Identification of dysregulated non-coding small RNA in canine tumor

(犬の腫瘍における発現異常ノンコーディングスモール RNA の同定)

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Abstract

In recent years, not only mRNA (messenger RNA) but also other small non-coding RNA have focused on molecular diagnosis and therapy in oncology fields. Especially in human medicine, many studies elucidate the ability and function of many microRNAs, which are small non-coding RNAs. However, there are still not many studies in the veterinary field. In my PhD study, I focused on the non-coding small RNA in canine oncology fields.

In the first chapter, I studied the dysregulated micro RNA in canine oral melanoma. At first, I performed the microarray-based miRNA profiling of canine malignant melanoma (CMM) tissue obtained from the oral cavity. Then, I also confirmed the differentially expressed microRNA by quantitative reverse transcription-PCR (qRT-PCR). An analysis of the microarray data revealed 17 dysregulated miRNAs; 5 were up-regulated, and 12 were down-regulated. qRT-PCR analysis was performed for 2 up-regulated (miR-204 and miR-383), 3 down-regulated (miR-122, miR-143, and miR-205) and 6 additional oncogenic miRNAs (oncomiRs; miR-16, miR-21, miR-29b, miR-92a, miR-125b and miR-222). The expression levels of seven of the miRNAs, miR-16, miR-21, miR-29b, miR-122, miR-125b, miR-204, and miR-383 were significantly up-regulated, while the expression of miR-205 was down-

regulated in CMM tissues compared with normal oral tissues. The microarray and qRT-PCR analyses validated the up-regulation of two potential oncomiRs, miR-204 and miR-383. I also constructed a protein interaction network and a miRNA–target regulatory interaction network using STRING and Cytoscape. In the proposed network, *CDK2* was a target for miR-383, *SIRT1* and *TP53* were targets for miR-204, and *ATR* was a target for both. The miR-383 and miR-204 were potential oncomiRs that may be involved in regulating melanoma development by evading DNA repair and apoptosis.

In my second chapter, I focused on non-coding RNA other than microRNA, and I compared canine hepatocellular carcinomas (HCC) and hepatocellular adenomas (HCA). I elucidated the differential expression of Y RNA-derived fragments because Y RNA-derived fragments have yet to be investigated in canine HCC and HCA. I used qRT-PCR to determine Y RNA expression in clinical tissues, plasma, and plasma extracellular vesicles, and two HCC cell lines (95-1044 and AZACH). Y RNA was significantly decreased in tissue, plasma, and plasma extracellular vesicles for canine HCC versus canine HCA and healthy controls. Y RNA was decreased in 95-1044 and AZACH cells versus normal liver tissue and

in AZACH versus 95-1044 cells. In plasma samples, Y RNA levels were decreased in HCC versus HCA and Healthy controls and increased in HCA versus Healthy controls. Receiver operating characteristic analysis showed that Y RNA could be a promising biomarker for distinguishing HCC from HCA and healthy controls. Overall, the dysregulated expression of Y RNA can distinguish canine HCC from HCA. However, further research is necessary to elucidate the underlying Y RNA-related molecular mechanisms in hepatocellular neoplastic diseases. To the best of my knowledge, this is the first report on the relative expression of Y RNA in canine HCC and HCA.

In conclusion, I have demonstrated the up-regulation of potential oncomiRs, miR-16, miR-21, miR-29b, miR-122, miR-125b, miR-204 and miR-383 in CMM tissues. In particular, the strong up-regulation of miR-383 in CMM tissues compared with normal oral tissues identified by microarray screening was confirmed by qRT-PCR. I conclude that miR-383 and miR-204 may promote melanoma development by regulating the DNA repair/checkpoint and apoptosis. Then, I also demonstrated the Y RNA dysregulation in the cHCC. Especially to my knowledge, this is the first report on Y RNA in canine tumors. Interestingly, this ncRNA has distinctive characteristics and differentiates malignant tumors (HCC) from benign

tumors (HCA). The expression pattern of Y RNA is consistent across clinical samples and cell lines. Thus, Y RNA has promising potential for differentiating HCC from HCA. Further research is required to fully elucidate the role of Y RNA in the development and progression of canine HCC and HCA.

General Introduction

In recent years, not only mRNA (messenger RNA) but also other small non-coding RNA have focused on molecular diagnosis and therapy in oncology fields. Especially in human medicine, many studies elucidate the ability and function of many microRNAs, which are small non-coding RNAs. However, there are still not many studies in the veterinary field. In my PhD study, I focused on the non-coding small RNA in canine oncology fields. In the first chapter, I focused on canine oral melanoma, and I also focused on canine hepatocellular carcinoma in the second chapter of my PhD study. Both tumors have a relatively high incidence in dog patients and are highly malignancy, which makes them difficult for treatment.

In addition to the different tumors of the experimental target, I used two current technologies for transcriptome analysis, one of microarray and the other of next-generation sequence, to identify the unique target of interest. Both methods successfully identify the new target in the veterinary oncology field. Then, I used qPCR (real-time PCR) technology to validate the expression of target RNA species further. The relatively new technology introduced to the veterinary oncology field is crucial and valuable, and I can strongly emphasize it because of the successful identification of attractive

new targets.

As we consider the transcriptome, mRNA has been mainly focused on dysregulation because mRNA translates to a protein that is a minimum functional unit for our biofunction. However, in the last two decades, non-coding RNA (ncRNA) species other than mRNA have been investigated. ncRNA categories encompass diverse transcripts, including miRNAs, long non-coding RNAs, and other RNA-like snoRNA, snRNA, tRNA fragments, and Y RNA fragments. In human medicine, the number of studies investigating these ncRNA is dramatically increasing. In contrast, there is still a need to condense investigation about ncRNA in veterinary oncology.

I first focus on the microRNAs (miRNAs), which are non-coding small RNAs that post-transcriptionally regulate the expression of target genes (mRNA) by binding to the 3′-untranslated regions of mRNAs, causing destabilization, degradation, or translation inhibition [1]. Because dysregulation of miRNA expression has been identified in several cancers, some miRNAs are categorized as oncogenic miRNAs or 'oncomiRs,' a term used to describe either tumor suppressors or oncogenes [2-5]. In addition, miRNAs have been investigated as potential therapeutic targets for

several malignant cancers as well. As such, I focused on microRNA dysregulation in canine malignant melanoma first.

Studies on miRNA involvement in HCC growth have been the subject of in-depth research in dogs by Dr. Miura's laboratory. However, no reports on ncRNAs in canine liver tumors have addressed other ncRNA species. As such, my second study intensively focused on ncRNA rather than miRNA.

Chapter 1

Identification of dysregulated microRNAs in canine malignant melanoma

1. Abstract

Inhibiting aberrantly up-regulated microRNAs (miRNAs) has become a new focus for therapeutic intervention in human melanoma. Thus, identifying up-regulated miRNAs is essential for obtaining additional melanoma-related therapeutic targets. Here, microarray-based miRNA profiling of canine malignant melanoma (CMM) tissue obtained from the oral cavity was performed and differential expression was confirmed by quantitative reverse transcription-PCR (qRT-PCR). An analysis of the microarray data revealed 17 dysregulated miRNAs; 5 were up-regulated and 12 were down-regulated. qRT-PCR analysis was performed for 2 up-regulated (miR-204 and miR-383), 3 down-regulated (miR-122, miR-143, and miR-205) and 6 additional oncogenic miRNAs (oncomiRs; miR-16, miR-21, miR-29b, miR-92a, miR-125b and miR-222). The expression levels of seven of the miRNAs, miR-16, miR-21, miR-29b, miR-122, miR-125b, miR-204 and miR-383 were significantly up-regulated while, the expression of miR-205 was down-regulated in CMM tissues compared with normal oral tissues. The microarray and qRT-PCR analyses validated the up-regulation of two potential oncomiRs miR-204 and miR-383.I also constructed a protein interaction network and a miRNA–target regulatory interaction network using STRING and Cytoscape. In the

proposed network, *CDK2* was a target for miR-383, *SIRT1* and *TP53* were targets for miR-204 and *ATR* was a target for both. I concluded that, miR-383 and miR-204 were potential oncomiRs that may be involved in regulating melanoma development by evading DNA repair and apoptosis.

2. Introduction

MicroRNAs (miRNAs) are small endogenous non-coding RNAs that post-transcriptionally regulate the expression of target genes by binding to the 3′-untranslated regions of mRNAs, causing destabilization, degradation, or translation inhibition [1]. Because dysregulation of miRNA expression has been identified in a number of cancers, some miRNAs are categorized as oncogenic miRNAs or 'oncomiRs', a term used to describe either tumor suppressors or oncogenes [2-5]. Consequently, miRNAs have been investigated as potential therapeutic targets for several malignant cancers including melanoma [6-7]. The tumor burden in mice with liver melanoma metastasis was found to be reduced by anti-miR-182 oligonucleotides that inhibited the up-regulated miR-182 in the tumor cells [6]. Inhibition of miR-383 over-expression suppressed the proliferation, cell cycle progression and invasion of human epithelial ovarian cancer (EOC) and immortal EOC cell lines [8]. Over-expression of miR-203 sensitized malignant melanoma cells to temozolomide drug by targeting glutaminase, which opened new opportunities for chemotherapy-resistant malignant melanoma patients [9]. Thus, profiling dysregulated miRNA expression in cancers is an important approach for detecting potential therapeutic targets.

Simpson et al. 2013 [10] suggested significant overlapping may exist in the clinical and histopathological features of canine and human mucosal melanomas. MiRNA expression has been investigated in different canine tumors, including B and T-cell lymphoma [11], lymphocytic leukemia [12], transitional cell carcinoma [13], mammary cancer [14], prostate cancer [15] and melanoma [16-18].These studies indicated that the expression patterns of specific miRNAs in specific cancers were similar to those in corresponding human cancers. For example, the up-regulation of miR-21 and miR-29b in canine mammary cancer is consistent with their up-regulation in human breast cancer [14,19-20] and melanoma [21-22] and miR-145, miR-203, and miR-205 were found to be down-regulated in both canine malignant melanoma (CMM) and human malignant melanoma (HMM) [16-17]. In the Noguchi et al. [17] studies of HMM, a total of seven down-regulated miRNAs were detected by microarray analysis; three of them were confirmed by quantitative reverse transcription PCR (qRT-PCR). In almost all HMM tumors that have been studied, up-regulated miRNA expression has been reported, including the miR-17-92 cluster, miR-222/221, miR-21 and miR-155 [23].Therefore, it is likely that some miRNAs will be up-regulated in oral CMM, similar to what Starkey et al. [18] reported in canine uveal melanoma.

