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Spontaneously arising disease

Characterization of canine intestinal microRNA expression in inflammatory bowel disease and T-cell lymphoma

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ABSTRACT

Differentiating between canine inflammatory bowel disease (IBD) and intestinal T-cell lymphoma by histopathological examination of endoscopically-derived intestinal biopsies can be challenging and involves an invasive procedure requiring specialized equipment and training. A rapid, non-invasive method of diagnosis, such as blood or faecal analysis for a conserved and stable biomarker, would be a useful adjunct or replacement. Studies on dogs and humans with various types of lymphoma have shown altered microRNA (miRNA) expression patterns in blood, faeces and tissues indicating their potential use as biomarkers of disease. The present study used residual archived endoscopically-derived, formalin-fixed, paraffin-embedded (FFPE) duodenal tissue taken from pet dogs undergoing routine investigation of gastrointestinal disease. The dogs had previously been diagnosed with either normal/minimal intestinal inflammation, severe IBD or intestinal T-cell lymphoma. Next generation sequencing with qPCR validation was used to elucidate differentially expressed miRNAs between groups. Our results show that miRNA can be extracted from archived endoscopically-derived FFPE tissues from the canine duodenum and used to differentiate normal/minimally inflamed canine duodenal tissue from severe lymphoplasmacytic IBD and T-cell lymphoma.

1. Introduction

Canine inflammatory bowel disease (IBD) and intestinal lymphoma may result in similar clinical presentations, including chronic vomiting, diarrhoea and weight loss. Canine IBD comprises a group of idiopathic disorders characterized by chronic gastrointestinal disease and diagnosed histopathologically by the type of inflammation in the lamina propria of the small and/or large intestine. In humans and animals, the cause of IBD is multifactorial but has been shown in both to have a genetic component [1]. Some breeds of dog are more prone to intestinal lymphoma, which also indicates a genetic component [2]. Canine intestinal lymphoma is typically T cell and morphologically classified into large cell lymphoma (LCL) and small cell lymphoma (SCL) [3], and can be graded using mitotic count [4]. To align canine intestinal lymphoma classification with that of humans, the term enteropathy-associated T-cell lymphoma (EATL) has been recently introduced. However, despite canine LCL sharing common features with human EATL type I, canine SCL cells and human EATL type II differ in their immunophenotype [3].

Differentiation between IBD and gastrointestinal lymphoma is based on histopathological and immunohistopathological evaluation of intestinal biopsies. However, this involves an invasive procedure and diagnosis can be difficult on the basis of histopathology alone [5]. This is due to several factors, including technical difficulties in achieving high-quality diagnostic samples that allow adequate microscopic assessment of the entire mucosal depth, tissue crushing and processing artefacts, sample orientation issues and poor proportional representation of disease within the entire length of the gastrointestinal tract [6]. Furthermore, there may be

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superimposition of lymphocytic inflammation alongside neoplastic lymphocytes, as intestinal lymphoma is hypothesized to develop from chronic lymphocytic inflammation [5]. SCL is characterized by proliferation of monomorphic small neoplastic lymphocytes, accompanied by infiltration of non-neoplastic plasma cells [3], which may resemble inflammation rather than appearing overtly neoplastic. Differentiating emerging SCL within a background of pre-existing inflammation is particularly challenging. To assist with differentiation, clonality testing by polymerase chain reaction (PCR) for antigen receptor rearrangement (PARR) in biopsy samples is frequently used as an adjunct to histopathology. However, the sensitivity of this test is suggested in one study on canine intestinal lymphoma to be only 66.7% [5]. The consequences of misdiagnosis are significant, because misdiagnosis of lymphoma as IBD can result in missed opportunities for appropriate treatments and management, such as chemotherapy, resulting in continued intestinal tissue destruction and a decreased survival time. Finding rapid, potentially non-invasive molecular methods for accurate diagnosis of IBD and lymphoma through faecal or blood sampling would therefore be highly desirable, and microRNAs (miRNAs) may offer this opportunity.

miRNAs are small, single-stranded, non-coding RNA strands that regulate post-transcriptional gene expression [7] and studies on dogs with various types of lymphoma have shown altered miRNA patterns in serum [8], cell lines [9] and formalin-fixed, paraffin-embedded (FFPE) tissue [10]. miRNAs are often well conserved across species [11] and are commonly tissue specific [12,13]. There is increasing evidence of disease-specific miRNAs in humans [14] and a synthetic miRNA inhibitor is currently in phase 1 trials for human patients with cutaneous T-cell lymphoma [15]. miRNA is stable in human and canine blood [8,16] and in human and canine faeces [17,18] and can be successfully extracted from FFPE samples [19]. Differentially expressed faecal and serum mRNAs may be able to distinguish between gastrointestinal cancer and chronic inflammatory enteropathy in dogs [20].

