การแสดงออกของโปรตีนกาเลกตินสามในกล้ามเนื้อหัวใจและการตรวจวัดระดับพลาสมากาเลกติน สามในสุนัขที่เป็นโรคลิ้นหัวใจไมทรัลเสื่อม

นางสาวศิริวรรณ สาครินทร์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาอายุรศาสตร์สัตวแพทย์ ภาควิชาอายุรศาสตร์
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EXPRESSION OF GALECTIN-3 IN CARDIAC MUSCLES AND DETERMINATION LEVEL OF PLASMA GALECTIN-3 IN DOGS WITH DEGENERATIVE MITRAL VALVE DISEASE

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A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science Program in Veterinary Medicine
Department of Veterinary Medicine
Faculty of Veterinary Science
Chulalongkorn University
Academic Year 2014

Thesis Title	EXPRESSION OF GALECTIN-3 IN CARDIAC MUSCLES AND DETERMINATION LEVEL OF PLASMA GALECTIN-3 IN DOGS WITH DEGENERATIVE MITRAL VALVE DISEASE
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ศิริวรรณ สาครินทร์ : การแสดงออกของโปรตีนกาเลกตินสามในกล้ามเนื้อหัวใจและการตรวจวัดระดับพลาสมากาเลกตินสามในสุนัขที่เป็นโรคลิ้นหัวใจไมทรัลเสื่อม (EXPRESSION OF GALECTIN-3 IN CARDIAC MUSCLES AND DETERMINATION LEVEL OF PLASMA GALECTIN-3 IN DOGS WITH DEGENERATIVE MITRAL VALVE DISEASE) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ผศ. สพ.ญ. ดร. สิริลักษณ์ สุรเชษฐพงษ์, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: รศ. น.สพ. ดร. อนุเทพ รังสีพิพัฒน์, 81 หน้า.

โรคลิ้นหัวใจไมทรัลเสื่อมเป็นโรคหัวใจที่เกิดขึ้นภายหลังกำเนิดสามารถพบได้บ่อยในสุนัขพันธุ์เล็กที่มีอายุมาก พยาธิกำเนิดของโรคนี้มีความคล้ายคลึงกับโรคลิ้นหัวใจโป่งในมนุษย์ โรคลิ้นหัวใจโป่งและโรคหัวใจอื่นๆ ในมนุษย์สามารถทำให้เกิดภาวะไฟโบรซิสที่กล้ามเนื้อหัวใจ สุนัขที่ป่วยเป็นโรคลิ้นหัวใจไมทรัลเสื่อมสามารถพบภาวะไฟโบรซิสที่กล้ามเนื้อหัวใจได้เช่นเดียวกับมนุษย์ กาเลกตินสามมีการนำมาใช้เป็นตัวชี้วัดภาวะไฟโบรซิสที่กล้ามเนื้อหัวใจในมนุษย์ โดยพบว่าการแสดงออกของกาเลกตินสามเพิ่มขึ้นทั้งในกล้ามเนื้อหัวใจและในกระแสเลือดของผู้ป่วยที่มีภาวะหัวใจล้มเหลว บทบาทของกาเลกตินสามในการเป็นตัวชี้วัดภาวะไฟโบรซิสที่กล้ามเนื้อหัวใจในสุนัขที่ป่วยเป็นโรคลิ้นหัวใจไมทรัลเสื่อมยังไม่มีการศึกษา ดังนั้นการศึกษามีวัตถุประสงค์เพื่อทำการประเมินการแสดงออกของกาเลกตินสามในกล้ามเนื้อหัวใจและเพื่อตรวจวัดระดับพลาสมากาเลกตินสามในสุนัขที่ป่วยเป็นโรคลิ้นหัวใจไมทรัลเสื่อม การศึกษาประกอบด้วยกล้ามเนื้อหัวใจสุนัขที่ป่วยเป็นโรคลิ้นหัวใจไมทรัลเสื่อม 12 ตัว และสุนัขปกติ 10 ตัว ที่เข้ารับการผ่าซาก โดยสุนัขทั้งหมดเป็นสุนัขพันธุ์เล็ก น้ำหนักน้อยกว่า 15 กิโลกรัม และอายุมากกว่า 6 ปี เพื่อทำการประเมินภาวะไฟโบรซิสที่กล้ามเนื้อหัวใจและการแสดงออกของกาเลกตินสามโดยการย้อมสีพิเศษมาสซองไตรโครมและย้อมสีอิมมูโนฮิสโตเคมีของกาเลกตินสามตามลำดับ ระดับของพลาสมากาเลกตินสามทำการวัดจากสุนัขปกติ 19 ตัว และสุนัขที่ป่วยเป็นโรคหัวใจไมทรัลเสื่อม 27 ตัว โดยสุนัขทั้งหมดมีอายุ ขนาดและพันธุ์ที่ใกล้เคียงกัน ด้วยชุดทดสอบอีไลซ่า อายุและน้ำหนักไม่มีความสัมพันธ์กับภาวะไฟโบรซิสที่กล้ามเนื้อหัวใจและการแสดงออกของกาเลกตินสาม สุนัขที่ป่วยเป็นโรคลิ้นหัวใจไมทรัลเสื่อมมีภาวะไฟโบรซิสที่กล้ามเนื้อหัวใจ ($p < 0.01$) และการแสดงออกของกาเลกตินสาม ($p < 0.01$) มากกว่าสุนัขปกติโดยเฉพาะบริเวณส่วน sub-endocardium ระดับพลาสมากาเลกตินสามมีค่าสูงในสุนัขที่ป่วยเป็นโรคลิ้นหัวใจไมทรัลเสื่อมมากกว่าสุนัขปกติอย่างมีนัยสำคัญ อายุ น้ำหนัก และค่าต่างๆ จากการอัลตราซาวน์หัวใจไม่มีความสัมพันธ์กับระดับพลาสมากาเลกตินสาม โดยสรุปกาเลกตินสามอาจเป็นตัวเลือกหนึ่งที่ใช้เป็นตัวชี้วัดภาวะไฟโบรซิสที่กล้ามเนื้อหัวใจในสุนัขที่ป่วยเป็นโรคลิ้นหัวใจไมทรัลเสื่อม

ภาควิชา อายุรศาสตร์	ลายมือชื่อนิสิต .....
สาขาวิชา อายุรศาสตร์สัตวแพทย์	ลายมือชื่อ อ.ที่ปรึกษาหลัก .....
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5575320031 : MAJOR VETERINARY MEDICINE
KEYWORDS: CARDIAC FIBROSIS / CONGESTIVE HEART FAILURE / DEGENERATIVE MITRAL VALVE DISEASE / GALECTIN-3

SIRIWAN SAKARIN: EXPRESSION OF GALECTIN-3 IN CARDIAC MUSCLES AND DETERMINATION LEVEL OF PLASMA GALECTIN-3 IN DOGS WITH DEGENERATIVE MITRAL VALVE DISEASE. ADVISOR: ASST. PROF. DR. SIRILAK SURACHETPONG, D.V.M., Ph.D., CO-ADVISOR: ASSOC. PROF. DR. ANUDEP RUNGSIPIPAT, D.V.M., Ph.D., 81 pp.