However, until now, no up-regulated miRNAs in oral CMM have been reported. To investigate this hypothesis, I examined the expression of miRNAs in CMM tissues obtained from the oral cavity using microarray and qRT-PCR analyses. Here I report the up-regulation of seven miRNAs in CMM tissues. To understand the biological relevance of miRNAs it is necessary to identify the target genes with which they interact. Protein–protein interactions are essential for cells to maintain systemic biological functions such as replication of DNA, transcription, translation and signal transduction [24]. Dysregulation of proteins may collapse the homeostasis process leading to complex diseases and miRNAs may act as master regulators by maintaining the stability of protein–protein interaction networks [25]. So, determining the interactions between the proteins encoded by targets of dysregulated miRNAs and other proteins is very important. In this study, I drew a miRNA–target regulatory interaction network with tumor suppressor genes, which revealed miR-383 and miR-204 may play roles in the development of melanoma by avoiding DNA repair and apoptosis.

3. Ethics statement

Informed consent to use the specimens in this study was obtained from the dog patient's owners. This study was approved by the Kagoshima University's Laboratory Animal Committee (A10031).

4. Materials and Methods

Sample collection.

The CMM tissues used in this study were obtained from dogs (n=10) that had undergone biopsy or surgical resection for diagnosis or treatment at the Veterinary Teaching Hospital, Kagoshima University, Japan. All melanoma samples were obtained from the oral cavity and were histopathologically diagnosed by two pathologists. Normal oral tissues were obtained from healthy laboratory beagle dogs (n=12). In addition to the CMM and normal oral tissues, I obtained a total of 21 canine tumors and normal tissues to use as microarray reference samples as follows: mammary tubulopapillary carcinoma (n=4), mammary benign mixed tumor (n=4), hepatic cell carcinoma (n=1), squamous cell carcinoma (n=1), lymphoma (n=1), adenosquamous carcinoma (n=1), mast cell tumor (n=1), malignant peripheral nerve sheath tumor (n=1), normal mammary gland tissue (n=4) and normal hepatic tissue (n=3).

Isolation of total RNA.

All the tissues were preserved in RNA*later* (Thermo Fisher Scientific Inc. Waltham, MA, USA) immediately after biopsy or surgical resection until used

for RNA isolation. Total RNA was isolated from the stored tissues using a mirVana™ miRNA Isolation Kit (Thermo Fisher Scientific Inc.) according to the manufacturer's instructions. RNA quantity was measured using either an ND-1000 spectrophotometer (Thermo Fisher Scientific Inc.) or a NanoPhotometer™ Pearl (Implen GmbH. München, Germany). RNA quality was verified using an Agilent 2100 Bioanalyzer (Agilent Technologies. Santa Clara, CA, USA) and RNA integrity numbers were determined [26].

Microarray analysis.

Three assays were performed (n=3) using the miRCURY™ LNA microRNA Array, version 11.0 (ExiqonInc.,Woburn, MA, USA). In each assay, Hy3 labeled miRNAs from different CMM tissues but the same references Hy5 labeled miRNAs were used. The reference miRNAs comprised equal amounts of RNA from 21 reference samples from 10 different tissues (listed in the *Sample collection* section), all of which were pooled. Two-color miRNA-microarrays with 264 identical canine miRNA probes were used. Signal extraction was performed using Feature Extraction 10.7.3.1 software (Agilent Technologies). To minimize error, each miRNA was spotted at four different locations on the array and the average signal intensity value of the four spots

was used and variable coefficients were calculated (standard deviation (SD) of signal intensity of four spots/average values). MiRNAs with signal intensity variable coefficients >0.5 or with low signal intensity (<100) in both the CMM and reference tissues were excluded from further analysis. The average values of the Hy3/Hy5 (fold change; FC) ratio between the CMM and reference tissues were compared using the Lowess normalization method [27]. MiRNAs that had FC ratios>2.0 or <0.5 were considered to be dysregulated.

qRT-PCR assays.

CMM tissues (n=10) and normal oral tissues (n=12) were used in the qRT-PCRs, which were performed in duplicate using TaqMan microRNA Assays (Thermo Fisher Scientific Inc.; see Table I for assay details) with 2 ng/μl total RNA, according to the optimal reagent concentrations and reaction conditions described in the manufacturer's instructions. The canine miRNA sequences used for the PCRs were identical to the corresponding human miRNA sequences (Table I).The qRT-PCRs were carried out using an Applied Biosystems 7300 Real-Time PCR System (Thermo Fisher Scientific Inc.). RNU6B, U6 small nuclear RNA, was used as a quantitative normalization control [13, 14]. Relative expression levels were calculated using the

comparative delta Cq method ($2^{-\Delta\Delta Cq}$) [28]. Cq values >36.0 were considered as absence of miRNA expression. The relative expression levels of miRNAs in the CMM tissues were calculated relative to the average values in the normal oral tissues, which were assigned a value of1.0.

Statistics.

In the microarray experiments, *P* values and false discovery rates (FDRs) were analyzed using Welch's test and the Benjamini-Hochberg correction for multiple hypotheses testing using R software [29]. For the qRT-PCRs, the miRNA expression levels between CMM and normal oral tissues were analyzed using the Mann Whitney U-test. Statistical analyses were performed with JMP 10.0 (SAS Institute). *P* values <0.05 were considered significant.

Network construction.

MiRNA targets were predicted using TargetScan 7.1 [30] and 1021 human tumor suppressor genes (with basic annotations) from the Tumor Suppressor Gene Database (TSGene; https://bioinfo.uth.edu/TSGene/). A miRNA–target interaction network was drawn using Cytoscape v3.5

(http://www.cytoscape.org/) [31] and a protein-protein interaction network of tumor suppressor genes was constructed using STRING (confidence score 0.9) (http://string-db.org/) [32]. The two networks were merged within Cytoscape and interconnected nodes were separated to obtain a co-ordinate network. Analysis of basic network parameters (degree, betweenness, centroid value and Eigenvector) was done using Centiscape 2.2.[33]. In the network, a node represents a protein (encoded by a target mRNA) or a miRNA and a line represents an interaction between a protein and a miRNA.

5. Results

Screening of differentially expressed miRNAs by microarray analysis.

The microarray analysis revealed 17 dysregulated miRNAs in the CMM tissues based on the FC ratios (Table II). Of the 17 miRNAs, 5 were up-regulated (FC ratios >2.0) with no significant FDRs and 12 were down-regulated (FC ratios<0.5) and 4 of them had significant FDRs (P<0.05) (Table II).

Confirmation of differentially expressed miRNAs by qRT-PCR.

qRT-PCRs were performed to validate some of the dysregulated miRNAs from the microarray analysis (Table II). Because none of the up-regulated miRNAs had significant FDRs, I selected the two most highly up-regulated miRNAs, miR-204 and miR-383, for validation. From among the down-regulated miRNAs, I selected three miRNAs (miR-122, miR-143 and miR-205) that had the most significant FDRs. I also selected six other miRNAs (miR-16, miR-21, miR-29b, miR-92a, miR-125b and miR-222) for validation because they were reported to be dysregulated in cancers other than CMM [13, 14, 34-36].

I found that seven miRNAs were significantly up-regulated (P values from 0.0001 (miR-21) to 0.025 (miR-29b)), but miR-205 was the only significantly

down-regulated miRNA (P<0.0001) in the CMM tissues compared with normal oral tissues (Fig. 1). No significant differences were detected in the expression of miR-92a, miR-143 and miR-222 between the CMM and normal oral tissues (Fig. 1).

Of the 17 dysregulated miRNAs identified by microarray analysis (Table II), only miR-204, miR-383 and miR-205 were found to be highly differentially expressed by qRT-PCR. The average FCs for miR-204 and miR-383 were 15.3 and 152.7, respectively, but for miR-205 the average FC was 0.01 (Fig. 1).

The relative expression patterns of miR-204, miR-383 and miR-205 were consistent between the qRT-PCR and microarray results, but there were discrepancies for some of the other miRNAs. For example, miR-122 was down-regulated (FC <0.5) in the microarray analysis but significantly up-regulated in the qRT-PCR analysis and miR-143 was down-regulated (FC of 0.244) in the microarray analysis but was not found to be significantly differentially expressed by qRT-PCR (Fig. 1).

MiRNA–target regulatory interaction network.

In the STRING protein interaction network, I found that miR-383 and miR-204 interacted with several common genes (proteins) as was reported

previously (Fig. 2A) [37, 38]. When I separated the connected network and calculated the basic parameters (degree, betweenness, centroid value and eigenvector) by Centiscape 2.2 through Cytoscape (Fig. 2B), I found all the basic parameters of TP53 (Fig. 3A) had higher value than any of the others. Further, the basic parameters of miR-383, miR-204, SIRT1, CDK2 and ATR (Fig. 3B–F) were higher than the average values, implying these miRNAs and proteins were the hub nodes of this biological network. In the separated miRNA–target interaction network I found that ATR and CDK2 were targets of miR-383 and miR-204 (Fig. 2B). Moreover, miR-204 could regulate the network through TP53 mediated by SIRT1. RBBP7, SMARCB1, and CREBBP were also connected with several nodes and may be related to the regulation of a small cluster network.

6. Discussion

Some of the dysregulated miRNAs identified in the CMM tissues by microarray analysis were validated by qRT-PCR. The up-regulation of seven miRNAs in CMM, namely miR-16, miR-21, miR-29b, miR-122, miR-125b, miR-204 and miR-383 was demonstrated here for the first time. In particular, miR-204 and miR-383 showed extra ordinarily high expression levels in the microarray and qRT-PCR analyses.

Down-regulation of miR-145, miR-205 and miR-203 was detected in the microarray analysis, which is consistent with previous studies on CMM [16, 17]. However, I did not detect dysregulation of other miRNAs that have been reported previously to be down-regulated [17]. These inconsistencies might be because different microarray platforms and/or samples were used in the two studies. Noguchi et al. [17] used a CombiMatrix array, whereas I used a miRCURY™ LNA microRNA Array. Thus, there were differences in the miRNAs that were spotted on the arrays. I used CMM tissues from three different dogs and Noguchi et al. [17] used CMM tissue from only one dog. Finally, in the previous study, miRNA expression was compared between CMM tissue and normal oral mucosal tissue [17], whereas I compared CMM tissues with reference miRNAs from several cancers and normal tissues. I used mixed

miRNA reference samples to avoid biases from low signal intensities in the microarray data. Using miRNAs from several different origins means different miRNAs will be included because miRNA expression is highly dependent on the tissue origin and status. My approach should cover a broad range of miRNAs, thus avoiding misleading FC ratios as a result of weak signals [39]. However, because my reference tissues were mostly tumor samples (70.8%), using this kind of miRNA reference samples may have caused miRNAs that are commonly dysregulated in tumors to be overlooked but, importantly, may have revealed miRNAs that are specifically dysregulated in melanoma.

In this study, the microarray and qRT-PCR results were consistent for the relative expressions of miR-204, miR-383 and miR-205. However, the discrepant expressions of miR-122 and miR-143 between the microarray and qRT-PCR results may be explained by differences in the control samples that were used in the two experiments; that is, a mixed sample reference in the microarray analysis and normal oral tissues in the qRT-PCRs. For the same reason, differential expression of miR-16, miR-21, miR-29b and miR-125b was not detected in the microarray analysis but was detected by qRT-PCR. MiR-21 and miR-29b are known to be up-regulated in several tumors; for example, miR-21 in mouse BL/6 melanoma cells [40], miR-29b in human breast cancer

[20] and both miRNAs in canine mammary cancer [14]. These findings indicate that miR-21 and miR-29b are common oncomiRs in several species. Thus, the microarray screening method that I used may have masked the differential expression of these miRNAs because they are not specific to melanoma but commonly shared among several kinds of tumors.

