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Evaluation of serum miR-216a and miR-375 as biomarkers in dogs with acute pancreatitis

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Abstract

Background: Serum microRNAs have emerged as biomarkers of various diseases. Overexpression of serum miR-216a and miR-375 occurs in dogs with experimentally induced acute pancreatitis (AP).

Objectives: To identify the possibility of using serum miR-216a and miR-375 as biomarkers for the diagnosis and evaluation of treatment response in dogs with naturally occurring AP.

Animals: Twenty-one dogs with AP and 20 healthy dogs.

Methods: Cross-sectional study. The relative expression of serum hsa-miR-216a-5p, cfa-miR-216a, and cfa-miR-375 were analyzed using reverse transcription and real-time PCR.

Results: A significant difference in the serum expression of cfa-miR-375 was found between dogs with AP (median [interquartile range] 3.59 [1.55-24.52]-fold) and healthy dogs (0.81 [0.54-2.21]-fold, $P < .001$), and no significant differences were observed in hsa-miR-216a-5p and cfa-miR-216a ($P > .05$). The area under the receiver operating characteristic curve of serum cfa-miR-375 for differentiating between AP dogs and healthy dogs was 0.84 (95% confidence interval [CI]: 0.71-0.96). The expressions of hsa-miR-216a-5p and cfa-miR-375 were positively correlated with the concentrations of serum C-reactive protein ($r_s = .46$, $r_s = .48$, respectively), but not with the serum specific canine pancreatic lipase. The expression of cfa-miR-375 was significantly less after treatment in dogs with AP ($P = .02$).

Conclusions and Clinical Importance: Serum cfa-miR-375 could be a potential biomarker for the diagnosis and evaluation of treatment response of AP in dogs. In addition, miR-216a and miR-375 could be associated with inflammatory processes in dogs with AP.

KEYWORDS
canine, miRNA, pancreatic inflammation, serum biomarkers

Abbreviations: AP, acute pancreatitis; AUC, area under the curve; CI, confidence interval; CRP, C-reactive protein; Ct, cycle threshold; IQR, interquartile range; miRNAs, microRNAs; ROC, receiver operating characteristic; Spec cPL, specific canine pancreatic lipase.

1 | INTRODUCTION

Acute pancreatitis (AP) is the most common pancreatic disease in dogs and is an important differential diagnosis for dogs with signs of gastrointestinal disease such as acute vomiting and abdominal pain.[1-3] Clinical diagnosis of AP is generally based on clinical signs, abnormal laboratory findings, and abdominal imaging findings.[4,5] At present, the serum-specific canine pancreatic lipase (Spec cPL), which reflects the release of pancreatic lipase into the circulation caused by pancreatic acinar cell damage, is widely used for the diagnosis of AP in dogs. Although this test shows high sensitivity (71.7%-90.9%) and specificity (74.1%-88.8%),[6,7] false positives might occur in some circumstances; for example, Spec cPL might increase abnormally in 22.2% of dogs with an acute abdominal disease without AP,[8] 60% of dogs with hyperadrenocorticism,[9] and 45% of dogs with intervertebral disc disease without signs of gastrointestinal disease.[10] Therefore, an accurate and reliable blood biomarker for diagnosis of AP in dogs is needed.

MicroRNAs (miRNAs) are small and noncoding RNAs that control gene expression by binding to messenger RNAs.[11] They are tissue-specific and can be released into body fluids with tissue injury.[12,13] Additionally, miRNAs are particularly stable in serum, which makes them possible biomarkers for the diagnosis and monitoring of various diseases such as cancer, viral infections, and inflammation.[14] The miR-216a and miR-375 are abundant in the pancreatic tissue of dogs[15,16] and the expression of serum miR-216a and miR-375 significantly increased in response to pancreatic injury in dogs with cerulein-induced AP.[15-17] However, there has been no study evaluating the serum expression levels of these miRNAs in dogs with naturally occurring AP. Therefore, we sought to identify the possibility of using serum miRNAs, including hsa-miR-216a-5p, cfa-miR-216a, and cfa-miR-375, as biomarkers for the diagnosis and treatment response of AP in dogs.

The primary objective of this study was to compare the expression of selected miRNAs (hsa-miR-216a-5p, cfa-miR-216a, and cfa-miR-375) in the serum of dogs with AP and healthy dogs, and to evaluate the accuracy of these selected miRNAs in differentiating between dogs with AP and healthy dogs. Secondary objectives were: (i) to analyze the difference in serum levels of the aforementioned miRNAs in dogs with AP showing high clinical severity scores, those showing low clinical severity scores, and clinically healthy dogs; (ii) to evaluate the correlation between expression levels of these miRNAs and specific canine pancreatic lipase (Spec cPL), which is a conventional serum biomarker indicative of pancreatic injury, C-reactive protein (CRP), which is an indicator of acute inflammation, or clinical severity score; and (iii) to assess how the expression levels of these miRNAs are influenced by AP treatment.

