 are suitable biomarker candidates for melanoma. I also successfully demonstrated the aberrant expression of a number of novel sRNAs in exosomes from COM cell lines and patient plasma. These findings also included several newly detected transcripts. In further analysis, I identified five of these differentially expressed sRNAs as candidate liquid biopsy biomarkers for diagnosing COM. Taking the results together, my study represents an expansion to existing knowledge of novel exosomal sRNAs, and sheds light on their diagnostic utility of these sRNA species as promising biomarkers for canine oral melanoma.

Appendix

Appendix 1. Data characterizing the melanoma patients

No	Age (Years)	Sex	Breed	WHO Stage	Metastasis (M) or Non-metastasis (N)
T1	12.4	Female	Miniature	Ⅳ	M
T2	14.6	Female	Miniature	Ⅱ	N
T3	15.2	Female	Mongrel	Ⅳ	N
T4	12.11	Male	Miniature	Ⅳ	M
T5	12.4	Male	Shiba and Miniature cross	Ⅳ	M
T6	15.2	Female	Mongrel	Ⅳ	N
T7	10.8	Male	Miniature	Ⅳ	N
T8	16.5	Male	Miniature-dachshund cross	N/A	M
T9	12.9	Female	Beagle	N/A	N/A

Appendix 2. Differentially expressed miRNAs between KMEC exosomes and its cells of origin

Upregulated miRNAs in KMEC exosomes		
miRNAs	**Fold Change**	**FDR**
let-7a-1//let-7a-3	8.65	4.048E-30
let-7f-2	5.90	1.483E-12
let-7i	2.13	3.48E-03
mir-100	86.55	2.749E-36
mir-101-1	9.95	1.014E-11
mir-101-1//mir-101-2	5.32	1.166E-25
mir-101-2//mir-101-1	16.76	2.242E-82
mir-103b-1//mir-103b-2	38481.53	9.53E-157
mir-107	8.22	1.59E-38
mir-10a	12.47	7.79E-36
mir-10b	2.62	4.28E-11
mir-122	89.56	8.504E-19
mir-1224	5.54	7.597E-09
mir-122b	581.54	2.834E-22
mir-1246	5.09	2.408E-13
mir-125b-2	3.39	8.893E-12
mir-126	10.33	3.178E-35
mir-127	75.57	4.091E-72
mir-1271	5.17	1.68E-04
mir-128-1//mir-128-2	4.67	8.786E-24
mir-1307	13.05	1.785E-25
mir-133a	20.39	2.743E-28
mir-133c	5.15	1.718E-06
mir-136	976.50	1.656E-32
mir-140	2.04	2.607E-05
mir-142	34.31	5.102E-28
mir-143	8.69	2.91E-39
mir-144	680.01	2.491E-27
mir-145	18.05	2.051E-36
mir-1468	22.15	2.774E-14
mir-146a	7.39	5.137E-25
mir-146b	7.55	4.619E-10
mir-148a	13.04	7.034E-50
mir-151b	33.95	6.423E-50
mir-153-1//mir-153-2	3.69	6.76E-04
mir-15a	16.48	1.057E-78
mir-15b	4.26	7.23E-19
mir-16-1//mir-16-2	2.00	5.067E-06
mir-181c	3.40	4.346E-12
mir-182	6.24	7.644E-05

mir-1839	5.19	7.937E-18
mir-184	3.08	2.897E-07
mir-1842	2.54	1.764E-05
mir-188	9.48	1.716E-23
mir-18b	5.32	2.64E-04
mir-190b	3.40	1.27E-03
mir-192	11.20	2.377E-45
mir-193a	5.44	1.043E-14
mir-195	2.45	9.67E-04
mir-199-1//mir-199-2//mir-199-3	10.16	1.919E-40
mir-19a	3.50	3.519E-15
mir-19b-1//mir-19b-2	12.13	4.207E-45
mir-203b	971.36	9.124E-35
mir-208b	130.21	4.755E-49
mir-21	2.08	1.098E-06
mir-210	2.06	5.729E-05
mir-215	627.98	1.709E-29
mir-218-1//mir-218-2	14.17	8.305E-07
mir-219-1//mir-219-2	12.45	1.739E-08
mir-219b	297.61	2.47E-14
mir-22	2.18	4.507E-07
mir-221	11.29	5.101E-53
mir-2355	2.56	1.887E-06
mir-23a	124.97	4.861E-78
mir-23b	160.36	3.567E-72
mir-24-1//mir-24-2	6.90	3.665E-21
mir-25	3.55	3.382E-16
mir-26b	2.93	5.493E-07
mir-27a	12.24	7.381E-68
mir-27b	10.54	9.011E-55
mir-29a	2.38	3.153E-09
mir-29c//mir-29c-1//mir-29c-2	4.64	6.582E-14
mir-301a	6.28	2.146E-28
mir-301b	4.99	6.176E-19
mir-3074	613.90	6.64E-122
mir-3085	3.16	6.51E-04
mir-30a	2.37	1.063E-08
mir-30e	2.42	6.249E-09
mir-3184	504533.85	2.65E-126
mir-32	63.22	1.027E-30
mir-320c-1//mir-320c-2	9.04	2.376E-28
mir-320d-1//mir-320d-2	17.49	2.001E-29
mir-323	23.70	5.412E-10
mir-335	11.21	8.568E-33
mir-339-1	8.74	7.618E-34
mir-33a	36.25	2.171E-18
mir-33b	43.21	7.897E-53
mir-340	5.29	1.012E-24

mir-345	5.49	1.206E-09
mir-34a	5.49	3.097E-11
mir-34c	5.02	5.548E-07
mir-3529	366.00	1.321E-18
mir-362	450.42	3.01E-21
mir-369	577.04	3.123E-75
mir-371a	27893.44	5.17E-116
mir-374c	789.83	2.102E-31
mir-376c	83.04	9.617E-14
mir-378a//mir-378	2.25	5.085E-08
mir-378c	4.67	1.087E-10
mir-378d-2//mir-378d-1	12.32	2.62E-36
mir-378e	24.15	2.914E-22
mir-378f	6.53	3.583E-15
mir-378h	14.75	3.308E-21
mir-378i	4.33	1.917E-17
mir-380	108.29	9.945E-72
mir-381	24.09	8.049E-30
mir-3958	93.00	4.895E-16
mir-409	4.26	1.729E-12
mir-410	51.87	2.211E-78
mir-411	8.99	1.285E-09
mir-421	2.94	1.298E-10
mir-422a	5.27	1.495E-15
mir-424	6.12	2.348E-16
mir-425	4.14	3.994E-14
mir-432	348.16	3.308E-18
mir-4486	416.15	2.357E-12
mir-450b	13.09	1.706E-42
mir-451	85.43	6.125E-66
mir-455	2.94	2.966E-10
mir-4634	704.44	9.462E-22
mir-486	1973.11	2.21E-134
mir-486-1//mir-486-2	2.20	1.921E-05
mir-494	42.68	1.829E-10
mir-499	19.14	5.107E-42
mir-499b	2762.02	8.159E-70
mir-502	2.51	1.584E-08
mir-503	5.50	2.1E-06
mir-532	2.73	1.465E-10
mir-541	174.87	3.526E-12
mir-542	2.19	1.20E-04
mir-551a	24.32	1.053E-09
mir-574	3.68	2.974E-13
mir-592	5.69	2.948E-15
mir-628	2.19	4.17E-02
mir-652	3.37	2.387E-08
mir-660	10.11	9.583E-44

mir-708	4.04	3.544E-10
mir-7-1	2.49	5.909E-05
mir-744	3.76	3.32E-12
mir-7975	63.57	2.584E-17
mir-8884	2.18	3.32E-03
mir-9-1//mir-9-2//mir-9-3	3.11	1.13E-04
mir-98	8.65	9.607E-22
mir-9985	6.46	7.625E-12
mir-99a	4.97	1.634E-10
mir-99a-1//mir-99a-2	3.03	4.087E-10

Downregulated miRNAs in KMEC exosomes

miRNAs	Fold Change	FDR
let-7a-1//let-7a-3//let-7a-2	-6.87	0
let-7a-2	-2.72	2.05E-04
let-7b	-5.11	0
let-7d	-12.66	0
let-7e	-10.94	0
let-7f-1//let-7f-2//let-7f	-5.57	0
mir-10395	-633.61	3.15425E-73
mir-103a-1//mir-103-1//mir-103-2	-19.83	0
mir-106a	-2.17	2.39E-03
mir-106b	-3.99	6.87761E-14
mir-107	-21.46	2.57143E-72
mir-10a	-9.94	1.70486E-23
mir-10b	-21.37	0
mir-1249	-47.26	9.44253E-48
mir-125a	-148.20	1.4701E-115
mir-125b-2//mir-125b-1	-8.50	0
mir-1260a	-41.50	1.05434E-78
mir-1260b	-12.45	1.18573E-24
mir-128-1	-10.94	2.72859E-20
mir-129-2	-6.25	2.31897E-20
mir-1296	-2.49	2.28144E-06
mir-1301	-18.03	1.90944E-37
mir-1306	-37.47	2.03384E-46
mir-1307	-4.56	1.98863E-15
mir-130b	-11.08	9.95899E-26
mir-1343	-12.74	3.67166E-25
mir-135b	-2.13	1.83E-02
mir-143	-11.82	6.55808E-12
mir-145	-3.19	4.03724E-10
mir-149	-5.15	1.71848E-06
mir-151a//mir-151	-4.25	0
mir-152	-2.73	1.78238E-08
mir-155	-14.07	0
mir-15a	-6.21	4.89009E-20
mir-15b	-30.59	9.27409E-85

mir-17	-3.40	1.69966E-09
mir-181a-1	-25.23	1.3564E-72
mir-181a-2	-46.08	8.37774E-65
mir-181a-2//mir-181a-1	-3.42	2.60126E-10
mir-181b-1//mir-181b-2	-5.94	0
mir-182	-3.67	2.77361E-14
mir-183	-11.18	1.86779E-37
mir-185	-2.87	2.73055E-05
mir-18a	-4.20	5.64027E-11
mir-190a	-2.67	4.78E-02
mir-191	-2.12	4.36598E-07
mir-193a	-4.33	5.71089E-19
mir-196a-2//mir-196a-1	-3.02	1.20E-04
mir-197	-3.51	3.80954E-15
mir-205	-2.49	1.79548E-07
mir-21	-13.31	5.08496E-15
mir-219a-1	-2.09	3.96E-04
mir-222	-4.44	1.00886E-17
mir-2387	-5.47	2.40177E-12
mir-23a	-36.43	1.26157E-40
mir-23b	-26.99	1.2175E-37
mir-24-2	-5.10	2.09186E-24
mir-25	-12.21	2.58155E-16
mir-26a-2//mir-26a-1	-7.38	0
mir-26b	-4.31	4.82412E-09
mir-27a	-71.44	4.82998E-84
mir-27b	-13.18	2.15683E-38
mir-28	-4.80	0
mir-29b-1	-10.88	3.47765E-26
mir-29b-2//mir-29b-1	-2.18	6.47139E-05
mir-30b	-4.31	1.36744E-06
mir-30c-1	-11.37	3.71134E-28
mir-30c-2	-5.11	7.87982E-08
mir-30c-2//mir-30c-1	-16.74	4.7128E-46
mir-30d	-2.65	7.87982E-08
mir-30e	-2.22	7.35E-04
mir-31	-8.56	1.04682E-15
mir-3135b	-222.79	2.75586E-36
mir-324	-2.51	1.43E-03
mir-330	-4.52	2.97315E-14
mir-331	-57.76	1.26389E-79
mir-339	-2.40	4.71179E-06
mir-33b	-37.94	4.87317E-37
mir-340	-17.21	1.24841E-21
mir-342	-2.07	3.15E-04
mir-361	-5.04	3.35752E-19
mir-362	-28.66	1.81048E-30
mir-3651	-790.16	2.5917E-33

