Screening for circulating miR-208a and -b in different cardiac arrhythmias of dogs

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Abstract

Introduction: In recent years, the high sensitivity and specificity of novel miRNA biomarkers have been utilised for early diagnosis and treatment monitoring of various diseases. Previous reports showed that abnormal expression of miR-208 in mice resulted in the development of an aberrant cardiac conduction system and consecutive arrhythmias. On the other hand, a study on infarcted human heart tissue showed upregulation of miR-208a in subjects with ventricular tachyarrhythmias compared to healthy controls. We prospectively investigated the expression of miR-208a and -208b in the serum of dogs presenting different cardiac arrhythmias. **Material and Methods:** A total of 28 dogs with atrial fibrillation (n = 8), ventricular premature contractions (n=6), conduction system disturbances (n = 7), and free of heart conditions (as controls) (n = 7) were enrolled in the study. Total RNA was extracted from serum samples and miR-208a and -b, miR-16 as well as a cel-miR-39-5p spike-in were analysed with qPCR and ddPCR. **Results:** miR-208a and miR-208b were not expressed in any of the samples. The calculated ddPCR miR-16 relative expression (normalised with cel-miR-39 spike-in) showed a good correlation (r = 0.82; P < 0.001) with the qPCR results. **Conclusion:** This outcome warrants further investigation, possibly focusing on tissue expression of miR-208 in the canine heart.

Keywords: dogs, heart, arrhythmias, biomarkers, microRNAs.

Introduction

Heart rhythm disorders are a serious clinical problem in dogs, often causing medical symptoms which are worrisome for the owner. They are reported in a significant percentage of dogs with organic heart diseases such as mitral valve disease (MVD) and dilated cardiomyopathy (DCM) as well as in those with congenital heart defects. The development of tachycardiomiopathy in dogs with cardiac arrhythmias such as focal atrial tachycardia (FAT), atrioventricular reentrant tachycardia (AVRT), or atrial fibrillation has also been documented (9). Atrial fibrillation and ventricular premature complexes are among the most common arrhythmias in dogs (10).

MicroRNAs (miRNA) are a class of small (~22 nucleotides in length) noncoding RNA molecules functioning as post-transcriptional gene regulators. They act by binding to the 3'-untranslated region (UTR) of target mRNA attenuating gene expression and, eventually, protein translation (1). MiRNAs participate in various biological processes such as cell differentiation, proliferation, metabolism, and apoptosis.

In recent years, there has been an emergence of highly sensitive and specific novel miRNA biomarkers utilised for the early diagnosis or treatment monitoring of various diseases. MiRNA expression can be evaluated in tissues, plasma/serum, and other body fluids. Circulating miRNAs have proven biostability in mammalian blood, even in unfavourable conditions (8). Unlike synthetic miRNA, serum miRNAs were found to be protected from endogenous RNase activity. This is due to their association with lipid (microvesicles, exosomes), protein, or lipoprotein (HDL and LDL cholesterol) complexes (14).

The role of miRNAs has been studied extensively in numerous cardiovascular pathologies including atherosclerosis, coronary artery disease, myocardial infarction, chronic heart failure, and cardiac arrhythmias (6). Of numerous miRNA families, the miR-208 family is one of the most important heart-enriched miRNAs involved in the pathogenesis of various cardiac diseases.

MiRNA-208a has been found to influence the development of the conduction system, while its dysregulation has been associated with atrial fibrillation and atrioventricular blocks (3). In a study conducted on human patients after myocardial infarction, the upregulation of miRNA-208a correlated with ventricular tachyarrhythmias (2).

In contrast to human medicine, there is little evidence on the usefulness of circulating miRNAs as novel biomarkers in canine cardiovascular diseases. Available studies have assessed the expression of miRNAs in the serum of Doberman Pinschers with dilated cardiomyopathy and in the plasma of dachshunds with different stages of chronic degenerative valvular disease (5, 13). To the best of the authors' knowledge, this is the first study which evaluates microRNAs in cardiac arrhythmias in dogs. Furthermore, this is one of the few canine-focused studies utilising ddPCR, which has been proved to have superior diagnostic performance when used in the analysis of serum microRNA biomarkers compared to qPCR. The main advantages of ddPCR over qPCR are partitioning of single PCR reactions before amplification into thousands of droplets and end-point data acquisition, which allow for absolute quantification of target DNA or cDNA copy number without the need of standard curve preparation and minimises the influence of sample-to-sample PCR efficiency differences on the data. In addition to that, the ddPCR was shown to be more reproducible and precise when used for the measurement of low abundant targets (4).