2 | MATERIALS AND METHODS

2.1 | Study group

This cross-sectional study involved 21 client-owned dogs with AP and 20 client-owned clinically healthy dogs. Dogs with AP and healthy dogs visited our institution between May 2019 and April 2022. Acute pancreatitis was clinically diagnosed if all of the following enrollment criteria were met: (a) at least two of the following acute clinical signs (duration ≤3 days): abdominal pain, diarrhea, vomiting, and hyporexia[18]; (b) ultrasonographic findings consistent with AP, such as hypoechoic and enlarged pancreatic parenchyma with irregular margins and irregular shape, hyperechoic mesentery, or presence of localized abdominal fluid[19]; and (c) serum Spec cPL (IDEXX Reference Laboratory Inc., Westbrook, ME) concentrations >400 μg/L (RI, 0-200 μg/L).[7] For the healthy controls group, client-owned clinically healthy dogs which were presented for routine health examination, were included in the study based on the unremarkable findings on physical examination, CBC, serum biochemistry profiles, Spec cPL, serum electrolyte analysis, urinalysis, survey radiography, and abdominal ultrasonography. This study obtained ethics approval from the Ethics Committee of Chungbuk National University (CBNUA-1710-22-01).

2.2 | Clinical severity grouping

A clinical severity scoring scheme was applied based on the results of blood analyses and clinical signs at the first visit. The clinical severity score of AP evaluated nine variables (endocrine, hepatic, renal, hematopoietic, local complications, cardiac, respiratory, intestinal integrity, and vascular forces) according to a previously used method (Appendix S1)[20,21] with possible minimum and maximum total scores of 0 and 24, respectively. Dogs with AP were divided into two groups according to the total clinical severity score: Dogs with below 50% clinical severity scores were classified as the low clinical severity group (group L, n = 11), whereas dogs with above 50% were classified as the high clinical severity group (group H, n = 10). Group L had ≤5 points, while group H had ≥6 points.

2.3 | Post-treatment assessment

The expression of selected serum miRNAs after treatment (n = 9) was compared with that of before treatment. After treatment was defined as the resolution of clinical signs consistent with AP and normalization of serum Spec cPL concentrations (≤200 μg/L). After treatment samples were obtained at the time of discharge or the next follow-up examination, which was conducted 3 to 15 days after discharge. Dogs with AP were medically treated with a combination of the following: intravenous fluid therapy, analgesics, anti-emetics, antiacids, and nasogastric tube for enteral feeding.[22,23] Dogs with pyrexia and left-shifted neutropenia were treated with broad-spectrum antibiotics.[22,23]

2.4 | Serum collection and RNA extraction

Blood samples were carefully collected in serum-separating tubes from the jugular or cephalic veins. After the samples were centrifuged

at 2000*g* for 10 minutes, the serum was carefully separated and stored at −80°C within 1 hour of collection until measurement. Total RNA was extracted from the serum samples using commercial kits for RNA extraction (Qiagen, Valencia, CA) in accordance with the manufacturer's instructions. Briefly, RNA was isolated from 200 μL of serum using 1 mL lysis reagent and 200 μL chloroform. After centrifugation at 12 000*g* for 15 minutes at 4°C, the upper aqueous phase was transferred into a collection tube and mixed with 100% ethanol (1.5 mL). RNA was purified using a commercial RNA purification kit (Qiagen, Valencia, CA) and eluted in 14 μL RNase-free water.

2.5 | Reverse transcription and real-time quantitative PCR

After RNA extraction, cDNA was synthesized using the miRCURY LNA RT kit (Qiagen, Valencia, CA). Reverse transcription and real-time quantitative PCR (RT-qPCR) for relative quantification was conducted using the miRCURY LNA SYBR Green PCR Kit (Qiagen, Valencia, CA) and miRCURY LNA miRNA PCR assay (Qiagen, Valencia, CA). All analyses were performed using the StepOnePlus Real-Time PCR System (Applied Biosystems, Waltham, MA), and all samples were analyzed in triplicates. MicroRNA sequences for the study were: (a) hsa-miR-216a-5p (UAAUCUCAGCUGGCAACUGUGA), (b) cfa-miR-216a (UAAUCUCAGCUGGCAACUGUG), and (c) cfa-miR-375 (UUUGUUCGUUCGGCUCGCGUGA) and were chosen based on the current literature on experimentally induced pancreatic injury.[15-17] All samples were normalized to the synthetic spike-in control, cel-miR-39-3p (UCACCGGGUGUAAAUCAGCUUG). All processes were performed according to the manufacturer's instructions.

2.6 | Analysis of miRNA expression

The results of RT-qPCR were expressed as cycle threshold (Ct), which was determined as the second derivative maximum cycle and was calculated automatically. If miRNAs were not detected after 40 cycles of RT-qPCR, the Ct value was regarded as equivalent to 40.[24] The relative expression of each miRNA (fold change, $2^{-\Delta\Delta Ct}$) was normalized to the expression levels of the control gene (cel-miR-39-3p) according to the formula: ΔCt = Ct value of the target gene − Ct value of the control gene, ΔΔCt = ΔCt value of the target sample − average of ΔCt value of the control sample, and the estimated expression ratio = $2^{-\Delta\Delta Ct}$.[25]

