



Galectin-3 in cardiac muscle and circulation of dogs with degenerative mitral valve disease



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KEYWORDS

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Abstract *Objectives:* This study aimed to determine the association of cardiac fibrosis with the galectin-3 (Gal-3) expression, a fibrosis marker in the myocardium and to compare plasma Gal-3 levels in normal and degenerative mitral valve disease (DMVD) dogs.

Animals: Studies of muscle expression and plasma levels of Gal-3 were performed in separate groups of dogs. The tissue study was performed on cardiac tissues collected from 22 dogs. The plasma study was performed on 46 client-owned dogs.

Methods: Papillary muscle and left ventricular (LV) wall obtained from 10 normal and 12 DMVD dogs were stained with Masson trichrome and Gal-3 immunohistochemistry to determine fibrosis areas and Gal-3 expression. Plasma samples were collected from 19 normal and 27 DMVD dogs for Gal-3 measurement by ELISA.

Results: Percentage of fibrosis was higher in papillary muscle and LV wall of DMVD dogs ($66.13 \pm 5.58\%$; $52.98 \pm 8.45\%$) than in normal dogs ($35.40 \pm 8.46\%$; $27.41 \pm 7.91\%$; $p < 0.0001$). Gal-3 was higher in papillary muscle and LV wall of DMVD dogs ($27.95 \pm 6.94\%$; $17.25 \pm 8.76\%$) than in normal dogs ($1.08 \pm 0.67\%$; $0.52 \pm 0.42\%$; $p < 0.0001$). Fibrosis areas correlated strongly with the Gal-3 expression ($r = 0.821$, $p < 0.0001$). Plasma Gal-3 levels were increased in DMVD dogs (1.50 ; 0.87 – 2.36 ng/mL) compared to normal dogs (0.42 ; 0.27 – 0.63 ng/mL; $p < 0.0001$).

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Conclusions: Gal-3 expression in cardiac muscle was associated with cardiac fibrosis and was higher in DMVD dogs than in normal dogs. DMVD dogs had higher plasma Gal-3 concentrations than normal dogs. Tissue Gal-3 is a candidate of fibrosis biomarker in DMVD; however, further investigation of associations between plasma Gal-3 and myocardial fibrosis is necessary.

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Abbreviations

CHF	congestive heart failure
CV	coefficient of variation
DMVD	degenerative mitral valve disease
ELISA	enzyme-linked immunosorbant assay
Gal-3	galectin-3
HRP	horse radish peroxidase
LA/Ao	the ratio of left atrium to aorta dimension
LV	left ventricular
LVEDd	left ventricular end diastolic diameter
MT	Masson trichrome
PIIINP	pro collagen type III amino-terminal propeptide
PBS	phosphate buffer saline
RPM	revolutions per minutes
SD	standard deviation

Introduction

Degenerative mitral valve disease (DMVD) is the most prevalent acquired cardiac disease and a common cause of left sided congestive heart failure (CHF) in adult small- to medium-sized dog breeds including Cavalier King Charles Spaniel, Miniature Poodle, Pomeranian, Chihuahua and Pekingese.¹ Degenerative mitral valve disease is a progressive degeneration of the mitral valve resulting in mitral regurgitation, cardiac volume overload, and cardiac structural remodeling. Echocardiography is a noninvasive technique used to assess the valve morphology, cardiac structural remodeling and function. However, echocardiography cannot directly evaluate the degree of myocardial fibrosis. Previous histopathological studies demonstrated collagen deposition in remodeled cardiac tissues of dogs affected with DMVD, similar to humans with mitral valve prolapse and other cardiovascular diseases.^{2–6}

Histopathology is considered the gold standard for detecting cardiac fibrosis. However, this technique is not clinically practical. Measurement of a

circulating biomarker of cardiac fibrosis might be a more suitable diagnostic tool. Currently, a few markers of cardiac fibrosis have been evaluated in veterinary medicine. These include matrix metalloproteinases, tissue inhibitors of metalloproteinases and procollagen type III amino-terminal propeptide (PIIINP). Matrix metalloproteinases and tissue inhibitors of metalloproteinases are nonspecific markers produced from several organs within the body.⁷ PIIINP is widely used to detect cardiac fibrosis in humans. Several studies indicate that PIIINP may not be a good marker to determine the fibrosis in dogs with DMVD.^{8,9}

Galectin-3 (Gal-3), a soluble β -galactoside-binding lectin, has been used as a biomarker of cardiac fibrosis in human patients. Gal-3 plays an important role in cardiac fibrosis by stimulating fibroblasts to change their phenotype to myofibroblasts and increase collagen synthesis.^{10–13} Several studies demonstrated an up-regulation of Gal-3 in murine and human hearts with CHF.^{10,13} Increased circulating Gal-3 concentration in humans directly correlated with the amount of collagen deposition in human hearts.¹¹ Circulating Gal-3 correlates with the incidence of CHF, disease progression and risk of mortality; and thus is an overall prognostic indicator for human patients with cardiovascular diseases.^{11,14,15}

Gal-3 expression in the canine heart has not been studied and the potential of circulating Gal-3 as a biomarker for cardiac fibrosis in dogs with DMVD is not known. We hypothesized that Gal-3 would be up-regulated in cardiac muscle of dogs with DMVD and that its expression would correlate with the extent of cardiac fibrosis, similar to human patients affected with mitral valve prolapse or other cardiovascular diseases. We further hypothesized that Gal-3 would be increased in the plasma of dogs with DMVD compared to normal dogs. This study aimed to correlate the abundance of Gal-3 with the degree of cardiac fibrosis in cardiac muscle and to measure plasma Gal-3 in dogs with DMVD compared to healthy normal dogs.