The aim of this study was to evaluate the miRNA-208 family expression in the serum of dogs with heart rhythm disturbances.

Material and Methods

A total of 28 dogs were prospectively enrolled in the study. All the dogs underwent a clinical, electrocardiographic (BTL-08, BTL Industries, U.K.), and echocardiographic (Aloka F37, Hitachi, Japan) evaluation and were assigned to groups by specific affliction: of atrial fibrillation (n = 8), numerous ventricular premature contractions (VPC) (n = 6), advanced 2nd and 3rd atrioventricular block (n = 7), and controls with healthy hearts (n = 7). Detailed study population characteristics are presented in Table 1.

Blood samples were drawn from the cephalic vein into plain sample tubes, using a 21-gauge needle. All the dogs were fasted at least 12 h prior to blood collection. Samples were allowed to clot for approximately 30 min at room temperature and immediately centrifuged at $3,000 \times g$ for 15 min. The serum was separated and used in routine blood tests, the remaining part being frozen at −80°C in 200 μL aliquots until miRNA screening.

Total RNA isolation was performed using the MagMAX mirVana Total RNA Isolation Kit (Thermo Fisher Scientific, USA) according to the manufacturer's protocol with minor modifications. Samples were thawed on ice and centrifuged at $16,000 \times g$ for 5 min at 4°C to remove cellular nucleic acids attached to cell debris. A 100 μL serum sample was transferred to a deep well plate (Thermo Fisher Scientific) and was digested with proteinase K at 65°C for 30 min. A 100 μL sample of a lysis binding mix containing an additional 3.5 μL of synthetic *Caenorhabditis elegans* cel-miR-39-3p at a concentration of 1.6×10^8 copies/μL from the Qiagen (Germany) miRNeasy serum/plasma spike-in control was added to the sample. A total of 20 μL of RNA Binding Beads (Thermo Fisher Scientific) was then added, and the sample was mixed. The spike-in was added to measure the RNA isolation efficiency. An equal volume of isopropanol (Chempur, Poland) was added to promote the binding of the RNA to the magnetic beads. With the use of the magnetic stand, beads were washed and DNA was digested using the TURBO DNase Solution (Thermo Fisher Scientific) (15 min, room temperature). Subsequent steps were performed according to the manufacturer's protocol. RNA was eluted with 50 μL of a preheated elution buffer.

cDNA was synthesised using the TaqMan MicroRNA Reverse Transcription Kit (Thermo Fisher Scientific) according to the modified supplementary Protocol for Creating Custom RT and Preamplification Pools utilising TaqMan MicroRNA Assays (Applied Biosystems, USA). Each reverse transcription (RT) reaction contained 0.75 μL of 4 TaqMan MicroRNA 5x RT Primer (miR-208, miR-208b, cel-miR-39-5p, and miR-16), 0.3 μL of 100 mM dNTPs, 3 μL of multiscribe reverse transcriptase (50U/μL), 1.5 of 10 × RT buffer, 0.19 μL of RNase inhibitor (20U/μL), 2.01 μL of nuclease-free water, and 5 μL of the RNA sample. The complete reaction mix was incubated on ice for 5 min and then underwent thermocycling (16°C for 30 min, 42°C for 30 min, 85°C for 5 min, and 4°C for ∞). The cDNA was stored at −20°C until use.

Quantitative PCR was performed to approximate the miR-208, miR-208b, cel-miR-39-5p, and miR-16 concentrations in the samples before the droplet digital PCR (ddPCR). Each 10 μL reaction on a 96-well plate (Bio-Rad, USA) contained 0.5 μL 20 × TaqMan MicroRNA Assay (Thermo Fisher Scientific), 2 μL 4 × diluted cDNA in nuclease-free water, 5 μL of TaqMan™ Universal PCR Master Mix, No AmpErase™ UNG (2x) (Thermo Fisher Scientific), and 2.5 μL of nuclease-free water. The reaction was conducted on the CFX Connect™ Real-Time PCR Detection System (Bio-Rad, USA) in accordance with the TaqMan Small RNA Assay Protocol (Applied Biosystems).