2.7 | Statistical analysis

Statistical analyses were performed using the commercial statistical software Prism 6 (GraphPad Software Inc., La Jolla, CA). The Shapiro-Wilk test was performed to determine normal distributions. Statistical analyses were conducted using nonparametric tests, and data were expressed as median and interquartile range (IQR) because the data were not normally distributed. The Mann-Whitney *U*-test was used to compare miRNA expression levels between dogs with AP and healthy dogs. The correlation between the concentrations of CRP, Spec cPL, or clinical severity score and the expression of serum miRNAs was assessed using Spearman's correlation test. Wilcoxon signed-rank tests were performed to compare the expression of miRNAs before and after treatment. Receiver operating characteristic (ROC) curve analysis was used to assess the diagnostic utility of each miRNA sequence in differentiating between dogs with AP and healthy dogs. The diagnostic accuracy was assessed using the area under the curve (AUC) of the ROC curve. Sensitivity and specificity were calculated and the optimal cut-off was selected as the value with the highest Youden index (sensitivity + specific − 1).[26] *P* value <.05 was considered statistically significant.

TABLE 1 Study group characteristics and demographics

	Healthy dogs (n = 20)	AP dogs (n = 21)
Age (years)	6.1 (4.0-8.5)	10.7 (9.1-14.2)
Body weight (kg)	4.8 (4.1-9.5)	3.6 (3.0-7.0)
Sex (number), IF/SF/IM/CM	5/3/3/9	2/10/1/8
Spec cPL (ng/mL)	37.0 (30.0-59.0)	1236.0 (500.3-1970.0)
CRP (mg/L)	5.7 (5.0-6.9)	79.5 (45.6-186.9)
Clinical score	NA	5 (4-7)

Note: Data are expressed as medians (interquartile ranges).
Abbreviations: AP, acute pancreatitis; CM, castrated male; CRP, C-reactive protein; IF, intact female; IM, intact male; NA, not applicable; SF, spayed female; Spec cPL, specific canine pancreatic lipase.

3 | RESULTS

3.1 | Study group

The dogs with AP consisted of 6 Maltese, 3 Poodles, 2 Yorkshire terriers, 2 Chihuahuas, 2 mixed-breed dogs, and 1 each of Shih Tzu, Pomeranian, Dachshund, Beagle, Golden Retriever, and Jindo dogs. Healthy dogs consisted of 6 Maltese, 3 Poodles, 3 Pomeranian, 2 Cocker Spaniels, 2 mixed-breed dogs, and 1 each of Yorkshire terriers, Chihuahuas, Spitz, and Labrador Retriever. Other demographic characteristics, serum Spec cPL and CRP concentrations, and clinical severity scores of the studied dogs are presented in Table 1. The dogs with AP were significantly older than healthy dogs ($P < .001$). Of the 21 dogs with AP, 15 dogs survived and were discharged from the hospital and 6 dogs died. The median hospitalization time was 3 (IQR 2-7) days in dogs with AP.

3.2 | Comparison of expression level of serum miRNAs between dogs with AP and healthy controls

No significant differences were observed in the expression of serum hsa-miR-216a-5p (dogs with AP: median [IQR] fold change 2.37

[0.52-12.54], healthy dogs: median [IQR] fold change 0.89 [0.34-2.10], $P = .17$; Figure 1A) and cfa-miR-216a (dogs with AP: median [IQR] fold change 2.57 [0.31-22.17], healthy dogs: median [IQR] fold change 0.74 [0.18-2.13], $P = .31$; Figure 1B) between dogs with AP and healthy dogs. A significant difference in the expression of serum cfa-miR-375 was found between dogs with AP (median [IQR] fold change 3.59 [1.55-24.52]) and healthy dogs (median [IQR] fold change 0.81 [0.54-2.21], $P < .001$; Figure 1C).

3.3 | AUC of miRNAs to differentiate between dogs with AP and healthy controls

The ability of miRNAs to differentiate between dogs with AP and healthy dogs was assessed using ROC curves. The AUCs of hsa-miR-216a-5p, cfa-miR-216a, and cfa-miR-375 were 0.63 (95% confidence interval [CI] = 0.45-0.81), 0.59 (95% CI = 0.41-0.77), and 0.84 (95% CI = 0.71-0.96), respectively. Based on the Youden index, the optimal cut-off of cfa-miR-375 was a fold change of 1.06, with a sensitivity of 95.2% (95% CI = 76.2%-99.9%) and specificity of 70.0% (95% CI = 45.7%-88.1%; Figure 2). The cut-off cfa-miR-375 with the second and third highest Youden index was 1.59 and 2.91, respectively, with a sensitivity and specificity of 76.2% (95% CI = 52.8%-91.8%) and 75.0% (95% CI = 50.9%-91.3%), 61.9% (95% CI = 38.4%-81.9%), and 85.0% (95% CI = 62.1%-96.8%), respectively.