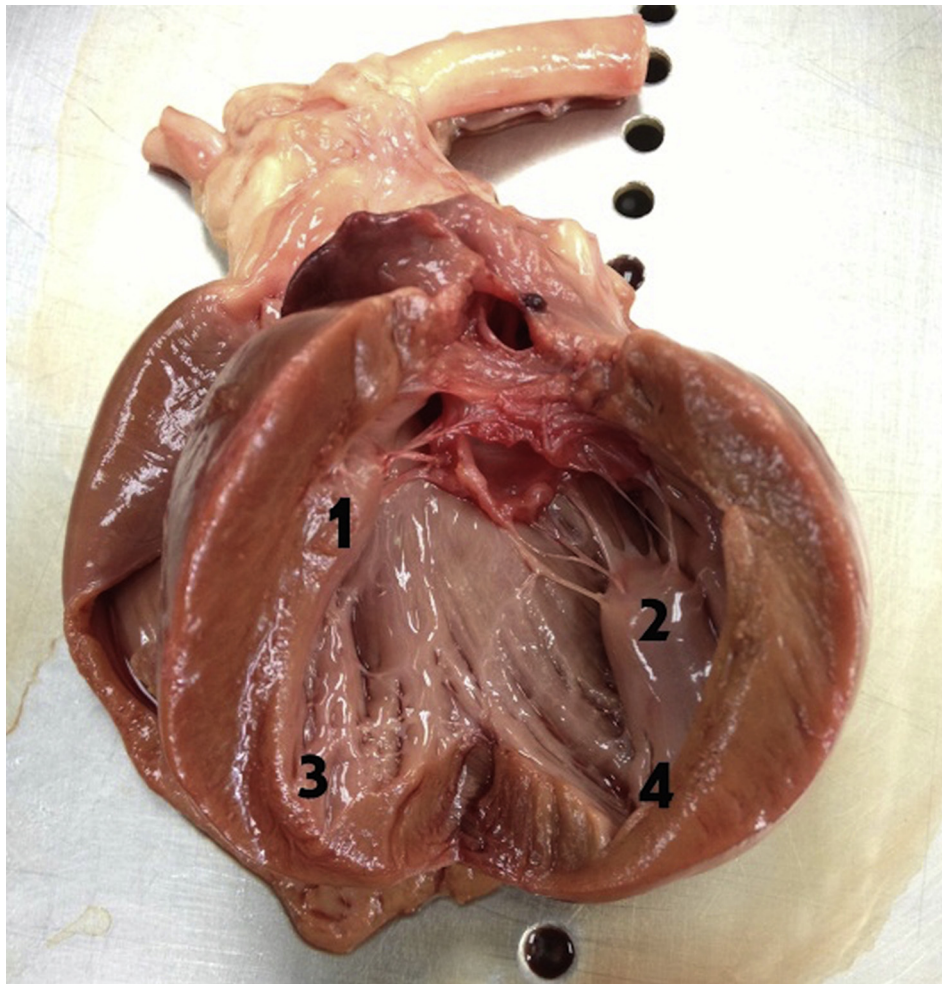


Fig. 1 Sampling sites of left ventricular myocardium from the heart of a degenerative mitral valve disease dog; papillary muscles (1, 2) and left ventricular free walls (3, 4).

Animals, materials and methods

Expression of collagen and galectin-3 in cardiac muscles

Dogs

Cardiac specimens were collected from dogs of 6 years of age or older and <15 kg body weight presented for necropsy to the Department of Pathology, Faculty of Veterinary Science, Chulalongkorn University. All dogs were necropsied within 72 h after spontaneous death. Cardiac samples from dogs without evidence of mitral valve degeneration based on mitral valve thickness <1 mm were assigned to the normal group. Dogs with mitral valve thickness >2 mm were selected into the DMVD group.¹⁶

The cardiac necropsy protocol was modified from previous studies by harvesting only myocardial tissue of papillary muscle and ventricular

wall from the left ventricle.^{4,5} One cm³ sections of papillary muscle (two per dog) and left ventricular (LV) wall (2/dog) with total four sections per dog were collected (Fig. 1). The tissues were preserved in 10% formalin for 24 h and embedded in paraffin blocks. Four µm thickness tissue sections were then stained with hematoxylin and eosin for general histological assessment and Masson trichrome (MT) for detection of cardiac fibrosis.

Tissue sections were deparaffinized in xylene, rehydrated in serial alcohol dilutions, pretreated with citrate buffer (0.01 M, pH 6.0) and heated in an autoclave at 121 °C for 5 min. The endogenous peroxidase activity was blocked with 3% hydrogen peroxide for 10 min at room temperature. Slides were washed with phosphate-buffered saline (PBS; pH 7.4) and blocked a nonspecific antibody binding by incubating with bovine serum albumin for 20 min at 37 °C. The slides were washed with PBS and incubated with a monoclonal mouse antibody

against human Gal-3^c at 4 °C overnight. Slides were incubated with a commercial available labeled polymer conjugated with horse radish peroxidase (HRP)^d for 45 min at 37 °C and washed again with PBS. The peroxidase activity was developed by incubation with 3,3'-diaminobenzidine tetrahydrochloride (1:50)^d for 3 min at room temperature. Lastly, slides were counterstained with Mayer's hematoxylin and then mounted.^{17,18} The positive control slides were prepared by using canine mammary adenoma.¹⁷ Negative controls for each sample were created by substituting universal negative control for N-series mouse antibody.^e All procedures were performed by a single investigator.

Area of collagen deposition and Gal-3 expression were microscopically examined under light microscopy and randomly photographed with a photomicroscope^f for 10 areas under 20× magnification by one investigator. The area of collagen deposition and Gal-3 expression was calculated as the percentage of collagen deposition area (blue stained areas) or Gal-3 positive area (brown stained areas) to total examined area by using an image analyzer software^g and a protocol modified from previous studies by calculating the percentage of positive area to total examined area not to total section area.^{18,19}

Measurement of plasma galectin-3

Dogs

Plasma samples were collected from dogs 6 years of age or older and <15 kg body weight presented to the Small Animal Hospital, Faculty of Veterinary Science, Chulalongkorn University with permission of the owners. The study protocol was approved by Animal Care and Use Committee, Faculty of Veterinary Science, Chulalongkorn University (Animal Use Protocol No. 1431033). General history, physical examination, blood collection, thoracic radiography and echocardiography were performed on dogs on the same day. Only dogs without previous history of cardiac disease were recruited. Dogs were subsequently assigned to 2 groups. The DMVD group consisted of dogs newly diagnosed with DMVD with or without clinical signs of heart failure (exercise intolerance, cough, and dyspnea). The

blood chemistry profile values were within normal limits^{20,21} and dogs had not previously received cardiovascular drugs. Dogs with other cardiac diseases detected by echocardiogram or other systemic diseases screened by radiography and blood profiles were excluded. Normal healthy dogs without evidence of DMVD were included into the normal group.