The aim of this study was to compare miRNA expression in archived duodenal FFPE samples from dogs previously diagnosed with no or mild histopathological evidence of gastrointestinal disease, severe IBD or intestinal T-cell lymphoma. The objectives were to profile differentially expressed miRNAs between the groups using next generation sequencing and alignment to the canine genome, and then use qPCR for validation of the sequencing results. Any differences identified could form the basis of further work to develop non-invasive miRNA tests for blood or faeces.

2. Materials and methods

2.1. Ethics statement

This research was approved by the Clinical Research Ethical Review Board (CRERB) of the Royal Veterinary College (URN 2017 1728-2) as a retrospective study using archived residual diagnostic material previously obtained from routine veterinary clinical practice.

2.2. Extraction of RNA from archived FFPE endoscopic grab biopsies of duodenum from canine patients

Samples were retrospectively selected from the archives of two histopathology laboratories, where the owners or submitting veterinary surgeons had given informed consent for residual tissue to be used in research. Inclusion criteria were that the samples were from the duodenum, had been retrieved via endoscopic biopsy and for the histopathology report to have given a diagnosis of either no or minimal inflammation (n = 12), denoted as the Minimal group (samples prefixed M [M1–12], severe lymphoplasmacytic inflammation (n = 10), denoted as the Severe group (samples prefixed S [S1–10], or T-cell lymphoma (n = 9), denoted as the Lymphoma group (samples prefixed L [L1–9]. T-cell lymphoma was confirmed using immunohistochemistry for CD3 (T cells) and CD20 and/or CD79a (B cells) at the time of diagnosis (Fig. 1). Samples were microsectioned on a rotary microtome (OS-315 Rotary Microtome; GT Vision, https://gtvision.co.uk) to collect eight slices at 4 μm thickness. Paraffin was removed using mineral oil (CAS 8042-47-5; Sigma-Aldrich, www.sigmaaldrich.com) heated to 80°C for 3 min with a heating block (Thermomixer 5436; Eppendorf, www.eppendorf.com). RNA was extracted using Qiagen miRNeasy FFPE Kit (Qiagen, www.qiagen.com) as per the manufacturer's protocol. The quantity and quality of the RNA was assessed using spectrophotometry (DS-11 Spectrophotometer; DeNovix, www.denovix.com) and stored at −80°C. Due to the availability of material, samples were processed as above in two separate batches. Additionally, remaining stored frozen eluted RNA of two severe cases (samples S4 and S5) and three lymphoma cases (samples L1, L4 and L5) from the first batch were sent with the second batch to test for reproducibility of results.

2.3. Sequencing and miRNA profiling

Five hundred and fifty ng of total RNA from each sample were subjected to next generation sequencing (NextSeq 500 System; Illumina, www.illumina.com), with 25 μl of RNAase-free water (9012; Takara, www.takarabio.com) used as a control. cDNA libraries were generated as part of a custom small RNA library preparation and raw reads were assessed for quality using FastQC and the seqTools package in R version 2.15.3 [21]. Adapter sequences were trimmed with Cutadapt [22] and the miRNAs were aligned to the canine genome (cfa, CanFam3.1) using Bowtie2 with the very-sensitive local preset [23]. Reads mapping to canine miRNAs in the MiRBase database version 22.1 (https://www.mirbase.org) were counted using the featureCounts function from the Rsubread package (www.rdocumentation.org).

2.4. Quantitative reverse transcriptase polymerase chain reaction

3.75 μl of a subset of samples from the first batch, M1, M2, M5, S2, S3, S6, L4, L5 and L6, were subjected to reverse transcription into cDNA using the Mir-X miRNA First-Strand Synthesis Kit (Takara) and a C1000 Touch thermocycler (Bio-Rad, www.bio-rad.com) according to the manufacturer's protocol. The quantity and quality of the cDNA were assessed using spectrophotometry (DS-11 Series Spectrophotometer; DeNovix, www.denovix.com), diluted to 600 ng cDNA per duplicate in RNAase-free water (Takara) and stored at −80°C prior to qPCR. Two 96-well plates were completed and run as per Mir-X miRNA First-Strand Synthesis and SYBR qRT-PCR Kit (Takara) protocol using a Bio-Rad CFX Maestro qPCR machine and software. Each sample and controls were run in duplicate. U6 snRNA supplied with the kit was used as a positive control analogous to a 'house-keeping gene' miRNA in each plate. Experimental miRNA sequences (cfa-miR-200c, cfa-mi-R363, cfa-miR-146b, cfa-miR-105a, cfa-miR-20b and cfa-miR-885) were selected from the sequencing results as those most consistently under- or overexpressed across samples and between groups. Primers were created using miRbase database version 22.1 (https://www.mirbase.org) and supplied by Eurofins Genomics (https://