3.4 | Comparison of serum miRNA expression levels among dogs with AP and high clinical severity scores, those showing low clinical severity scores, and healthy dogs

Multiple comparisons were conducted to identify differences in the expression of serum miRNAs between dogs with AP showing high (group H, n = 10), low clinical severity scores (group L, n = 11) and healthy dogs (n = 20). In group H, the number of dogs showing clinical severity scores 6, 7, 8, and 9 was 1, 6, 2, and 1, respectively. In group L, the number of dogs representing the clinical severity scores 2, 3, 4, and 5 was 1, 3, 3, and 4, respectively. Group H had a case fatality of 50% (5 of 10) compared with 9.1% (1 of 11) in group L. The median time of hospitalization was 5 (IQR 3-7) days in group H and 3 (IQR 1-7) days in group L. The expression of serum hsa-miR-216a-5p in group H (median [IQR] fold change 12.54 [2.06-138.50]) was significantly different from that of group L (median [IQR] fold change 1.18 [0.16-4.33]; $P = .01$) and healthy controls (median [IQR] fold change 0.89 [0.34-2.10]; $P = .01$; Figure 3A), respectively. The expression of serum cfa-miR-216a in group H (median [IQR] fold change 16.51 [2.08-295.28]) was significantly different from that of group L (median [IQR] fold change 0.62 [0.08-3.71]; $P = .01$) and healthy dogs (median [IQR] fold change 0.74 [0.18-2.13]; $P = .04$; Figure 3B), respectively. However, there were no significant differences in the expression of hsa-miR-216a-5p and cfa-miR-216a between group L and healthy controls. When the expression of serum miR-375 was compared among the three groups, a significant difference was found only between group H (median [IQR] fold change 11.21 [3.45-165.94]) and healthy dogs (median [IQR] 0.81 [0.51-2.21]; $P < .001$; Figure 3C).

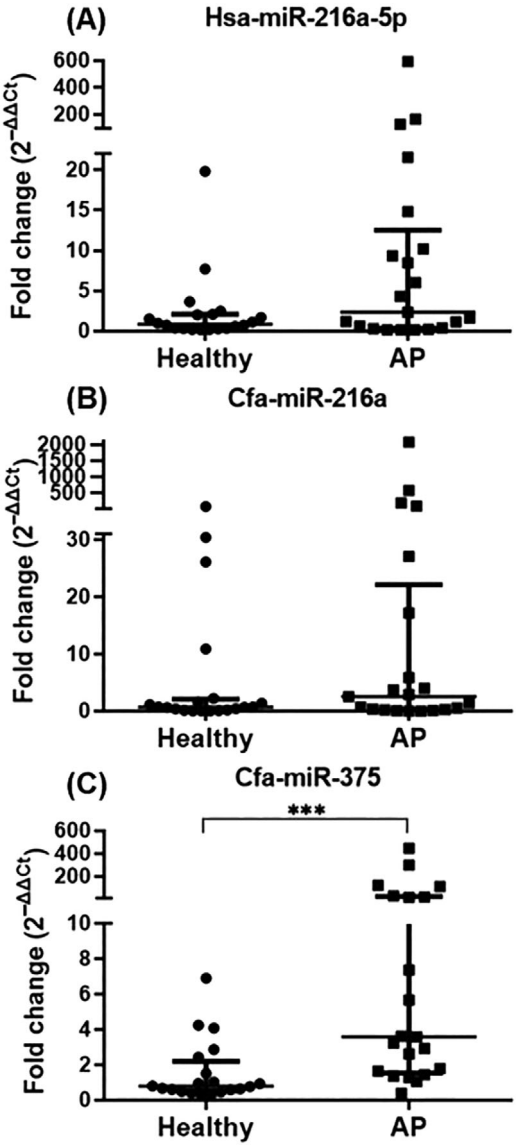

FIGURE 1 Scatterplot of expression levels of serum (A) hsa-miR-216a-5p ($P = .17$), (B) cfa-miR-216a ($P = .31$), and (C) cfa-miR-375 ($P < .001$) in healthy dogs (n = 20) and dogs with AP (n = 21). The horizontal bars show the medians and interquartile ranges from the first to the third quartiles. The Mann-Whitney *U* test. ***$P < .001$. AP, acute pancreatitis

3.5 | Correlation of miRNA expression with C-reactive protein, Spec cPL, and clinical severity scores

Serum CRP concentrations were positively correlated with the expression of serum hsa-miR-216a-5p ($r_s = .46$, 95% CI: 0.02-0.75, $P = .04$; Figure 4A) and cfa-miR-375 ($r_s = .48$, 95% CI: 0.05-0.76, $P = .03$; Figure 4C). No correlation was observed between serum CRP

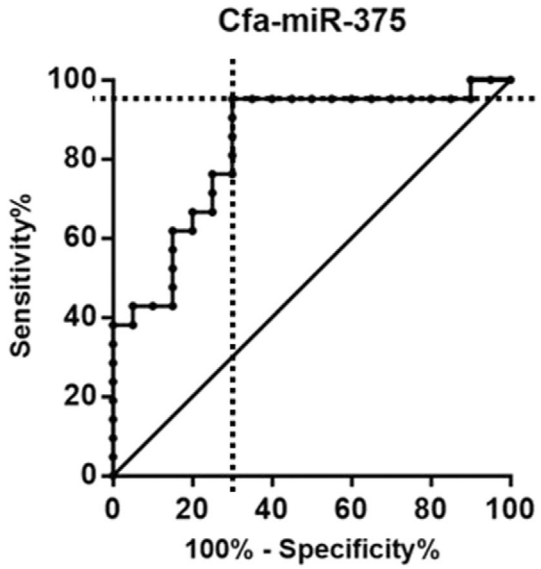

FIGURE 2 Receiver operating characteristic curve differentiating between dogs with AP (n = 21) and healthy dogs (n = 20) based on the expression levels of serum cfa-miR-375. The AUC was .84 (95% CI = 0.71-0.96). The point of intersection represents the optimal cut-off value of fold change 1.06, with a sensitivity of 95.2% (95% CI = 76.2%-99.9%) and specificity of 70.0% (95% CI = 45.7%-88.1%). AUC, area under the receiver operating characteristic curve; AP, acute pancreatitis; CI, confidence interval