Degenerative mitral valve disease (DMVD) is a common acquired cardiac disease in older small breed dogs. The pathophysiology of this disease is similar to mitral valve prolapse (MVP) in humans. MVP and other cardiovascular diseases in human can cause cardiac fibrosis. DMVD dogs also have cardiac fibrosis like human. Gal-3, has been used as a cardiac fibrosis marker in humans. Gal-3 is up-regulated in cardiac muscles and blood circulation of CHF patients. A role of Gal-3 as a cardiac fibrosis marker in dogs with naturally occurring DMVD has not been studied. The aims of this study were to determine expression of Gal-3 in cardiac muscles and measure level of plasma Gal-3 in DMVD dogs. Twelve DMVD and ten normal cardiac muscles from small breed less than 15 kilograms and older than 6 years old necropsy dogs were collected to determine cardiac fibrosis and Gal-3 expression by Masson trichrome and Gal-3 immunohistochemistry staining, respectively. Plasma Gal-3 concentration was measured from 19 normal and 27 aged, sized and breed matched DMVD dogs by ELISA test kits. Age and weight were not correlated with cardiac fibrosis and Gal-3 expression. DMVD dogs had more cardiac fibrosis ($p < 0.01$) and overexpressed of Gal-3 ($p < 0.01$) than normal dogs particularly in sub-endocardium. Plasma Gal-3 concentration was significantly higher in DMVD than normal dogs ($p < 0.01$). Age, weight and echocardiographic indices showed no correlation with plasma Gal-3 concentration. In conclusion, Gal-3 might be a potential candidate of cardiac fibrosis markers in dogs with DMVD.

Department:	Veterinary Medicine	Student's Signature	.....
Field of Study:	Veterinary Medicine	Advisor's Signature	.....
Academic Year:	2014	Co-Advisor's Signature	.....

ACKNOWLEDGEMENTS

Firstly, I am much honored to be supported by a grant from Chulalongkorn University Graduate Commemorate the 72nd Anniversary of His Majesty King Bhumibol Adulyadej and the 90th Anniversary of Chulalongkorn University fund (Ratchadaphiseksomphot Endowment Fund).

I would like to sincerely thank my advisor, Assistant Professor Dr. Sirilak Surachetpong and my co-advisor Associate Professor Dr. Anudep Rungsipipat for their kindness instruction, advice, guidance, assistance and support during the time of performing this thesis. I would like to thank Associate Professor Dr. Somsak Pakpinyo, Associate Professor Achara Tawatsin, Associate Professor Dr. Rosama Pusoonthornthum and Assistant Professor Dr. Walasinee Moonarmart as members of thesis committee for their valuable comments and suggestions on this thesis.

I would like to thank Assistant Professor Dr. Sayamon Srisuwattanasagul for her guidance on using Image-ProPlus software. Miss Katriya Chankow for her kindness helps in sample collection. Mr. Sittichoke Lachroj for his helps in immunohistochemistry and special staining technique suggestions. I would like to express my appreciation to all my teachers and staffs in Department of Veterinary Medicine, Department of Veterinary Pathology and Department of Anatomy, Faculty of Veterinary Science, Chulalongkorn University, my student colleagues and dogs' owners from Small Animal Hospital, Faculty of Veterinary Science, Chulalongkorn University for their support, encouragement, extensive help and participation in this thesis.

Lastly, I would like to express thanks to my lovely family and friends for their kindness support and cheerfulness throughout this study.

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CHAPTER I
INTRODUCTION

Degenerative mitral valve disease (DMVD) is the most common acquired cardiac disease in adult small to medium sized breed dogs. DMVD is also the most frequent cause of congestive heart failure (CHF) in dogs. The prevalence is age related, especially in old small sized breed dogs. DMVD occurs in males more frequently than females (Borgarelli and Buchanan, 2012). The predisposing breeds include small to medium sized breed dogs such as Cavalier King Charles spaniels, Miniature poodles, Cocker spaniels, Miniature schnauzers, Dachshunds, Pomeranians, Chihuahuas, Pekingese, Fox terriers, Boston terriers and mixed breed dogs (Disatian, 2010). This disease is a progression of valve degeneration. The affected mitral valve leaflets are nodular and thickening. The chordae tendineae become elongated and occasional rupture. Thickening of valve leaflets results in valve regurgitation. These pathological changes cause regurgitant flow from left ventricle to left atrium during systole. The progression of mitral valve insufficiency leads to remodeling of left atrium and left ventricle by myocardial hypertrophy and chamber dilatation. The affected dogs will eventually develop CHF and need to be treated by either surgical or medical therapy (Haggstrom et al., 2004).

Mitral valve prolapse (MVP) is one of cardiovascular diseases in human caused by abnormality of mitral valve (Hayek et al., 2005). The pathophysiology of this disease in human is similar to DMVD in dogs (Pedersen and Haggstrom, 2000). MVP has been suggested to be involved with cardiac remodeling called cardiac dilatation or eccentric hypertrophy (Cohn et al., 2000). Cardiac remodeling is caused by abnormal hemodynamic and neurohormonal activation (Cohn et al., 2000). Histopathological studies on cardiac muscles in MVP patients found abnormal endocardium and interstitial fibrosis (Mason et al., 1978). Many human researches

indicated that cardiac fibrosis is one of the pathological changes of cardiac remodeling resulted from cardiovascular diseases (Cohn et al., 2000; Lijnen and Petrov, 2000; Burlew and Weber, 2002). However, there are few studies about cardiac fibrosis in dogs with DMVD (Falk et al., 2006; Falk et al., 2010; Falk et al., 2013). Dogs with left ventricular dilatation have more collagen deposition in left ventricular myocardium (Weber et al., 1990). DMVD dogs have more fibrosis in myocardium than normal dogs and mainly found in sub-endocardium and papillary muscles of the left ventricle (Falk et al., 2006; Falk et al., 2010). The survival times of DMVD dogs may be associated with the severity of fibrosis in cardiac muscles (Falk et al., 2010). Cardiac fibrosis can be detected by histopathology of myocardium or measurement of serum cardiac fibrosis markers.