mir-365-2//mir-365-1	-4.16	0
mir-365a	-2.00	9.47E-03
mir-365b	-3.23	1.50977E-08
mir-371b	-2.63	2.42553E-06
mir-374a	-2.50	2.42E-04
mir-378a	-2.43	5.9524E-07
mir-423	-2.36	1.20721E-07
mir-425	-2.49	9.70519E-09
mir-4286	-6.08	2.1221E-19
mir-449a	-2.01	4.39E-02
mir-4508	-4.05	1.18E-02
mir-454	-12.09	2.42716E-16
mir-455	-20.18	2.65452E-44
mir-491	-5.93	2.3797E-05
mir-500a	-2.52	5.90422E-09
mir-502	-3.35	4.94E-04
mir-505	-2.84	4.28328E-11
mir-532	-3.88	2.45872E-14
mir-5701-1//mir-5701-2//mir-5701-3	-1215.05	1.2952E-112
mir-615	-6.77	5.0747E-21
mir-652	-2.34	1.45E-04
mir-6529	-2.41	8.11E-04
mir-664	-4.35	2.49E-03
mir-671	-6.02	0
mir-708	-2.18	9.47E-04
mir-7-1//mir-7-2//mir-7-3	-72.86	7.28137E-44
mir-7180	-62.79	4.46282E-43
mir-744	-6.90	0
mir-769	-2.20	7.78035E-07
mir-7977	-9.40	1.34806E-28
mir-874	-6.67	1.46471E-10
mir-8803	-56.57	1.85474E-27
mir-8829	-6.85	1.01652E-06
mir-8865	-4.88	1.10817E-17
mir-8890	-27.69	1.21189E-62
mir-8903	-2.40	2.27E-04
mir-9-2//mir-9-3//mir-9-1	-2.44	1.23431E-06
mir-92a-1	-6.39	2.41932E-06
mir-92a-1//mir-92a-2	-4.37	3.80954E-15
mir-92b	-8.68	0
mir-93	-2.42	3.27747E-05
mir-935	-10.18	9.79039E-18
mir-940	-6.62	1.25929E-06
mir-98	-3.17	1.64853E-08
mir-99a	-19.34	2.24507E-85
mir-99b	-3.09	8.27627E-13

FDR, False discovery rates

Appendix 3. Differentially expressed miRNAs between LMEC exosomes and its cells of origin

Upregulated miRNAs in LMEC exosomes		
miRNAs	**Fold Change**	**FDR**
mir-100	93.99	7.04632E-36
mir-101-1	3.05	2.41682E-06
mir-101-2//mir-101-1	2.31	1.93849E-11
mir-107	7.39	5.13426E-75
mir-10a	3.73	3.49942E-23
mir-122	128.00	6.68017E-21
mir-1224	17.15	1.44369E-57
mir-122b	138.93	3.08772E-21
mir-124-1//mir-124-2//mir-124-3	6.04	1.02659E-10
mir-1246	28.71	9.34167E-34
mir-126	211.47	1.58281E-74
mir-127	921.39	1.49847E-81
mir-1271	3.17	1.50218E-09
mir-129-1//mir-129-2	2.62	1.50678E-14
mir-130a	2.45	2.6579E-13
mir-133a	1034.20	4.7332E-110
mir-133a-1//mir-133a-2	35.46	9.44681E-09
mir-133c	228.18	2.06169E-41
mir-134	17.59	5.25152E-08
mir-136	353.07	9.30715E-45
mir-138-2//mir-138a	3.03	2.72705E-15
mir-139	4.20	2.80285E-06
mir-140	5.25	7.39556E-32
mir-141	2.51	1.20E-02
mir-142	6.16	2.37285E-19
mir-143	170.75	1.5923E-52
mir-144	187.86	5.52455E-45
mir-145	533.84	6.20371E-16
mir-1468	127.39	4.67803E-36
mir-146a	4.47	4.50673E-12
mir-146b	9.19	1.53833E-97
mir-148a	2.75	1.26566E-15
mir-150	2.52	1.19E-02
mir-151a	2.12	9.93367E-09
mir-151b	7.00	7.66401E-41
mir-15a	5.76	1.32179E-42
mir-15b	3.25	1.70521E-19
mir-181a-1//mir-181a-2	2.28	1.11994E-10
mir-181c	3.82	3.63515E-22
mir-1838	2.54	2.15E-02
mir-1839	3.66	3.31681E-23

mir-184	9.44	1.53575E-14
mir-1840	2.32	2.65E-03
mir-1844	2.33	7.1647E-05
mir-188	3.89	8.57965E-18
mir-190b	2.62	4.01E-03
mir-193a	4.51	1.08582E-15
mir-193b (hsa-3p)	3.78	2.56035E-08
mir-193b (hsa/cfa-5p)	3.83	8.53373E-15
mir-196a-2	3.21	3.90E-04
mir-199-1//mir-199-2//mir-199-3	4.10	1.01266E-22
mir-19b-1//mir-19b-2	5.41	7.67215E-39
mir-205	6.83	3.31391E-12
mir-208b	31.58	7.3393E-53
mir-210	6.37	7.05262E-18
mir-214	87.04	5.9123E-18
mir-215	696.78	6.66316E-50
mir-219a-2//mir-219-1//mir-219-2	3.61	1.63013E-08
mir-219b	3.84	1.64501E-09
mir-22	3.62	4.00376E-13
mir-221	10.43	5.67284E-90
mir-23a	2.20	1.15186E-10
mir-23b	26.33	1.2165E-132
mir-24-1//mir-24-2	2.96	2.47972E-15
mir-27a	5.05	2.10812E-35
mir-27b	3.99	5.13018E-26
mir-29a	2.37	7.16814E-09
mir-29c//mir-29c-1//mir-29c-2	2.12	6.51957E-07
mir-301a	3.32	1.40734E-17
mir-301b	2.38	2.23254E-09
mir-3074	2.42	1.19898E-10
mir-3085	6.78	1.48534E-15
mir-30a	4.43	3.51492E-31
mir-30d	3.44	2.58218E-20
mir-3120	2.23	1.81E-02
mir-32	18.21	9.35745E-18
mir-320a//mir-320	4.46	3.27961E-30
mir-320c-1//mir-320c-2	4.10	1.36621E-17
mir-320d-1//mir-320d-2	4.53	1.46394E-14
mir-323	280.87	1.89056E-26
mir-324	2.00	8.08249E-06
mir-331	2.06	3.39678E-07
mir-335	4.24	4.07821E-24
mir-339-1	4.71	6.14757E-34
mir-33a	4.44	4.17779E-12
mir-33b	7.08	4.51221E-38
mir-345	9.69	1.01534E-36
mir-34c	4.28	6.20901E-06
mir-362	11.25	1.21321E-15

mir-369	1750.27	1.3893E-140
mir-376c	972.20	1.87845E-26
mir-378c	8.85	1.97059E-22
mir-378d-2//mir-378d-1	5.69	2.35534E-37
mir-378e	2.73	6.0533E-09
mir-378f	7.39	1.70968E-22
mir-378h	18.30	5.54242E-35
mir-378i	5.91	3.39136E-35
mir-380	565.14	1.2401E-111
mir-381	54.00	2.26288E-64
mir-383	4.14	9.54307E-05
mir-3958	47.05	6.62503E-19
mir-409	54.83	6.69307E-54
mir-410	678.69	2.115E-111
mir-411	8.52	3.03417E-16
mir-421	3.15	1.11683E-19
mir-422a	6.38	1.04286E-23
mir-432	1006.27	2.07913E-33
mir-433	126.35	3.69524E-09
mir-4486	362.65	4.17939E-20
mir-4502	4.69	1.40E-03
mir-451	558.47	1.54938E-79
mir-4634	4.46	7.237E-08
mir-486	2.22	2.70921E-09
mir-487b	76.59	5.32883E-15
mir-491	2.54	8.56E-03
mir-494	1068.93	1.80226E-32
mir-497	2.51	1.9515E-06
mir-499	3.68	4.93611E-20
mir-499b	3.80	1.13854E-20
mir-500	4.48	1.07423E-12
mir-502	4.40	8.87871E-31
mir-503	2.03	1.98E-02
mir-532	2.29	1.96565E-11
mir-541	219.65	5.82569E-21
mir-574	2.75	4.48193E-06
mir-592	3.21	1.87667E-15
mir-628	2.10	4.73374E-05
mir-6516	4.22	4.55401E-05
mir-652	6.69	4.59097E-27
mir-656	302.31	5.03671E-16
mir-660	3.66	1.59083E-23
mir-665	145.08	3.57639E-10
mir-676	2.32	4.12E-02
mir-7975	2422.18	6.24184E-52
mir-877	2.22	4.77109E-05
mir-8859b	2.91	1.36879E-17
mir-889	9.91	3.44009E-10

mir-9985	6.20	4.99547E-28
mir-99a	239.39	7.92157E-13
mir-99a-1//mir-99a-2	3.59	1.42919E-19
Downregulated miRNAs in LMEC exosomes		
miRNAs	**Fold Change**	**FDR**
let-7a-1//let-7a-3//let-7a-2	-5.68	0
let-7a-2	-2.72	9.73E-04
let-7b	-3.39	0
let-7d	-7.07	0
let-7e	-7.66	0
let-7f-1	-2.81	1.33945E-12
let-7f-1//let-7f-2//let-7f	-23.23	0
let-7i	-2.79	2.06806E-14
mir-100	-7.08	3.28786E-05
mir-103a-1//mir-103-1//mir-103-2	-6.61	0
mir-103b-1//mir-103b-2	-2.30	6.90101E-11
mir-107	-12.56	1.2006E-36
mir-10a	-8.76	2.2027E-72
mir-10b	-23.06	0
mir-1224	-3.51	2.08644E-06
mir-1249	-34.78	2.94061E-83
mir-125a	-14.08	0
mir-1260a	-49.39	3.75485E-88
mir-1260b	-9.15	1.68659E-20
mir-128-1	-21.84	1.28595E-97
mir-129-2	-2.11	1.06793E-07
mir-1298	-2.22	2.80629E-09
mir-1301	-3.42	2.47949E-06
mir-1306	-4.78	2.00092E-20
mir-1307	-2.80	4.62565E-11
mir-130b	-18.26	2.74007E-59
mir-132	-3.59	6.99041E-09
mir-1343	-3.16	6.50868E-15
mir-135a-2//mir-135a-1	-17.06	5.5348E-07
mir-135b	-18.50	2.87764E-72
mir-142	-2.15	7.33643E-06
mir-148a	-3.65	0
mir-148b	-4.65	0
mir-149	-16.51	1.4531E-13
mir-151a//mir-151	-7.09	0
mir-152	-3.70	6.60402E-13
mir-155	-4.13	0
mir-15a	-15.90	5.4887E-112
mir-15b	-32.95	7.1834E-135
mir-16-1	-6.85	7.73813E-14
mir-17	-5.51	0
mir-181a-2	-16.03	1.76729E-12