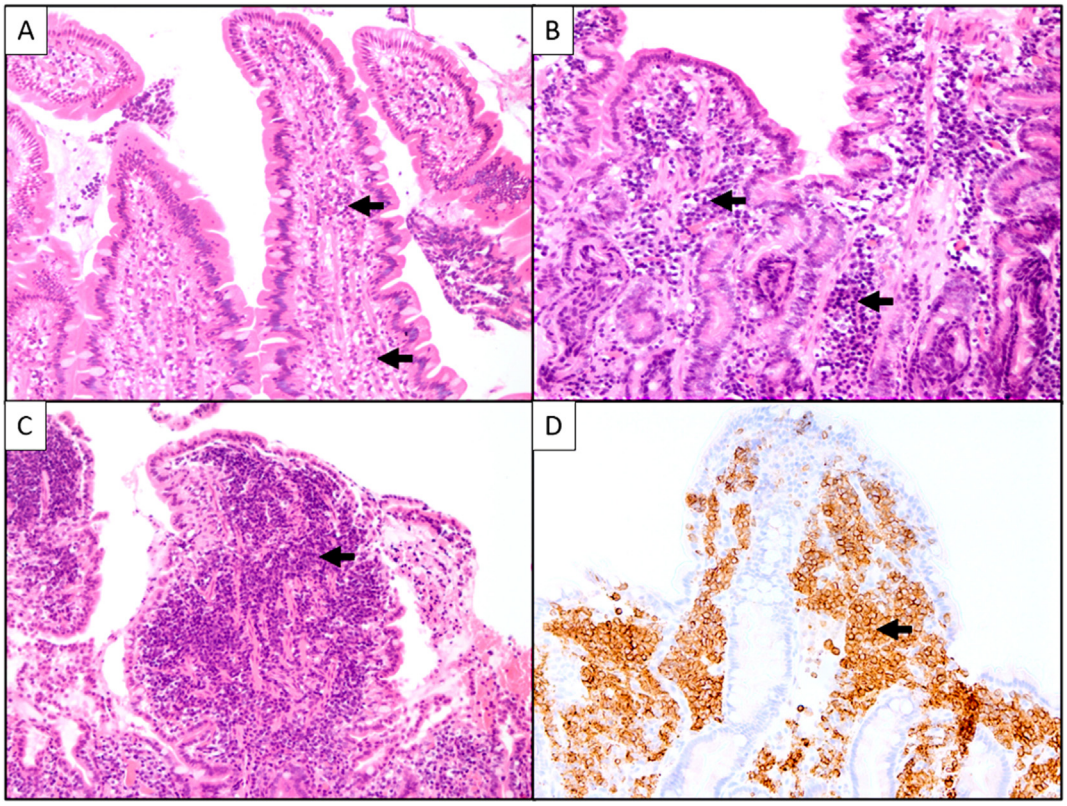

Fig. 1. Duodenum, dog. **(A)** Minimal inflammation. Tall, thin villi with minimal numbers of lymphocytes and plasma cells in lamina propria (arrows). HE. ×200. **(B)** Severe lymphoplasmacytic inflammatory disease. Lamina propria infiltrated by high numbers of lymphocytes and plasma cells (arrows). HE. ×200. **(C)** T-cell lymphoma. Infiltration of lamina propria and/or epithelium by large numbers of neoplastic T cells (arrow). HE. ×200. **(D)** Almost universal immunolabelling of CD3 in neoplastic T cells (arrow). IHC. ×200.

eurofinsgenomics.com). The entire sequence of the miRNA was used as the miRNA-specific, 5′ primer.