Galectin-3 (Gal-3) is a soluble β-galactoside-binding lectins. Interestingly, one of the effects of Gal-3 is promoting fibrosis. According to previous studies, Gal-3 is suggested to play an important role in cardiac fibrosis and pathophysiology of CHF (Sharma et al., 2004; de Boer et al., 2009; Lok et al., 2010; Ho et al., 2012). Several studies indicated that Gal-3 is up-regulated in rat, murine and human hearts with CHF (Sharma et al., 2004; de Boer et al., 2009). In human, circulating Gal-3 concentration has been proved as a marker of cardiac fibrosis and can be used as a prognostic marker in CHF human patients (Lok et al., 2010; Ho et al., 2012). Recently, Gal-3 expression in canine heart has not been studied and the concentration of Gal-3 in circulation of dogs with DMVD is unknown. The aims of this study were to determine the expression of Gal-3 in cardiac muscles and to measure level of plasma Gal-3 in CHF dogs with DMVD. The short term goal of this study is to prove a role of Gal-3 as a marker of cardiac fibrosis in DMVD dogs. The long term goal is to use plasma Gal-3 concentration for prognosis or monitoring the response to therapies.

Objectives of Study

1. To determine the expression of Gal-3 in cardiac muscles of normal and DMVD dogs.
2. To measure plasma Gal-3 levels in normal and DMVD dogs.

Hypothesis

1. Gal-3 is up-regulated in cardiac muscles of DMVD dogs compared to normal dogs.
2. Gal-3 concentration is increased in DMVD dogs compared to normal dogs.

Keywords: cardiac fibrosis, congestive heart failure, degenerative mitral valve disease, Galectin-3

Advantages of Study

The advantage of this study is to prove a role of Gal-3 as a marker of cardiac fibrosis in DMVD dogs. In case that Gal-3 can be used as a marker of cardiac fibrosis in dogs affected with DMVD, circulating Gal-3 concentration may be used for prognosis or monitoring the response to therapies.

CHAPTER II

LITERATURE REVIEWS

Degenerative mitral valve disease

Degenerative mitral valve disease (DMVD) is the most common acquired cardiac disease in adult small to medium sized breed dogs. DMVD can be called in other names such as myxomatous mitral valve disease, endocardiosis, chronic valvular disease and chronic myxomatous valve disease. About 97% of 9 years old and older dogs develop severe lesions of mitral valve degeneration (Borgarelli and Buchanan, 2012). The prevalence is age related and varied from 5% to 75% in dogs less than 1 year old to 16 years old (Borgarelli and Buchanan, 2012). Males are more frequently affected than females (Borgarelli and Buchanan, 2012). The predisposing breeds include small to medium sized breed dogs such as Cavalier King Charles spaniels, Miniature poodles, Cocker spaniels, Miniature schnauzers, Dachshunds, Pomeranians, Chihuahuas, Pekingese, Fox terriers, Boston terriers and mixed breed (Disatian, 2010). The heredity was suspected as an important cause to pass on the DMVD from generation to generation (Haggstrom et al., 2004). The previous study confirmed that genetic factors are the cause of this disease in Cavalier King Charles spaniels and Dachshunds (Haggstrom et al., 2004).

DMVD is a progression of valve degeneration. The affected mitral valve leaflets are nodular and thickening. The chordae tendineae become elongated and occasional rupture. Rupture of chordae tendineae usually occurs at minor chords. Rupture of major chords conduces to acute mitral valve regurgitation (Haggstrom et al., 2009). The thickening valve can cause valve regurgitation. Chronic mitral valve regurgitation leads to cardiac remodeling characterized by left atrial and left

ventricular dilatation. Left atrial enlargement may cause atrial fibrillation and worsening clinical outcome (Haggstrom et al., 2009). The pathological changes of valve apparatus cause regurgitant flow from left ventricle to left atrium during systole resulting in reduction of forward stroke volume. All of these changes stimulate the neurohormonal activation in order to maintain sufficient forward stroke volume, blood pressure and tissue perfusion by increasing sodium and water retention as well as vasoconstriction. However, the excessive responses of neurohormonal systems lead to cardiac volume overload and increased afterload. Finally, the affected dogs will develop left sided congestive heart failure (Oyama, 2009). There are several neurohormonal systems involved in pathophysiology of congestive heart failure such as sympathetic nervous system, Renin-Angiotensin-Aldosterone System (RAAS) and endothelin (Bernay et al., 2010). RAAS is a system that plays a major role in response to a decrease of cardiac output and renal blood flow by stimulating the release of renin from kidney. Renin stimulates conversion of angiotensinogen produced by liver to angiotensin I (ATI). ATI are then converted into angiotensin II (ATII) by angiotensin converting enzyme (ACE) from pulmonary system (Oyama, 2009). The biological actions of ATII are to stimulate sympathetic activity, increase sodium and chloride reabsorption, augment potassium excretion, increase fluid retention, constrict blood vessels and accelerate adrenal glands and pituitary gland to secret aldosterone and anti-diuretic hormone (ADH), respectively. Increasing of ATII and aldosterone in long term contributes to increase heart workload and cardiac remodeling (Mochel et al., 2013). The elevation of aldosterone causes many negative effects including promoting myocardial fibrosis and cardiac remodeling by stimulate collagen synthesis (Bernay et al., 2010), decreasing baroreceptor sensitivity and inducing endothelial dysfunction (Struthers, 2004).