The ddPCR was performed using the QX200 Droplet Digital PCR System (Bio-Rad). Before the reaction, the cDNA was diluted 4 × in nuclease-free water. The reaction mixture contained 10 μL of ddPCR Supermix for probes (No dUTP), 1 μL of the TaqMan MicroRNA Assay (Thermo Fisher Scientific), 4 μL of nuclease-free water, and 5 μL of diluted cDNA.

Table 1. Detailed characteristics of the study group

Study group	Breed	Age (year)	Weight (kg)	Sex	Others
healthy	boxer	5.5	27.4	M	-
healthy	beagle	2.4	12.5	F	-
healthy	fox terrier	9.2	15.4	M	-
healthy	beagle	2.7	12	M	-
healthy	beagle	4.4	11.6	M	-
healthy	mix	8.9	13.9	M	-
healthy	boxer	8.2	24.4	F	-
n = 7	mean:	5.9	16.74	-	-
atrial fibrillation	Labrador retriever	9.3	45	M	tachycardiomyopathy
atrial fibrillation	Labrador retriever	12.8	50	M	tachycardiomyopathy
atrial fibrillation	Rhodesian ridgeback	5.5	55	M	DCM
atrial fibrillation	dogue de Bordeaux	10.6	46	M	tachycardiomyopathy
atrial fibrillation	dachshund	13.9	10.8	M	mitral valve disease ACVIM Dc
atrial fibrillation	German shepherd	2.4	74	M	tachycardiomyopathy
atrial fibrillation	German shepherd	9.5	46.7	F	CHF secondary to PDA
atrial fibrillation	German shepherd	8.2	48.5	M	DCM
n = 8	mean:	9.03	47	-	-
VPC	Amstaff	10.8	30	M	no primary heart disease, hypothyroidism
VPC	Labrador retriever	14.3	35	M	no primary heart disease
VPC	boxer	13.4	29.8	F	arrhythmogenic right ventricular cardiomyopathy
VPC	boxer	5	25.6	M	-
VPC	mix	11.1	7.8	F	mitral valve disease ACVIM Dc
VPC	boxer	8.5	30.5	F	-
n = 6	mean:	10.52	26.45	-	-
III* AVB	Dalmatian	13.6	27	F	-
III* AVB	Labrador retriever	5.7	29.7	F	-
III* AVB	mix	14.3	14.6	M	-
III* AVB	boxer	10.8	27.6	M	-
II* AVB	shih-tzu	13.8	8	F	mitral valve disease ACVIM Cc
II* AVB	Saarloos wolfdog	2.5	39	M	-
tachy-brady syndrome	miniature schnauzer	9.8	7.4	F	-
n = 7	mean:	10.07	21.9	-	-

DCM – dilated cardiomyopathy, ACVIM – American College of Veterinary Internal Medicine classification for mitral valve disease, CHF – chronic heart failure, PDA – patent ductus arteriosus, AVB – atrioventricular block

The droplet generator cartridge (Bio-Rad) sample wells were filled with 20 μL of each ddPCR reaction, and 70 μL of the droplet generation oil for probes (Bio-Rad) was loaded into the oil wells. The reaction volume was divided into droplets using the QX200 droplet generator (Bio-Rad). The emulsion (40 μL) was carefully transferred to a 96-well PCR plate (Bio-Rad) with a multichannel pipette and heat-sealed with aluminium foil. Forty PCR cycles were conducted in the T100 Thermocycler (Bio-Rad) according to the manufacturer's two-step protocol, using a ramp rate of 2°C/s and an annealing/extension temperature of 60°C, as well as final enzyme inactivation at 98°C for 10 min. The no template control (NTC) and no reverse transcription control (NRT) were included for each assay. Single ddPCRs were prepared for each sample and miRNA. The data were accrued with a QX200 droplet reader and Quantasoft software (Bio-Rad).

The choice of miR-16 as a reference miRNA was based on several reports showing its stable level in human and dog serum both in healthy individuals and those with cardiovascular disease (5, 12).

Statistica for Windows, version 10.0 (StatSoft, USA) was used to analyse the data. The data were compared using the two-proportion Z test and chi-square independence test. The differences in the expression of miRNA between the study groups were analysed using the Kruskal–Wallis test. A Spearman's rank correlation test was used to assess the relationship between two sets of data. A P-value below 0.05 was considered to be statistically significant. The results are presented as mean ±SEM.