While the significant down-regulation of miR-205 can be explained, up-regulation of miR-204 and miR-383 expression has not been reported in CMM until now. Indeed, miR-204 was reported to be up-regulated in old HMM patients compared with young HMM patients [41]; however, no comparison between melanoma and normal tissue was performed and the target mRNA was not defined. In another study, miR-204 was found to be down-regulated in malignant melanoma compared with benign nevi [42], but the age of the patients was not considered and the comparisons were between malignant melanoma and benign nevi tissues. In prostate cancer and breast cancer studies, miR-204 was reported to be both up- and down-regulated [43-47], maybe because of different experimental designs and individual identity.

TP53 is a well-known tumor suppressor gene located in the center of the network with a high centroid value (Fig. 3A). SIRT1, an indirect regulator of TP53, is a direct target of miR-204 in the network and has been reported to be

down-regulated in canine melanoma [48]. SIRT1 acts as a tumor suppressor via ß-catenin and has reminiscent effects on TP53 in colon cancer [49]. Abnormal expression of ß-catenin was reported in melanoma [50, 51], so the miR-204-mediated down-regulation of SIRT1 revealed in the network may cause ß-catenin-mediated cell survival by evading TP53 in melanoma.

Up-regulation of miR-383 expression has been observed in primary HMM tumor cell lines compared with normal human epidermal melanocytes [52]. In their study, Mueller et al. [52] found that miR-383 was down-regulated in snail stable knockdown melanoma cells by transfection of an antisense snail plasmid construct, named as-snail, compared with the parental melanoma cell line. Snail belongs to the snail superfamily of zinc finger transcription factors and is involved in the development of malignant melanoma through direct repression of E-cadherin expression [53]. Indeed, the transcriptional profile of the as-snail cells was reported to be more similar to normal melanocytes than malignant melanoma cells [52]. However, the detailed biological functions of miR-383 have not been reported so far. In my study, miR-383 was up-regulated in CMM tissues. Liao et al. [54] showed that ATR was the direct target of miR-383 and ATR was found to play a central role in the ATM/ATR pathway involved in DNA damage recognition and initial phosphorylation [55]. Liao et

al. [54] also showed that GADD45γ, MDC1, and H2AX were all negatively correlated with miR-383 expression. Moreover, a recent study showed that loss of function or mutations of ATR lead to the development of melanoma [56]. In testicular embryonal carcinoma miR-383 overexpression was found to reduce CDK2 expression at the protein level, which was also found to be necessary for proper DNA repair [57]. Furthermore, CREB binding protein, a known co-activator of TP53, was found to be a direct target of miR-383 [58]. There is also a possibility that miR-383 has indirect control over apoptosis via TP53 inhibition through CDK2. So, my network analysis and the above discussion suggest that miR-383 may be involved in DNA damage repair and apoptosis phenomena in melanoma. In this study, I demonstrated the dysregulation of 17 miRNAs in CMM and investigated the probable biological functions of these miRNAs based on their target genes. My study is valid not only for dog but also for human because dog has been considered as a good preclinical model for human melanoma [10]. Further studies are required to clarify the functions of the dysregulated miRNAs by for example, detecting the actual target genes and their pathways and analyzing their differential expression patterns in established canine melanoma cell lines [59, 60] to determine the roles of the miRNA–target interactions in CMM tumor genesis and therapy.

7. Conclusion

I have demonstrated the up-regulation of potential oncomiRs, miR-16, miR-21, miR-29b, miR-122, miR-125b, miR-204 and miR-383 in CMM tissues. In particular, the strong up-regulation of miR-383 in CMM tissues compared with normal oral tissues identified by microarray screening was confirmed by qRT-PCR. I conclude that miR-383 and miR-204 may promote melanoma development by regulating both the DNA repair/checkpoint and apoptosis. To identify therapeutic targets in melanoma, further studies are required to verify the biological significance of the miRNA target genes.

8. Figures and Tables

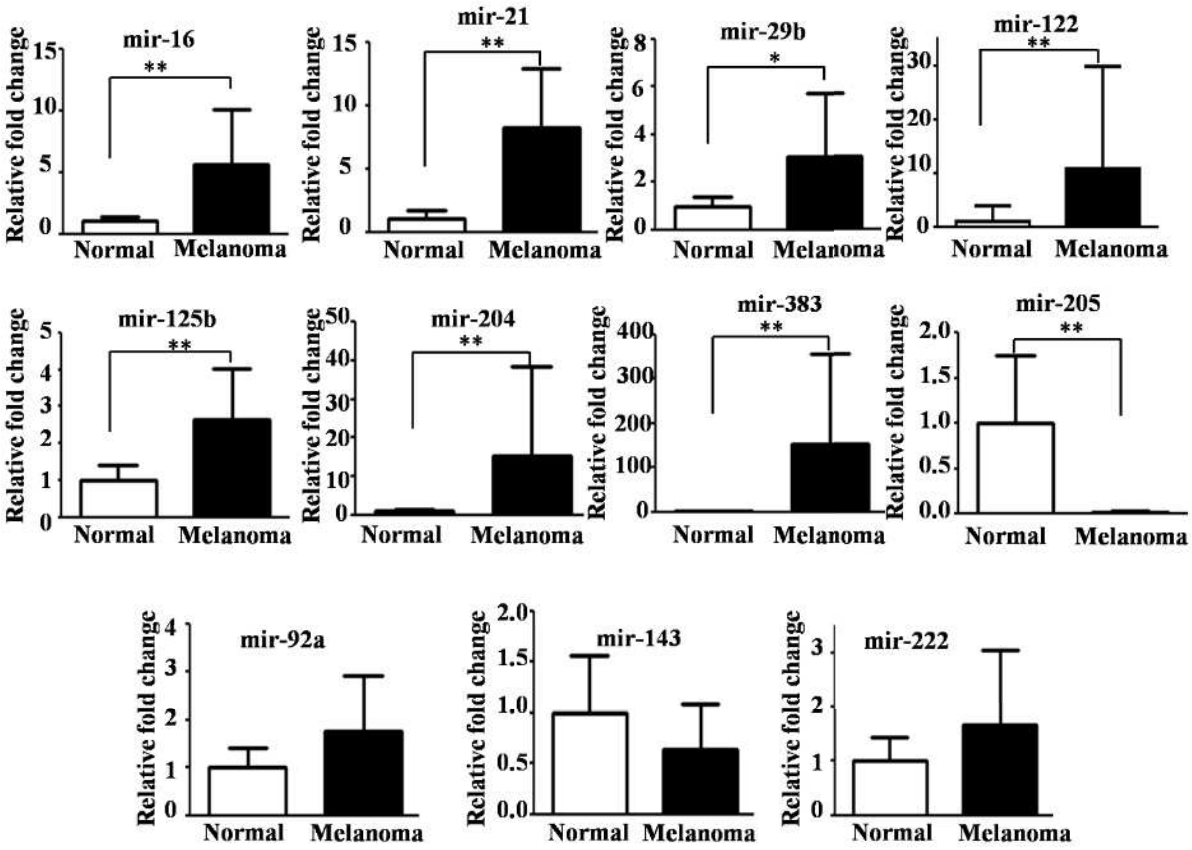

Figure 1. qRT-PCR validation of five dysregulated microRNAs from the microarray assays and six other cancer-related miRNAs. Relative expression levels in CMM tissues (Melanoma) and normal oral tissues (Normal) are shown. The mean expression levels of the Normal samples were set to 1.0. *P* values were determined by the Mann Whitney U-test (**P*<0.05, ***P*<0.01). The bars indicate standard error.

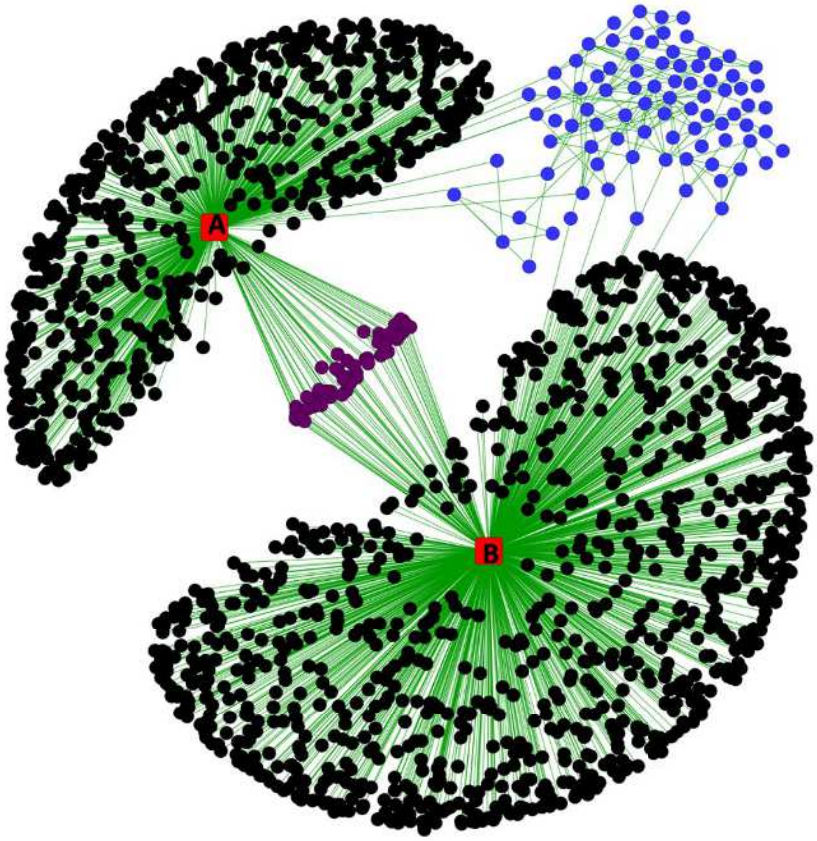

Figure 2-A. MicroRNA–target regulatory interaction network. MiRNA–target regulatory network merged with the tumor suppressor genes protein interaction network. The red squares indicate miRNA nodes (A: miR-383; B: miR-204). Black circles indicate targets (mRNAs) of single miRNAs, purple circles indicate targets shared by miRNAs and blue circles indicate tumor suppressor genes predicted to be targeted by one or both of the miRNA. The edges (lines) connecting two nodes are indicative of regulation (interaction).