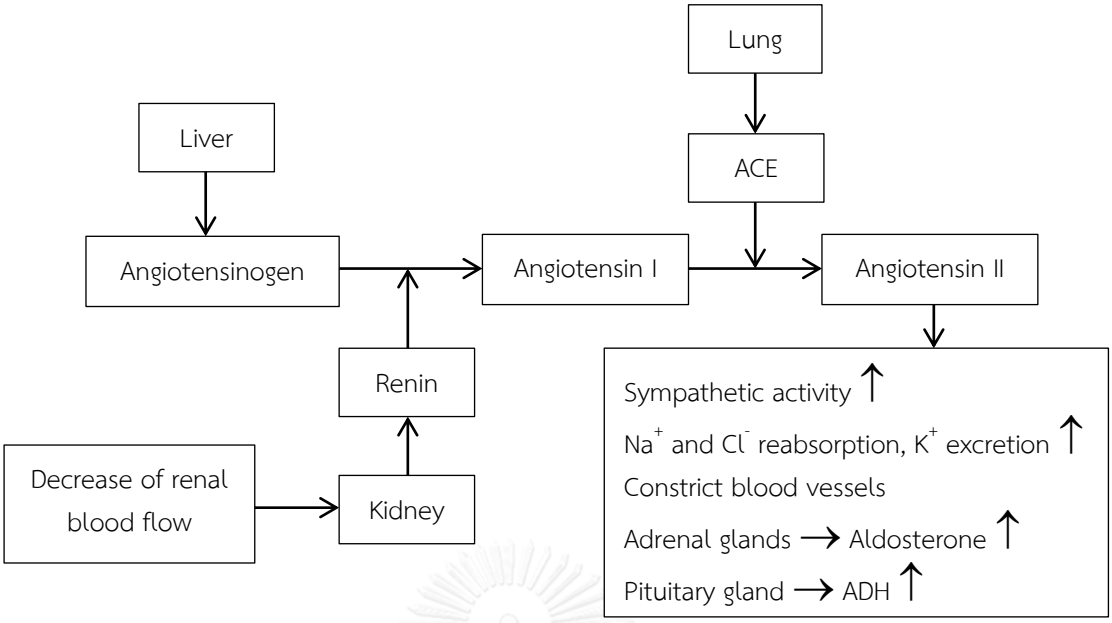

Figure 1: Renin angiotensin aldosterone system

The diagnosis of dogs with DMVD can be performed by history taking, physical examination, thoracic radiography, electrocardiography and echocardiography.

- Signalment: Adult small to medium sized breed dogs are predisposing to this disease. Cavalier King Charles spaniels can have this disease at younger ages and develop CHF faster than other breeds (Borgarelli and Buchanan, 2012).
- History taking: The clinical signs of DMVD dogs include signs of left sided CHF such as cough, exercise intolerance, dyspnea and occasional syncope (Disatian, 2010). Some DMVD dogs may not have clinical sign.
- Physical examination: The left apical systolic murmur loudest at mitral area is usually found. However, dogs with mild DMVD may not have murmur heart sound (Borgarelli and Buchanan, 2012). Intensity of heart murmur is an indirect indicator of mitral regurgitation severity (Haggstrom et al., 2009). The precordial thrill can be palpable in dogs with severe mitral valve regurgitation (Haggstrom et al., 2004).

- Thoracic radiography: Left atrial and ventricular enlargement can be found in dogs with severe DMVD. Vascular enlargement and pulmonary edema can be seen in dogs with left sided CHF (Boswood, 2008; Disatian, 2010).
- Electrocardiography: The findings from electrocardiography are non-specific. Supraventricular arrhythmias, sinus tachycardia and atrial fibrillation can be found (Oliveira et al., 2014).
- Echocardiography: Echocardiography is a gold standard tool to detect dogs with early DMVD (Haggstrom et al., 2004). Two dimensional and M-mode echocardiography are valuable non-invasive procedures that provide information of mitral valve lesions, severity of mitral valve regurgitation, cardiac function and cardiac remodeling (Haggstrom et al., 2004; Boswood, 2008; Disatian, 2010). Nodular, thickening and irregular valves are common echocardiographic findings. Chordae tendineae rupture can be seen in severe cases (Chetboul and Tissier, 2012). Doppler color flow on two dimensional echocardiography is commonly used to evaluate severity of mitral valve regurgitation by determining ratio of regurgitant jet area to left atrial area during systole. The severity of regurgitation is divided into mild (< 20-30%), moderate (≥ 20-30% but ≤ 70%) and severe (> 70%) (Chetboul and Tissier, 2012).

DMVD is a progressive disease. Dogs with mild mitral valve regurgitation may not have clinical sign called asymptomatic DMVD. When severity of regurgitation is increased, decompensated dogs start to show clinical signs of CHF called symptomatic DMVD (Häggström et al., 2004). The identification of stages and disease severity are useful to guide prognosis and select treatment protocols (Atkins and Häggström, 2012). American College of Veterinary Internal Medicine (ACVIM) classified DMVD into 4 stages as follow (Atkins et al., 2009; Atkins and Häggström, 2012):

- Stage A: Dogs that are at high risk for developing heart disease but not yet have cardiac structural changes such as Cavalier King Charles spaniels.
- Stage B: Dogs that have cardiac structural changes with murmur heart sound but have no clinical sign of CHF. This stage is subdivided into B1 and B2.
    - Stage B1: Dogs in this stage have no radiographic and echocardiographic evidence referring to cardiac remodeling.
    - Stage B2: Dogs in this stage have radiographic and echocardiographic evidence of cardiac remodeling such as left sided heart enlargement.
- Stage C: Dogs that have cardiac structural changes with murmur heart sound and have clinical signs of CHF such as cough, exercise intolerance, dyspnea and syncope.
- Stage D: Dogs with end stage CHF and refractory to standard therapy (e.g. Angiotensin Converting Enzyme Inhibitor (ACEI) and furosemide).

The affected dogs with CHF need to be treated by either surgical or medical therapy. The surgical therapy can be done to pause progression of valve degeneration or enhance valve function by valve replacement or valve repair (Griffiths et al., 2004). However, the surgical procedures need to be performed by experienced veterinary surgeons. The cost of the operation is quite high. To increase the success rate of the operation, the procedure has to be done before the dogs have clinical signs of CHF (Haggstrom et al., 2004). Therefore, the surgical therapy for heart valve disease is available in only a few hospital case dogs. The medical therapy is used as a palliative treatment because currently there is no drug that can inhibit or prevent progression of valve degeneration. The targets of medical therapy are to improve quality of life, clinical signs and survival time (Haggstrom et al., 2004). Commonly, veterinarians treat CHF dogs with ACEI and furosemide. This combination is able to prolong survival time and improve quality of life in DMVD dogs (de Madron

et al., 2011). ACEI can suppress RAAS but cannot completely inhibit aldosterone production. Although, aldosterone level decreases in response to ACEI, its level can increase again after several months of treatment, i.e. aldosterone escape (Struthers, 2004). Aldosterone has numerous negative effects promoting progression of heart failure as described above, especially the direct effect on myocardial and endothelial fibrosis.