mir-182	-3.59	0
mir-183	-2.21	1.86382E-05
mir-185	-3.49	2.40445E-14
mir-188	-3.82	1.18609E-05
mir-18a	-3.46	2.37086E-12
mir-190a	-20.83	3.99751E-22
mir-1911	-3.17	1.51063E-09
mir-194-1//mir-194-2//mir-194	-4.04	4.5456E-18
mir-194-2	-13.08	4.82929E-05
mir-196b	-2.74	9.15042E-05
mir-197	-2.84	9.88399E-06
mir-19a	-5.18	1.2069E-07
mir-19b-1	-5.56	7.08236E-06
mir-20a	-13.78	2.43415E-15
mir-2113	-4.08	3.41734E-16
mir-212	-11.22	1.15752E-33
mir-215	-6.43	5.10085E-20
mir-219a-1	-3.70	6.18928E-18
mir-222	-16.79	0
mir-23a	-7.99	1.92939E-07
mir-23b	-19.10	4.9098E-131
mir-24-1	-6.93	5.47085E-11
mir-24-2	-6.90	0
mir-25	-22.01	2.12364E-81
mir-26a-1	-118.32	3.27503E-08
mir-26a-2	-10.24	7.05036E-14
mir-26a-2//mir-26a-1	-7.16	0
mir-26b	-2.91	0
mir-27a	-145.55	4.379E-210
mir-27b	-29.78	5.8536E-127
mir-28	-3.85	0
mir-29b-1	-5.24	4.99725E-10
mir-29b-2	-5.78	1.47E-04
mir-29b-2//mir-29b-1	-5.08	0
mir-29c	-2.25	2.54E-02
mir-301a	-18.36	1.50686E-13
mir-3065	-3.97	0.000755099
mir-30a	-2.05	1.84572E-08
mir-30b	-23.64	1.29025E-27
mir-30c-1	-85.84	3.56494E-85
mir-30c-2	-10.06	1.03131E-19
mir-30c-2//mir-30c-1	-26.62	0
mir-30d	-4.42	0
mir-30e	-8.02	0
mir-32	-5.91	3.29277E-05
mir-330	-10.04	1.36204E-92
mir-331	-10.81	5.08321E-67
mir-335	-5.41	0

mir-338	-7.13	2.09963E-05
mir-339	-3.62	0
mir-33b	-52.91	5.34777E-96
mir-340	-26.38	1.11475E-27
mir-3529	-29.55	4.06057E-48
mir-3613	-9.31	4.67803E-36
mir-362	-28.47	1.16942E-37
mir-374a	-6.88	1.59392E-35
mir-378b	-7.40	6.74E-04
mir-423	-2.08	1.76407E-08
mir-4286	-3.06	2.37185E-07
mir-4454	-2.91	1.53E-04
mir-4484	-981.66	2.70521E-33
mir-449a	-2.41	4.87693E-06
mir-450a	-4.50	1.02943E-14
mir-450a-1//mir-450a-2	-3.24	1.3465E-13
mir-450b	-6.35	6.20607E-33
mir-454	-8.98	1.80828E-20
mir-4677	-185.88	8.24833E-09
mir-491	-3.69	1.06793E-07
mir-500a	-2.16	2.81761E-09
mir-502	-3.81	5.20399E-08
mir-504	-15.27	1.89E-100
mir-505	-30.42	4.16061E-29
mir-5701-1//mir-5701-2//mir-5701-3	-59.55	6.81709E-23
mir-582	-4.09	1.0927E-05
mir-590	-4.09	0
mir-615	-2.37	2.81E-03
mir-652	-20.58	4.92813E-09
mir-6529	-3.43	8.67519E-09
mir-664	-2.26	1.69E-02
mir-671	-5.27	0
mir-7-1//mir-7-2//mir-7-3	-533.40	2.8613E-122
mir-7180	-264.66	1.49605E-78
mir-744	-4.89	0
mir-769	-2.20	7.2623E-08
mir-7977	-80.16	6.49121E-59
mir-874	-6.42	4.60774E-19
mir-8803	-15.74	5.05338E-16
mir-8829	-3.09	7.06257E-08
mir-8865	-6.89	9.15061E-23
mir-8890	-15.30	5.89142E-96
mir-92a-1	-24.14	8.76719E-51
mir-92b	-5.71	0
mir-93	-4.05	3.29716E-17
mir-9-3//mir-9-2//mir-9-1	-10.19	0
mir-940	-4.44	7.37E-03
mir-98	-5.48	0

mir-99a	-24.89	0

FDR, False discovery rates

Appendix 4 Differentially expressed miRNAs between Reference and KMEC cell line exosomes

Upregulated miRNAs		
miRNAs	**Fold Change**	**FDR**
let-7b	3.15	1.39E-02
let-7c	4.88	1.75E-02
let-7e	36.77	4E-14
let-7f-1	4.02	8.00E-04
mir-10b	28.97	1.01E-16
mir-122	704.85	7.25E-09
mir-1224	28.90	8.97E-09
mir-122b	735.15	4.85E-09
mir-1246	28.50	8.96E-10
mir-1249	4.57	9.61E-03
mir-125a	14.86	5.07E-11
mir-125b-1	1733.24	3.58E-35
mir-125b-2	25.16	2.43E-13
mir-125b-2//mir-125b-1	29.76	2.15E-14
mir-127	5.46	1.96E-02
mir-129-2	2223.21	1.23E-17
mir-1296	3.30	1.61E-02
mir-1301	7.34	5.59E-04
mir-1307	5.03	1.31E-03
mir-130a	9.41	3.32E-09
mir-132	4.51	3.07E-04
mir-133c	3.15	3.94E-02
mir-135b	206.85	3.58E-06
mir-136	3.66	4.23E-02
mir-138-2//mir-138a	11.81	3.39E-06
mir-143	21.55	1.22E-10
mir-145	6.51	8.98E-04
mir-146b	3.67	1.28E-02
mir-148a	3.51	6.92E-04
mir-149	82.37	3.12E-09
mir-151b	3.32	5.64E-03
mir-152	80.17	7.54E-16
mir-155	15.53	5.58E-10
mir-181b-1//mir-181b-2	4.65	3.13E-04
mir-181d	14.57	1.05E-07
mir-182	38.93	1.19E-16
mir-183	234.40	3.37E-32
mir-1843	6.00	8.45E-05
mir-185	9.83	5.22E-06
mir-193a	14.56	5.81E-08
mir-193b (hsa-3p)	124.81	1.72E-15
mir-193b (hsa/cfa-5p)	79.78	3.62E-11

mir-195	12.51	2.01E-07
mir-197	4.63	1.07E-03
mir-199-1//mir-199-2//mir-199-3	47.40	2.56E-18
mir-199a-1//mir-199a-2	64.36	8.97E-09
mir-199a-1//mir-199a-2//mir-199b	42.56	4.61E-15
mir-204	6.28	2.83E-03
mir-208b	11.01	3.03E-06
mir-21	13.43	7.86E-11
mir-210	3.97	7.73E-04
mir-212	3.86	1.86E-02
mir-214	5.19	1.38E-03
mir-22	7.37	3.33E-07
mir-221	9.02	3.02E-07
mir-222	18.93	4.62E-09
mir-2355	3.19	6.04E-03
mir-2387	202.27	4.33E-12
mir-23a	5.14	3.78E-05
mir-23b	3.88	6.39E-04
mir-24-1//mir-24-2	10.78	7.08E-10
mir-27a	4.49	1.70E-04
mir-27b	4.42	1.88E-04
mir-28	4.09	1.61E-04
mir-29a	5.59	3.33E-06
mir-29b-1	31.01	1.78E-04
mir-3074	9.26	6.74E-09
mir-3085	85.94	9.5E-10
mir-30a	27.93	1.65E-15
mir-30d	3.53	9.64E-03
mir-3184	2.69	3.28E-02
mir-320a//mir-320	5.77	6.18E-06
mir-320c-1//mir-320c-2	14.18	1.45E-07
mir-320d-1//mir-320d-2	11.46	4.13E-06
mir-323	12.14	2.28E-04
mir-324	8.11	8.21E-07
mir-330	3.93	7.05E-04
mir-335	7.76	4.88E-05
mir-34a	4.66	1.97E-03
mir-3529	4.22	1.30E-02
mir-365-2//mir-365-1	2.75	1.86E-02
mir-365a	588.15	1.02E-10
mir-365b	11.14	1.61E-06
mir-369	160.52	1.01E-18
mir-371	5.59	4.79E-04
mir-376c	29.16	1.67E-05
mir-378a	4.51	2.29E-03
mir-378a//mir-378	10.10	2.56E-07
mir-378c	8.65	1.12E-05
mir-378d-2//mir-378d-1	54.33	4.51E-17

mir-378e	68.52	1.98E-13
mir-378f	9.85	1.29E-06
mir-378g	4.44	5.23E-03
mir-378h	17.72	6.29E-07
mir-378i	6.71	6.88E-06
mir-380	31.68	1.7E-05
mir-409	7.69	1.07E-03
mir-410	12.25	1.11E-05
mir-421	4.20	4.91E-04
mir-422a	29.24	4.33E-12
mir-4286	5.95	3.01E-04
mir-432	12.22	3.88E-04
mir-455	46.01	4.81E-10
mir-494	16.33	3.55E-04
mir-497	8.03	7.81E-07
mir-541	7.91	1.38E-02
mir-551a	7.56	2.26E-03
mir-574	25.85	2.4E-13
mir-615	19.43	8.98E-05
mir-6529	2.99	3.11E-02
mir-671	2.69	3.57E-02
mir-708	11.15	6.81E-05
mir-7-1//mir-7-2//mir-7-3	7.74	6.22E-04
mir-744	4.05	4.83E-03
mir-769	10.75	2.85E-04
mir-874	173.05	3.91E-24
mir-877	11.52	2.08E-06
mir-8859a	170.64	6.74E-20
mir-8859b	8.97	2.1E-07
mir-8865	7.39	5.06E-05
mir-8890	176.85	3.46E-12
mir-8903	11.14	2.28E-04
mir-8908a-1//mir-8908a-2//mir-8908a-3//mir-8908a-4	589.21	7.42E-09
mir-9-1//mir-9-2//mir-9-3	41.27	3.04E-09
mir-92b	2.94	3.16E-02
mir-96	100.04	1.19E-16
mir-99a	7.74	2.16E-03
mir-99a-1//mir-99a-2	16.60	4.7E-12
mir-99b	190.80	3.54E-31

Downregulated miRNAs

miRNAs	Fold Change	FDR
let-7g	-3.43	5.29E-03
let-7i	-2.49	3.91E-02
mir-101-1//mir-101-2	-2.49	3.71E-02
mir-101-2//mir-101-1	-3.21	1.74E-03
mir-103-1//mir-103-2	-4.98	1.09E-02
mir-106b	-5.24	1.70687E-05

mir-10a	-4.73	3.72189E-05
mir-126	-61.57	0
mir-130b	-3.19	2.23E-02
mir-141	-173.69	2.20026E-13
mir-142	-289.23	0
mir-144	-172.53	2.01083E-18
mir-1468	-21.83	9.2621E-05
mir-146a	-4.08	1.04E-03
mir-150	-987.46	4.78026E-28
mir-15a	-4.08	1.61E-04
mir-15b	-5.11	1.46843E-05
mir-16-1//mir-16-2	-3.96	1.61E-04
mir-17	-4.41	2.79E-03
mir-1844	-24.72	2.91722E-09
mir-18a	-7.69	2.29641E-07
mir-191	-4.37	5.8945E-05
mir-192	-12.52	2.265E-10
mir-194-1//mir-194-2//mir-194	-34.49	3.5983E-12
mir-19a	-3.93	5.95E-04
mir-19b-1//mir-19b-2	-4.25	1.63E-04
mir-203a//mir-203	-2.65	4.18E-02
mir-203b	-2.69	3.66E-02
mir-218-1//mir-218-2	-12.40	2.356E-08
mir-223	-1510.54	2.40263E-17
mir-25	-5.72	2.19823E-06
mir-26a-2//mir-26a-1	-6.41	5.29E-03
mir-26b	-13.87	7.71E-04
mir-29c//mir-29c-1//mir-29c-2	-10.10	9.05131E-09
mir-30b	-2.55	2.96E-02
mir-30e	-2.85	8.51E-03
mir-32	-13.87	3.11659E-10
mir-326	-110.13	1.22023E-10
mir-339-1	-2.20	3.99E-02
mir-342	-3.83	9.78E-04
mir-371a	-1078.08	7.88078E-08
mir-371b	-1746.82	2.97001E-08
mir-374a	-3.90	6.10E-03
mir-383	-199.74	6.58238E-10
mir-425	-8.18	2.4786E-08
mir-4486	-10.80	3.19E-03
mir-450a	-15.31	2.78E-04
mir-450a-1//mir-450a-2	-4.83	3.00E-02
mir-450b	-2.71	4.19E-02
mir-451	-681.51	0
mir-4634	-7.45	4.46E-03
mir-486	-608.92	0
mir-486-1//mir-486-2	-544.68	0
mir-500a	-2.61	1.34E-02

mir-551b	-1523.39	2.50384E-15
mir-628	-2.75	2.25E-02
mir-652	-4.53	1.41E-04
mir-7975	-8.88	3.17E-03
mir-92a-1//mir-92a-2	-2.29	3.91E-02
mir-93	-17.29	7.54299E-14