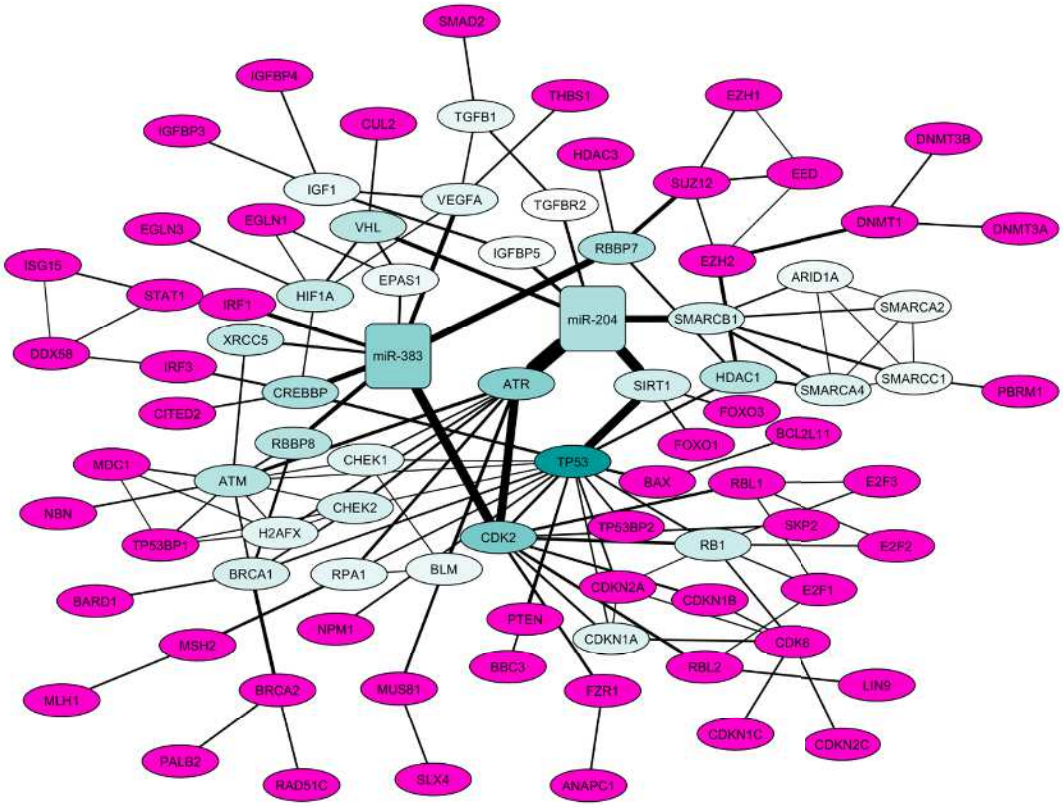

Figure 2-B. MicroRNA–target regulatory interaction network. MiRNA–target. Separated co-ordinate network showing the interactions between microRNAs and tumor suppressor genes. The node colors indicate the centroid value (CV); pink gradient indicates CVs lower than average; blue gradient indicates CVs higher than average. Edge width indicates the betweenness measurement.

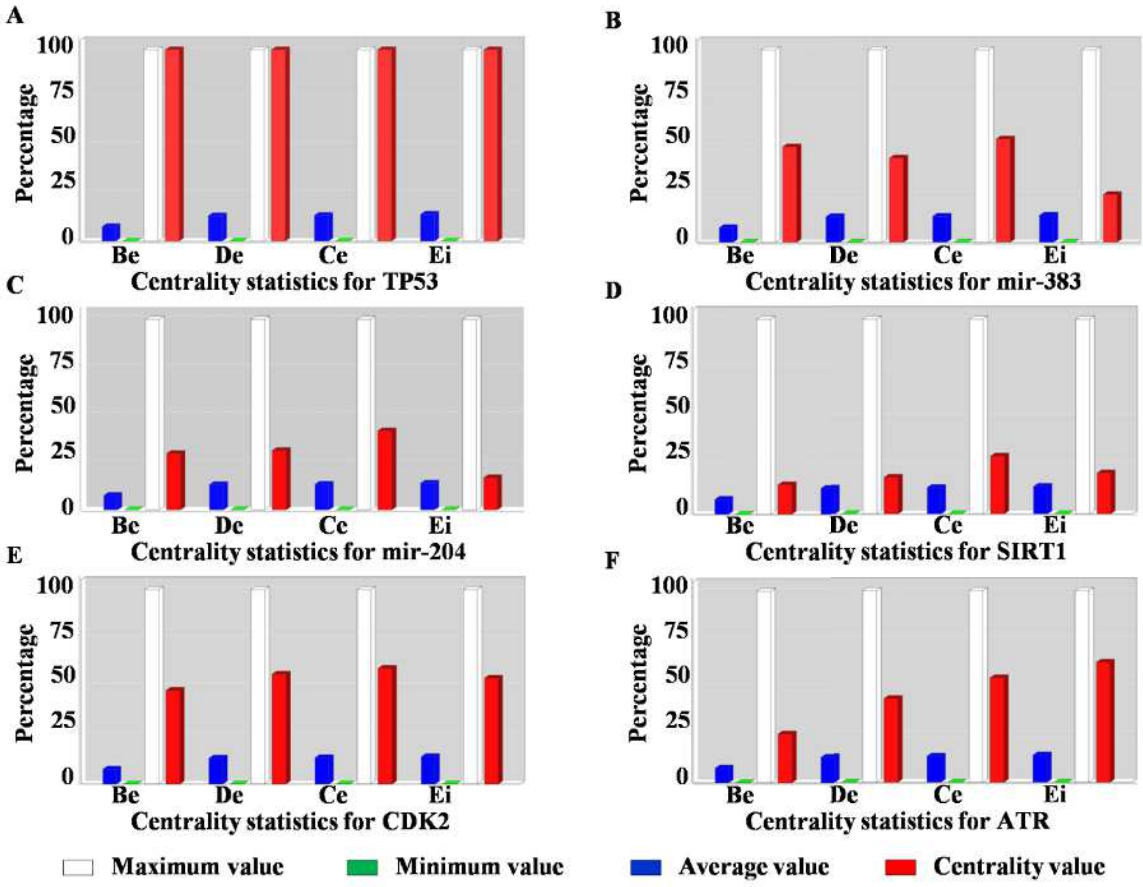

Figure 3. Centrality (Betweenness (Be), degree (De), centroid value (Ce) and eigenvector (Ei)) measures of the hub nodes in the microRNA–target regulatory network.

A). Centrality measures for TP53; B). Centrality measures for miR-383; C.) Centrality measures for miR-204; D). Centrality measures for SIRT1; E.) Centrality measures for CDK2; F.) Centrality measures for ATR.

Table I

MicroRNAs used in the qRT-PCR assays in this study

Assay Name	Assay ID	Mature microRNA Sequence	miRBase Accession Number
hsa-miR-16	000391	UAGCAGCACGUAAAUAUUGGCG	MI0000070
hsa-miR-21	000397	UAGCUUAUCAGACUGAUGUUGA	MI0000077
hsa-miR-29b	000413	UAGCACCAUUUGAAAUCAGUGUU	MI0000105
hsa-miR-92a	000431	UAUUGCACUUGUCCCGGCCUGU	MI0000093
hsa-miR-122	002245	UGGAGUGUGACAAUGGUGUUUG	MI0000442
hsa-miR-125b	000449	UCCCUGAGACCCUAACUUGUGA	MI0000446
hsa-miR-143	002249	UGAGAUGAAGCACUGUAGCUC	MI0000459
hsa-miR-204	000508	UUCCCUUUGUCAUCCUAUGCCU	MI0000284
hsa-miR-205	000509	UCCUUCAUUCCACCGGAGUCUG	MI0000285
hsa-miR-222	002276	AGCUACAUCUGGCUACUGGGU	MI0000299
hsa-miR-383	000573	AGAUCAGAAGGUGAUUGUGGCU	MI0000791

Assay Name	Assay ID	Control Sequence	NCBI Accession Number
RNU6B	001093	CGCAAGGATGACACGCAAATTCGTGAAGCGTTCCATATTTTT	NR_002752

Table II

Dysregulated microRNAs identified in CMM tissues by microarray analysis

Up-regulated (FC > 2.0)			Down-regulated (FC < 0.5)		
microRNA	FC*[1]	FDR*[2]	microRNA	FC	FDR
miR-9	2.420	> 0.05	miR-10	0.486	<0.05
miR-149	2.022	> 0.05	miR-101	0.446	> 0.05
miR-204	2.781	> 0.05	miR-122	0.060	<0.05
miR-326	2.056	> 0.05	miR-142	0.385	> 0.05
miR-383	3.581	> 0.05	miR-143	0.244	<0.05
			miR-195	0.391	> 0.05
			miR-200c	0.382	> 0.05
			miR-205	0.100	<0.05
			miR-328	0.299	> 0.05
			miR-487b	0.430	> 0.05
			miR-652	0.457	> 0.05
			miR-875	0.264	> 0.05

*[1]FC, fold change; *[2]FDR, false discovery ratio

Chapter 2

Novel Y RNA-Derived Fragments Can Differentiate Canine Hepatocellular Carcinoma from Hepatocellular Adenoma

1. Abstract

Hepatocellular carcinomas (HCC) are common tumors, whereas hepatocellular adenomas (HCA) are rare, benign tumors in dogs. The aberrant expression of noncoding RNAs (ncRNAs) plays a pivotal role in HCC tumorigenesis and progression. Among ncRNAs, micro RNAs have been widely researched in human HCC, but much less widely in canine HCC. However, Y RNA-derived fragments have yet to be investigated in canine HCC and HCA. This study targeted canine HCC and HCA patients. I used qRT-PCR to determine Y RNA expression in clinical tissues, plasma, and plasma extracellular vesicles, and two HCC cell lines (95-1044 and AZACH). Y RNA was significantly decreased in tissue, plasma, and plasma extracellular vesicles for canine HCC versus canine HCA and healthy controls. Y RNA was decreased in 95-1044 and AZACH cells versus normal liver tissue and in AZACH versus 95-1044 cells. In plasma samples, Y RNA levels were decreased in HCC versus HCA and Healthy controls and increased in HCA versus Healthy controls. Receiver

operating characteristic analysis showed that Y RNA could be a promising biomarker for distinguishing HCC from HCA and healthy controls. Overall, the dysregulated expression of Y RNA can distinguish canine HCC from HCA. However, further research is necessary to elucidate the underlying Y RNA-related molecular mechanisms in hepatocellular neoplastic diseases. To the best of my knowledge, this is the first report on the relative expression of Y RNA in canine HCC and HCA.

2. Introduction

Hepatocellular adenoma (HCA) and hepatocellular carcinoma (HCC) can occur in both canine and human patients [61]. HCAs are rare, benign tumors that derive from proliferating hepatocytes, whereas HCCs are common, malignant tumors that can develop from HCAs [62]. HCC accounts for 50-59.4% of hepatic tumors in dogs [63]and is the sixth most common cancer in humans worldwide [64]. Canine HCC frequently occurs in patients from the age of ten years and these tumors are mainly common in males [65]. Distinguishing HCA from HCC can be complicated [66,67], but correct tumor identification is crucial because the indicated treatment and prognosis differ between these two tumor types. Therefore, there is a need for a minimally invasive diagnostic technique for differentiating HCA from HCC on a molecular basis for canine patients.