Cardiac fibrosis

In human, mitral valve prolapse (MVP) is one of cardiovascular diseases caused by an abnormality of mitral valve. The myxomatous degeneration causes valve thickening and redundancy leading to mitral valve regurgitation. Patients with severe mitral valve regurgitation will have left ventricular dilatation and left atrial enlargement (Hayek et al., 2005). The pathophysiology of this disease in human is similar to DMVD in dogs (Pedersen and Haggstrom, 2000). MVP has been suggested to be involved with cardiac remodeling called cardiac dilatation or eccentric hypertrophy (Cohn et al., 2000). Cardiac remodeling is caused by abnormal hemodynamic and neurohormonal activation (Cohn et al., 2000). Histopathological studies on cardiac muscles in MVP patients found an abnormal endocardium and interstitial fibrosis (Mason et al., 1978; Burlew and Weber, 2002).

Myocardium is consisted of cardiomyocytes, cardiofibroblasts, endothelial cells and smooth muscle cells. Cardiac fibroblast is the major cell type found in myocardium (Fan et al., 2012). It has function to regulate collagen turnover in normal heart. Disturbance of homeostasis between collagen synthesis and degradation leads to structural and functional abnormalities of the heart (Kong et al., 2014). Collagen type I and III are mainly found in myocardium. These collagen networks contribute cardiac strength and play a role in connection of myocytes.

There are several conditions stimulating collagen deposition in myocardium such as age, pressure overload, volume overload, hypertrophic cardiomyopathy, toxic substance and metabolic disturbance. The replacement of fibrous tissues in myocardium or myocardial fibrosis occurs secondary to the death of myocytes from cardiac volume overload or cardiac injury by cytotoxic effects of angiotensin II and aldosterone (Mason et al., 1978; Cohn et al., 2000). ATII stimulates cardiac fibroblast proliferation and promotes collagen synthesis by inducing an up-regulation of myocardial procollagens type I and III through transforming growth factor β (TGF-β) and interfering matrix metalloproteinase (MMP) to break down collagen (Passino et al., 2014). Actions of aldosterone on cardiac fibrosis are promoting pro-inflammatory effects, converting macrophages to fibroblasts, activating cardiomyocyte-derived fibrogenic signals and stimulating fibroblast proliferation (Kong et al., 2014). Moreover, chronic elevation of aldosterone causes an increase of potassium loss in urine and leads to a decrease of intracellular potassium. The reduction of intracellular potassium causes cardiac myocyte necrosis following by cardiac fibrosis (Lijnen and Petrov, 2000).

Many human researches indicated that cardiac fibrosis is one of the pathological changes of cardiac remodeling resulting from cardiovascular diseases (Cohn et al., 2000; Lijnen and Petrov, 2000; Burlew and Weber, 2002). A Post-mortem study in humans with cardiovascular diseases found an accumulation of collagen in cardiac muscles called cardiac fibrosis (Pearlman et al., 1982; Huysman et al., 1989; Beltrami et al., 1994; Rossi, 1998). The information of cardiac fibrosis in dogs are mostly obtained from histopathological studies of myocardium in post-mortem or experimental dogs. A previous study demonstrated an increased collagen deposition in left ventricular myocardium of experimental dogs with cardiac dilatation (Weber et al., 1990). A post-mortem study demonstrated that DMVD dogs had more fibrosis in myocardium than normal dogs. The fibrosis was mainly found in sub-endocardium

and papillary muscles of the left ventricle (Falk et al., 2006; Falk et al., 2010; Falk et al., 2013). The survival time of DMVD dogs is associated with the severity of fibrosis in cardiac muscles (Falk et al., 2010).

Cardiac muscles with fibrosis are stiffness and inflexible resulting in systolic and diastolic dysfunction (Kong et al., 2014). Detection of cardiac fibrosis in human patients consists of invasive and non-invasive techniques. Each technique has advantages and disadvantages (de Jong et al., 2012).

Detection of cardiac fibrosis

Invasive techniques

For invasive techniques, cardiac biopsy by catheterization or surgery is widely used to investigate structural changes in myocardium. However, this technique has the risk effect and cannot provide information of overall structural changes. It is representative only the area of collected tissues. Histologically, Masson's trichrome and Picriosirius Red are the routine and cheap techniques for detecting collagen. Fluorescent labeled antibodies, immunohistochemistry and western blot are also used for more specific collagen detection. Because these methods need high cost antibodies and advanced equipments which are not available in all laboratories, the dye staining technique is more wildly used (de Jong et al., 2012).

Non-invasive techniques

- Circulating fibrosis biomarkers: There are several cardiac fibrosis biomarkers that can be detected fibrosis non-invasively including N-terminal procollagen type III (PIIINP) and C- and N- terminal procollagen type I (PICP and PINP), the products of cleaved procollagen as well as matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs), collagen degradation biomarkers. However, these biomarkers are nonspecific makers of cardiac

fibrosis. The levels of these biomarkers can be increased secondary to other sites of fibrosis (de Jong et al., 2012).

- MRI: MRI is a tool that can be used to detect cardiac collagen deposition indirectly by using gadolinium-based contrast agent. However, this technique can detect only large area of fibrosis (de Jong et al., 2012).
- Echocardiography and electrocardiography are ineffective tools for detecting cardiac fibrosis (Falk et al., 2010).

As described above, the histopathology is the gold standard method used to detect cardiac fibrosis. However, this method is not practical to perform in alive patients. The measurement of cardiac fibrosis markers which is a non-invasive method may be more suitable to use specially in alive patients. To date, there are a few cardiac fibrosis markers used in veterinary medicine (Boswood, 2009). PIIINP is a fibrosis marker that has been used widely to detect cardiac fibrosis in humans. However, several studies indicated that PIIINP might be not a good marker to determine the fibrosis status in DMVD dogs (Schuller et al., 2006; Hezzell et al., 2012). Galectin-3 (Gal-3) is a new biomarker associated with fibrosis in humans which is not currently used in veterinary medicine.