concentrations and serum cfa-miR-216a expression (P = .09; Figure 4B). There was no correlation between Spec cPL concentration and the expression levels of hsa-miR-216a-5p (P = .07; Figure 4D), cfa-miR-216a (P = .26; Figure 4E), and cfa-miR-375 (P = .86; Figure 4F). The clinical severity score was positively correlated with the expression levels of serum hsa-miR-216a-5p (r_s = .64, 95% CI: 0.28-0.85, P = .002; Figure 4G), cfa-miR-216a (r_s = .51, 95% CI: 0.09-0.78, P = .02; Figure 4H), and cfa-miR-375 (r_s = .45, 95% CI: 0.01-0.75, P = .04; Figure 4I).

3.6 | Comparison of pre- and post-treatment serum miRNA expression levels in dogs with AP

No significant difference in the expression of serum hsa-miR-216a-5p (P = .2; Figure 5A) and cfa-miR-216a (P = .16; Figure 5B) was found between pre- and post-treatment (n = 9). However, a significant difference in expression of serum cfa-miR-375 was observed between before (median [IQR] fold change 3.59 [2.15-71.29]) and after treatment (median [IQR] fold change 1.03 [0.65-1.67]; P = .02; Figure 5C) in dogs with AP.

4 | DISCUSSION

Our study showed that the expression level of serum cfa-miR-375 differed significantly between dogs with AP and healthy dogs. The area

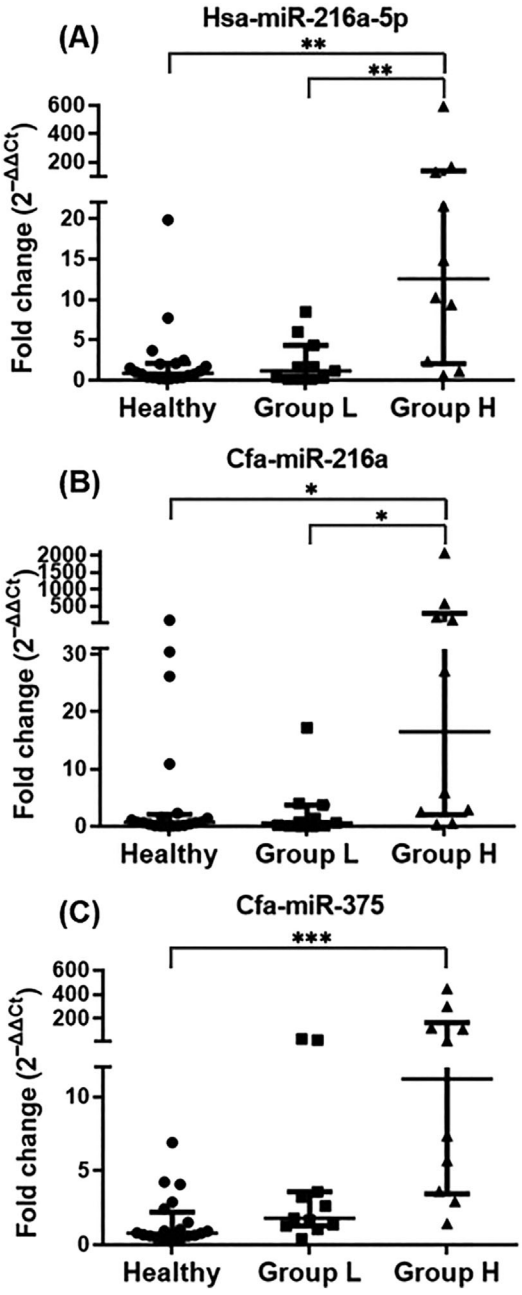

FIGURE 3 Scatterplot of expression levels of serum (A) hsa-miR-216a-5p, (B) cfa-miR-216a, and (C) cfa-miR-375 in healthy dogs (n = 20) and AP dogs with high (Group H, n = 10) and low (Group L, n = 11) clinical severity scores. The horizontal bars show the medians and interquartile ranges from the first to the third quartiles. The Kruskal-Wallis test with Dunn's multiple comparison test. *P < .05, **P < .01, ***P < .001. AP, acute pancreatitis

under the receiver operating characteristic curve of serum cfa-miR-375 for differentiating between AP dogs and healthy dogs was 0.84, and the optimal cut-off of cfa-miR-375-fold change was 1.06, with a sensitivity of 95.2% and specificity of 70.0%. These findings suggested that cfa-miR-375 could be a potential biomarker in dogs for the diagnosis of naturally occurring AP. In addition, serum expressions of hsa-miR-216a-5p and cfa-miR-216a were higher in dogs with AP