FDR, False discovery rates

Appendix 5 Differentially expressed miRNAs between Reference and LMEC cell line exosomes

Upregulated miRNAs		
miRNAs	**Fold Change**	**FDR**
let-7e	25.30	4.2235E-12
let-7f-1	3.48	2.70E-03
let-7g	3.48	7.66E-03
mir-10b	10.44	7.2678E-10
mir-122	623.62	1.4356E-08
mir-1224	173.85	2.5362E-19
mir-122b	676.80	6.8115E-09
mir-124-1//mir-124-2//mir-124-3	1242.02	1.1366E-11
mir-1246	54.88	5.2014E-13
mir-125a	16.08	1.1386E-11
mir-125b-1	430.16	4.7122E-26
mir-125b-2//mir-125b-1	21.04	2.1192E-12
mir-127	5.61	1.69E-02
mir-129-1//mir-129-2	4.35	1.79E-03
mir-129-2	1212.47	2.2849E-14
mir-1298	9.84	2.57E-03
mir-130a	19.17	4.3156E-14
mir-132	5.24	6.4016E-05
mir-133a-1//mir-133a-2	9.41	2.09E-02
mir-133c	3.13	3.77E-02
mir-134	67.36	1.37E-02
mir-135b	176.17	9.2155E-06
mir-136	4.01	2.69E-02
mir-138-2//mir-138-1//mir-138a	7.52	1.21E-04
mir-139	7.25	1.85E-02
mir-146b	23.95	2.4123E-09
mir-148a	4.69	2.5584E-05
mir-149	13.03	1.47E-04
mir-151a	2.95	5.12E-03
mir-151b	4.08	9.76E-04
mir-152	112.15	5.9398E-13
mir-155	23.38	2.6052E-06
mir-181c	5.32	7.26E-03
mir-181d	24.17	5.3694E-10
mir-182	26.19	3.376E-14
mir-183	105.23	7.5592E-26
mir-185	9.30	7.3781E-06
mir-187	601.94	9.7644E-11
mir-188	3.90	4.67E-02
mir-1911	474.54	7.4511E-09
mir-193a	12.57	1.9342E-07
mir-193b (hsa 3p)	141.40	3.2545E-16

mir-193b (hsa/cfa 5p)	55.98	8.1902E-10
mir-195	7.25	3.5162E-05
mir-196a-2	129.82	2.85E-04
mir-197	4.26	2.00E-03
mir-199-1//mir-199-2//mir-199-3	72.76	2.5035E-21
mir-199a-1//mir-199a-2	143.94	4.812E-11
mir-199a-1//mir-199a-2//mir-199b	116.20	5.8663E-21
mir-208b	11.57	1.6969E-06
mir-21	16.94	1.8664E-12
mir-210	23.29	2.0268E-13
mir-2113	967.99	3.0311E-12
mir-212	23.49	5.2335E-09
mir-214	38.83	5.3984E-11
mir-22	6.34	2.0046E-06
mir-221	42.18	8.1227E-16
mir-222	93.37	1.4619E-16
mir-2355	2.96	1.05E-02
mir-2387	112.55	1.3314E-09
mir-23a	4.15	2.85E-04
mir-23b	5.71	1.0034E-05
mir-24-1//mir-24-2	14.62	5.5032E-12
mir-27a	8.44	1.0416E-07
mir-27b	7.65	3.4265E-07
mir-28	2.66	9.64E-03
mir-29a	5.76	2.0616E-06
mir-29b-1	17.23	2.76E-03
mir-29b-2//mir-29b-1	2.29	4.89E-02
mir-3074	12.59	6.0148E-11
mir-3085	80.40	1.1941E-09
mir-30a	20.49	1.5335E-13
mir-3120	34.51	2.2216E-10
mir-320a//mir-320	8.23	6.0604E-08
mir-320b-1//mir-320b-2	2.91	3.02E-02
mir-320c-1//mir-320c-2	7.77	3.5665E-05
mir-320d-1//mir-320d-2	5.23	2.06E-03
mir-323	13.08	1.08E-04
mir-324	6.28	1.3839E-05
mir-330	6.14	6.0611E-06
mir-335	25.39	4.6442E-10
mir-33b	2.44	3.63E-02
mir-34a	9.86	1.4836E-06
mir-3529	4.00	1.55E-02
mir-361	2.87	1.09E-02
mir-365-2//mir-365-1	3.53	2.59E-03
mir-365a	270.07	2.9876E-07
mir-369	180.20	1.6835E-19
mir-375	2.75	4.35E-02

mir-376c	36.19	2.0679E-06
mir-378a	3.84	6.58E-03
mir-378a//mir-378	6.18	3.4941E-05
mir-378c	8.25	1.4139E-05
mir-378d-2//mir-378d-1	47.06	2.939E-16
mir-378e	60.80	7.2009E-13
mir-378f	10.25	6.943E-07
mir-378h	13.45	5.9938E-06
mir-378i	5.87	2.5115E-05
mir-380	34.50	1E-05
mir-409	5.00	1.11E-02
mir-410	14.96	2.0616E-06
mir-422a	17.80	2.3139E-09
mir-432	13.49	1.38E-04
mir-448	316.34	8.3235E-07
mir-4502	16.56	1.48E-03
mir-491	224.76	5.0657E-05
mir-494	18.27	1.57E-04
mir-497	4.42	3.63E-04
mir-504	19.48	5.3332E-06
mir-541	12.45	1.09E-03
mir-574	91.25	1.6951E-21
mir-615	73.47	9.76E-04
mir-656	24.15	9.171E-05
mir-671	85.11	2.1309E-13
mir-676	105.33	2.38E-03
mir-708 (hsa/cfa-5p)	241.88	3.3454E-21
mir-708 (hsa-3p)	26.97	4.5967E-14
mir-7-1//mir-7-2//mir-7-3	7.80	4.83E-04
mir-769	19.04	1.2559E-06
mir-874	61.19	2.3495E-17
mir-8826	214.85	2.5035E-21
mir-8859a	80.16	1.4103E-15
mir-8859b	2.78	1.58E-02
mir-8865	4.34	3.07E-03
mir-8876	4.81	1.73E-02
mir-8884	3.15	2.87E-02
mir-889	24.38	1.21E-04
mir-8890	92.51	1.5769E-09
mir-8903	5.22	2.02E-02
mir-8908a-1//mir-8908a-2//mir-8908a-3//mir-8908a-4	172.26	6.7524E-05
mir-9-1//mir-9-2//mir-9-3	46.08	5.2417E-10
mir-935	224.27	6.0148E-11
mir-96	133.39	1.3788E-18
mir-99a	5.35	1.17E-02
mir-99a-1//mir-99a-2	13.28	8.9016E-11
mir-99b	164.32	6.39E-30

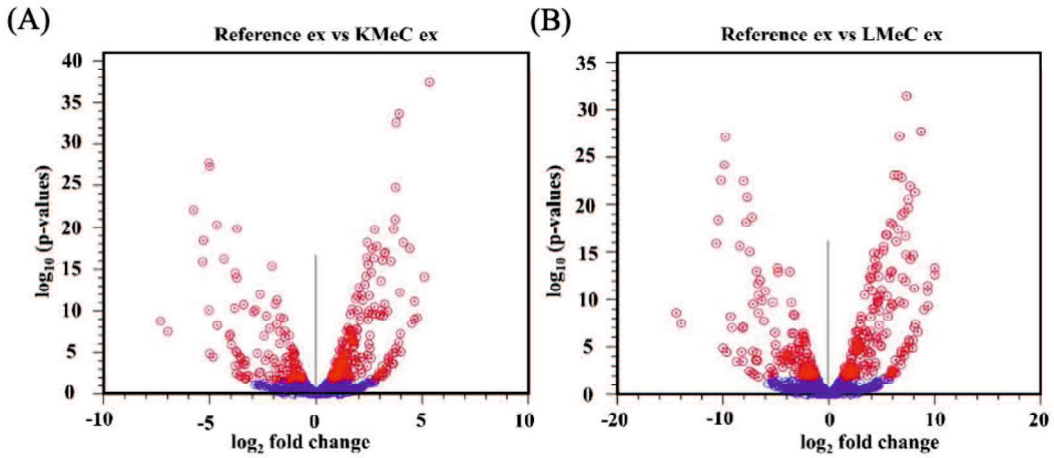
Appendix

Downregulated miRNAs		
miRNAs	**Fold Change**	**FDR**
let-7c	-9.71	6.69E-04
mir-101-1//mir-101-2	-2.40	4.50E-02
mir-101-2//mir-101-1	-3.21	1.65E-03
mir-106b	-5.15	1.79E-05
mir-10a	-2.83	6.06E-03
mir-126	-160.31	0
mir-1260a	-11.72	2.18E-04
mir-1260b	-5.03	5.98E-03
mir-140	-5.36	5.29064E-06
mir-141	-62.44	1.06822E-10
mir-142	-226.32	0
mir-143	-27.64	5.11723E-12
mir-144	-206.11	1.87017E-19
mir-145	-28.52	4.34973E-13
mir-1468	-15.38	3.76E-04
mir-150	-870.96	7.83347E-28
mir-15a	-2.67	9.36E-03
mir-15b	-5.60	4.20967E-06
mir-16-2//mir-16-1	-3.37	8.07E-04
mir-181a-1//mir-181a-2	-9.34	6.1028E-08
mir-181a-2//mir-181a-1	-5.49	2.28E-02
mir-184	-11.51	2.64659E-06
mir-18a	-4.19	1.89E-04
mir-191	-4.59	2.82882E-05
mir-192	-2.41	2.67E-02
mir-194-1//mir-194-2//mir-194	-6.97	2.21521E-05
mir-19b-1//mir-19b-2	-2.63	1.28E-02
mir-203a//mir-203	-4.99	5.04E-04
mir-203b	-5.25	2.82E-04
mir-205	-4.20	1.52E-03
mir-218-1//mir-218-2	-3.29	5.68E-03
mir-223	-1379.68	1.86871E-17
mir-25	-4.88	1.43238E-05
mir-26a-2//mir-26a-1	-4.98	1.58E-02
mir-26b	-9.13	4.15E-03
mir-30b	-3.26	4.89E-03
mir-30c-2//mir-30c-1	-10.21	3.80159E-08
mir-30e (cfa-3p)	-7.09	1.21E-04
mir-30e (hsa-5p)	-4.10	2.71E-04
mir-3184	-7.05	1.33E-04
mir-32	-11.59	2.07461E-09
mir-326	-97.68	8.68381E-11
mir-331	-3.46	2.51E-03
mir-340	-4.12	1.72E-02
mir-363	-266.62	2.50352E-21

mir-371	-920.19	1.4871E-22
mir-371a	-17900.09	2.15996E-11
mir-371b	-20916.81	2.70706E-11
mir-383	-141.69	2.48551E-09
mir-423//mir-423a	-7.05	1.33E-04
mir-424	-2.94	4.09E-02
mir-425	-4.44	5.62357E-05
mir-4454	-7.85	7.81216E-06
mir-4486	-32.73	3.4941E-05
mir-450a	-14.75	2.67E-04
mir-450a-1//mir-450a-2	-5.18	2.24E-02
mir-450b	-3.41	1.08E-02
mir-451	-731.62	0
mir-4634	-36.07	1.63678E-06
mir-486	-220.36	0
mir-486-1//mir-486-2	-150.77	0
mir-500a	-2.55	1.57E-02
mir-505	-2.56	3.24E-02
mir-542	-2.83	3.00E-02
mir-551b	-1523.39	2.13563E-15
mir-628	-2.85	1.60E-02
mir-652	-3.47	1.57E-03
mir-7975	-15.30	2.65E-04
mir-93	-12.83	8.01261E-12

FDR, False discovery rates

Appendix 6



Appendix 6 Differential exomiRs in COM. (A-B) Volcano plot analysis represents the differentially expressed exomiRs between the (A) Reference and KMEC exosomes and (B) Reference and LMEC exosomes.