2.5. Statistical analysis

For combined analysis of the two batches of sequencing data, differentially expressed miRNAs among the three groups were identified using edgeR [24] including a coefficient for sample batch in the generalized linear model. Significant results were selected on the basis of a Benjamini-Hochberg adjusted *P*-value (FDR) threshold of 0.05. Data was normalized and transformed to $\log_2$ counts per million mapped reads (logCPM) in edgeR and $\log_2$ fold changes ($\log_2$FC) are expressed relative to the Minimal group. For visualization, the batch effect was corrected using RUVg normalization from the RUVSeq R package [25] and the set of 100 miRNAs with the lowest absolute fold change (FDR >0.05) between groups in the edgeR analysis. Principal component analysis and hierarchical clustering of normalized miRNA counts were conducted using custom scripts in R, and heatmaps were generated using the pheatmap R package [26]. For qPCR data, the delta−delta Ct method was used with the U6 primer supplied with the Mir-X miRNA First-Strand Synthesis Kit (Takara) utilized as a positive control analogous to a 'house-keeping gene' miRNA. Log transformed qPCR expression values were tested for normality by the Shapiro−Wilk test and ANOVA was used to assess statistical significance with the Minimal group as the control. Where qPCR was performed with cfa-miR-363, which had been shown to be increased in the Severe group but not the Lymphoma group, Dunnet's post hoc test was performed following ANOVA to confirm which group had the statistically significant change.

3. Results

3.1. Genome sequencing

3.1.1. Batch 1

Principal component analysis and hierarchical clustering of sequenced canine miRNAs revealed distinct miRNA expression profiles for the Minimal group compared with the Severe and Lymphoma groups, but no clear separation between the Severe and Lymphoma groups (Fig. 2a). In the first batch of sequencing, 11 canine miRNAs were identified as differentially expressed in the Severe versus Minimal comparison and 13 in the Lymphoma versus Minimal comparison. Of these, five differentially expressed miRNAs were shared between the comparisons (Fig. 2b). In the Severe versus Minimal comparison (Fig. 3a), eight miRNAs had statistically significant (FDR-adjusted P <0.05) increased expression in the Severe group and three had decreased expression. Of the miRNAs that had increased expression in the Severe group compared with the Minimal group, the largest fold changes were seen with cfa-miR-122, cfa-miR-363 and cfa-miR-20b at $\log_2$FC = +6.3, +3.0 and +2.9, respectively. In the Lymphoma versus Minimal comparison (Fig. 3b), seven miRNAs had increased expression in the Lymphoma group compared with the Minimal group and six had decreased expression. Of particular note are cfa-miR-9, cfa-miR-155, cfa-miR-146b, cfa-miR-105a and cfa-miR146a, which had increased expression in the Lymphoma group compared with the Minimal group with a fold change of $\log_2$FC = +2.5, +2.4, +2.0, + 1.9 and +1.6, respectively. cfa-miR-885 and cfa-miR-200c were downregulated in both the Severe and Lymphoma groups when compared with the Minimal group, with a fold change of $\log_2$FC −1.3 and $\log_2$FC −1.3, and −0.89 and

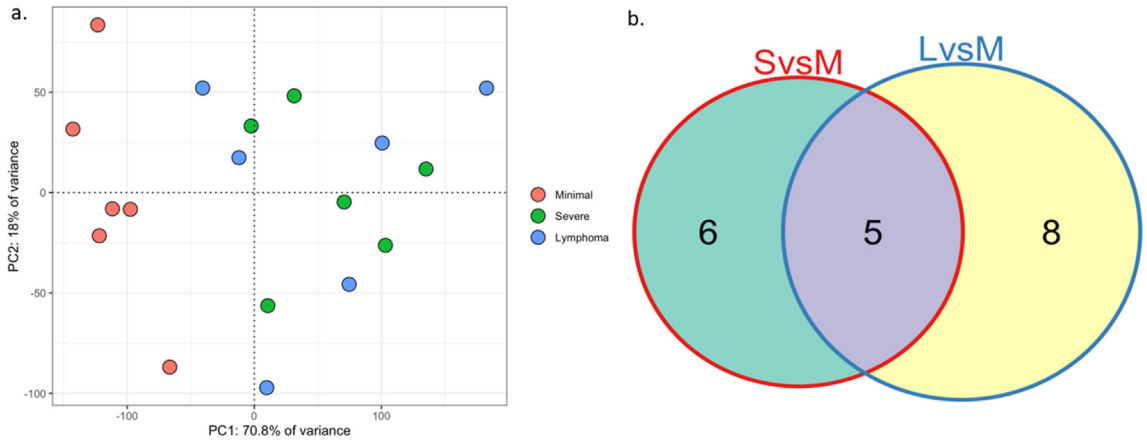

Fig. 2. Results of genome sequencing data analysis. **(a)** Principal component analysis reveals distinct miRNA expression profiles for the Minimal group compared to the Severe and Lymphoma groups, but no clear separation between the Severe and Lymphoma groups. **(b)** Eleven canine miRNAs were identified as differentially expressed in the Severe versus Minimal comparison and 13 in the Lymphoma versus Minimal comparison, with five differentially expressed miRNAs shared between the comparisons. PC1, principal component 1; PC2, principal component 2; SvsM, Severe versus Minimal groups; LvsM, Lymphoma versus Minimal groups.