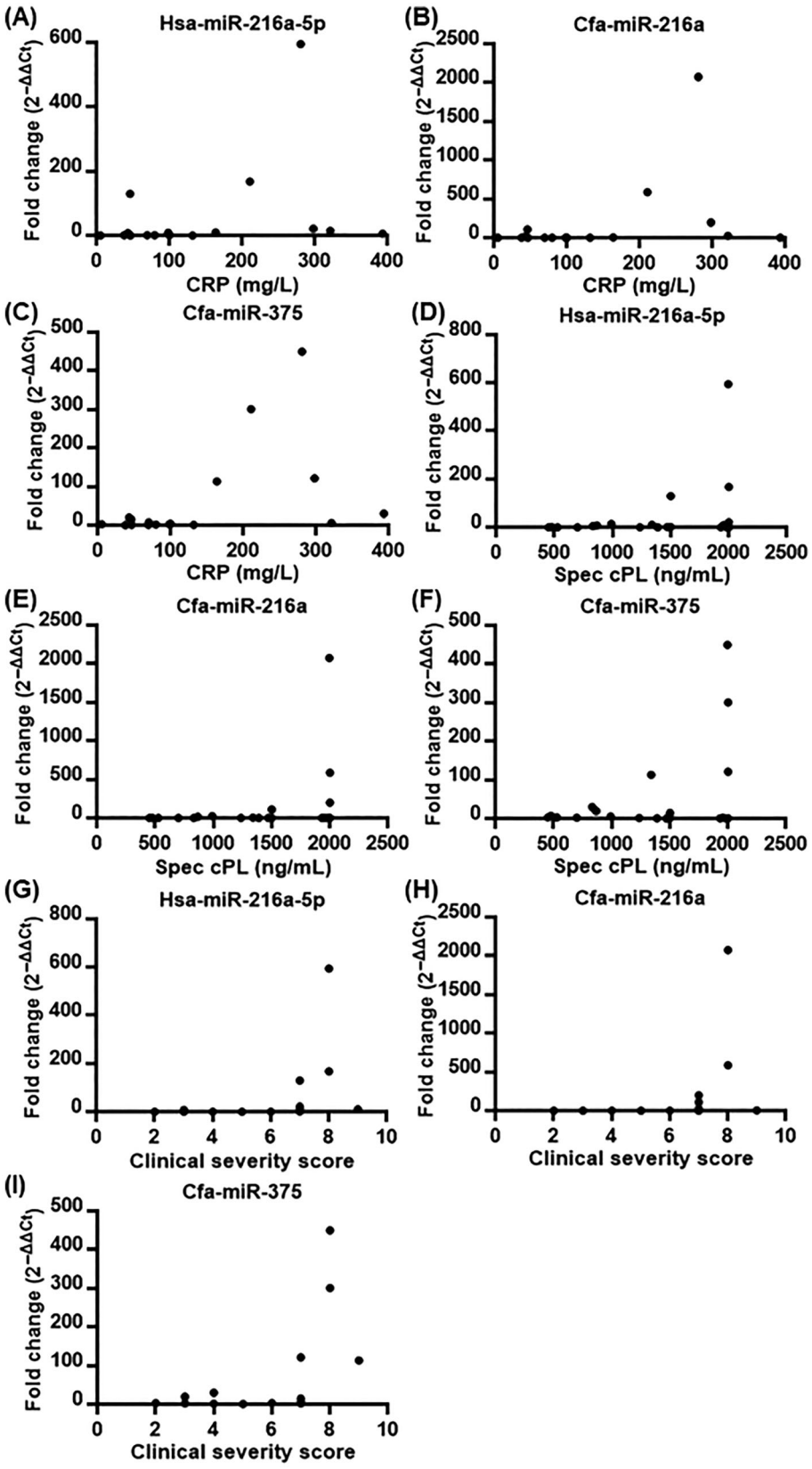

FIGURE 4 Correlations between concentrations of serum CRP and expression of serum miRNAs [(A) hsa-miR-216a-5p ($r_s = .46$, $P = .04$), (B) cfa-miR-216a ($r_s = .38$, $P = .09$), (C) cfa-miR-375 ($r_s = .48$, $P = .03$)], correlations between concentrations of serum Spec cPL and expression of serum miRNAs [(D) hsa-miR-216a-5p ($r_s = .40$, $P = .07$), (E) cfa-miR-216a ($r_s = .26$, $P = .26$), (F) cfa-miR-375 ($r_s = .04$, $P = .86$)], and correlations between clinical severity score and expression of serum miRNAs [(G) hsa-miR-216a-5p ($r_s = .64$, $P = .002$), (H) cfa-miR-216a ($r_s = .51$, $P = .02$), (I) cfa-miR-375 ($r_s = .45$, $P = .04$)] in dogs with AP. The Spearman's rank test. AP, acute pancreatitis; CRP, C-reactive protein; miRNA, microRNA; Spec cPL, specific canine pancreatic lipase

and a high clinical severity than in dogs with AP and a low clinical severity, implying that these miRNAs could be biomarkers for the severity of AP in dogs.