Galectin-3

Galectin-3 (Gal-3) is a soluble β-galactoside-binding lectins, with a protein size of 29 to 35 kilodalton (Dumic et al., 2006). Gal-3 consisted of two functional domains including N-terminal domain that have tandem repeated short amino acid segments (110-130 amino acids, depending on species) and C-terminal carbohydrate-recognition domain with 130 amino acids. Gal-3 has high homology of amino acid and gene sequences between different species (Dumic et al., 2006). Gal-3 is widely found in epithelial cells, fibroblasts, monocytes, macrophages, eosinophils,

neutrophils, mast cells and dendritic cells (de Boer et al., 2009). In tissues, Gal-3 overexpressed in lung, spleen, stomach, colon, adrenal gland, uterus and ovary; on the other hand, it expressed at a low level in kidney, heart, cerebrum, pancreas and liver (de Boer et al., 2010). Because Gal-3 has no signal peptide for vesicle-mediated exocytosis secretory pathway, it is localized in cytoplasm and occasionally in nucleus. Gal-3 can move from cytoplasm to nucleus by passive and active pathway (de Boer et al., 2010). Gal-3 has no specific receptors. It can interact with both cell surface receptors (e.g. macrophage) and extracellular receptors (e.g. collagen IV) containing suitable oligosaccharides (de Boer et al., 2010). Gal-3 can be secreted via non-classical secretory pathway by activated macrophages, mast cells, eosinophils and fibroblasts into extracellular space then bind to cell surface receptors to initiate transmembrane signaling pathway leading to inflammation and fibrosis (de Boer et al., 2009; de Boer et al., 2011). Gal-3 is involved in many biological functions including cell differentiation, proliferation, adhesion, inflammation, immune response, cancer and promoting fibrosis.

Location of Gal-3 expression can be defined its biological roles. There are two types of Gal-3 including intracellular Gal-3 and extracellular Gal-3.

Intracellular Gal-3

Intracellular Gal-3 can be found both in cytoplasm and nucleus. Gal-3 in cytoplasm is regulated cell proliferation, differentiation, survival and death. Nuclear Gal-3 is regulated gene transcription and required as a pre-mRNA splicing factor (Haudek et al., 2010). Gal-3 can stimulate cell proliferation in experimental fibroblast cell culture. An up-regulation and down regulation of Gal-3 affect the B-cell differentiation between memory cells and plasma cells (de Boer et al., 2009). Gal-3 has both pro-apoptotic (extracellular Gal-3) and anti-apoptotic (intracellular Gal-3) properties (Dumic et al., 2006; de Boer et al., 2009; Haudek et al., 2010).

Extracellular Gal-3

Extracellular Gal-3 can be found on cell surface, in extracellular matrix and biological fluids. Gal-3 is exported to outside of the cell via vesicles containing Gal-3. Extracellular Gal-3 is mediated cell adhesion, cell activation and chemoattraction via autocrine and paracrine pathway (Dumic et al., 2006; de Boer et al., 2009).

The location of Gal-3 in cardiac tissues has not been reported. Immunohistochemistry and confocal laser microscopy studies revealed the localization of Gal-3 in fibroblasts and macrophages in myocardial matrix and at the sites of fibrosis (de Boer et al., 2009; de Boer et al., 2010).

Gal-3 in cardiovascular disease

There are several conditions attributed to cardiac injury leading to cardiac fibrosis. Fibroblasts, myofibroblasts and macrophages are key cells involved in initiation and progression of cardiac fibrosis. When cardiac injury occurs, activated macrophages are infiltrated to the site of injury and secreted Gal-3 into extracellular space. Gal-3 in extracellular space bound to intracellular receptors of fibroblasts then activated resting fibroblasts to active fibroblasts (Inohara et al., 1998; Sharma et al., 2004; de Boer et al., 2009). Active fibroblasts secreted procollagen into extracellular matrix. Amino and carboxy-peptide terminals of procollagen are then removed by protease enzymes to form mature collagen leading to collagen deposition in myocardium (Hundae and McCullough, 2014; Passino et al., 2014).

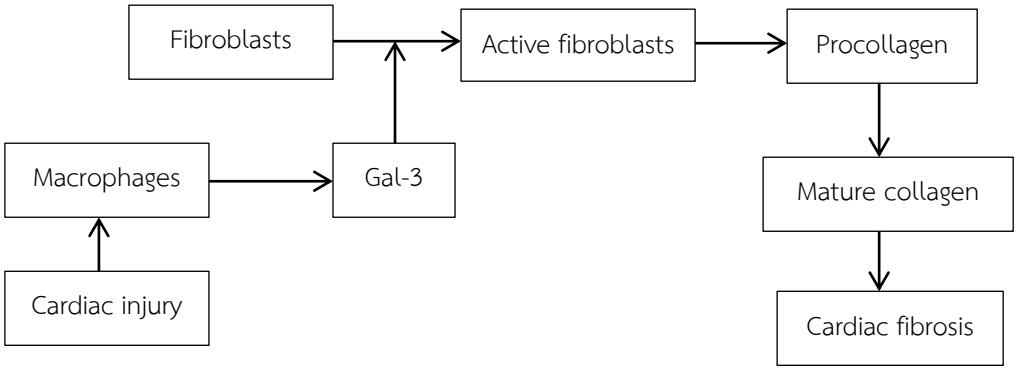

Figure 2: Roles of Gal-3 on cardiac fibrosis (Modified from Hundae and McCullough, 2014)

Expression of Gal-3 in cardiac muscles

Immunohistochemistry is an useful technique to detect Gal-3 expression in cardiac muscles (Sharma et al., 2004; de Boer et al., 2009) and other tissues (Kim et al., 2006; Nio et al., 2006; Kim et al., 2008; Kim et al., 2009; Nio-Kobayashi et al., 2009; Park et al., 2012; Haddad Kashani et al., 2013). This method provides distribution, localization and location of Gal-3 expression in tissue samples. Moreover, it can be showed level and intensity of Gal-3 expression in different tissues (Hofman and Taylor, 2001).

Immunohistochemistry demonstrated an up-regulation of Gal-3 in CHF rat, murine and human hearts with cardiac fibrosis (Sharma et al., 2004; de Boer et al., 2009; de Boer et al., 2010; Beiras-Fernandez et al., 2013; Yu et al., 2013; Passino et al., 2014). There are several studies about relationship between Gal-3 and cardiovascular diseases. Gene expression studies indicated that Gal-3 gene was overexpressed in CHF rat hearts. The expression of Gal-3 was 5-fold higher in decompensated than in compensated CHF rat hearts (Sharma et al., 2004; de Boer et al., 2010).

Expression of Gal-3 in early stage hypertrophic heart was increased only in rats that later developed CHF but not in rats that do not develope CHF (Sharma et al., 2004). Human patients with aortic stenosis with the depression of ejection fraction overexpressed Gal-3 in myocardium (Sharma et al., 2004).