Noncoding RNAs (ncRNAs) are potentially implicated in hepatocellular tumorigenesis and may serve as a diagnostic marker for these tumors [68,69]. ncRNA categories encompass diverse transcripts, including miRNAs, long

noncoding RNAs, and other RNA-like Y RNA fragments. Studies on miRNAs in human HCA are limited [62,70], and only one report on miRNAs involved in canine HCA [71]. miRNA involvement in HCC growth has been the subject of in-depth research in dogs and humans [71-75]. For example, my group has previously reported miR-1 dysregulation in canine HCC [72]. However, none of the reports on ncRNAs in canine liver tumors have addressed Y RNA.

Y RNA is first reported in patients with systemic lupus erythematosus in 1981 [76]. Despite being highly conserved molecules, Y RNAs exist in all vertebrate species [77], and the number of Y RNA transcripts varies between species [78]. Y RNAs are a type of regulatory RNA that have a sequence of 80-110 nucleotides [79]. They are identified by a stem-loop structure formed by complementary 5' and 3' ends [78]. Y RNAs may follow the miRNA's biogenesis pathways due to having a stem-loop structure of both Y RNAs and miRNAs [78]. Another study suggests that Y RNAs do not enter the miRNA biogenesis pathway and also do not bind to argonaut complex protein [80]. Y RNAs are transcribed by the enzyme RNA polymerase III. These RNAs are

bound to the polyuridine tail of the La protein, also known as small RNA binding exonucleolytic protection factor. This binding ensures nuclear retention and safeguards them from degradation [81]. Additionally, Y RNAs are also bound to RO60, also known as SSA, which promotes nuclear export and makes them more stable [82]. Y RNA-derived fragments (YRFs) are formed as a result of the partial breakdown of Y RNAs during apoptosis, which is carried out via the caspase-3-dependent pathway [80]. YRFs have been detected in both normal and cancerous tissues [83].

The dysregulation of Y RNAs may contribute to the development of tumors, affect cell growth, and promote inflammation [84]. Y RNAs are crucial in initiating DNA replication, maintaining RNA stability, and responding to cell stress [78,85]. Y RNAs are responsible for cellular processes such as cell proliferation [78]. Y RNAs and YRFs might be involved in signaling or a gene regulation function [86,87]. Y RNAs have not previously been investigated in human or canine HCC and HCA.

Y RNAs have been found in substantial amounts in plasma and serum from human patients [88,89], other biofluids [90], and extracellular vesicles [91]. Y RNAs have been established as reliable diagnostic biomarkers for a range of human cancers, including prostate [92] and bladder [93] cancers, melanoma [88], head, and neck squamous cell carcinoma (HNSCC) [84], breast cancer [89], lung cancer [94] and clear cell renal cell carcinoma [95]. Regarding evidence from dogs, my group has found decreased Y RNA-fragment expression in canine mammary gland tumors [96].

Extracellular vehicles (EVs) are of potential interest for the quantification of Y RNA fragments and other ncRNAs. They are small structures released by cells to facilitate the transportation of vital components such as DNA, RNA, and proteins for effective intercellular communication [97]. EV-derived ncRNAs have great significance for the early diagnosis of HCC due to their presence in circulation at an early stage of the disease, and they also have implications for any drug delivery system used in the treatment of HCC [98,99]. Recent studies have shown that EV-derived Y

RNA is abundant in human small-cell lung cancer [100], melanoma [101], brain tumors [102]. However, EV-derived Y RNA has yet to be studied in either canine or human HCC or HCA.

Similar gene expression patterns, such as the significance of TGF-beta, seem to be evident in the development of HCC in dogs and humans [103]. That is why exploring the role of Y RNA presents a promising avenue for gaining significant insights into the development of hepatic diseases.

Accordingly, in this study, I aimed to determine relative Y RNA expression levels in dogs with HCC and HCA using qRT-PCR analysis targeting tumor tissues, plasma, and plasma EVs from clinical samples, and HCC cell lines, to evaluate Y RNAs as diagnostic biomarkers for these two types of liver tumor in dogs.

3. Ethics statement

Informed consent to use the specimens in this study was obtained from the dog patient's owners. This study was approved by the ethics committee of the Kagoshima University Veterinary Teaching Hospital (Approval No. KVH220001).

4. Materials and Methods

Study population (clinical samples)

The clinical samples evaluated in this study had been obtained from a population of 28 dogs (age range: 8-14 years) diagnosed histopathologically with HCA (n=15) or HCC (n=13) by a veterinary pathologist when undergoing surgery at the Kagoshima University Veterinary Teaching Hospital or an affiliated clinic, between September 2012 and December 2022. The owner of each dog gave informed consent for using samples in this research. Samples were also collected from nine healthy adult laboratory beagle dogs to include as healthy controls in the evaluation provided by Shin Nippon Biomedical Laboratories, Ltd. [72].

Tumor tissue samples were collected at the time of surgery from the clinical patients, and biopsy samples were collected from the livers of healthy controls. Plasma samples were obtained from a subset of the study population (n=20; Healthy controls: n=6; HCA: n=5; HCC n=9). Full details of the HCA and HCC patients are summarized in Table 1. Tissue samples were immersed in RNAlater immediately after collection and stored at -80°C for long-term preservation. Blood samples were collected in anticoagulant-treated tubes (Terumo Venoject tubes 3.2% sodium

citrate) and centrifuged at 3000*g for 10 minutes to remove the cell debris. The plasma samples were separated and centrifuged again at 16000 Ïg at 4°C to remove the debris. The supernatant was transferred to new Eppendorf tubes and stored at -80°C as plasma samples.

Cell lines and cell culture

In this study, I evaluated two HCC cell lines, 95-1044 (a fast-proliferating cell line) and AZACH (an intermediate-proliferating cell line) [72,104]. Cell lines were preserved using a CultureSure freezing medium and stored in liquid nitrogen (Wako Pure Chemical Industries, Ltd., Osaka, Japan). D-MEM medium (Sigma-Aldrich, St. Louis, Missouri), 5% fetal bovine serum (Thermo Fisher Scientific, Waltham, Massachusetts), 5% L-glutamine (Sigma-Aldrich), and 3.5 µg/mL spectinomycin (Sigma-Aldrich) were used to culture the cells. All cells were cultured in a humidified incubator with 5% CO_2 at 37°C. Cold phosphate-buffered saline (PBS) and 0.25% trypsin or 0.1% EDTA were applied for detaching the cells. Cells were counted using an automated cell counter (LUNAII, Logos).

EV isolation

The Total Exosome RNA and Protein Isolation Kit (Invitrogen, Thermo Fisher Scientific) was used to isolate EVs from plasma, following the manufacturer's protocol. In brief, 300 µl plasma samples were mixed with a half volume of 1X PBS. 90 µl of exosome precipitation reagent was then added, and the resultant mixture was vortexed thoroughly and centrifuged at 10,000*g for 5 minutes. The supernatant was discarded, and the tube was centrifuged again at 1000Ïg for 30 seconds to remove the residual reagent. Finally, the pellet was reconstituted in 150 µl 1X PBS and stored at -80°C for further analysis.

RNA isolation of clinical samples and HCC cell lines

A mirVana™ RNA Isolation Kit (Thermo Fisher Scientific, Waltham, MA, USA) was used to extract total RNA from tissues and cells in accordance with the manufacturer's protocol. A mirVana PARIS Kit (Thermo Fisher Scientific) was used to isolate total RNAs from plasma samples and EVs. Before extraction, 5 µl (5 femtomoles) of synthetic cel-miR-39 was added to every plasma and plasma EV sample for normalization. Briefly, each tissue sample or the relevant HCC cell

preparation was mixed with the required amount of lysis buffer. A 300 μL aliquot of each plasma sample was mixed with an equivalent amount of 2x denaturation solution. A 1:10 ratio of a miRNA homogenate additive was added to the tissue or cell lysate, then kept on ice for 10 min. A 600 μL Acid-phenol: chloroform (Ambion®) was added to the tissue, cell lysate, or plasma, with subsequent thorough vortex-mixing and then centrifugation at 15000Ïg for 5 min at room temperature. The supernatant was then collected carefully in an Eppendorf tube, to which a 1.25-fold amount of molecular-grade ethanol (99.9% in purity) was added (and the amount recorded), and the tube contents were filtered using centrifugation. In the final step, total RNA was obtained as sediment in the tube using an elution solution pre-heated to 95°C. The NanoDrop 2000c spectrophotometer was used to measure the concentration of total RNA (Thermo Fisher Scientific). To evaluate the quality and integrity of RNA, an Agilent 2100 Bioanalyzer was utilized (Agilent Technologies, Santa Clara, CA, USA). The cells and tissues had RNA Integrity Numbers ranging from 8.5 to 9.5.

ncRNAs selection and qRT-PCR

Y RNA was selected based on a previously published NGS dataset (SRA: PRJNA716131) for canine mammary gland tumors [96]. The qRT-PCR protocol was described previously [105-107]. Briefly, 2 ng (for tissues and cell lines) or 1.25 µl (for plasma and plasma EVs) of total RNA were reverse transcribed to cDNA using the TaqMan MicroRNA Reverse Transcription Kit (Thermo Fisher Scientific) with a T100 thermal cycler, following the manufacturer's protocol. For qRT-PCR, a TaqMan First Advanced Master Mix Kit and a Quant Studio 3 real-time PCR system (Thermo Fisher Scientific) were applied. Each experiment was conducted two times to ensure accuracy. To evaluate the expression level, the $2^{-\Delta\Delta CT}$ method was used. RNU6B was used as an internal control for tissues and plasma, miR-16 was for the plasma, and miR-186 was for EVs [108]. The TaqMan primer sequences are as follows; 5′-GGCTGGTCCGAGTGCAGTGGTGCTTAC-3′ YRNA fragments (Ensembl ID: ENSCAFT00000034244.1).

Statistical analysis

GraphPad Prism 9 (https://www.graphpad.com/) was used for

statistical analysis and graph visualization. A Mann–Whitney U test and a one-way analysis of variance (ANOVA) followed by the Kruskal-Wallis test were used to assess the qRT-PCR results where applicable. ROC curves and AUCs were plotted using Wilson/Brown method. A P-value <0.05 was considered statistically significant.

5. Result

Relative expression in the clinical tissue samples

The relative expression of Y RNA was investigated in HCA and HCC tissue samples. YRNA was significantly decreased in HCC [fold change (FC) =0.43, P=0.008] versus healthy controls (Fig. 1 A, B). In addition, Y RNA was preferentially decreased in HCC (FC=0.39, P=0.001) versus HCA. However, the Y RNA expression level did not significantly differ between healthy controls and HCA. Thus, the expression profile for Y RNA in HCC differed to those in healthy controls and HCA.

Relative expression in plasma

I evaluated the expression of the selected Y RNA in plasma samples. Y RNA was significantly decreased in HCC (FC=0.02, P=0.002) and significantly increased in HCA (FC=2.50, P=0.002) versus Healthy controls (Fig. 2). Furthermore, Y RNA expression was decreased in HCC (FC=0.009, P=0.030) versus HCA. Taken together, my findings indicate that Y RNA expression may differentiate HCC and HCA from Healthy controls and HCC from HCA.