In this study, the expression of selected circulating miRNAs was analyzed in dogs with AP. The criteria for selection of specific miRNAs were pancreatic specificity, serum abundance of dogs with

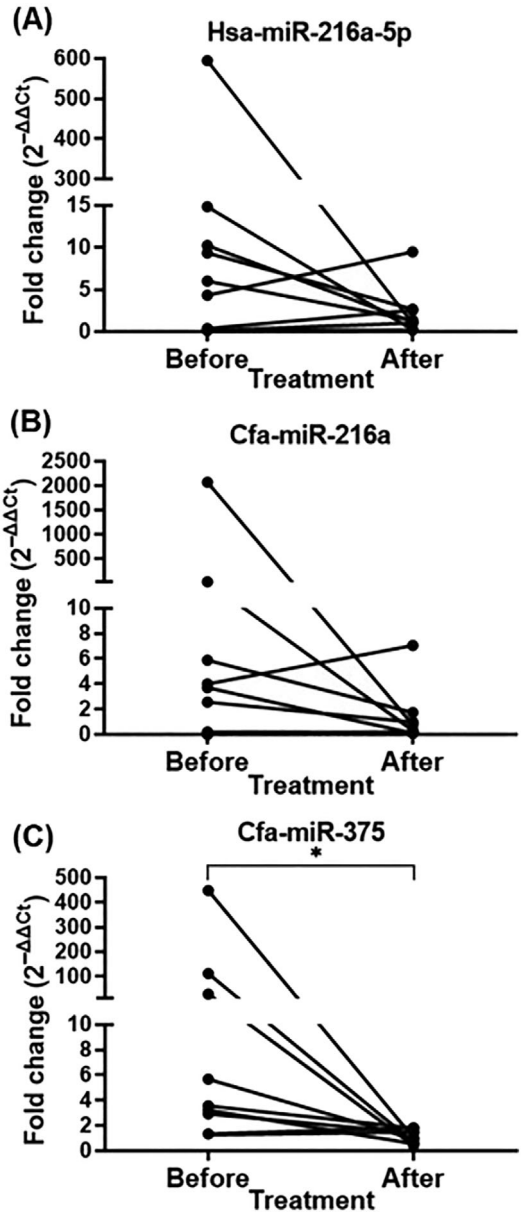

FIGURE 5 Comparisons of expression levels of serum (A) hsa-miR-216a-5p ($P = .20$), (B) cfa-miR-216a ($P = .16$), and (C) cfa-miR-375 ($P = .02$) between before and after treatment in dogs with AP (n = 9). Wilcoxon-signed rank sum test. *$P < .05$. AP, acute pancreatitis; miRNA, microRNA

experimentally induced AP,[15-17] and availability of commercial primers. Taking these criteria into consideration, we selected 3 miRNAs: hsa-miR-216a-5p, cfa-miR-216a, and cfa-miR-375. Hsa-miR-216a-5p, 1 of human miRNAs, shares homology to the cfa-miR-216a of canine miRNAs.[16,27] In the pancreas of dogs, the most abundant isomer of miR-216a was hsa-miR-216a-5p, whereas cfa-miR-216a were present at somewhat lower levels.[16] Our study included both hsa-miR-216a-5p and cfa-miR-216a because there was no previous study in which of the isomers of miR-216a was more abundant in serum of dogs. Expressions of serum hsa-miR-216a-5p and cfa-miR-216a were not detected in 23.8% (5/21) and 19.0% (4/21) of dogs with AP, and 45.0% (9/20) and 30.0% (6/20) of healthy dogs, respectively (data not shown). In contrast, the expression of serum miR-375 was detected in all dogs with AP and in healthy dogs in this study. In this study, cel-miR-39-3p was chosen as spike-in control as it has been used in many studies regarding the expression of serum miRNAs in dogs; additionally, the primer for cel-miR-39-3p is commercially available.[28-30]

MiR-375 is highly expressed in the pancreatic islets of Langerhans and regulates beta cell function.[16,31,32] The results of our study are in line with those of a previous report on upregulated serum miR-375 compared with controls in murine and canine models of AP.[15,17] Receiver operating characteristic curve analyses in our study supported that expression of serum miR-375 could be a biomarker for the diagnosis of AP in dogs. The optimal cut-off for serum miR-375 expression was a fold-change of 1.06, as it exhibited the highest sensitivity (95.2%) and specificity (70.0%) for AP in dogs. This information suggests that serum miR-375 expression could be a useful screening tool for detecting AP in dogs; however, the 1.06-fold cut-off might be small to be used clinically. Cut-off values with the second and third highest Youden index (1.59- and 2.91-fold, respectively) might be clinically useful to differentiate dogs with AP. However, caution should be used when strictly applying the miR-375 cut-off to differentiate between dogs with AP and healthy dogs because this study included a small sample size.

In this study, after treatment expression of serum miR-375 showed a significant decrease compared with pre-treatment with AP, suggesting the potential role of cfa-miR-375 in monitoring the treatment response of dogs with naturally occurring AP. Several studies of dogs with cerulein-induced AP analyzed the expression levels of circulating miR-375 at multiple time points after the administration of cerulein.[15-17] Serum levels of miR-375 increased within 30 to 60 minutes of administration of cerulein,[16,17] peaked at 4 to 8 hours,[15,17] and decreased close to baseline until approximately 24 hours.[15,17] In other words, miR-375 increased rapidly in pancreatic injury and showed a gradual decrease in the absence of additional damage to the pancreas, suggesting its potential as a useful biomarker for evaluating the response to treatment. Unfortunately, serial measurements of serum miRNA levels during hospital management were not performed in this study. Additional studies involving larger number of dogs and serial measurements of serum levels of miRNA over time in hospitalized dogs are necessary to identify the alterations of serum levels of miRNAs in naturally occurring AP, and its usefulness in a clinical setting.