Appendix 7 Differentially expressed miRNAs in between melanoma cell lines exosomes (KMEC and LMEC)

Upregulated miRNAs		
miRNAs	**Fold Change**	**FDR**
mir-10a	6.76	1.12E-02
mir-1224	6.01	5.82E-05
mir-124-1//mir-124-2//mir-124-3	268.05	1.66E-11
mir-1298	45.31	6.78E-06
mir-130b	4.96	3.37E-03
mir-146b	6.53	7.08E-04
mir-1838	4.48	2.35E-02
mir-1844	15.38	1.17E-06
mir-187	4.18	9.07E-03
mir-1911	474.54	1.86E-10
mir-192	5.20	6.89E-05
mir-194-1//mir-194-2//mir-194	4.95	3.55E-03
mir-196b	31.80	1.75E-04
mir-210	5.87	3.94E-05
mir-2113	8.87	4.72E-05
mir-212	6.09	6.24E-04
mir-214	9.38	9.27E-06
mir-218-1//mir-218-2	3.77	1.02E-02
mir-221	4.68	9.44E-04
mir-222	4.93	3.04E-03
mir-29c//mir-29c-1//mir-29c-2	5.01	1.65E-04
mir-301b	2.89	1.65E-02
mir-3120	9.46	1.63E-05
mir-335	3.27	4.45E-02
mir-448	20.77	6.35E-05
mir-486	2.76	4.06E-02
mir-486-1//mir-486-2	3.61	6.65E-03
mir-491	9.35	4.86E-03
mir-504	35.43	4.56E-08
mir-574	3.53	6.20E-03
mir-582	3.63	5.00E-02
mir-708 (hsa/cfa-5p)	21.69	6.83E-11
mir-708 (hsa-3p)	14.26	9.82E-10
mir-8826	478.96	2.12E-24
mir-8884	4.62	1.02E-02
mir-935	9.40	1.69E-04
Downregulated miRNAs		
miRNAs	**Fold Change**	**FDR**
let-7b	-3.70	1.37E-02
let-7c	-47.38	3.59354E-07
mir-106a	-44.78	6.82546E-11
mir-10b	-2.78	2.07E-02

mir-1249	-7.10	4.57E-04
mir-125b-1	-4.03	2.94E-03
mir-125b-2	-27.96	1.7774E-13
mir-1296	-3.93	1.37E-02
mir-140	-5.16	3.46826E-05
mir-143	-595.62	0
mir-145	-44.31	3.19769E-09
mir-149	-6.32	1.63E-02
mir-181a-1	-14.09	2.54E-03
mir-181a-1//mir-181a-2	-14.33	1.2395E-09
mir-181a-2//mir-181a-1	-6.37	3.78E-02
mir-181b-1//mir-181b-2	-4.92	6.50E-04
mir-184	-7.18	4.62E-04
mir-18b	-66.84	8.02075E-07
mir-197	-4.71	1.78E-02
mir-199b	-4.57	2.21E-02
mir-205	-7.74	1.94048E-05
mir-20b	-52.32	2.53242E-07
mir-25	-3.58	4.60E-02
mir-30c-1	-5.24	1.91E-02
mir-3184	-5.05	5.27E-03
mir-33a	-3.26	3.57E-02
mir-363	-147.50	8.74695E-18
mir-365b	-4.82	2.50E-03
mir-371	-5145.05	1.69585E-30
mir-371a	-16.60	2.88E-02
mir-423//mir-423a	-5.05	5.27E-03
mir-4286	-4.68	1.23E-03
mir-4454	-12.75	1.61775E-07
mir-455	-594.05	9.16805E-19
mir-551a	-191.72	2.05114E-06
mir-615	-5.73	1.70E-02
mir-6529	-3.73	8.27E-03
mir-7977	-65.70	5.54825E-07
mir-874	-2.83	3.54E-02
mir-877	-5.34	2.87E-03
mir-8859b	-3.22	1.59E-02
mir-92a-1	-6.15	4.66E-02
mir-99a	-9.66	2.05114E-06

FDR, False discovery rates

Appendix 8 Commonly decreased exomiRs in all groups (Reference vs KMEC, Reference vs LMEC and KMEC vs LMEC)

miRNA	Fold Change			FDR		
	P vs K	P vs L	K vs L	P vs K	P vs L	K vs L
mir-371a	-1078.08	-17900.1	-16.6037	7.88E-08	2.16E-11	2.88E-02

FDR, False discovery rates

Appendix 9 ExomiRs commonly increased in melanoma cell lines exosomes

Feature ID	miRNAs	Resource	Match type
AACCCGTAGATCCGAACTTGTG	mir-100	Homo sapiens	Mature 5'
AGCAGCATTGTACAGGGCTAT	mir-107	Canis familiaris	Mature 3'
TGGAGTGTGACAATGGTGTTTG	mir-122	Canis familiaris	Mature 5'
GTGAGGACTCGGGAGGTGG	mir-1224	Homo sapiens	Mature 5'
AAACACCATTGTCACACTCCAC	mir-122b	Homo sapiens	Mature 3'
CATCATCGTCTCAAATGAGTCT	mir-136	Homo sapiens	Mature 3'
ACCACAGGGTAGAACCACGGA	mir-140	Canis familiaris	Mature 3'
CCCATAAAGTAGAAAGCACTA	mir-142	Canis familiaris	Mature 5'
TACAGTATAGATGATGTACT	mir-144	Homo sapiens	Mature 3'
GGATTCCTGGAAATACTGTTCT	mir-145	Homo sapiens	Mature 3'
CTCCGTTTGCCTGTTTTGCTGAT	mir-1468	Canis familiaris	Mature 5'
TAGCAGCACATCATGGTTTA	mir-15b	Canis familiaris	Mature 5'
AACATTCAACCTGTCGGTGAGT	mir-181c	Homo sapiens	Mature 5'
AAGGTAGATAGAACAGGTCTTG	mir-1839	Canis familiaris	Mature 5'
CATCCCTTGCATGGTGGAGGGT	mir-188	Canis familiaris	Mature 5'
AACTGGCCTACAAAGTCCCAGT	mir-193a	Homo sapiens	Mature 3'
TGTGCAAATCCATGCAAAACTG	mir-19b-1//mir-19b-2	Canis familiaris	Mature 3'
ATGACCTATGAATTGACAGAC	mir-215	Homo sapiens	Mature 5'
AAGCTGCCAGTTGAAGAACTGT	mir-22	Homo sapiens//Canis familiaris	Mature 3'
ATCACATTGCCAGGGATTTCC	mir-23a	Homo sapiens	Mature 3'
TAGCACCATCTGAAATCGGTTA	mir-29a	Homo sapiens//Canis familiaris	Mature 3'
CAGTGCAATAGTATTGTCAAAGC	mir-301a	Canis familiaris	Mature 3'
TCTGGCTGCTATGGCCCCCTC	mir-3085	Homo sapiens	Mature 3'
AAAAGCTGGGTTGAGAGGGT	mir-320c-1//mir-320c-2	Homo sapiens	Mature 3'
AAAAGCTGGGTTGAGAGGA	mir-320d-1//mir-320d-2	Homo sapiens	Mature 3'
CACATTACACGGTCGACCTCT	mir-323	Canis familiaris	Mature 3'
TCAAGAGCAATAACGAAAAATGT	mir-335	Canis familiaris	Mature 5'
CCTGAACTAGGGGTCTGGAGG	mir-345	Canis familiaris	Mature 3'
AGGCAGTGTAGTTAGCTGATTGC	mir-34c	Canis familiaris	Mature 5'
AACACACCTATTCAAGGATTCA	mir-362	Homo sapiens	Mature 3'
AATAATACATGGTTGATCTTT	mir-369	Homo sapiens	Mature 3'
AACATAGAGGAAATTCCACGT	mir-376c	Homo sapiens	Mature 3'
ACTGGACTTGGAGTCAGAAGAGTGG	mir-378c	Homo sapiens	Mature 5'

Appendix

ACTGGACTTGGAGTCAGAAA	mir-378d-2//mir-378d-1	Homo sapiens	Mature 3'
ACTGGACTTGGAGCCAGAAG	mir-378f	Homo sapiens	Mature 3'
ACTGGACTTGGTGTCAGATGG	mir-378h	Homo sapiens	Mature 5'
ACTGGACTAGGAGTCAGAAGG	mir-378i	Homo sapiens	Mature 5'
TATGTAATATGGTCCACGTCT	mir-380	Canis familiaris	Mature 3'
TATACAAGGGCAAGCTCTCTGT	mir-381	Canis familiaris	Mature 3'
CAGATATTGCACGGTTGATCTCT	mir-3958	Canis familiaris	Mature 3'
ATAGTAGACCGTATAGCGTACG	mir-411	Canis familiaris	Mature 5'
ATCAACAGACATTAATTGGGCG	mir-421	Canis familiaris	Mature 3'
ACTGGACTTAGGGTCAGAAGGC	mir-422a	Homo sapiens	Mature 5'
TCTTGGAGTAGGTCATTGGGTGG	mir-432	Canis familiaris	Mature 5'
GCTGGGCGAGGCTGGCA	mir-4486	Homo sapiens	Mature 5'
AAACCGTTACCATTACTGAGTT	mir-451	Canis familiaris	Mature 5'
CGGCGCGACCGGCCCGGGG	mir-4634	Homo sapiens	Mature 5'
TGAAACATACACGGGAAACCTC	mir-494	Canis familiaris	Mature 3'
AATGCACCTGGGCAAGGATTCA	mir-502	Homo sapiens//Canis familiaris	Mature 3'
TAGCAGCGGGAACAGTACTG	mir-503	Canis familiaris	Mature 5'
CATGCCTTGAGTGTAGGACCGT	mir-532	Canis familiaris	Mature 5'
TGGTGGGCACAGAATCTGGACT	mir-541	Homo sapiens	Mature 3'

Appendix 10 ExomiRs specifically increased in primary melanoma cell lines exosomes