Relative expression in plasma EVs

In plasma, the relative Y RNA expression was significantly decreased in HCC (FC=0.21, P=0.001) versus Healthy controls (FC=0.21, P=0.001), and HCA (FC=0.06, P=0.001) [Fig. 3]. However, Y RNA expression did not significantly differ between HCA and Healthy controls. Thus, Y RNA expression could distinguish HCC and HCA from Healthy controls. The Y RNA expression profile in plasma was consistent with that in clinical tumor tissue samples.

Relative expression in canine HCC cell lines

The relative expression of Y RNA was evaluated in a fast-proliferative 95-1044 and intermediate-proliferating AZACH cell lines. Y RNA was significantly decreased in 95-1044 (FC=0.03, P=0.0002) and AZACH (FC=0.24, P=0.0007) cells versus normal liver tissue (Fig. 4). In addition, Y RNA was significantly decreased in 95-1044 cells (FC=0.15, P=0.004) versus AZACH cells. My results thus suggest that Y RNA expression is substantially decreased in fast-proliferative HCC cell lines, which is consistent with the results for clinical tumor tissue samples.

Diagnostic value of Y RNA

Receiver operating characteristics (ROC) curves and areas under the curve (AUCs) were generated to investigate the diagnostic value of Y RNA. In plasma analyses, Y RNA yielded AUCs of 0.920 (P=0.028) and 1.00 (P=0.004) for HCA and HCC, respectively, when evaluated against Healthy controls (Fig. 5 A, B). Y RNA also differentiated HCC (AUC=1.00, P=0.004) from HCA in plasma samples (Fig. 5 C). In plasma EV analyses, Y RNA significantly distinguished HCC (AUC=0.963, P=0.003) from Healthy controls (Fig. 5 D).and from HCA (AUC=1.00, P=0.005; Fig. E); however, it could not distinguish HCA from Healthy controls (AUC=0.833, P=0.088). In summary, Y RNA could discriminate HCC and HCA from Healthy controls and HCC from HCA in plasma and plasma EVs.

6. Discussion

The ncRNAs play a pivotal role in HCC development and progression, and evidence exists to support their utility as diagnostic and prognostic biomarkers for this disease [109-111]. Among ncRNAs, miRNAs have showed similar expression patterns in extensive studies on human and canine HCC [71-75]. miRNAs are less studied in human HCA [62,70], and have featured in only one study in canine HCA, in which dysregulation was found [75]. In contrast, Y RNA-derived fragments have not previously been studied in human or canine HCC or HCA, and here I report original findings (to my knowledge) on Y RNA expression in these two types of liver tumors in canine patients.

In key findings, Y RNA expression was significantly decreased in canine HCC tumor tissue versus healthy controls, and HCA tumor tissue, and the same pattern was noted in plasma EV samples. In plasma samples, Y RNA was significantly decreased in canine HCC and significantly increased in HCA versus Healthy controls. I also investigated Y RNA in two canine HCC cell lines and found it was significantly decreased in fast-proliferating 95-1044 cells and intermediate proliferating AZACH cells versus normal liver tissue.

The expression pattern of Y RNA in HCC cell lines was similar to that in clinical tissues. Y RNA was found to be decreased in HCC and in 95-1044 and AZACH cells, versus the control liver samples. This findings is interesting because results for the HCC cell lines reflected those in clinical tissue HCC samples, in comparisons against the same control liver samples. ROC analyses revealed that Y RNA could distinguish HCC from the Healthy controls and HCA patients in plasma and plasma EV analyses.

Altered Y RNA and YRFs expression levels are potentially implicated in carcinogenesis, and there is evidence that they act as diagnostic and prognostic biomarkers for several cancers [84,92,112]. Oncologists focussing on the human prostate have found that RNY1, RNY3, RNY4, and RNY5 are downregulated in prostate adenocarcinoma versus normal tissue and benign prostate hyperplasia [92]. These Y RNAs (RNY1, RNY3, RNY4, and RNY5) are reportedly similarly downregulated in human bladder cancer versus normal urothelial bladder tissue and act as a prognostic indicator for this condition [93]. RNY3P1, RNY4P1, and RNY4P25 show significantly higher expression in stage 0 human melanoma than at more advanced stages [88]. YRNA1 and YRNA5 are downregulated in human HNSCC, for which YRNA1 is regarded as a

potential biomarker [84]. Deep sequencing and bioinformatics analysis-based study has reported that dysregulated Y RNAs are also abundant in serum of human breast cancer patients [89]. YRNA-RNY1 is downregulated in human lung cancer patients compared to normal patients, whereas YRNA-RNY1 is found to be upregulated in lung cancer patients suffering from tuberculosis compared to normal controls [94]. In clear cell renal cell carcinoma, hY3 and hY4 show altered expression compared to normal renal tissue [95]. hY1 and hY3 RNA are highly abundant and upregulated in colon cancer patients than in healthy controls [113]. A set of Y RNAs (hY1, hY3, and hY4) are shown an increase in human cervix cancer [113]. I are currently compiling evidence that Y RNA is substantially decreased in metastasized canine mammary gland tumors versus those classified as benign mixed tumors [96]. A recent study revealed that hY4 RNA fragments are upregulated in human small-cell lung cancer-derived EVs and it inhibits tumor development by inhibiting MAPK/NF-$_k$B signaling [100]. Deep sequencing-based studies have shown that EV-derived Y RNAs are abundant in human melanoma [101]. Y RNAs are also found to be abundant in human brain tumors-derived EVs [102]. RNY4 fragments are highly abundant in non-Hodgkin lymphoma-derived EVs [114]. hY5 RNA is shown to be enriched in blood cancer-derived EVs

(K562 cells, and myelogenous leukemia) [115]. Overall, the findings in this study are consistent with several reports on human cancer and canine MGT, indicating that Y RNA expression is decreased in malignant tumors (such as canine HCC) relative to benign tumors (such as canine HCA) and healthy controls.

A recent study revealed that canine HCA transforms into HCC, which means recurrence may occur [116]. Therefore, this study demonstrated that Y RNA has a high potential for distinguishing canine HCA from HCC. I believe these findings provided insights into comprehending the knowledge of differential diagnoses among hepatic diseases.

The functional roles of Y RNA in canine HCC and HCA and its participation in the relevant underlying molecular mechanisms have yet to be fully elucidated. Here, I have demonstrated the aberrant expression of this ncRNA in canine HCC and HCA patients. I posit that Y RNA might be involved in cancer malignancy through its downregulated expression in HCC. Y RNA could be a biomarker distinguishing malignant tumors (HCC) from benign tumors (HCA) and tumor-free patients. However, this study still has some limitations. First, my study sample was relatively saml. I need to validate Y RNA in a large cohort sample to strengthen my

findings further. Second, the roles of Y RNA in canine HCC development need to be investigated in a bio-functional study.

Dogs have great potential utility for comparative oncology clinical trials, partly because they maintain an intact immune system and experience natural co-evolution of the tumor microenvironment [117]. Humans and dogs are known to develop cancer through aberrations occurring for the same genes [118]. Therefore, this study has great potential to enhance my understanding of the expression of Y RNA in hepatic diseases.

7. Conclusion

To my knowledge, this is the first report on Y RNA in canine HCC and HCA. This ncRNA has distinctive characteristics and differentiates malignant tumors (HCC) from benign tumors (HCA). Notably, its expression pattern is consistent across clinical samples and cell lines. I thus consider that Y RNA has promising potential for differentiating HCC from HCA. My findings provide significant insights into how Y RNA contributes to the progression of hepatic disease in dogs. Further research is required to fully elucidate the role of Y RNA in the development and progression of canine HCC and HCA.

8. Figures and Table

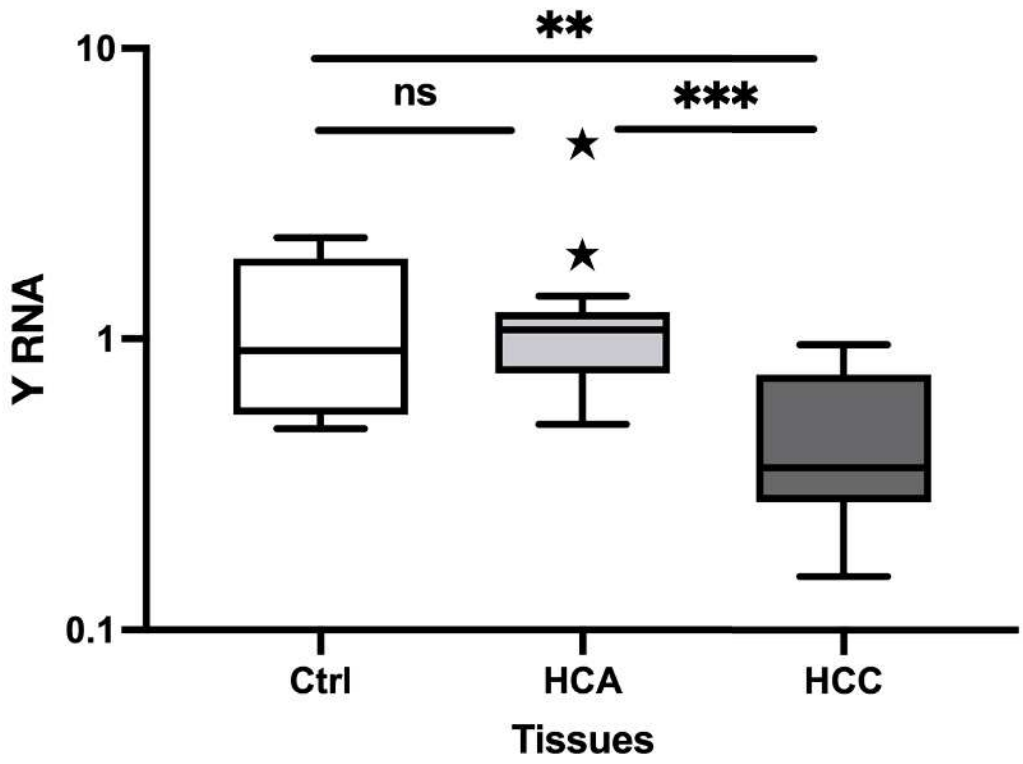

Figure 1. The relative expression of Y RNA in HCA and HCC tissue samples using qRT-PCR. The relative expression level of Y RNA in HCA (n=15) and HCC (n= 13) versus normal liver tissue (n = 9). The Y-axis represents relative noncoding RNA expression levels in log10 units. One-Way ANOVA (nonparametric) was performed, followed by the Kruskal-Wallis and Mann-Whitney tests. Differences were considered significant when the p-value was < 0.05 (*p < 0.05, **p < 0.01, ***p < 0.001). Ctrl: Control; HCA: Hepatocellular adenoma; HCC: Hepatocellular carcinoma.