Two thoracic radiographic views (ventrodorsal and right lateral) were performed in all dogs to evaluate heart size and shape, abnormality of the pulmonary parenchyma and the thoracic cavity. Echocardiography was performed to diagnose DMVD and determine the cardiac structural changes using an ultrasound machine^h with 6–10 multifrequency phased array and 5–6 MHz microconvex transducers by an experienced veterinarian. All unsedated dogs were manually restrained in right lateral recumbent position. Degenerative mitral valve disease was diagnosed by subjectively evaluating mitral valve lesions including thickening of the valve leaflet, valve prolapse and/or chordal rupture assessed on two dimensional echocardiography.²² Left ventricular remodeling and systolic function were evaluated by m-mode echocardiography. Echocardiographic indices including left ventricular end diastolic diameter (LVEDd), left ventricular end systolic diameter, wall thickness of left ventricular free wall during diastole and systole, interventricular septal thickness during diastole and systole, the ratio of left atrium to aorta dimension (LA/Ao) and percent of fractional shortening were determined.²² The echocardiographic values were indexed to body weight.²² All dogs with DMVD had to have mitral regurgitation assessed by color Doppler on two dimensional echocardiography. Severity of valve regurgitation was determined by the ratio of regurgitant jet area to left atrium area during systole. The severity of regurgitation was divided into mild (<20–30%), moderate (≥20–30% but ≤70%) and severe (>70%).²³ Degenerative mitral valve disease dogs were staged based on American College of Veterinary Medicine classification²⁴ including stage A: predisposing breed dogs with no murmurs, clinical signs or cardiac structural changes; stage B1: asymptomatic DMVD dogs with no sign of CHF i.e. pulmonary edema evaluated by radiography or cardiac remodeling i.e. LV dimension within normal limits and LA/Ao <1.5 assessed by echocardiography; stage B2: asymptomatic DMVD dogs with cardiac remodeling i.e. LV dimension greater

^c NCL-GSL3; Novocastra Laboratories, Newcastle, UK.

^d Envision (HRP Rabbit/Mouse); Dako, Hamburg, Germany.

^e Negative control for N-series mouse antibody; Dako, Carpinteria, CA, USA.

^f Olympus® BX50, Olympus Optical, Tokyo, Japan.

^g Image-Pro® Plus version 6.0; Media Cybernetics, Rockville, MD, USA.

^h Logic™ 5 Pro; GE Healthcare, Solingen, Germany.

than normal limits and LA/Ao >1.5 assessed by echocardiography and no sign of CHF on radiography; stage C: symptomatic DMVD dogs with evidence of CHF and cardiac remodeling assessed by radiography and echocardiography, respectively.

Plasma for analysis of Gal-3 was obtained from 1 mL of venous blood collected in ethylenediaminetetraacetic acid Eppendorf tubes, centrifuged at 1000g (or 3000 revolutions per minute) for 15 min and stored at -20°C until assay.²⁵ Samples for analysis of Gal-3 were batched and analysis was performed in duplicate. Gal-3 concentration was determined by the canine Gal-3 ELISA kit.¹ Briefly, samples and buffer were incubated with Gal-3-HRP conjugate in microplates for 1 h, wells were poured and washed to remove nonspecific binding, and then incubated with the substrate for HRP enzyme. The reaction was stopped by adding stop solution to the microplate. Spectrophotometry at 450 nm was used to measure the intensity of color. Validation of an ELISA test kit was performed by in-house laboratory. Spike and recovery analysis was determined using spiked plasma samples from a normal dog and five different manufacturer standard concentrations with dilution 1:1. The range of spike samples was 0.89–9.54 ng/mL. Recovery was assessed as the percentage of recovered Gal-3 spiked canine samples vs. expected standard Gal-3 at five different concentrations. The interassay coefficient of variation (CV) was calculated from an average CV of two standard control samples from three different ELISA plates. The intra-assay CV was calculated from the average of the individual CV of five standard duplicate samples of one plate. The analytical sensitivity and specificity were not performed in the present study. The manufacturer analytical range of the assay is 0.1–10 ng/mL.¹

Statistical analysis

Statistical analyses were performed using a computer based software.² Normality was tested by a Shapiro–Wilk test. The normally distributed data were presented as normal $\pm$ standard deviation (SD). Data with nonnormal distribution were presented as median (25th–75th percentiles). The difference of fibrotic areas and the Gal-3 expression between two groups was compared by an independent *t*-test. The difference of plasma Gal-

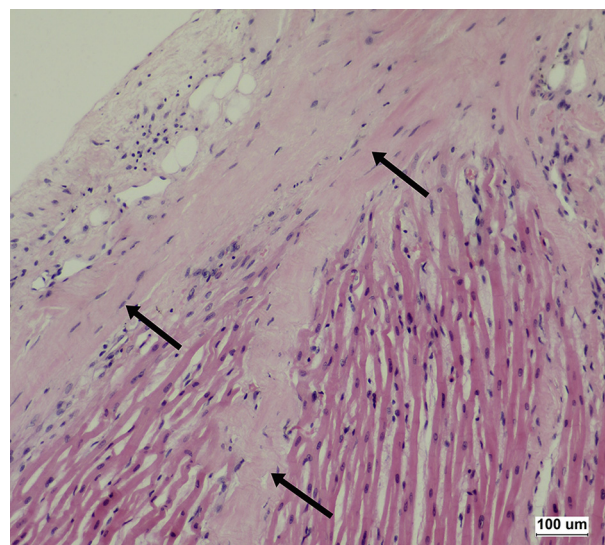


Fig. 2 Sub-endocardial fibrosis in the papillary muscle of a degenerative mitral valve disease dog (arrows) (H&E stain, 100 $\times$ magnification).