Feature ID	miRNAs	Resource	Match type
CTATACAATCTACTGTCTTTC	let-7a-1//let-7a-3	Homo sapiens	Mature 3'
CTATACAGTCTACTGTCTTTCC	let-7f-2	Homo sapiens	Mature 3'
CTGCGCAAGCTACTGCCTTGCT	let-7i	Homo sapiens	Mature 3'
CAGTTATCACAGTGCTGATGCT	mir-101-1	Homo sapiens	Mature 5'
TACAGTACTGTGATAACTGAA	mir-101-1//mir-101-2	Homo sapiens	Mature 3'
TACAGTACTGTGATAACTGA	mir-101-2//mir-101-1	Canis familiaris	Mature 3'
TCATAGCCCTGTACAATGCTGCT	mir-103b-1//mir-103b-2	Homo sapiens	Mature 5'
TACCCTGTAGATCCGAATTTGT	mir-10a	Canis familiaris	Mature 5'
TACCCTGTAGAACCGAATTTGTG	mir-10b	Homo sapiens	Mature 5'
AGTGCCTGCTATGTGCCAGGCA	mir-1271	Homo sapiens	Mature 3'
TCACAGTGAACCGGTCTCTTT	mir-128-1//mir-128-2	Homo sapiens//Canis familiaris	Mature 3'
TCGACCGGACCTCGACCGGCT	mir-1307	Homo sapiens	Mature 5'
TGAGAACTGAATTCCATGGGTT	mir-146a	Homo sapiens//Canis familiaris	Mature 5'
TGAGAACTGAATTCCATAGGCT	mir-146b	Canis familiaris	Mature 5'
TCAGTGCACTACAGAACTTTGT	mir-148a	Homo sapiens//Canis familiaris	Mature 3'
TCGAGGAGCTCACAGTCT	mir-151b	Homo sapiens	Mature 3'
TTGCATAGTCACAAAAGTGATC	mir-153-1//mir-153-2	Homo sapiens	Mature 3'
TAGCAGCACATAATGGTTTGT	mir-15a	Canis familiaris	Mature 5'
TAGCAGCACGTAAATATTGGCG	mir-16-1//mir-16-2	Homo sapiens//Canis familiaris	Mature 5'
TGGTTCTAGACTTGCCAACTA	mir-182	Homo sapiens	Mature 3'
TGGCTCTGCGAGGTCAGCTCA	mir-1842	Canis familiaris	Mature 5'
TAAGGTGCATCTAGTGCAGTTA	mir-18b	Canis familiaris	Mature 5'
TGATATGTTTGATATTGGGTTG	mir-190b	Homo sapiens	Mature 5'

CTGACCTATGAATTGACAGCC	mir-192	Homo sapiens//Canis familiaris	Mature 5'
TAGCAGCACAGAAATATTGGC	mir-195	Homo sapiens	Mature 5'
ACAGTAGTCTGCACATTGGTT	mir-199-1//mir-199-2//mir-199-3	Canis familiaris	Mature 3'
TGTGCAAATCTATGCAAAACTGA	mir-19a	Homo sapiens//Canis familiaris	Mature 3'
TAGTGGTCCTAAACATTTCACA	mir-203b	Homo sapiens	Mature 5'
ATAAGACGAACAAAAGGTTTGT	mir-208b	Canis familiaris	Mature 3'
TAGCTTATCAGACTGATGTTGA	mir-21	Homo sapiens//Canis familiaris	Mature 5'
CTGTGCGTGTGACAGCGGCTGA	mir-210	Homo sapiens	Mature 3'
TTGTGCTTGATCTAACCATGT	mir-218-1//mir-218-2	Canis familiaris	Mature 5'
TGATTGTCCAAACGCAATTCT	mir-219-1//mir-219-2	Canis familiaris	Mature 5'
AGAATTGCGTTTGGACAATCAGT	mir-219b	Homo sapiens	Mature 3'
AGCTACATTGTCTGCTGGGTTT	mir-221	Canis familiaris	Mature 3'
ATCCCCAGATACAATGGACAA	mir-2355	Homo sapiens	Mature 5'
ATCACATTGCCAGGGATTA	mir-23b	Canis familiaris	Mature 3'
TGGCTCAGTTCAGCAGGAACAG	mir-24-1//mir-24-2	Homo sapiens	Mature 3'
CATTGCACTTGTCTCGGTCTGA	mir-25	Homo sapiens//Canis familiaris	Mature 3'
TTCAAGTAATTCAGGATAGGT	mir-26b	Homo sapiens	Mature 5'
TTCACAGTGGCTAAGTTCCG	mir-27a	Canis familiaris	Mature 3'
TTCACAGTGGCTAAGTTCTGC	mir-27b	Homo sapiens//Canis familiaris	Mature 3'
TAGCACCATTTGAAATCGGTTA	mir-29c//mir-29c-1//mir-29c-2	Homo sapiens//Canis familiaris	Mature 3'
CAGTGCAATGATATTGTCAAAGC	mir-301b	Canis familiaris	Mature 3'
GTTCCTGCTGAACTGAGCCAG	mir-3074	Homo sapiens	Mature 5'
TGTAAACATCCTCGACTGGAAGC	mir-30a	Canis familiaris	Mature 5'
TGTAAACATCCTTGACTGGAAG	mir-30e	Homo sapiens	Mature 5'
AAAGTCTCGCTCTCTGCCCCTCA	mir-3184	Homo sapiens	Mature 3'

Appendix

TATTGCACATTACTAAGTTGCAT	mir-32	Canis familiaris	Mature 5'
TCCCTGTCCTCCAGGAGCT	mir-339-1	Canis familiaris	Mature 5'
GTGCATTGTAGTTGCATTGC	mir-33a	Canis familiaris	Mature 5'
GTGCATTGCTGTTGCATTGC	mir-33b	Canis familiaris	Mature 5'
TTATAAAGCAATGAGACTGATT	mir-340	Canis familiaris	Mature 5'
TGGCAGTGTCTTAGCTGGTTGT	mir-34a	Homo sapiens//Canis familiaris	Mature 5'
AACAACAAAATCACTAGTCTTCCA	mir-3529	Homo sapiens	Mature 3'
AAGTGCCGCCATCTTTTGAGTGT	mir-371a	Homo sapiens	Mature 3'
CACTTAGCAGGTTGTATTATAT	mir-374c	Homo sapiens	Mature 3'
ACTGGACTTGGAGTCAGAAGGC	mir-378a//mir-378	Homo sapiens//Canis familiaris	Mature 3'
ACTGGACTTGGAGTCAGGA	mir-378e	Homo sapiens	Mature 3'
CAGCAGCAATTCATGTTTTGAA	mir-424	Homo sapiens	Mature 5'
ATCGGGAATGTCGTGTCCGCCC	mir-425	Homo sapiens	Mature 3'
TTTTGCAATATGTTCCTGAAT	mir-450b	Canis familiaris	Mature 5'
TCGGGGCAGCTCAGTACAGGAT	mir-486	Canis familiaris	Mature 3'
TCCTGTACTGAGCTGCCCCGAG	mir-486-1//mir-486-2	Homo sapiens	Mature 5'
TTAAGACTTGCAGTGATGTTT	mir-499	Canis familiaris	Mature 5'
AACATCACTGCAAGTCTTAACA	mir-499b	Homo sapiens	Mature 3'
TGTGACAGATTGATAACTGAAA	mir-542	Homo sapiens//Canis familiaris	Mature 3'
GCGACCCACTCTTGGTTTCCA	mir-551a	Canis familiaris	Mature 3'
TGAGTGTGTGTGTGTGAGTGTGT	mir-574	Homo sapiens	Mature 5'
TTGTGTCAATATGCGATGATGT	mir-592	Homo sapiens	Mature 5'
ATGCTGACATATTTACTAGAGG	mir-628	Canis familiaris	Mature 5'
TACCCATTGCATATCGGAGTTG	mir-660	Canis familiaris	Mature 5'
CAACTAGACTGTGAGCTTCTAG	mir-708	Homo sapiens	Mature 3'
CAACAAATCACAGTCTGCCATA	mir-7-1	Homo sapiens	Mature 3'
CTGTTGCCACTAACCTCAACCT	mir-744	Homo sapiens	Mature 3'
TTTGATGGATTTGCTTAGCACC	mir-8884	Canis familiaris	Mature 3'

Appendix

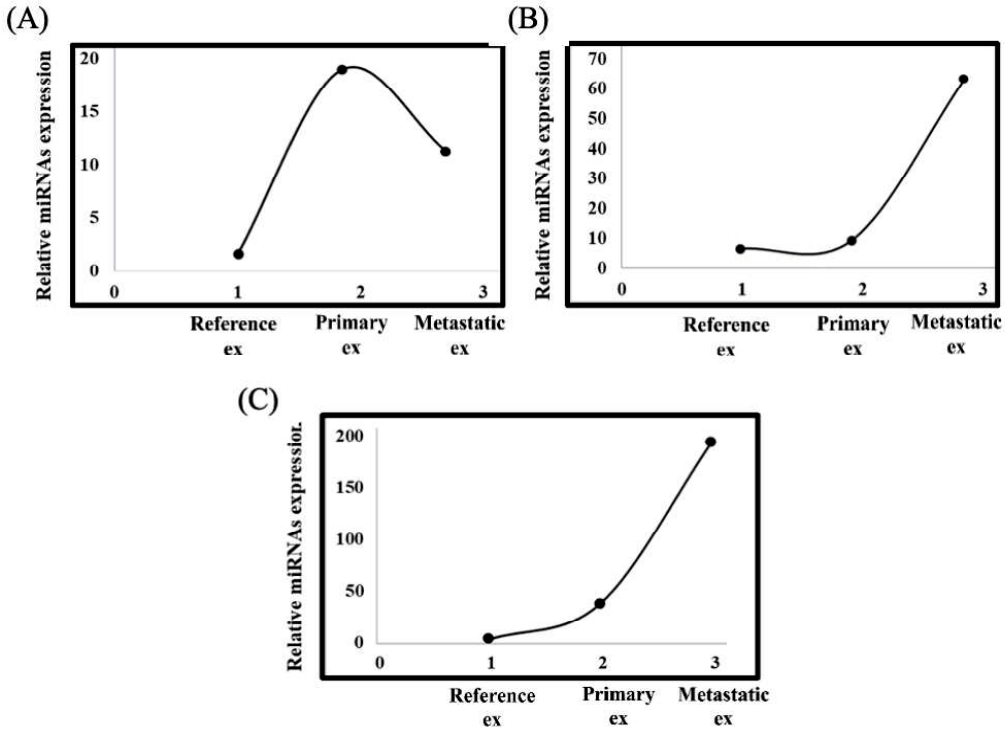
ATAAAGCTAGATAACCGAAAGT	mir-9-1//mir-9-2//mir-9-3	Homo sapiens	Mature 3'
CTATACAACTTACTACTTTCCC	mir-98	Homo sapiens	Mature 3'
TTCACAGTGGCTAAGCTAT	mir-9985	Homo sapiens	Mature 5'