Expression of Gal-3 in blood circulation

The gold standard method used to detect cardiac fibrosis is histopathology which is not practical in alive patients. Measurements of cardiac fibrosis markers may be more suitable. Gal-3 is a new biomarker of cardiac fibrosis used in human patients. Levels of circulating Gal-3 are associated with left ventricular mass related to cardiac fibrosis in human patients (Ho et al., 2012). Moreover, circulating Gal-3 concentration was higher in CHF than healthy people (Milting et al., 2008; Lok et al., 2010; Beiras-Fernandez et al., 2013). Once Gal-3 is elevated, its level is stable for a long period of time. Normal cut-off value of Gal-3 in humans is 17.7 ng/ml. Patients with subclinical stage of CHF had minor elevation of Gal-3 concentration (de Boer et al., 2011). Patients with higher baseline Gal-3 level tended to have more mortality rate than others (Lok et al., 2010). An increase of plasma Gal-3 was related to an increase risk of mortality from cardiovascular diseases (de Boer et al., 2011) and increased incidence of CHF (Ho et al., 2012). Moreover, Gal-3 level was increased along with cardiovascular disease progression and associated with severity outcome (Lopez-Andres et al., 2012). Based on the information mentioned previously, not only a cardiac fibrosis marker, but circulating Gal-3 concentration can also be used as a prognostic marker to predict an onset of CHF in asymptomatic patients as well as the time of hospitalization and death of CHF patients (de Boer et al., 2010; Lok et al., 2010; Ho et al., 2012).

There are 2 major methods used to determine circulation Gal-3 levels including automated immunoassay and enzyme-linked immunosorbent assay (ELISA). ELISA method takes more times to do than automated immunoassay. However, the sensitivity of these 2 methods are similar (Passino et al., 2014). ELISA is an easier and convenient method to evaluate value of Gal-3 concentration in each patient. To date, there are species specific ELISA kits providing more reliable results.

All of above information revealed the advantage of Gal-3 as a cardiac fibrosis marker in human patients. However, the expression of Gal-3 in canine cardiac muscles has not been studied and the concentration of circulating Gal-3 in dogs with DMVD is unknown. Thus, the present study aimed to determine the expression of Gal-3 in cardiac muscles and measure level of plasma Gal-3 in order to prove whether Gal-3 can be a candidate marker of cardiac fibrosis in dogs affected with DMVD.

CHAPTER III

MATERIALS AND METHODS

This study was divided into two parts. Part I was the determination of expression of Gal-3 in cardiac muscles and Part II was the determination of plasma Gal-3 concentration.

Part I: Histology

Tissue samples

22 samples from 6 years old or older with body weight less than 15 kilograms necropsy dogs presented at the Department of Veterinary Pathology, Faculty of Veterinary Science, Chulalongkorn University were collected. The hearts were removed and dissected to determine mitral valve thickness. Specimens were divided into 2 groups as following criteria.

- **DMVD group:** 12 dogs with mitral valve thickness more than 2 millimeters and left ventricular chamber dilatation were selected into this group.
- **Control group:** 10 dogs without lesion of mitral valve degeneration (valve thickness less than 2 millimeters) and cardiac remodeling were included into this group.

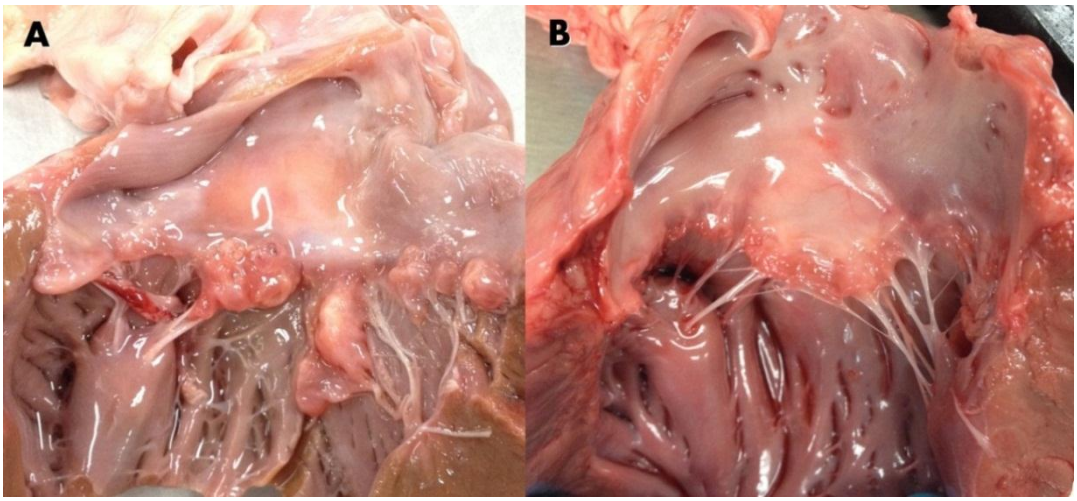

Figure 3: Degenerative mitral valve leaflets (A) and normal valve leaflets (B).

Tissue preparation

The hearts were removed and dissected in both ventricles from apex to base. After dissected, mitral valve leaflets were determined and measured by vernier caliper. Myocardial tissue samples were randomly collected at papillary muscles and left ventricular walls (4 pieces per dog). Size of tissue samples was approximately 1 cm^2 each. Samples were fixed in 10% formalin for 24 hours, histologically processed and then embedded in paraffin blocks. All tissue blocks were sectioned at 4 μm thickness and placed on silane-coated slides and stained with Hematoxylin and Eosin (H&E) for determining general appearance and Masson trichrome (MT) for evaluating collagen expression i.e. cardiac fibrosis.

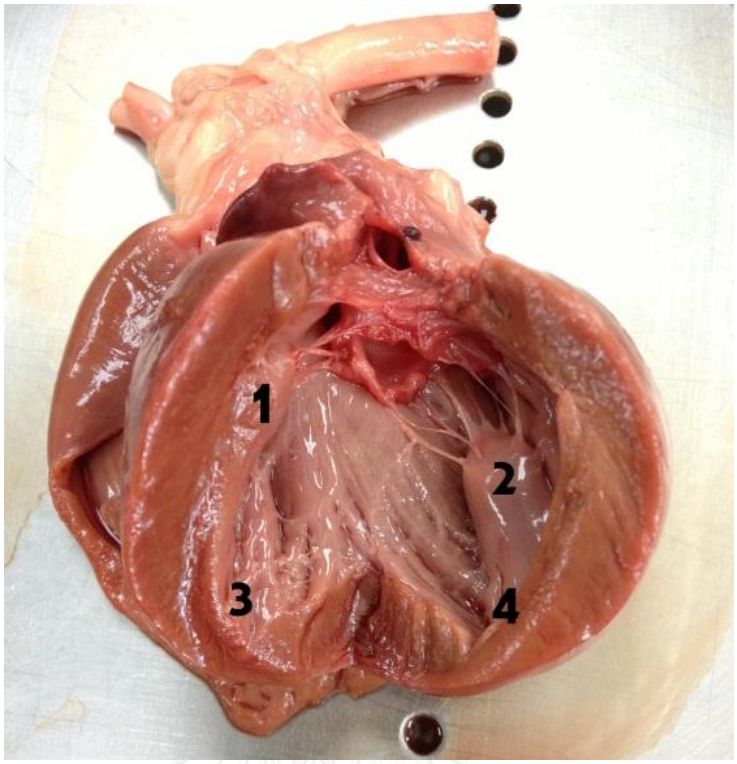

Figure 4: Sampling sites of left ventricle: papillary muscles (1, 2) and left ventricular walls (3, 4).