MiR-216a is predominantly expressed in acinar cells, which make up ~90% of pancreatic tissue[27] and play a key role in the exocrine pancreatic function.[16] Unlike a previous report documenting increased serum miRNA-216a expression in canine models of AP,[15-17] there was no significant difference in serum miRNA-216a expression between the AP group and healthy controls of the present study. However, a significant difference in miR-216a expression was found between healthy dogs and dogs with AP showing high clinical severity scores, and between dogs with AP showing low and those showing high clinical severity scores. This result is consistent with the result that the expression of serum miR-216a was significantly upregulated in human patients with severe AP compared with mild or moderate AP.[33]

Therefore, the results of our study suggest that serum expression of miR-216a could be a potential marker for evaluating the severity of AP in dogs and could thus be markers for severe AP.

Traditionally, overexpression of miR-216a and miR-375 in the serum of dogs with pancreatic injury was thought to be caused by direct leakage from the damaged pancreatic tissue. In a study of canine models of AP, the expressions of serum miR-216 and miR-375 were positively correlated with the degree of histopathological pancreatic injury, which support this assumption.[17] However, another study of murine models of AP showed no correlation between the expression of serum miR-216a and the degree of histopathologic necrotic injury, which contradicts the assumption.[33] Interestingly in our study, the expressions of serum hsa-miR-216a-5p and miR-375 were positively correlated with the concentrations of serum CRP, an inflammatory marker, but not with the concentrations of serum Spec cPL, a leakage marker. Therefore, overexpression of serum hsa-miR-216a-5p and miR-375 could not be entirely explained by leakage from the pancreatic tissue, and there was a possibility of involvement of factors other than cell leakage, such as inflammation. Recent studies have provided evidence to support that miR-216a is involved in regulation of the inflammatory process.[34-37] Transforming growth factor-β, a multifunctional cytokine, induced up-regulation of miR-216a, which is exacerbated AP in mice.[36] Furthermore, miR-216a-5p regulates the expression of the FOS transcription factor complex, which has been a modulator in the inflammatory process.[37] In addition, a study of murine models of AP revealed that miR-375 could accelerate inflammation and apoptosis of pancreatic acinar cells by regulating autophagy-related protein 7 (ATG7), which is a gene encoding protein involved in autophagy.[38] Our results are similar to previous studies[33,37,38] supporting a potential pathophysiological link between the inflammatory process in canine AP and the expression of both miR-216a and miR-375. Further studies might provide additional insight into the pathophysiology of canine AP.

Our study had several limitations. First, the sample size of each group was small. The negative findings of our study might have been because of a type II error caused by the small sample size. Second, the healthy dogs in the present study were younger than dogs with AP. Unlike some miRNAs related with aging,[39,40] there are no reports of associations between the age and miR-216a or miR-375. However, the unmatched ages of the two groups can potentially affect the results; thus, studies using healthy and AP dogs of similar ages would be necessary to minimize this limitation. Additionally, our study group was skewed toward small breed dogs. Third, our study did not compare the utility of serum miRNAs for the diagnosis of AP with that of serum Spec cPL. As Spec cPL was included in the diagnostic criteria of this study, comparison of the diagnostic utility of miRNAs and Spec cPL for AP was not performed. Further studies including dogs with a definitive diagnosis of AP based on the histopathological examinations are needed to compare the diagnostic utility of these two assays. Fourth, although all dogs with AP had acute clinical signs (duration ≤3 days), the presentation to the hospital for consultation after the onset of clinical signs was variable (few hours to 3 days); therefore, pancreatic damage might have already resolved by the time of collecting serum, which might have contributed to the lack of differences in miRNAs expression. Finally, based on our results, miRNAs could be useful in differentiating healthy dogs from dogs with AP. However, it could be more important to clinically distinguish dogs with acute signs of gastrointestinal disease caused by AP from other nonpancreatic acute gastrointestinal diseases, which would provide more meaningful information about miRNAs in clinical use.

In conclusion, serum cfa-miR-375 could potentially be a biomarker for diagnosing and evaluating the treatment response to AP in dogs. Furthermore, the expression of serum hsa-miR-216a-5p and cfa-miR-375 were positively correlated with serum CRP concentration, unlike serum Spec cPL concentration. These results suggest that miR-216a and miR-375 could potentially be associated with inflammatory processes in canine AP, and additional studies are necessary to identify the potential roles of miRNAs in inflammatory regulation in dogs with AP.

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CONFLICT OF INTEREST DECLARATION

Authors declare no conflict of interest.

OFF-LABEL ANTIMICROBIAL DECLARATION

Authors declare no off-label use of antimicrobials.

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE (IACUC) OR OTHER APPROVAL DECLARATION

Approved by the Chungbuk National University Animal Care Committee and was carried out according to the Guide for Care and Use of Animals (Chungbuk National University Animal Care Committee, CBNUA-1710-22-01).

HUMAN ETHICS APPROVAL DECLARATION

Authors declare human ethics approval was not needed for this study.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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