Appendix 11 ExomiRs specifically increased in metastatic melanoma cell lines

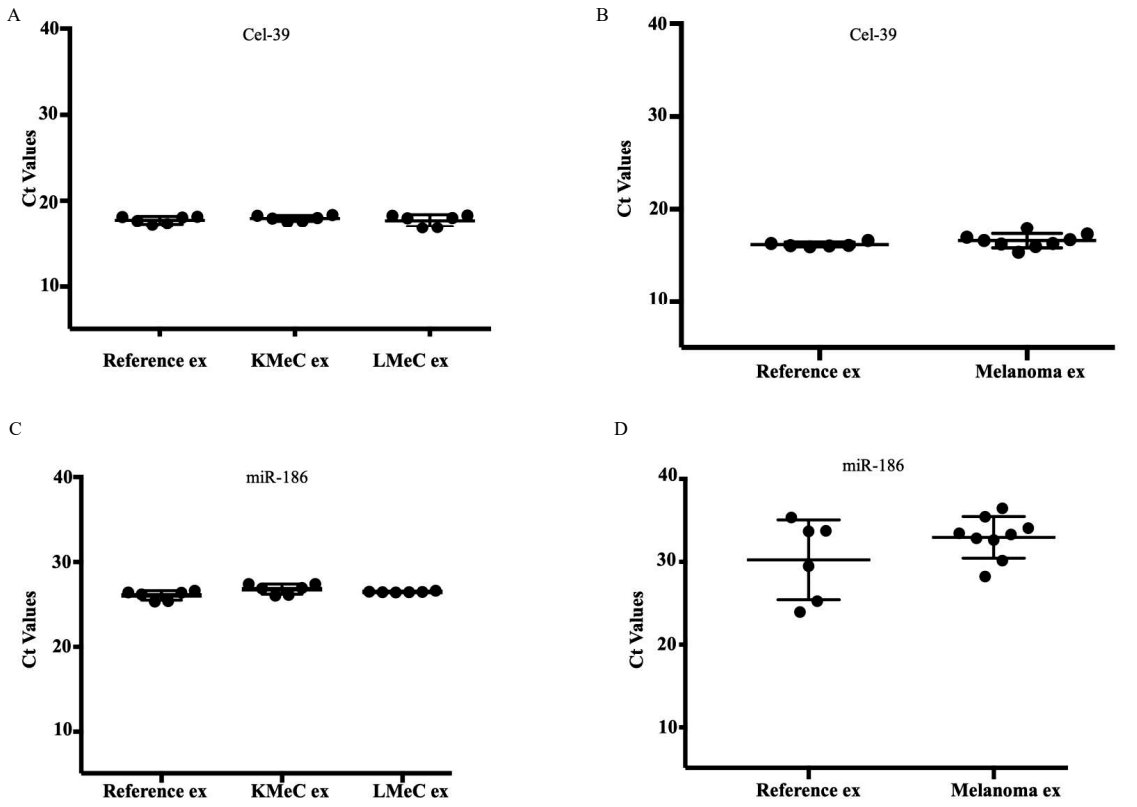
Feature ID	miRNAs	Resource	Match type
AATGGATTTTTGGAGCAGG	mir-1246	Homo sapiens	Mature 5'
CATTATTACTTTTGGTACGCG	mir-126	Canis familiaris	Mature 5'
TCGGATCCGTCTGAGCTTGGCT	mir-127	Homo sapiens//Canis familiaris	Mature 3'
CTTGGCACCTAGTAAGCACT	mir-1271	Canis familiaris	Mature 5'
CTTTTTGCGGTCTGGGCTTGC	mir-129-1//mir-129-2	Canis familiaris	Mature 5'
CAGTGCAATGTTAAAAGGGCAT	mir-130a	Homo sapiens//Canis familiaris	Mature 3'
TTGGTCCCCTTCAACCAGCTGT	mir-133a	Canis familiaris	Mature 3'
TTTGGTCCCCTTCAACCAGCTG	mir-133a-1//mir-133a-2	Homo sapiens	Mature 3'
TTGGTCCCCTTCAACCAGCTG	mir-133c	Canis familiaris	Mature 3'
TGTGACTGGTTGACCAGAGGGG	mir-134	Canis familiaris	Mature 5'
AGCTGGTGTTGTGAATCAGGCCG	mir-138-2//mir-138a	Homo sapiens//Canis familiaris	Mature 5'
TAACACTGTCTGGTAAAGATGG	mir-141	Homo sapiens	Mature 3'
TGAGATGAAGCACTGTAGCTC	mir-143	Homo sapiens//Canis familiaris	Mature 3'
TCTCCCAACCCTTGTACCAGTG	mir-150	Canis familiaris	Mature 5'
CTAGACTGAAGCTCCTTGAGG	mir-151a	Homo sapiens	Mature 3'
AACATTCAACGCTGTCGGTGAG	mir-181a-1//mir-181a-2	Canis familiaris	Mature 5'
CCACCAGCTGGCGTTCCCTGG	mir-1838	Canis familiaris	Mature 5'
TGGACGGAGAACTGATAAGGGT	mir-184	Canis familiaris	Mature 3'
TCACGTGACGGGCCTCGGCG	mir-1840	Canis familiaris	Mature 3'
TGATATGTTTGATATTGGGTT	mir-190b	Canis familiaris	Mature 5'
AACTGGCCCTCAAAGTCCCGCT	mir-193b	Homo sapiens	Mature 3'
CGGGGTTTTGAGGGCGAGATGA	mir-193b	Homo sapiens	Mature 5'
CGGCAACAAGAAACTGCCTGAG	mir-196a-2	Homo sapiens	Mature 3'
TCCTTCATTCCACCGGAGTCTG	mir-205	Canis familiaris	Mature 5'
ACAGCAGGCACAGACAGGCAGT	mir-214	Canis familiaris	Mature 3'
TGTAAACATCCTCGACTGGAAG	mir-30a	Homo sapiens	Mature 5'
TGTAAACATCCCCGACTGGAAG	mir-30d	Homo sapiens	Mature 5'
CCTGTCTGTGCCTGCTGTACA	mir-3120	Homo sapiens	Mature 5'
TATTGCACATTACTAAGTTGCA	mir-32	Homo sapiens	Mature 5'
AAAAGCTGGGTTGAGAGGGCGA	mir-320a//mir-320	Homo sapiens//Canis familiaris	Mature 3'

Appendix

CCCACTGCCCCAGGTGCTGCTGG	mir-324	Homo sapiens	Mature 3'
CTAGGTATGGTCCCAGGGATCC	mir-331	Homo sapiens	Mature 5'
AGATCAGAAGGTGATTGTGGCT	mir-383	Canis familiaris	Mature 5'
GAATGTTGCTCGGTGAACCCCT	mir-409	Homo sapiens	Mature 3'
AATATAACACAGATGGCCTGT	mir-410	Canis familiaris	Mature 3'
ATCATGATGGGCTCCTCGGTGT	mir-433	Canis familiaris	Mature 3'
GCTGATGATGATGGTGCTGAAG	mir-4502	Homo sapiens	Mature 3'
AATCGTACAGGGTCATCCACTT	mir-487b	Homo sapiens//Canis familiaris	Mature 3'
CTTATGCAAGATTCCCTTCTA	mir-491	Canis familiaris	Mature 3'
CAGCAGCACACTGTGGTTTGT	mir-497	Homo sapiens//Canis familiaris	Mature 5'
ATGCACCTGGGCAAGGATTCT	mir-500	Canis familiaris	Mature 3'
CACGCTCATGCACACACCCACA	mir-574	Homo sapiens//Canis familiaris	Mature 3'
ATTGTGTCAATATGCGATGATGT	mir-592	Canis familiaris	Mature 5'
TCTAGTAAGAGTGGCAGTCGA	mir-628	Homo sapiens	Mature 3'
ATCATGTATGATACTGCAAACA	mir-6516	Homo sapiens	Mature 3'
AATATTATACAGTCAACCTCT	mir-656	Homo sapiens	Mature 3'
ACCAGGAGGCTGAGGCCCCT	mir-665	Homo sapiens	Mature 3'
CTCTTCAATCTCAGGACTCGC	mir-676	Canis familiaris	Mature 5'
GTAGAGGAGATGGCGCAGGG	mir-877	Homo sapiens	Mature 5'
GGTCGGATTCCGTGCCTGGAGT	mir-8859b	Canis familiaris	Mature 3'
TTAATATCGGACAACCATTGT	mir-889	Homo sapiens	Mature 3'
CAAGCTCGCTTCTATGGGTCTG	mir-99a	Homo sapiens	Mature 3'



Appendix 12 Hypothetical expression pattern of exosomal miRNAs according to the site of disease. (A) ExomiRs increased at primary site of melanoma. (B) ExomiRs only increased at metastatic site of melanoma. (C) ExomiRs gradually increased at metastatic site of melanoma. X-axes indicates reference, as well as primary and metastatic site of melanoma. Y-axes indicates the relative miRNAs expression levels.



Appendix 13 CT values of Cel-39 and miR-186 among reference, melanoma cell line and patients' plasma exosomes. (a-d) Cel-39 was spiked before exosome RNA isolation. Graph represents the consistent ct values among reference exosomes, KMeC and LMeC cell line derived exosomes (a); and in between the reference exosomes and melanoma patients' plasma exosomes (b). Ct values of miR-186 were also consistent among the reference, KMeC and LMeC exosomes (c); and between the reference and patients' plasma exosomes (d). The Y-axes represent the ct values. n = 6 in reference and cell lines groups whereas, n = 9 in melanoma group. Ex, exosome; KMeC, primary melanoma cell line; LMeC, metastatic melanoma cell line.

References

1. Vlassov A V., Magdaleno S, Setterquist R, Conrad R. Exosomes: Current knowledge of their composition, biological functions, and diagnostic and therapeutic potentials. Biochimica et Biophysica Acta - General Subjects. 2012.
2. Srivastava A, Filant J, Moxley K, Sood A, McMeekin S, Ramesh R. Exosomes: A Role for Naturally Occurring Nanovesicles in Cancer Growth, Diagnosis and Treatment. Current Gene Therapy. 2015;
3. Isola A, Chen S. Exosomes: The Messengers of Health and Disease. Current Neuropharmacology. 2016;
4. Taylor DD, Gercel-Taylor C. The origin, function, and diagnostic potential of RNA within extracellular vesicles present in human biological fluids. Frontiers in Genetics. 2013.
5. Aguilera-Rojas M, Badewien-Rentzsch B, Plendl J, Kohn B, Einspanier R. Exploration of serum- and cell culture-derived exosomes from dogs. BMC Veterinary Research. 2018;
6. Wang Z, Chen JQ, Liu J lu, Tian L. Exosomes in tumor microenvironment: Novel transporters and biomarkers. Journal of Translational Medicine. 2016.
7. Mashouri L, Yousefi H, Aref AR, Ahadi AM, Molaei F, Alahari SK. Exosomes: Composition, biogenesis, and mechanisms in cancer metastasis and drug resistance. Molecular Cancer. 2019;18(1):1–14.
8. Jiao Y, Xu P, Shi H, Chen D, Shi H. Advances on liver cell-derived exosomes in liver diseases. Vol. 25, Journal of Cellular and Molecular Medicine. 2021.
9. Gao PF, Huang D, Wen JY, Liu W, Zhang HW. Advances in the role of exosomal non-coding RNA in the development, diagnosis, and treatment of gastric cancer (Review). Molecular and Clinical Oncology. 2020;13(2):101–8.
10. LU X, ZHANG Y, XIE G, DING Y, CONG H, XUAN S. Exosomal non-coding RNAs: Novel biomarkers with emerging clinical applications in gastric cancer (Review). Vol. 22, Molecular Medicine Reports. 2020.
11. Javadi M, Rad JS, Farashah MSG, Roshangar L. An Insight on the Role of Altered Function and Expression of Exosomes and MicroRNAs in Female Reproductive Diseases. Reproductive Sciences. 2021.
12. Kok VC, Yu CC. Cancer-derived exosomes: Their role in cancer biology and biomarker development. International Journal of Nanomedicine. 2020;15:8019–36.
13. O'Brien J, Hayder H, Zayed Y, Peng C. Overview of microRNA biogenesis, mechanisms of actions, and circulation. Frontiers in Endocrinology. 2018.
14. Liu SL, Sun P, Li Y, Liu SS, Lu Y. Exosomes as critical mediators of cell-to-cell communication in cancer pathogenesis and their potential clinical application. Translational Cancer Research. 2019.
15. Desmond BJ, Dennett ER, Danielson KM. Circulating extracellular vesicle microRNA as diagnostic biomarkers in early colorectal cancer—A review. Cancers. 2020.