3 concentrations between normal and DMVD groups was analyzed using a Mann–Whitney *U* test. The difference of plasma Gal-3 in various stages of DMVD and mitral regurgitation severity was determined by the Kruskal–Wallis test. The difference between pairs of stages and regurgitation severity was analyzed by the Mann–Whitney test. A Bonferroni correction was performed for multiple comparisons. The correlations were built testing effects of age and weight on fibrotic areas, Gal-3 expression and plasma Gal-3. The relationship between plasma Gal-3 and echocardiographic values was also evaluated. The correlations between fibrotic areas and the Gal-3 expression as well as between fibrotic areas or the Gal-3 expression and age and weight were assessed by Pearson's correlation. The correlations between plasma Gal-3 concentrations and age, weight or echocardiographic values were tested using a Spearman's rank correlation. A *p*-value of <0.05 was considered significant.

Results

Cardiac fibrosis and galectin-3 in cardiac muscles

Dogs

Specimens of cardiac muscle were harvested from 22 dogs that underwent necropsy. The normal group consisted of 10 dogs (one male and nine females). Breeds of dogs were Poodle ($n = 4$), Shih-Tzu ($n = 2$), French Bulldog ($n = 1$),

¹ BlueGene Biotech, Shanghai, China

² SPSS, IBM, Chicago, IL, USA.

Chihuahua ($n = 1$), Beagle ($n = 1$) and Pomeranian ($n = 1$). The DMVD group consisted of 12 dogs (four males and eight females). Breeds of dogs were Poodle ($n = 7$), Shih-Tzu ($n = 3$), Schnauzer ($n = 1$) and mixed breed ($n = 1$). The average age of dogs in the DMVD group (12.83 ± 4.03 years) was greater than that of dogs in the normal group (9.45 ± 3.18 years; $p=0.031$). The average body weight of dogs in the normal group (5.54 ± 2.0 kg) was not different from dogs in the DMVD group (6.61 ± 2.01 kg; $p=0.226$). Major cause of death of dogs in the DMVD group was cardiorespiratory failure ($n = 9$). Other dogs in this group died from causes unrelated to cardiac disease problems (septicemia = 2; hepatic failure = 1). In the DMVD group, seven dogs received enalapril, furosemide and pimobendan, two dogs received only enalapril and furosemide and three dogs did not receive cardiac drugs.

Cardiac fibrosis evaluation by MT staining

Microscopically, large areas of cardiac fibrosis were noted in DMVD dogs, especially in papillary muscles and sub-endocardial region of the LV wall (Fig. 2). Masson trichrome staining showed an increased area of collagen deposition in the sub-endocardium in the hearts of dogs in the DMVD group (Fig. 3). Percentage of fibrosis area was higher in the DMVD group (papillary muscles: $66.13 \pm 5.58\%$; LV walls: $52.96 \pm 8.49\%$) than in the normal group (papillary muscles: $35.40 \pm 8.46\%$; LV walls: $27.41 \pm 7.91\%$) dogs ($p < 0.0001$). The area

of collagen deposition was higher in papillary muscles (DMVD: $66.13 \pm 5.58\%$; normal: $35.40 \pm 8.46\%$) than in LV wall (DMVD: $52.98 \pm 8.45\%$; normal: $27.41 \pm 7.91\%$) in both normal and DMVD groups ($p=0.001$ and < 0.0001 , respectively). There was no correlation between percentage area of collagen deposition and age ($r = 0.059$, $p=0.796$) or weight ($r = 0.023$, $p=0.920$) when all dogs were analyzed together.

Galectin-3 expression by immunohistochemical staining

Gal-3 expression was observed in the cytoplasm of myocardial and large cells with few myofibrils and a vacuous cytoplasm i.e. Purkinje-like cells mainly found in the sub-endocardium of DMVD dogs (Figs. 4 and 5). Based on quantitative analysis by image analysis software⁸, percentage of Gal-3 expression in papillary muscles and LV walls was increased in specimens from the DMVD group (papillary: $27.95 \pm 6.94\%$; LV walls: $17.25 \pm 0.42\%$) compared to the normal group (papillary: $1.07 \pm 0.67\%$; LV walls: $0.52 \pm 0.67\%$; $p < 0.0001$ and 0.007 , respectively). Galectin-3 expression was higher in papillary muscle (DMVD: $27.95 \pm 6.94\%$; normal: $1.07 \pm 0.67\%$) than in LV wall (DMVD: $17.25 \pm 0.42\%$; normal: $0.52 \pm 0.67\%$) in normal and DMVD dogs ($p < 0.0001$ and < 0.0001 , respectively). There was no correlation between percentage of Gal-3 expression and age ($r = -0.354$, $p=0.106$) or weight ($r = 0.137$, $p=0.542$) of the entire population. A positive correlation was found