Results

Circulating blood miR-208a, miR-208b, miR-16 (as a reference miRNA), and synthetic cel-miR-39-5p as a spike-in control were measured. Based on the assessment of the ddPCR cel-miR-39, the average RNA isolation efficiency was $59.9 \pm 4.3\%$. One sample was excluded from the analysis due to low RNA isolation efficiency (<10%). The average Ct (threshold cycle number) for miR-16 was 24.63 ± 0.21 and for cel-miR-39-5p was 25.97 ± 0.21. The average number of copies per 20 μL of the ddPCR reaction was $44,598.52 \pm 5,022.16$ for miR-16 and $27,916.15 \pm 2,012.21$ for cel-miR-39-5p. No expression of either miR-208a or miR-208b was found. There were no statistically significant differences in the mean levels of cel-miR-39-5p, miR-16, or absolute serum miR-16 concentrations among the study groups (Fig. 1).
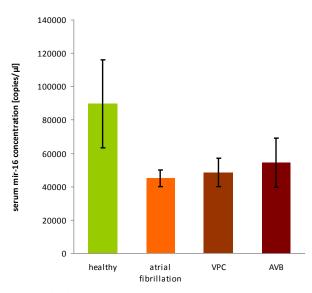
Fig. 1. Absolute serum miR-16 concentration in copies per microlitre normalised for RNA extraction efficiency based on the measured cel-miR-39 concentration (mean ±SEM)

Each ddPCR contained an amount of RNA extracted from 0.833 μL of canine serum, and in consequence of this the average number of miR-16 copies in the serum was estimated to be 58,964.10 ± 8,242.4 miRNA/μL. Amplification in the no template control and no reverse transcription samples was not observed for any target. The calculated ddPCR miR-16 relative expression (normalised with spike-in cel-miR-39) showed a good correlation (r = 0.82; P < 0.001) with the qPCR results.

Discussion

The study included 28 dogs presenting a number of cardiac rhythm disturbances, both tachy- and bradyarrhythmias. Some of those rhythm disturbances were primary in nature, while others resulted from ongoing primary heart disease such as dilated cardiomyopathy, mitral valve disease, or chronic heart failure secondary to a congenital heart defect. Therefore, the study group presented a wide variety of frequent cardiac diseases. MiR-208a and -b were not expressed in any of the samples. Thus, the results are in accordance with previous studies and support the finding that the miR-208 family is present in canine plasma neither in mitral valve disease (MVD) nor in numerous cardiac dysrhythmias or, possibly, other cardiac diseases (5). The authors of a previous study argued that the lack of plasma miR-208 expression may be due to differences in the aetiopathogenesis of MVD between dogs and humans. However, different aetiopathogenesis cannot explain the results of this study as the mechanisms behind arrhythmogenesis are identical in humans and dogs. This outcome warrants further investigation, possibly focusing on tissue expression of miR-208 in the canine heart.

One of the major difficulties in studies assessing circulating miRNA is its low abundance. Currently, qPCR is considered the gold standard for examining low level miRNA expression. In this study, a ddPCR was used, which proved to be of equal sensitivity and even higher precision and reproducibility than qPCR (4). Nevertheless, we were unable to detect circulating miR-208 family members in the canine serum samples. We hypothesise that one of the reasons for this outcome could be insufficient and variable miRNA isolation efficiency. This observation suggests that the development of new improved methodologies for isolation of circulating nucleic acid would expedite miRNA biomarker screening and discovery.

There is no consensus in the scientific community about the best normalisation strategy for analysis of circulating miRNA. There is growing support for the use of the mean expression value as the best normalisation strategy in microarray experiments measuring the whole miRNA profile. On the other hand, small-scale more sensitive PCR-based detection methods lack a similar precise and straight-forward normalisation approach (7). As in qPCR mRNA expression studies, the best system should include several stably expressed miRNAs. However, there are considerable difficulties in choosing these because the species-specific data on this topic remains scarce. In order to address the variability predicated on the specifics of any one ddPCR implementation when isolating circulating miRNA and assessing reverse transcription efficiency, it is highly desirable to include both endogenous and spike-in controls, such as cel-miR-39 in the normalisation strategy. This combined approach enables the control of differences in both the preanalytical sample handling and miRNA recovery (11).

In the present study, several limitations should be acknowledged. The size of the study groups was small, although comparable to the study samples in other published data. This result should not be extrapolated beyond the range of the data included in this analysis, mainly because of the diversity of arrhythmogenic mechanisms. However, these cardiac dysrhythmias were among the most commonly reported.

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