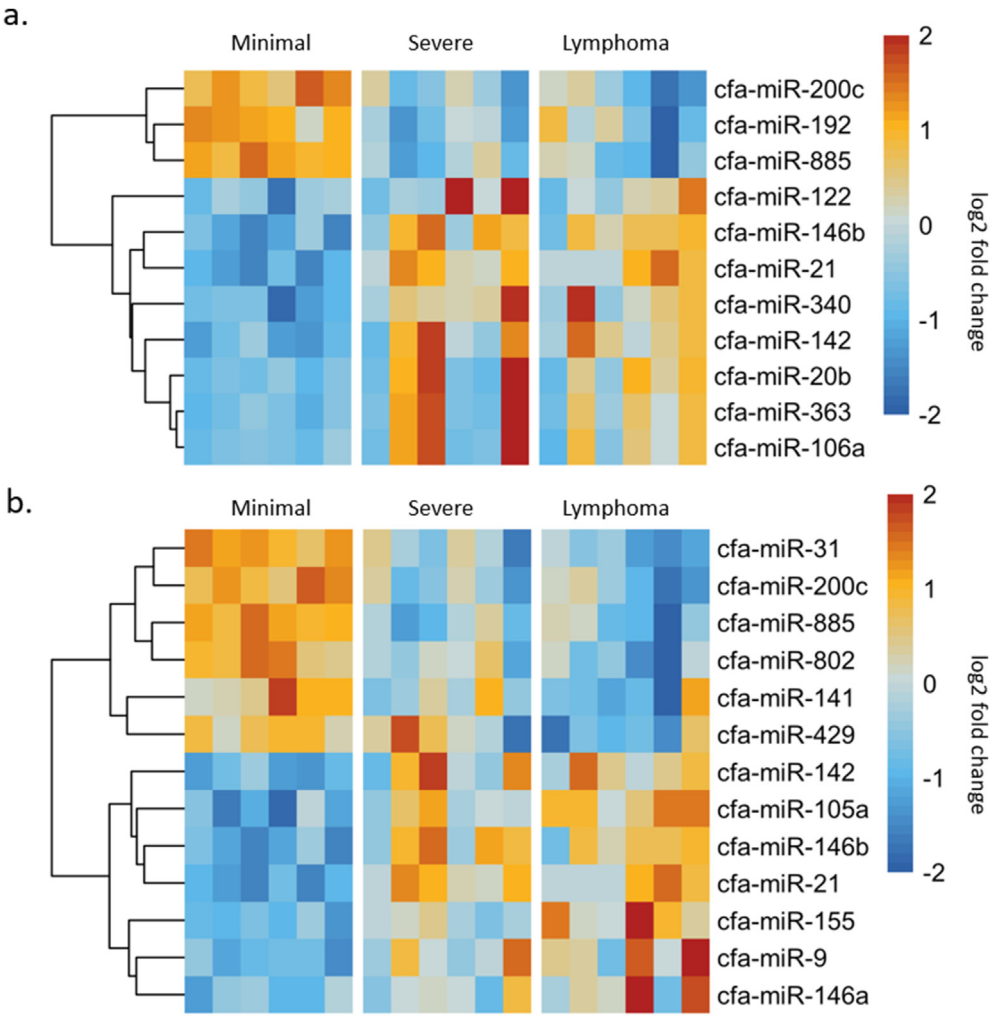

Fig. 3. Results of genome sequencing data analysis. Heatmaps depicting two-way hierarchical clustering of miRNAs and samples for Severe (green) versus Minimal (red) comparison **(a)** and Lymphoma (blue) versus Minimal (red) groups **(b)**. Each row represents one miRNA and each column represents a sample. Colour scale illustrates mean relative $\log_2$ fold change of miRNA expression: red, higher expression; blue, lower expression.