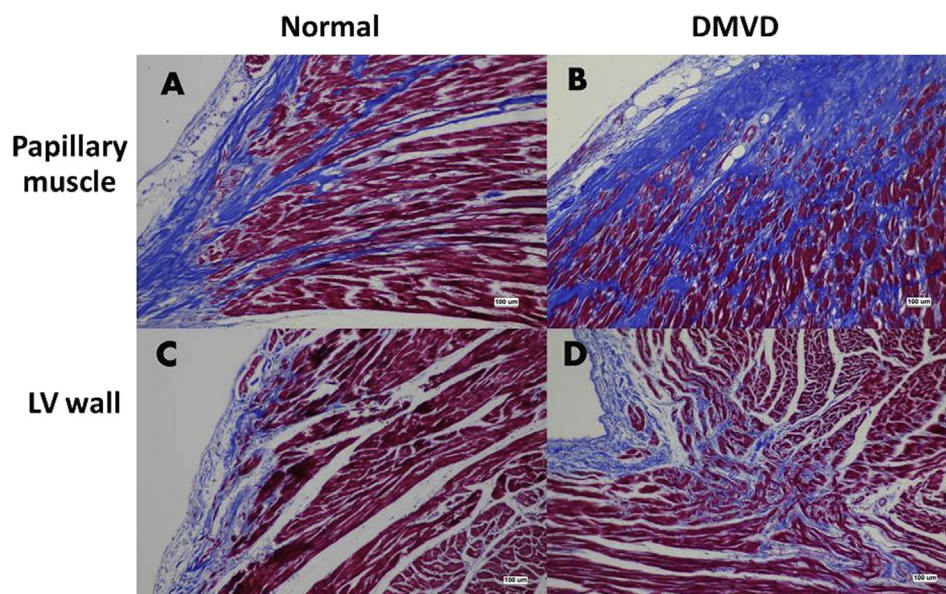


Fig. 3 Sub-endocardial fibrosis (blue color) in papillary muscles (A, B) and left ventricular wall muscles (C, D) of normal and degenerative mitral valve disease dogs, respectively. The collagen deposition (blue color) was mainly found in the sub-endocardium. Myocardium was stained in red color (Masson trichrome stain, 100 $\times$ magnification).

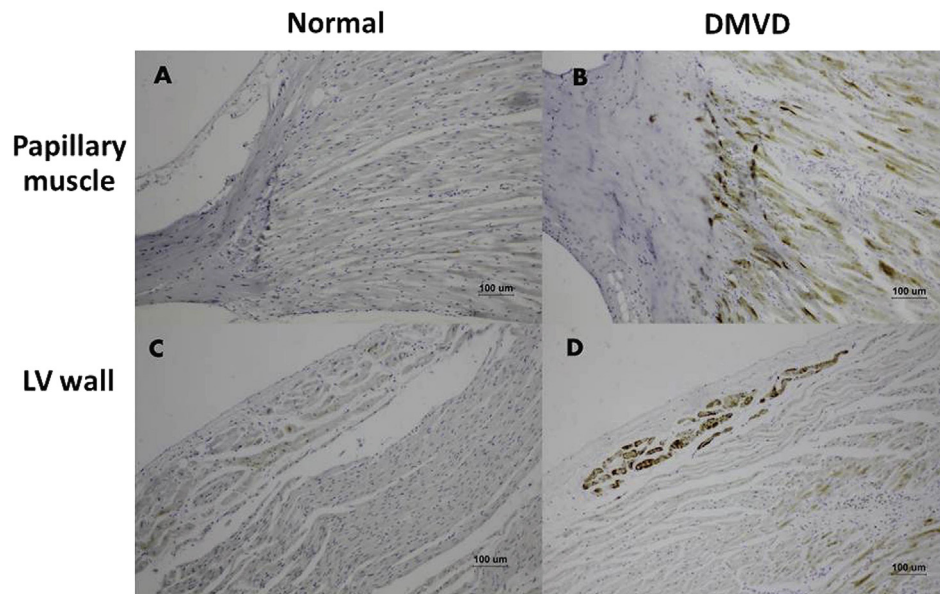


Fig. 4 Gal-3 expression in papillary muscles (A, B) and left ventricular muscles (C, D) of normal and degenerative mitral valve disease dogs, respectively. Galectin-3 expression (brown color) was mainly found in papillary muscles and sub-endocardial area. (Envision; HRP rabbit and mouse with 3,3'-diaminobenzidine tetrahydrochloride immunohistochemistry and Mayer's hematoxylin stain, 100× magnification).

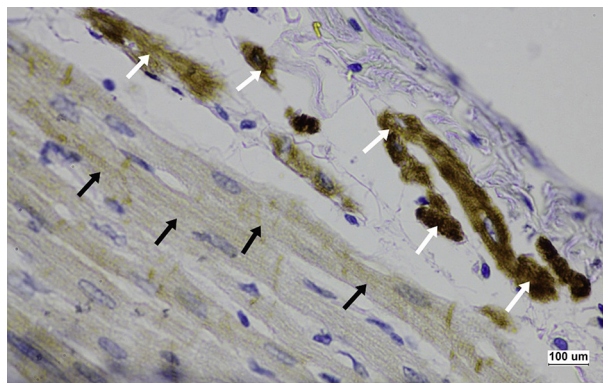


Fig. 5 Galectin-3 expression (brown color) in the cytoplasm of Purkinje-like cells (white arrows) and cardiomyocytes (black arrows) (Envision; HRP rabbit and mouse with 3,3'-diaminobenzidine tetrahydrochloride immunohistochemistry and Mayer's hematoxylin stain, 400× magnification).

between the percentage of fibrosis area and the percentage of Gal-3 expression of the entire population ($r = 0.821$, $p < 0.0001$; Fig. 6).

Plasma study: measurement of plasma galectin-3

Dogs

Plasma was collected from 54 dogs (31 DMVD and 23 normal dogs). Eight dogs (four DMVD and four

normal dogs) were excluded from the study because the blood chemistry profile values were outside of normal limits. Forty-six client-owned dogs were therefore included in the study and divided into normal ($n = 19$) and DMVD groups ($n = 27$). Nineteen dogs in the normal group included 13 females and 6 males. Breeds of dogs were Poodle ($n = 7$), Shih-Tzu ($n = 2$), Miniature Pinscher ($n = 2$), Yorkshire Terrier ($n = 2$), Chihuahua ($n = 1$), Pekingese ($n = 1$) and mixed breed ($n = 4$). Twenty-seven dogs in the DMVD group included 14 females and 13 males. Breeds of dogs were Poodle ($n = 12$), Pomeranian ($n = 3$), Cavalier King Charles Spaniel ($n = 2$), Chihuahua ($n = 2$), Shih-Tzu ($n = 2$), Schnauzer ($n = 1$) and Miniature Pinscher ($n = 1$) and mixed breed ($n = 4$). Average age of dogs in the DMVD group (11.41 ± 0.51 years) was greater than that of dogs in the normal group (8.42 ± 0.47 years; $p < 0.0001$). Body weight was not significantly different between normal (5.64 ± 0.63 kg) and DMVD dogs (5.17 ± 0.39 kg; $p = 0.521$).