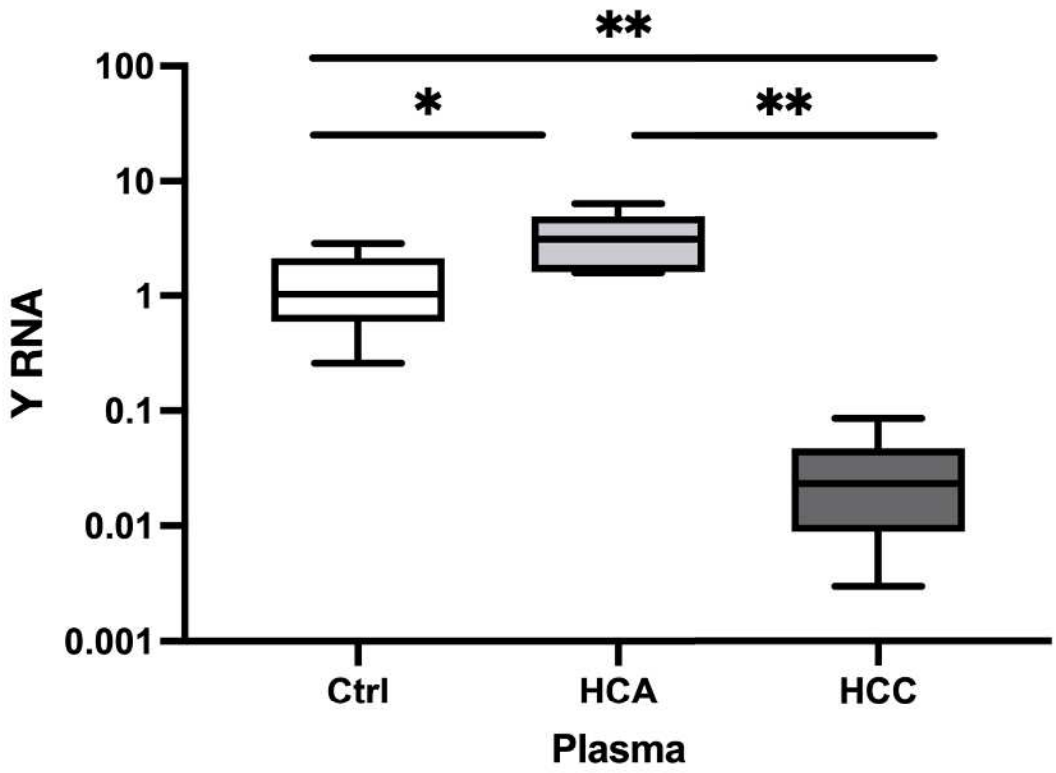

Figure 2. The relative expression of Y RNA in HCA and HCC plasma. The relative expression level of Y RNA in plasma HCA (n=5) and HCC (n= 7) versus Healthy controls (n = 6). The Y-axis represents relative noncoding RNA expression levels in log10 units. One-Way ANOVA (nonparametric) was performed, followed by the Kruskal-Wallis and Mann-Whitney tests. Differences were considered significant when the p-value was < 0.05 (*p < 0.05, **p < 0.01, ***p < 0.001). Ctrl: control; HCA: Hepatocellular adenoma; HCC: Hepatocellular carcinoma.

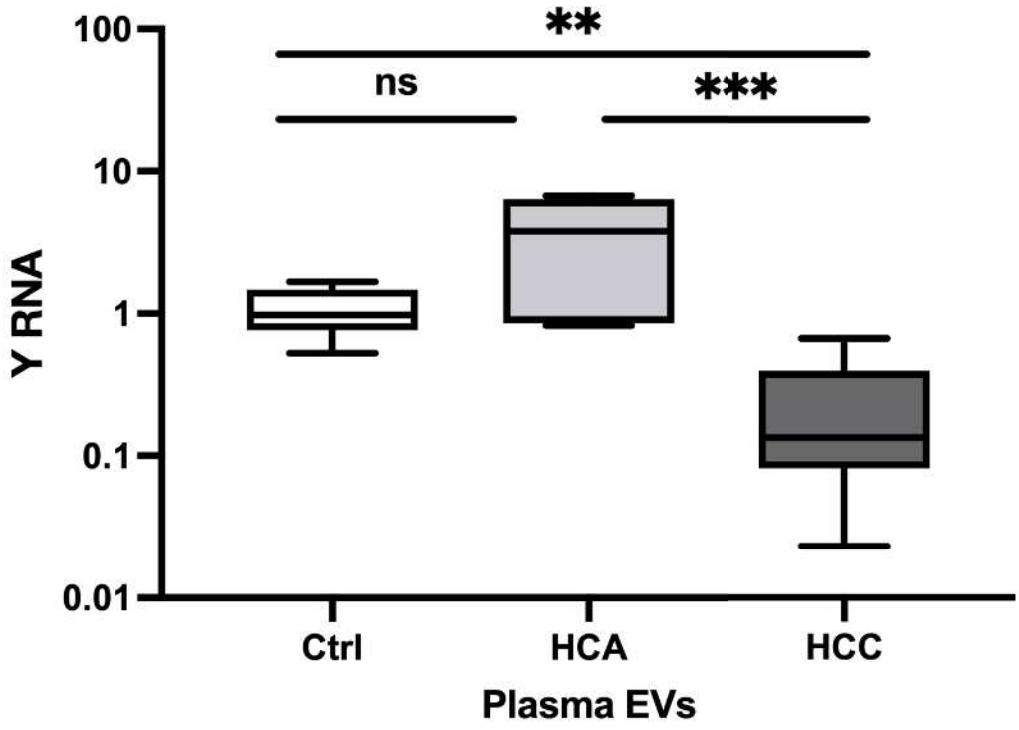

Figure 3. The relative expression of Y RNA in HCA and HCC plasma EV samples. The relative expression level of Y RNA in plasma HCA (n=5) and HCC (n= 9) versus Healthy controls (n = 6). The Y-axis represents relative noncoding RNA expression levels in log10 units. One-Way ANOVA (nonparametric) was performed, followed by the Kruskal-Wallis and Mann-Whitney tests. Differences were considered significant when the p-value was < 0.05 (*p < 0.05, **p < 0.01, ***p < 0.001). Ctrl; control, HCA; Hepatocellular adenoma, HCC; Hepatocellular carcinoma, EVs; Extracellular vesicles.

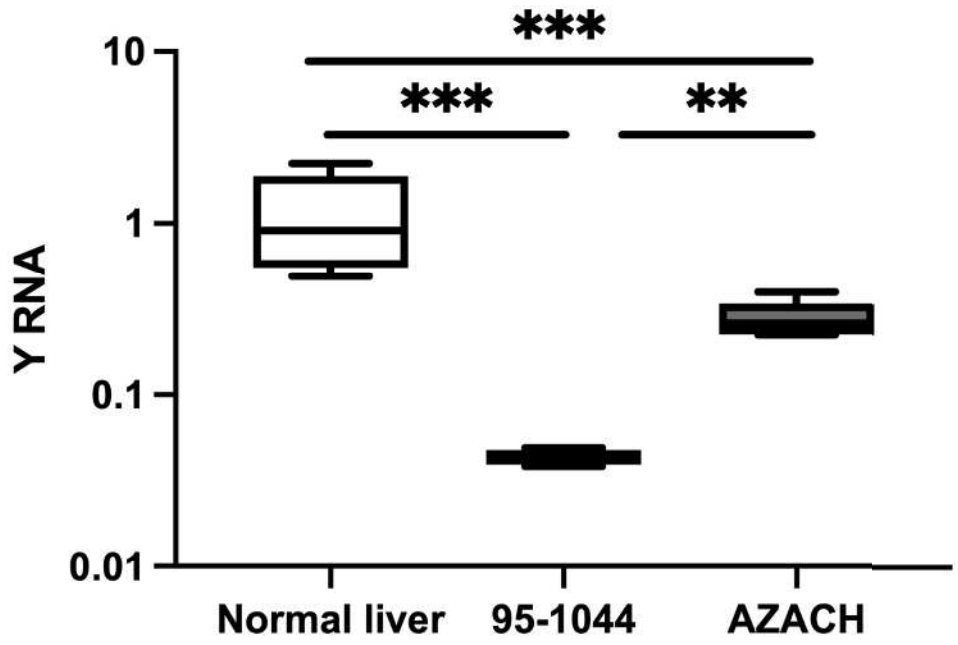

Figure 4. The relative expression of Y RNA in HCC cell lines. The relative expression level in HCC-1044 (n=6) and AZACH (n=6) versus normal liver tissue (n=9). The Y-axis represents relative noncoding RNA expression levels in log10 units. The Mann-Whitney U test was performed. Differences were considered significant when the p-value was < 0.05 (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