Table 1
Results of genome sequencing data analysis for Severe versus Minimal group after data were normalized and transformed to $\log_2$ counts per million (logCPM) using the R package 'DESeq2'

Genes	logFC	logCPM	F	*P*-value	FDR
cfa-miR-146b	2.23807931	9.69401175	31.4422574	1.84E-05	0.00606327
cfa-miR-885	−1.2807853	9.01057795	21.4515085	0.00016749	0.0276356
cfa-miR-363	2.98229652	10.6707495	18.3625154	0.00037306	0.03515155
cfa-miR-106a	2.8637104	8.26470712	16.3253746	0.00065813	0.03515155
cfa-miR-142	1.33147443	8.94501174	16.2867959	0.00066547	0.03515155
cfa-miR-21	0.80759134	17.8022814	15.9205562	0.00073982	0.03515155
cfa-miR-20b	2.93340995	8.04203	15.8936341	0.00074564	0.03515155
cfa-miR-122	6.31665871	6.87762538	15.3011982	0.00088739	0.03660485
cfa-miR-192	−1.2594069	16.4789585	14.0604163	0.00129162	0.04516603
cfa-miR-340	0.65408148	10.4990049	13.8733287	0.00136867	0.04516603
cfa-miR-200c	−0.8892425	14.6843286	13.5376914	0.00151998	0.04559925

logFC, $\log_2$-transformed fold change in expression (relative to Minimal group); F, fold change; FDR, *P*-value after correcting for multiple tests (Bonferroni method).

log2FC −1.01, respectively. All statistically significant differentially expressed miRNAs from the first batch, their fold changes and further data from the sequencing are summarized in Table 1 (Severe versus Minimal) and Table 2 (Lymphoma versus Minimal).

3.1.2. Batch 2 and replicates

Results of sequencing of the second batch of samples were largely concordant with the first batch (Supplementary Figs. S1a and b). Analysis of the sequencing results of the replicates, using the Pearson correlation coefficient (r), revealed good correlation with higher values between technical replicates than between biological replicates (Supplementary Figs. S2a, b and c).

3.2. Quantitative reverse transcriptase polymerase chain reaction

cfa-miR-200c, cfa-mi-R363, cfa-miR-146b, cfa-miR-105a, cfa-miR-20b and cfa-miR-885 were chosen for qPCR validation of the sequencing results because of their statistically significant changes between the groups. Results were analysed as fold change relative to expression in the Minimal group. qPCR results for cfa-miR-200c, cfa-miR-363 and cfa-miR-146b were concordant with the sequencing results. cfa-miR-200c was significantly downregulated (ANOVA $P = 0.0357$) in both severe IBD (log fold change 0.44) and T-cell lymphoma (log fold change 0.53) (Fig. 4a). cfa-miR-363 was significantly upregulated in severe IBD (log fold change 15.7648) but not significantly changed in T-cell lymphoma (log fold change 4.27) (ANOVA $P = 0.047$) (Fig. 4b). cfa-miR-146b was upregulated in both severe IBD and T-cell lymphoma but these results were not statistically significant ($P = 0.3$) (Fig. 4c). Results for cfa-miR-105a (Fig. 4d), cfa-miR-20b (Fig. 4e) and cfa-miR-885 (Fig. 4f) were discordant with the sequencing results but were not statistically significant (ANOVA $P = 0.716$, 0.36035 and 0.63232, respectively).

4. Discussion

IBD and intestinal T-cell lymphoma in dogs present with similar clinical signs and are often difficult to differentiate using histopathological examination alone. Molecular biomarkers are proposed as an adjunct to diagnosis. In this study, we have identified distinct miRNA expression patterns in normal/minimally inflamed canine duodenal tissue compared with cases of severe lymphoplasmacytic IBD and T-cell intestinal lymphoma. Specifically, our results show that (1) miRNA extracted from archived endoscopically derived FFPE tissues from the canine duodenum can be subjected to next-generation miRNA sequencing and (2) some sequencing results can be corroborated by qPCR. However, in our data, only three of the six miRNA primers used in qPCR validation were concordant with the sequencing results, two of which, cfa-miR-200c and cfa-miR-363, had statistically significant fold changes between groups in the qPCR data analysis. While a thorough discussion of discordant sequencing and qPCR results is beyond the scope of this paper, possible explanations include low miRNA concentration in the samples, incomplete reverse transcription, variable efficiency of primers and off target amplification or human error. Additionally, preanalytic variables such as length of time of fixation, storage conditions and time in archive may have affected the results. These variables are typical of stored FFPE tissues. Of the qPCR results that were discordant with the sequencing

Table 2
Results of genome sequencing data analysis for Lymphoma versus Minimal group after data were normalized and transformed to $\log_2$ counts per million (logCPM) using the R package 'DESeq2'