Clinical data

Based on clinical signs, radiographic findings and echocardiographic data, nine dogs in the DMVD group were classified as stage B1 (33.34%), five dogs were classified as stage B2 (18.52%) and 13 dogs were classified as stage C (48.15%) according to American College of Veterinary Medicine classification.^{24,26} The clinical signs of dogs in

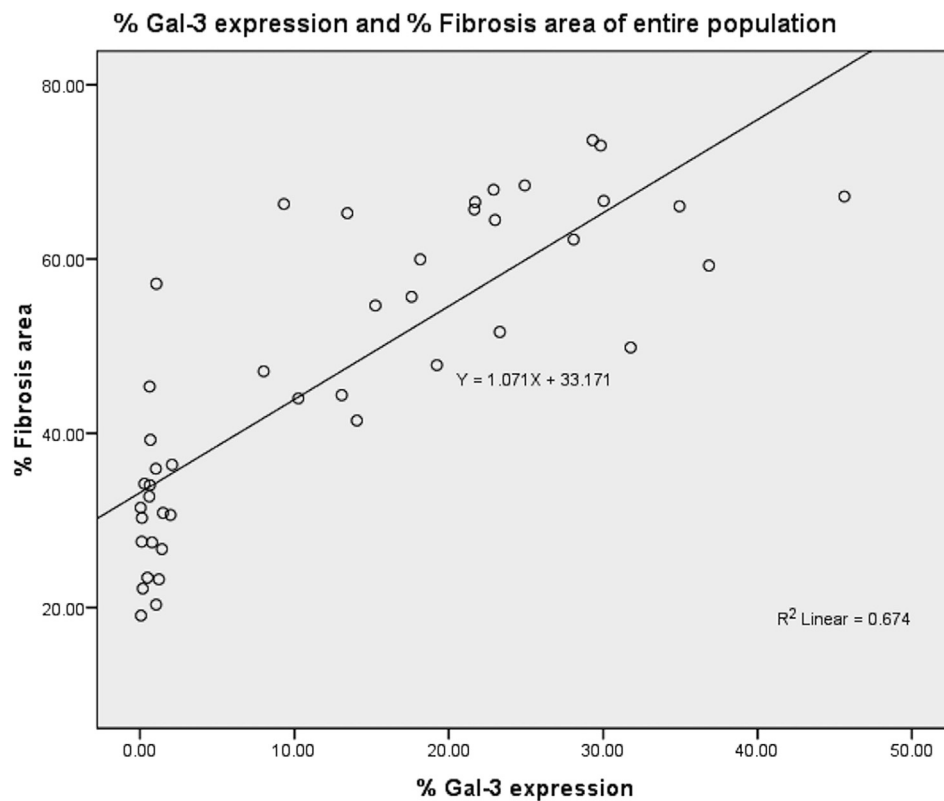


Fig. 6 Correlation between percent galectin-3 expression and percent fibrosis area of entire population ($r = 0.821$; $p < 0.0001$).

Table 1 Echocardiographic data of normal and DMVD groups.

Parameter	Normal	DMVD	p-value
Septum-d index	1.33 ± 0.52	1.5 ± 0.59	0.314
LV chamber-d index	4.43 ± 1.54	5.30 ± 1.68	0.081
LV wall-d index	1.21 ± 0.46	1.41 ± 0.56	0.215
Septum-s index	1.76 ± 0.68	2.26 ± 0.95	0.057
LV chamber-s index	2.63 ± 0.75	2.76 ± 0.93	0.626
LV wall-s index	1.95 ± 1.01	2.28 ± 0.89	0.249
%FS	38.41 ± 11.71	47.33 ± 8.98	0.005 ^a
Aorta index	2.25 ± 0.80	2.51 ± 0.97	0.335
LA index	3.31 ± 1.23	4.11 ± 1.53	0.030 ^a
LA/Ao	1.39 ± 0.21	1.62 ± 0.25	0.002 ^a

DMVD, degenerative mitral valve disease; FS, fractional shortening; LV, left ventricular; LA/Ao, the ratio of left atrium to aorta dimension.

Data presented as mean ± standard deviation.

The significant difference was assessed by an independent t-test.

^a Indicate statistical difference between normal and DMVD groups.

DMVD included cough (13 of 13), exercise intolerance (10 of 13) and dyspnea (1 of 13). The abnormalities from physical examination in the DMVD group on the first day of diagnosis included systolic heart murmur (27 of 27), pulmonary crackles (5 of 27), increased lung sounds (12 of 27), pale pink mucous membrane (5 of 27), tachypnea (6 of 27), dyspnea (2 of 27), and abdominal distention (2 of 27).

Echocardiographic data

Severity of mitral valve regurgitation assessed by echocardiographic examination in DMVD dogs was mild in 10 dogs, moderate in eight dogs and severe in nine dogs. Echocardiographic data of dogs in the normal and DMVD groups are shown as mean ± SD in Table 1.

Plasma galectin-3 concentration

Average recovery for the canine Gal-3 ELISA test was 92.50% (mean ± SD for recovery at low (0.67–1.01 ng/mL), medium (1.32–1.82 ng/mL) and high (2.14–9.81 ng/mL) concentrations were 90.39 ± 0.001%, 92.22 ± 0.025% and 93.85 ± 0.006%, respectively). Intra-assay and interassay CVs were 2.54% and 3.84%, respectively.