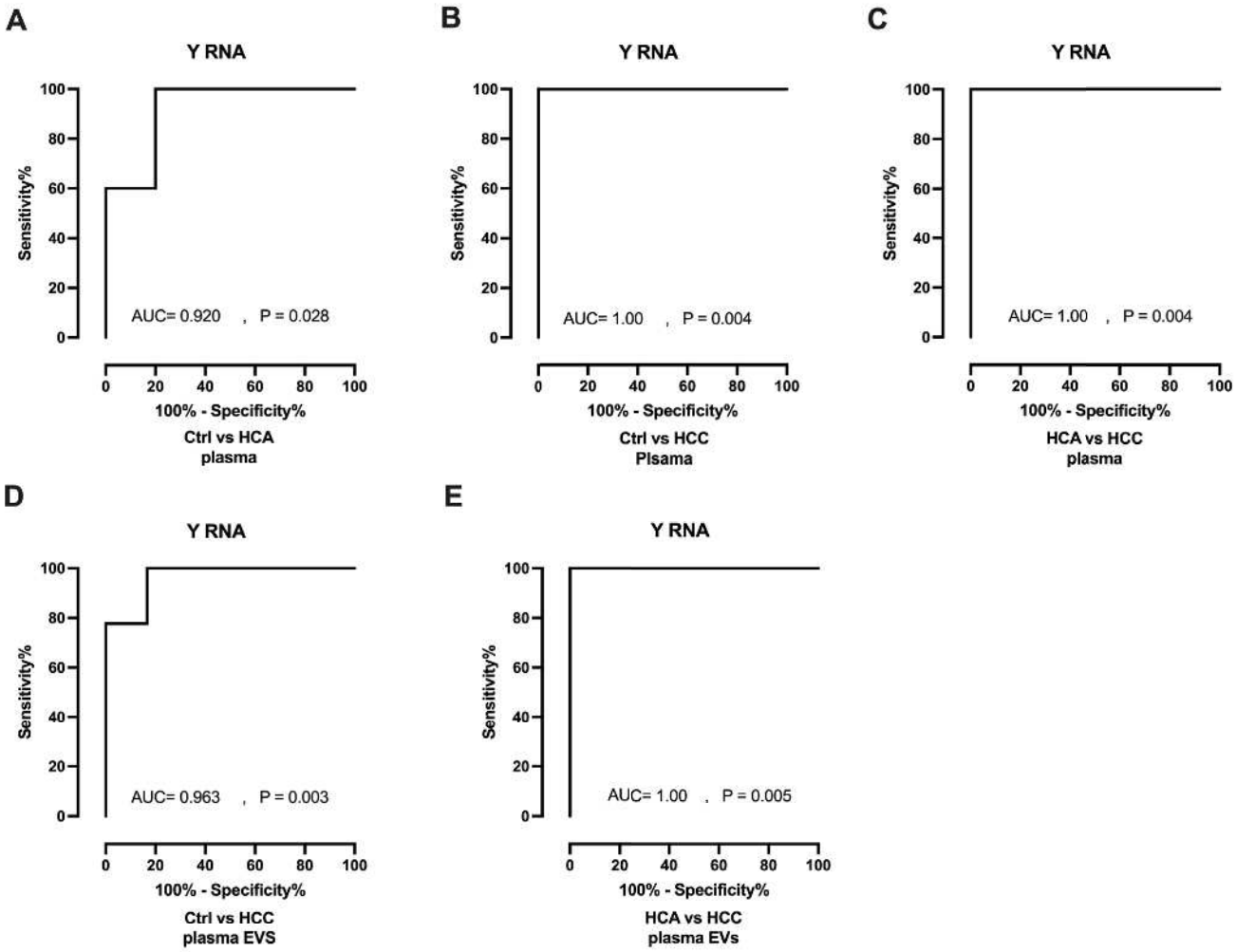

Figure 5. Diagnostic potential of Y RNA as a biomarker. **A-B.** ROC curve (plasma) of Y RNA for differentiating HCA (n=5) and HCC (n=7) group from Healthy controls (n=6). **C.** ROC curve of Y RNA for differentiating HCC (n=7) from HCA (n=5). **D.** ROC curve (plasma Evs)) of Y RNA for differentiating HCC (n=9) from Healthy controls (n=6) **E.** HCC (n=9) versus HCA (n=5). Ctrl: control; HCA: Hepatocellular adenoma; HCC: Hepatocellular carcinoma; EVs: Extracellular vesicles.

Table 1. HCA and HCC patient information.

Number	Age	Disease	Sex	Neutered	Breed	Tissue	Plasma
P1	11 Y 3 M	HCA	M	Yes	Crossbreed	15	5
P2	8 Y 1M	HCA	F	Yes	Miniature dachshund		
P3	10 Y 7 M	HCA	M	Yes	Toy poodle		
P4	12 Y 2 M	HCA	F	Yes	Shiba		
P5	11 Y 6 M	HCA	M	No	Miniature dachshund		
P6	12 Y 3 M	HCA	M	No	Miniature dachshund		
P7	11 Y 9 M	HCA	M	No	Crossbreed		
P8	13 5 M	HCA	F	Yes	Miniature dachshund		
P9	14 Y	HCA	M	No	Golden retriever		
P10	9 Y 2 M	HCA	F	Yes	Toy poodle		
P11	13 Y	HCA	F	Yes	Jack Russell terrier		
P12	11 Y 1 M	HCA	M	No	Miniature dachshund		
P13	12 Y 2 M	HCA	M	No	Crossbreed		
P14	12 Y 3 M	HCA	M	No	Crossbreed		
P15	10 Y 7 M	HCA	M	No	Shiba		
P16	12 Y 3 M	HCC	F	No	Chihuahua	13	9
P17	11 Y 3 M	HCC	F	Yes	Miniature dachshund		
P18	14 Y	HCC	F	Yes	Crossbreed		
P19	10 Y 8 M	HCC	M	Yes	Shiba		
P20	11 Y 7 M	HCC	M	Yes	Welsh corgi		
P21	10 Y 9 M	HCC	F	No	Crossbreed		
P22	10 Y 3 M	HCC	F	No	Beagle		
P23	10 Y 9 M	HCC	F	No	Yorkshire terrier		
P24	11 Y 6 M	HCC	M	No	Shiba		
P25	12 Y	HCC	F	No	Miniature schnauzer		
P26	11 Y 10 M	HCC	M	No	Yorkshire terrier		
P27	13 Y 10 M	HCC	F	No	Shetland sheepdog		
P28	11 Y 7 M	HCC	M	Yes	Crossbreed		

※ P; Patient, HCA; Hepatocellular adenoma, HCC; Hepatocellular carcinoma.

※ F; Female; M; Male

Conclusion

I have demonstrated the up-regulation of potential oncomiRs, miR-16, miR-21, miR-29b, miR-122, miR-125b, miR-204 and miR-383 in canine malignant melanoma (CMM) tissues. In particular, the strong up-regulation of miR-383 in CMM tissues compared with normal oral tissues identified by microarray screening was confirmed by qRT-PCR. I conclude that miR-383 and miR-204 may promote melanoma development by regulating the DNA repair/checkpoint and apoptosis.

Then, I also demonstrated the Y RNA dysregulation in the canine hepatocellular carcinoma (HCC). Especially to my knowledge, this is the first report on Y RNA in canine tumors. Interestingly, this ncRNA has distinctive characteristics and differentiates malignant tumors (HCC) from benign tumors (HCA). The expression pattern of Y RNA is consistent across clinical samples and cell lines. Thus, Y RNA has promising potential for differentiating HCC from HCA. Further research is required to fully elucidate the role of Y RNA in the development and progression of canine HCC and HCA.

Acknowledgement

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I appreciate grateful all animals, patients, and their owners for the cooperation in this study.

Finally, and for most, I would like to especially thank my family for always help to my life.

Appendix

Samples		Nano Drop			Bio Analyzer		
		ng/µl	260/280	260/230	ng/µl	rRNA ratio	RIN
C11	Melanoma	409.00	2.08	1.70		1.50	9.50
C32		198.70	2.05	2.03	111.00	1.60	8.30
A19		130.90	2.14	2.04	117.00	1.70	9.10
C47		1744.70				1.30	8.00
C93		82.90	1.96	1.76		1.80	9.70
C96		505.00	2.04	2.09		1.90	9.40
C97		589.00	2.02	2.06		1.60	8.00
C98		277.00	1.96	2.01		1.50	9.10
C100		411.00	2.05	2.07		2.00	9.00
C108		139.00	1.97	2.11		1.90	9.80
C67	Control	266.00				1.70	10.00
C68		65.70	1.94	1.40		1.90	9.60
C69		90.40	1.96	2.18		2.00	9.80
C70		126.00	1.96	2.19		1.70	9.50
C71		133.00	2.01	1.95		1.50	9.30
C72		292.00	2.05	1.94		1.60	9.10
C101		149.00	1.95	2.21		1.90	9.60
C102		102.00	1.97	2.12		2.00	8.90
C103		143.00	1.97	1.98		1.70	9.30
C104		100.00	1.95	1.74		1.90	8.40
C105		55.00	1.97	2.23			
C106		179	1.991	2.143		1.60	8.00

Samples	miR-16	miR-21	miR-29b	miR-122	miR-125b	miR-204	miR-205	miR-383
C11	14.91	27.55	39.33	797.37	8.63	46694.01	21.09	45384.12
C32	5.63	9.66	3.86	684.05	4.45	21688.61	102.59	8989.03
A19	1.47	6.55	1.00	54.04	3.83	388.08	345.02	15.53
C47	26.36	33.84	13.40	365.61	12.31	7723.02	1406.96	28348.80
C93	11.69	32.68	17.92	12027.09	9.45	4352.06	7.93	740.01
C96	9.12	14.92	15.72	20.04	6.40	3032.62	263.03	1123.20
C97	8.85	12.05	8.00	4787.48	3.15	466.83	1.00	2178.55
C98	7.08	26.56	13.20	313.08	11.47	6173.19	2345.07	26060.91
C100	31.26	26.48	10.26	2335.39	8.98	1736.66	3761.56	46.15
C108	5.47	4.43	3.58	12.65	1.54	3414.01	524.49	5584.84
C67	4.21	2.85	6.15	16.11	3.36	1364.64	105515.23	84.90
C68	2.00	2.19	4.90	2028.29	3.66	451.57	36068.87	17.63
C69	1.81	1.36	2.32	205.81	1.18	551.38	16949.05	39.95
C70	2.14	2.25	5.17	72.46	2.34	702.86	30182.86	39.21
C71	1.96	2.17	3.70	24.72	3.10	703.81	57256.69	181.90
C72	2.94	6.93	6.74	26.03	1.00	753.96	49583.30	79.28
C101	1.50	2.17	3.68	1.03	1.49	641.92	45022.75	29.62
C102	2.73	2.02	2.69	#VALUE!	4.56	413.43	113408.30	128.18
C103	1.27	1.00	2.48	1.65	2.43	330.77	24523.74	19.53
C104	1.00	1.10	2.41	1.00	2.54	501.52	31533.91	53.26
C105	2.21	1.79	4.33	24.86	3.69	572.20	46492.70	38.45
C106	2.27	2.60	4.71	2.63	2.69	522.36	168509.06	218.77

YRNA Expression	SamplID	Expression Value
Normal Liver	C187	0.521
	C190	2.084
	C191	2.237
	YC160	1.225
	YC169	0.560
	363	0.968
	357	1.830
	360	0.855
	376	0.492
	434	0.806

	SamplID	Expression Value
HCA	330	0.509
	319	1.955
	410	0.736
	195	0.555
	225	0.761
	323	1.401
	362	1.236
	375	1.027
	359	1.073
	356	1.194
	123	1.027
	162	1.181
	475	4.709
	145	0.932
	148	1.139

	SamplID	Expression Value
HCC	C73	0.961
	C92	0.344
	C99	0.826
	C119A	0.205
	C119B	0.693
	C154	0.258
	C162	0.676
	C176	0.295
	C195	0.628
	C199	0.153
	YC13	0.363
	YC90	0.885
	YC116	0.310

YRNA Expression	SamplID	Expression Value		SamplID	Expression Value		SamplID	Expression Value
Normal Liver	C187	0.521	1044	1044a	0.040	AZACH	220	0.398
	C190	2.084		1044b	0.038		221	0.223
	C191	2.237		1044c	0.047		222	0.283
	YC160	1.225		1044d	0.042		223	0.225
	YC169	0.560		1044e	0.044		224	0.261
	363	0.968		1044f	0.049			
	357	1.830						
	360	0.855						
	376	0.492						
	434	0.806						

YRNA Expression	SamplID	Expression Value		SamplID	Expression Value		SamplID	Expression Value
Control	YC247	0.259	HCC	144	6.392	HCC	13	0.087
	YC248	1.027		148	3.532		323	0.048
	YC249	2.882		319	3.127		449	0.009
	YC250	0.927		502	1.656		162	0.023
	YC251	1.405		504	1.598		330	0.021
							446	0.003
							362	0.031

YRNA Expression	SamplID	Expression Value		SamplID	Expression Value		SamplID	Expression Value
Control	YC248	0.909	HCC	144	5.974	HCC	123	0.023
	YC249	1.403		148	0.824		449	0.135
	YC250	0.843		319	0.893		330	0.668
	YC251	0.527		502	3.778		466	0.104
	YC 246	1.052		504	6.731		469	0.082
	YC 247	1.678					362	0.081
							162	0.595

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