16. Kumata Y, Iinuma H, Suzuki Y, Tsukahara D, Midorikawa H, Igarashi Y, et al. Exosome-encapsulated microRNA-23b as a minimally invasive liquid biomarker for the prediction of recurrence and prognosis of gastric cancer patients in each tumor stage. Oncology Reports. 2018;
17. Husna AA, Rahman MM, Lai Y, Chen H, Hasan MdN, Nakagawa T, et al. Identification of melanoma-specific exosomal miRNAs as the potential biomarker for canine oral melanoma. Pigment Cell & Melanoma Research. 2021 Jul 17;
18. Zimta AA, Sigurjonsson OE, Gulei D, Tomuleasa C. The malignant role of exosomes as nanocarriers of rare rna species. International Journal of Molecular Sciences. 2020;21(16).
19. Rahman MM, Lai YC, Husna AA, Chen HW, Tanaka Y, Kawaguchi H, et al. Aberrantly expressed snoRNA, snRNA, piRNA and tRFs in canine melanoma. Veterinary and Comparative Oncology. 2019;18(3):353–61.
20. Yi Q, Zou WJ. A novel four-snoRNA signature for predicting the survival of patients with uveal melanoma. Molecular Medicine Reports. 2019;19(2).
21. Atherton MJ, Morris JS, McDermott MR, Lichty BD. Cancer immunology and canine malignant melanoma: A comparative review. Veterinary Immunology and Immunopathology. 2016;169:15–26.
22. Sharma P, Diergaarde B, Ferrone S, Kirkwood JM, Whiteside TL. Melanoma cell-derived exosomes in plasma of melanoma patients suppress functions of immune effector cells. Scientific Reports. 2020;10(1):92.
23. Guy GP, Thomas CC, Thompson T, Watson M, Massetti GM, Richardson LC. Vital signs: Melanoma incidence and mortality trends and projections — United States, 1982–2030. Morbidity and Mortality Weekly Report. 2015;64(21):591–6.
24. Rahman MM, Lai YC, Husna AA, Chen HW, Tanaka Y, Kawaguchi H, et al. Transcriptome analysis of dog oral melanoma and its oncogenic analogy with human melanoma. Oncology Reports. 2020;43(1).
25. Smith SH, Goldschmidt MH, Mcmanus PM. A Comparative Review of Melanocytic Neoplasms. Vol. 39, Veterinary Pathology. 2002.
26. Bergman PJ. Canine Oral Melanoma. Clinical Techniques in Small Animal Practice. 2007;
27. Miller AJ, Mihm MC. Melanoma. New England Journal of Medicine. 2006.
28. Rahman MM, Lai YC, Husna AA, Chen HW, Tanaka Y, Kawaguchi H, et al. Micro RNA transcriptome profile in canine oral melanoma. International Journal of Molecular Sciences. 2019;20(19).
29. Bhome R, Del Vecchio F, Lee GH, Bullock MD, Primrose JN, Sayan AE, et al. Exosomal microRNAs (exomiRs): Small molecules with a big role in cancer. Cancer Letters. 2018.
30. Xiao D, Ohlendorf J, Chen Y, Taylor DD, Rai SN, Waigel S, et al. Identifying mRNA, MicroRNA and Protein Profiles of Melanoma Exosomes. PLoS ONE. 2012;7(10):e46874.
31. la Shu S, Yang Y, Allen CL, Maguire O, Minderman H, Sen A, et al. Metabolic reprogramming of stromal fibroblasts by melanoma exosome microRNA favours a pre-metastatic microenvironment. Scientific Reports. 2018;8(1):12905.

32. Mione M, Bosserhoff A. MicroRNAs in melanocyte and melanoma biology. Pigment Cell and Melanoma Research. 2015;
33. Wozniak M, Sztiller-Sikorska M, Czyz M. Expression of miRNAs as important element of melanoma cell plasticity in response to microenvironmental stimuli. Anticancer Research. 2015;
34. Wozniak M, Mielczarek A, Czyz M. miRNAs in Melanoma: Tumor Suppressors and Oncogenes with Prognostic Potential. Current Medicinal Chemistry. 2016;
35. Rahman MM, Lai YCYC, Husna AAAA, Chen HWHW, Tanaka Y, Kawaguchi H, et al. Transcriptome analysis of dog oral melanoma and its oncogenic analogy with human melanoma. Oncology Reports. 2020;43(1):16–30.
36. Chen B, Liu S, Wang H, Li G, Lu X, Xu H. Differential expression profiles and function prediction of transfer RNA-derived fragments in high-grade serous ovarian cancer. BioMed Research International. 2021;2021.
37. Xu R, Rai A, Chen M, Suwakulsiri W, Greening DW, Simpson RJ. Extracellular vesicles in cancer — implications for future improvements in cancer care. Nature Reviews Clinical Oncology. 2018;15(10):617–38.
38. Rahman MM, Lai YCYC, Husna AAAA, Chen HWHW, Tanaka Y, Kawaguchi, Hiroaki Kawaguchi, H., Miyoshi, N., Nakagawa, T., Fukushima, R., Miura N, et al. Micro RNA transcriptome profile in canine oral melanoma. International Journal of Molecular Sciences. 2019;20(19):4832.
39. Murillo OD, Thistlethwaite W, Rozowsky J, Subramanian SL, Lucero R, Shah N, et al. exRNA Atlas Analysis Reveals Distinct Extracellular RNA Cargo Types and Their Carriers Present across Human Biofluids. Cell. 2019;177(2):463–77.
40. Rappa G, Mercapide J, Anzanello F, Pope RM, Lorico A. Biochemical and biological characterization of exosomes containing prominin-1/CD133. Molecular Cancer. 2013;12:62.
41. Ushio N, Rahman MM, Maemura T, Lai YC, Iwanaga T, Kawaguchi H, et al. Identification of dysregulated microRNAs in canine malignant melanoma. Oncology Letters. 2019;17(1):1080–8.
42. Wang W, Wu L rong, Li C, Zhou X, Liu P, Jia X, et al. Five serum microRNAs for detection and predicting of ovarian cancer. European Journal of Obstetrics and Gynecology and Reproductive Biology: X. 2019;3:100017.
43. Ng EKO, Li R, Shin VY, Siu JM, Ma ESK, Kwong A. MicroRNA-143 is downregulated in breast cancer and regulates DNA methyltransferases 3A in breast cancer cells. Tumor Biology. 2014;35(3):2591–8.
44. Donnarumma E, Fiore D, Nappa M, Roscigno G, Adamo A, Iaboni M, et al. Cancer-associated fibroblasts release exosomal microRNAs that dictate an aggressive phenotype in breast cancer. Oncotarget. 2017;8(12):19592–608.
45. Jusufović E, Rijavec M, Keser D, Korošec P, Sodja E, Iljazović E, et al. Let-7b and miR-126 Are Down-Regulated in Tumor Tissue and Correlate with Microvessel Density and Survival Outcomes in Non-Small-Cell Lung Cancer. PLoS ONE. 2012;7(9):e45577.
46. Min L, Zhu S, Chen L, Liu X, Wei R, Zhao L, et al. Evaluation of circulating small extracellular vesicles derived miRNAs as biomarkers of early colon cancer: a

comparison with plasma total miRNAs. Journal of Extracellular Vesicles. 2019;8(1):1643670.
47. Mueller DW, Bosserhoff AK. Role of miRNAs in the progression of malignant melanoma. British Journal of Cancer. 2009;101(4):551–6.
48. Ohshima K, Inoue K, Fujiwara A, Hatakeyama K, Kanto K, Watanabe Y, et al. Let-7 microRNA family Is selectively secreted into the extracellular environment via exosomes in a metastatic gastric cancer cell line. PLoS ONE. 2010;5(10):13247.
49. Mirzaei H, Gholamin S, Shahidsales S, Sahebkar A, Jafaari MR, Mirzaei HR, et al. MicroRNAs as potential diagnostic and prognostic biomarkers in melanoma. European Journal of Cancer. 2016;53:25–32.
50. Felicetti F, de Feo A, Coscia C, Puglisi R, Pedini F, Pasquini L, et al. Exosome-mediated transfer of miR-222 is sufficient to increase tumor malignancy in melanoma. Journal of Translational Medicine. 2016;14:56.
51. Liu Y, Wang Y, Xu Q, Zhou X, Qin Z, Chen C, et al. Prognostic evaluation of microRNA-210 in various carcinomas. Medicine (United States). 2017;96(43):e8113.
52. Wang X, Wang T, Chen C, Wu Z, Bai P, Li S, et al. Serum exosomal miR-210 as a potential biomarker for clear cell renal cell carcinoma. Journal of Cellular Biochemistry. 2019;120(2):1492–502.
53. Chen M, Xu R, Rai A, Suwakulsiri W, Izumikawa K, Ishikawa H, et al. Distinct shed microvesicle and exosome microRNA signatures reveal diagnostic markers for colorectal cancer. PLoS ONE. 2019;14(1):e0210003.
54. Qin X, Guo H, Wang X, Zhu X, Yan M, Wang X, et al. Exosomal miR-196a derived from cancer-associated fibroblasts confers cisplatin resistance in head and neck cancer through targeting CDKN1B and ING5. Genome Biology. 2019;20(1):12.
55. Staton CA, Shaw LA, Valluru M, Hoh L, Koay I, Cross SS, et al. Expression of class 3 semaphorins and their receptors in human breast neoplasia. Histopathology. 2011;59(2):274–82.
56. Inoue K, Ohashi E, Kadosawa T, Hong SH, Matsunaga S, Mochizuki M, et al. Establishment and characterization of four canine melanoma cell lines. Journal of Veterinary Medical Science. 2004;66(11):1437–40.
57. Dsouza VL, Adiga D, Sriharikrishnaa S, Suresh PS, Chatterjee A, Kabekkodu SP. Small nucleolar RNA and its potential role in breast cancer – A comprehensive review. Vol. 1875, Biochimica et Biophysica Acta - Reviews on Cancer. 2021.
58. Zhu P, Yu J, Zhou P. Role of tRNA-derived fragments in cancer: novel diagnostic and therapeutic targets tRFs in cancer. Am J Cancer Res. 2020;10(2).
59. Yu M, Lu B, Zhang J, Ding J, Liu P, Lu Y. TRNA-derived RNA fragments in cancer: Current status and future perspectives. Vol. 13, Journal of Hematology and Oncology. 2020.
60. Zhang Y, Cai F, Liu J, Chang H, Liu L, Yang A, et al. Transfer RNA-derived fragments as potential exosome tRNA-derived fragment biomarkers for osteoporosis. International Journal of Rheumatic Diseases. 2018;21(9).
61. Zhu L, Li J, Gong Y, Wu Q, Tan S, Sun D, et al. Exosomal tRNA-derived small RNA as a promising biomarker for cancer diagnosis. Molecular Cancer. 2019;18(1).

62. Grafanaki K, Anastasakis D, Kyriakopoulos G, Skeparnias I, Georgiou S, Stathopoulos C. Translation regulation in skin cancer from a tRNA point of view. Vol. 11, Epigenomics. 2019.
63. Zhang PF, Wu J, Wu Y, Huang W, Liu M, Dong ZR, et al. The lncRNA SCARNA2 mediates colorectal cancer chemoresistance through a conserved microRNA-342-3p target sequence. Journal of Cellular Physiology. 2019;234(7).
64. Cao T, Rajasingh S, Samanta S, Dawn B, Bittel DC, Rajasingh J. Biology and clinical relevance of noncoding sno/scaRNAs. Vol. 28, Trends in Cardiovascular Medicine. 2018.
65. Ronchetti D, Todoerti K, Tuana G, Agnelli L, Mosca L, Lionetti M, et al. The expression pattern of small nucleolar and small Cajal body-specific RNAs characterizes distinct molecular subtypes of multiple myeloma. Blood Cancer Journal. 2012;2(11).
66. Lunavat TR, Cheng L, Kim DK, Bhadury J, Jang SC, Lässer C, et al. Small RNA deep sequencing discriminates subsets of extracellular vesicles released by melanoma cells – Evidence of unique microRNA cargos. RNA Biology. 2015;
67. Gong J, Li Y, Liu C jie, Xiang Y, Li C, Ye Y, et al. A Pan-cancer Analysis of the Expression and Clinical Relevance of Small Nucleolar RNAs in Human Cancer. Cell Reports. 2017;21(7).
68. Howe KL, Achuthan P, Allen J, Allen J, Alvarez-Jarreta J, Ridwan Amode M, et al. Ensembl 2021. Nucleic Acids Research. 2021;49(D1).