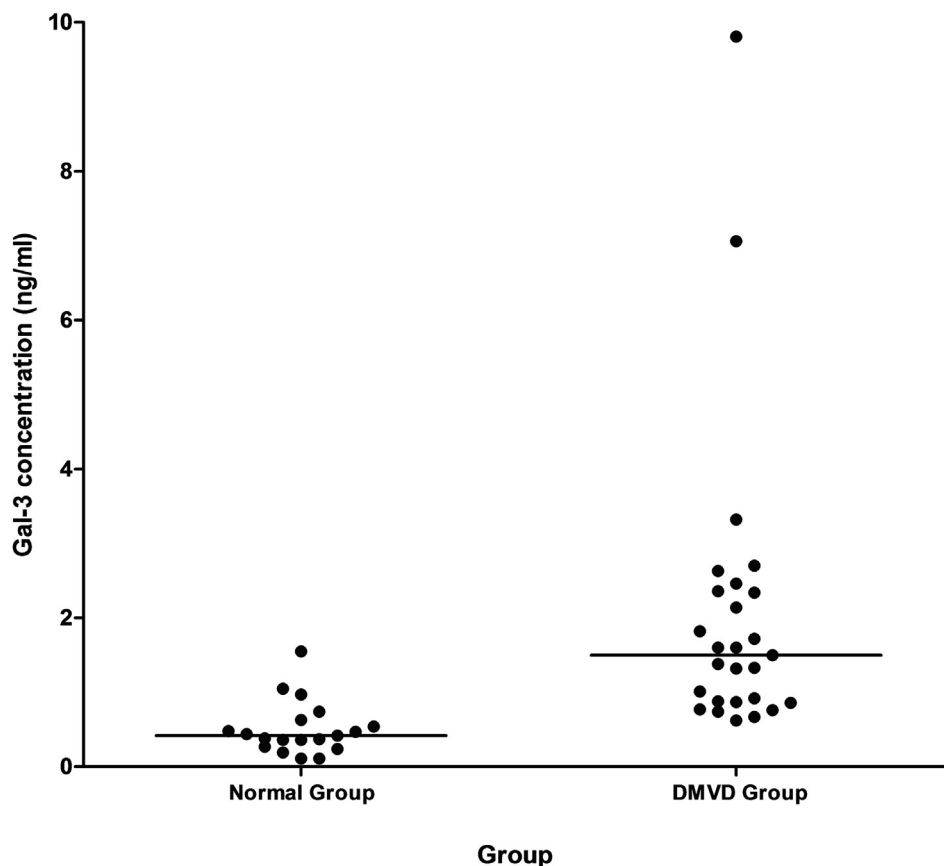


Fig. 7 Scatter plot of plasma galectin-3 concentration in normal (0.42 [0.87–2.36] ng/mL) and degenerative mitral valve disease (1.49 [0.87–2.36] ng/mL) dogs ($p < 0.0001$). Median values were presented as lines.

Median plasma Gal-3 concentration of dogs in the DMVD group (1.49 [0.87–2.36] ng/mL) was higher than that of dogs in the normal group (0.42 [0.27–0.63] ng/mL; $p < 0.0001$; Fig. 7). There was no difference of plasma Gal-3 concentration in dogs in the DMVD group based on functional classification (stage B1 1.38 [0.88–2.41 ng/mL], B2 1.58 [0.68–5.91 ng/mL] and stage C 1.55 [0.83–2.42 ng/mL]; $p=0.989$) or severity of mitral regurgitation (mild 1.49 [0.88–2.22 ng/mL] and moderate to severe 1.49 [0.81–2.49 ng/mL]; $p=0.980$). No correlation was observed between Gal-3 concentration and age ($r = 0.218$, $p=0.145$) or weight ($r = 0.011$, $p=0.944$) based on all dogs. There was no correlation between plasma Gal-3 concentration and echocardiographic values including interventricular septal thickness during diastole ($r = -0.022$, $p=0.883$), interventricular septal thickness during systole ($r = 0.152$, $p=0.314$), left ventricular end systolic diameter ($r = 0.109$, $p=0.472$), left ventricular end systolic diameter ($r = -0.074$, $p=0.627$), wall thickness of left ventricular free wall during diastole ($r = 0.108$, $p=0.474$), wall thickness of left ventricular free wall during systole ($r = 0.133$, $p=0.377$) and LA/Ao ($r = 0.202$, $p=0.312$).

Discussion

The major findings of this study include demonstration of increased cardiac fibrosis in the papillary muscles and LV wall of dogs with DMVD compared to dogs without DMVD, correlation of cardiac muscle Gal-3 expression with degree of cardiac muscle fibrosis, and increased plasma Gal-3 concentration in dogs with DMVD compared to dogs without DMVD.

DMVD is a common cardiac disease in adult small-to medium-sized breeds of dogs. The prevalence of DMVD is age related.²⁷ In this study, dogs in the DMVD and normal groups were well matched in terms of size, but dogs in the DMVD group were significantly older than normal dogs. However, no significant correlation between age and fibrotic area and Gal-3 expression in cardiac muscle was found, nor did circulating Gal-3 concentration correlate with age. Gender of dogs in tissue and plasma studies was not matched between normal and DMVD groups so a gender effect cannot be ruled out.

DMVD is a progressive disease causing valve regurgitation and cardiac structural remodeling

secondary to volume overload.²⁸ Cardiac fibrosis is an expected pathologic change associated with several cardiac diseases, including DMVD. The present study demonstrated an increased area of cardiac fibrosis in dogs with DMVD, especially in sub-endocardium regions of cardiac muscle. This finding is in agreement with a previous study.⁴ The amount of fibrosis in the papillary muscle was higher than in the LV wall which was also in agreement with another previous study.⁵ The sub-endocardial portion of cardiac muscle may be more susceptible to cardiac injury from cardiac ischemia because of decreased perfusion in this area.⁴ Cardiac fibrosis has been found to increase with both aging and cardiovascular disease in human patients and animal models. Rats have an increase in collagen deposition from 5.5% in young hearts to 12% in aging hearts.²⁹ In humans, the collagen deposition increases by approximately 50% in aging hearts.²⁹ Fibrosis in aging hearts has been characterized as reactive fibrosis; an increased collagen deposition without cardiomyocyte loss. Cardiac fibrosis in normal dogs in this study could be related to aging.^{30,31} The area of cardiac fibrosis related to aging in humans is usually small because it is associated with a reduction of collagen degradation rather than increased synthesis.³¹ Cardiac fibrosis associated with cardiac damage or cardiovascular disease in dogs and humans is often more extensive because it is the result of replacement fibrosis.^{29,30} In this study, cardiac fibrosis area was markedly larger in DMVD dogs than in normal aging dogs suggesting that the mechanism is likely related to cardiac injury and replacement fibrosis. However, aging may have some influence on fibrosis formation in these dogs.