Genes	logFC	logCPM	F	*P*-value	FDR
cfa-miR-155	2.44435077	11.2306119	27.5366328	4.11E-05	0.00735495
cfa-miR-105a	1.93136698	2.38207791	27.1591515	4.46E-05	0.00735495
cfa-miR-146b	1.9724069	9.69401175	24.8788693	7.40E-05	0.00814279
cfa-miR-885	−1.3454342	9.01057795	23.605852	9.94E-05	0.00820449
cfa-miR-31	−1.0179749	8.55602132	19.7902607	0.0002555	0.01464183
cfa-miR-9	2.51112976	11.1918823	19.6324796	0.00026622	0.01464183
cfa-miR-802	−1.0661692	8.49603424	18.4154769	0.00036775	0.01733694
cfa-miR-200c	−1.009969	14.6843286	17.3862653	0.00048761	0.02011404
cfa-miR-21	0.80436811	17.8022814	15.7953373	0.00076731	0.02813484
cfa-miR-429	−0.9263072	10.303545	14.9475037	0.0009861	0.03070014
cfa-miR-141	−1.0751347	9.56618059	14.8241408	0.00102334	0.03070014
cfa-miR-142	1.23407825	8.94501174	14.0578641	0.00129264	0.03554768
cfa-miR-146a	1.57537254	13.4752811	12.8082674	0.001917	0.04866241

logFC, log2-transformed fold change in expression (relative to Minimal group); F, fold change; FDR, P-value after correcting for multiple tests (Bonferroni method).