Histopathology is an efficient method of detecting the amount and location of cardiac fibrosis. The expression of Gal-3, a marker of fibrosis, can be performed by immunohistochemistry to determine the specific areas of cardiac fibrosis and cells involved in fibrosis formation. The present study demonstrated that Gal-3 expression was localized to the cytoplasm of cardiomyocytes and larger cells beneath the endocardium suspected to be Purkinje cells. Fibrotic Purkinje cells may lose their electrical conduction properties which in turn might affect electrical and mechanical functions of the heart. Previous studies have not reported a relationship between fibrosis and Purkinje cell function. Studies focusing on role of cardiac fibrosis on Purkinje cell function and electrophysiology should be further investigated. Gal-3 expression was mainly found in the sub-endocardium and sites of collagen deposition

identified by MT staining. Increased Gal-3 expression was found in DMVD dogs compared to normal dogs and was found to be strongly correlated with MT staining. These findings support that Gal-3 is a marker of cardiac fibrosis at a tissue level in dogs with DMVD.

The measurement of plasma Gal-3 concentration was performed in the different group of dogs in the present study. Plasma Gal-3 concentration in DMVD dogs was higher than in normal dogs similar to previous studies in humans with cardiovascular diseases.^{12,32,33} However, the reported magnitude of increased Gal-3 levels in human patients was greater than that seen in the DMVD dogs in this study. This is perhaps because of species differences between humans and dogs or that common pathophysiologic mechanisms of human cardiovascular diseases such as cardiac ischemia, hypertension and myocardial diseases result in more extensive cardiac fibrosis compared to dogs with DMVD. Galectin-3 is a useful prognostic marker for human patients with cardiovascular diseases correlating with both disease progression and severity.^{15,34} Plasma Gal-3 concentration predicts the onset of CHF in asymptomatic human patients.^{11,34} Levels of Gal-3 are related to incidence of heart failure and mortality in human patients with heart diseases such as coronary disease and aortic stenosis which are more prone to develop cardiac fibrosis than DMVD.^{11,12,14} This study showed that plasma Gal-3 concentration in DMVD dogs did not correlate with functional stage of disease or mitral regurgitation severity. The results did not meet statistical significance possibly because (1) the number of dogs in each stage and severity was too low, (2) the disease in stage C dogs might not be severe enough to cause the marked release of Gal-3 into the circulation, (3) the method used to quantify mitral valve regurgitation severity was subjective and imperfect, (4) Gal-3 may not increase linearly with disease progression, or (5) plasma Gal-3 does not reflect cardiac fibrosis during disease progression. In this study plasma Gal-3 concentration was not correlated with echocardiographic values, similar to the results in human studies.^{33,35} A previous study in dogs with DMVD showed that LVEDd normalized for body weight assessed by echocardiography increased with fibrosis score; however, the relationship was not strong and was suggested to be not clinically significant.³⁶ The echocardiography is considered to be a nonsensitive or effective tool for detecting cardiac fibrosis.⁵ In other words, cardiac structure assessed by echocardiography may not show a significant change,

even though cardiac fibrosis has already developed. Another explanation is that cardiac fibrosis may not be linearly associated with echocardiographic values i.e. cardiac remodeling. Fibrosis may occur suddenly at particular disease stages. The weak correlation between circulating levels of another marker of myocardial fibrosis, PIIINP, and fibrosis score in myocardial tissues and echocardiographic values has been reported in dogs with DMVD.³⁶ However, a direct association between plasma Gal-3 concentration and myocardial tissue fibrosis was not evaluated in the present study because these end points were measured in different groups of dogs, thus establishing a direct association which needs further studies to elucidate.

The major limitation of this study was that the evaluation of Gal-3 expression in the myocardium and Gal-3 concentration in circulation was performed in different groups of dogs. A significant correlation between serum Gal-3 levels and myocardial fibrosis estimated with cardiovascular magnetic resonance imaging by late gadolinium enhancement T1 mapping in humans with stable coronary artery disease has been reported.³⁷ A similar study in dogs with DMVD has not yet been performed. Further studies elucidating the relationship between concentration of plasma Gal-3 and expression of Gal-3 in the myocardium as well as other markers of collagen turnover such as procollagen type I carboxy-terminal propeptide, C-telopeptide for type I collagen, or tissue inhibitors of metalloproteinase 1 would provide further insight about the role of Gal-3 in the clinical setting. Another limitation of this study was the use of a monoclonal mouse antibody against human Gal-3 instead of an antibody against canine Gal-3 for immunohistochemistry study. However, this antibody has been used to detect canine mammary adenoma and canine splenic hemangiosarcoma.^{17,18} Moreover, canine Gal-3 is highly homologous to human Gal-3 in both the structure and its amino acid sequence.^{38,39} Thus, it is reasonable to use this antibody in the present study. Another limitation is that the population of dogs in this study was not gender matched; thus a gender influence on the results cannot be ruled out. In addition, plasma Gal-3 concentration was performed only at a single time point for each dog. There currently is a lack of information about the kinetics of Gal-3 in dogs. However, serial measurements of plasma Gal-3 concentration in human are stable⁴⁰ perhaps implying that a single test of plasma Gal-3 concentration would be sufficient for dogs. The analytical sensitivity and specificity tests were not performed. However, plasma Gal-3

concentration from all samples in this study was within detection range of the assay reported by the manufacturer. Lastly, spike recovery analysis was not performed with Gal-3 concentration lower than 0.89 ng/mL; therefore, the background noise effect was not ruled out.

In conclusion, dogs with naturally occurring DMVD developed more extensive cardiac fibrosis than size matched dogs without DMVD. Galectin-3 expression was correlated to the increased area of cardiac fibrosis in DMVD dogs. Plasma Gal-3 concentrations were higher in DMVD dogs than in normal dogs. Based on these results, Gal-3 might be a useful marker of cardiac fibrosis in dogs with DMVD. Because an association between plasma Gal-3 concentration and fibrosis within cardiac tissues was not evaluated in the same dogs, further studies are necessary to clarify.

Conflicts of interest

The authors do not have any conflicts of interest to disclose.

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