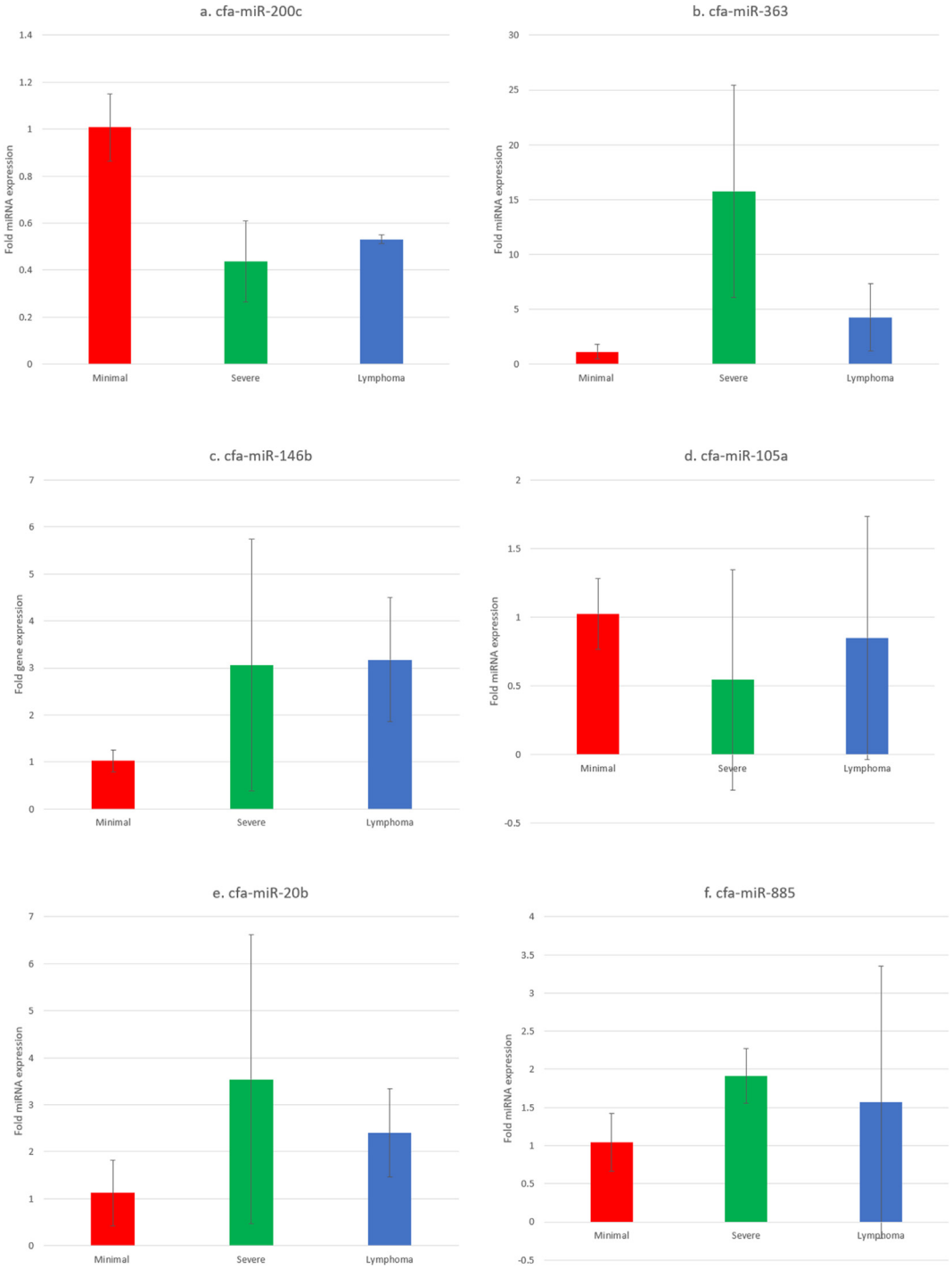

Fig. 4. Bar plots showing relative levels of miRNA expression from qPCR for selected canine miRNAs for validation of sequencing findings in Minimal, Severe and Lymphoma groups. Expression levels were normalized to the levels of the geometric mean of the U6 'housekeeping' gene using the delta–delta CT method. Values are presented as mean and standard deviation. Results are shown as fold change relative to expression in the Minimal group. cfa-miR-200c, cfa-miR-363, cfa-miR-146b are concordant with the sequencing results. **(a)** cfa-miR-200c is significantly downregulated in both severe inflammatory disease (IBD) and T-cell lymphoma (ANOVA, $P = 0.0357$). **(b)** cfa-miR-363 is significantly upregulated in severe IBD but not in T-cell lymphoma (ANOVA, $P = 0.047$). **(c)** cfa-miR-146b is upregulated in both severe IBD and T-cell lymphoma but not with statistical significance ($P = 0.3$). Results for cfa-miR-105a **(d)**, cfa-miR-20b **(e)** and cfa-miR-885 **(f)** are discordant with the sequencing results but are not statistically significant (ANOVA, $P = 0.716$, 0.36035 and 0.63232, respectively).

results, all three results were not statistically significant with large standard deviation suggesting erratic amplification.

We have shown that miRNA profiling can potentially be used to differentiate no/minimal inflammation from severe IBD and no/minimal inflammation from T-cell lymphoma. However, there was less clear differentiation when comparing candidate miRNAs for differentiation between severe IBD and T-cell lymphoma, although there were some evident differences in expression patterns between the three groups. Sequencing revealed 11 canine miRNAs identified as differentially expressed in the Severe versus Minimal comparison and 13 in the Lymphoma versus Minimal comparison. Eight miRNAs had increased expression in the Severe group and three had

decreased expression. Seven miRNAs had increased expression in the Lymphoma group and six had decreased expression. Two of the miRNAs overexpressed in the Lymphoma group, cfa-miR-9 and cfa-miR-155, have also been shown to be overexpressed in other lymphoma studies. Specifically, miR-9 was overexpressed in several human cancers and associated with invasive behaviour and spontaneous metastasis in canine mast cell tumours [27], and cfa-miR-155 has been shown to negatively correlate with survival in canine multicentric lymphoma [28]. cfa-miR-885 and cfa-miR-200c were downregulated in both the Severe and Lymphoma groups, and downregulation of cfa-miR-885 is correlated with nodal metastasis in canine mast cell tumours [29]. Interestingly, while no candidate miRNAs for distinguishing between severe IBD and T-cell lymphoma were confirmed by sequencing, when qPCR was performed with cfa-miR-363 it revealed a statistically significant increased expression in the Severe group, However, the increase in the Lymphoma group was much lower and not statistically significant, potentially indicating cfa-miR-363 as a candidate miRNA to differentiate between severe IBD and T-cell lymphoma in dogs. miR-363 is also overexpressed in peripheral blood of human patients with IBD [30]. In the second batch of samples sent for sequencing (Supplementary Fig. S1a) in our study, increased expression of cfa-miR-320 was found in the Minimal group compared with the Severe group, which is similar to what was found in the faeces of normal dogs when compared with dogs with gastrointestinal cancer and chronic enteropathy [20]. Further work towards the goal of an adjunctive diagnostic tool or non-invasive biomarker of canine intestinal T-cell lymphoma should aim to validate the source of miRNAs as the intestinal tissue in which inflammatory/neoplastic infiltrates reside, as performed in the current study, while simultaneously investigating miRNA signatures within faeces and serum.

5. Conclusion

In summary, we have demonstrated that miRNA can be retrieved from archived FFPE canine endoscopically derived samples at a quantity and quality appropriate for next generation sequencing. The results of the sequencing were reproducible, amenable to batch correction and could be corroborated by qPCR. Our results have identified potential candidate miRNA biomarkers for diagnosis and differentiation of severe IBD and T-cell lymphoma of the canine gastrointestinal tract.

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Declaration of competing interests

The authors declared no conflicts of interest with respect to the research, authorship or publication of this article.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jcpa.2023.03.186.

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