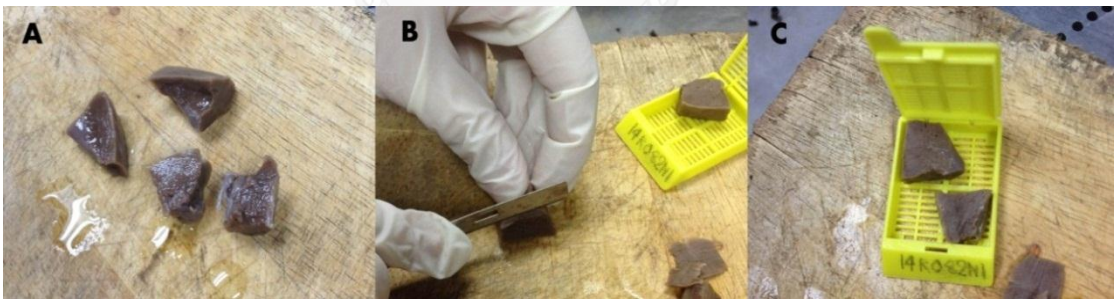

Figure 5: Pathological process of tissue sampling: A) Tissues were collected 4 pieces/dog at size approximately 1 cm^2 each, B) After 24 hours of formalin fixation, tissues were trimmed and then placed into cassettes (C) for histological process.

Cardiac fibrosis evaluation by Masson trichrome staining

All tissue sections were deparaffinized in xylene, rehydrated in absolute and 95% alcohol, immersed in Mordant in Bouin's solution overnight at room temperature then washed in running water followed by distilled water (DW). After that, the sections were stained with Weigert's iron hematoxylin solution for 10 minutes. After washing process, the sections were stained with Biebrich scarlet-acid fuchin solution for 3 minutes, washed with DW and then immersed in Phosphomolybdic-phosphotungstic acid for 15 minutes. Collagen was stained by Aniline blue solution for 20 minutes, washed with DW and immersed in 1% Gracial acetic solution for 5 minutes. Thereafter, all section slides were dipped in 100% alcohol and xylene for dehydrating and then mounted. All of these procedures were done by one person and same condition at the Department of Veterinary Pathology, Faculty of Veterinary Science Chulalongkorn University.

All slides were microscopically examined for myocardial fibrosis from Masson trichrome staining. Sections were randomly photographed under light microscope for 10 areas with Olympus BX50 photomicroscope under 20x magnification. The areas of blue staining (collagen) were calculated as a percentage of the total selected area by using Image-ProPlus software (Image – Pro® Plus version 6.0, Media Cybernetics, Inc., Rockville, MD, U.S.A.) (Linharattanaruksa et al., 2014; Park et al., 2014).

Galectin-3 expression by immunohistochemistry

4 μm thick sections were deparaffinized in xylene, rehydrated in different concentration alcohol (absoluted, 95%, 80% and 70% alcohol), and then pretreated with citrate buffer (0.01M, pH 6.0) heated in autoclave for 5 minutes. Endogenous peroxidase activity was blocked by 3% hydrogen peroxide for 10 minutes at room temperature. Slides were washed with phosphate-buffered saline (PBS; pH7.4) and

non-specific antibody was blocked by binding with normal bovine serum albumin for 20 minutes at 37°C. The slides were washed with PBS and incubated with primary antibody at 4°C overnight. Monoclonal mouse antibody against human galectin-3 (NCL-GSL3; Novocastra Laboratories, Newcastle, UK) was used as a primary antibody at 1 in 100 dilution. After washing with PBS, the slides were incubated with EnVision kit (Dako, Hamburg, Germany) for 45 minutes at 37°C and washed again with PBS. Peroxidase activity was developed by incubated with 3,3'-diaminobenzidine tetrahydrochloride (1:50 DAB) (Dako, Hamburg, Germany) for 3 minutes at room temperature. Finally, slides were counterstained with Mayer's Hematoxylin and then mounted (Choi et al., 2004; Johnson et al., 2007). The positive control slides were prepared by using canine mammary adenoma (Choi et al., 2004). All of these procedures were done by one person and same condition.

All slides were microscopically examined for the area of Gal-3 immunolabelling. Sections were randomly photographed under light microscope for 10 areas with Olympus BX50 photomicroscope under 20x magnification and calculated the mean score of Gal-3 expression. The expression of Gal-3 was quantitatively evaluated by image analyzer program. Score of Gal-3 expression was calculated as a percentage of positive area (brown) to total selected area by using Image-ProPlus software (Image – Pro® Plus version 6.0, Media Cybernetics, Inc., Rockville, MD, U.S.A) (Johnson et al., 2007).

Part II: Determination of plasma Gal-3

Animals

The study protocol was approved by Animal Care and Use Committee, Faculty of Veterinary Science, Chulalongkorn University (Animal Use Protocol No. 1431033). Blood samples were collected from 46 dogs with 6 years old or older and body weight less than 15 kilograms presented at the Small Animal Hospital, Faculty of Veterinary Science, Chulalongkorn University with the permission from the owners. All dogs were performed history taking, physical examination, blood collection, thoracic radiography and echocardiography. Dogs were then divided into 2 groups as follow.

- **DMVD group:** consisted of 27 DMVD dogs newly diagnosed for DMVD.

  Inclusion criteria

  To be included in this group, each dog had to be newly diagnosed with DMVD and had normal blood profiles for complete blood count (CBC) and blood chemistry. All dogs in this group had not been treated with cardiovascular medicine. Dogs with or without clinical sign of heart failure such as exercise intolerance, cough and dyspnea were included.

  Exclusion criteria

  Dogs were excluded, if they were on any cardiovascular medication. Dogs that have other cardiac diseases or systemic diseases such as hepatic diseases: alkaline phosphatase (ALP) of more than 5 times the upper limit of normal range and alanine aminotransferase (ALT) of more than 2-3 times the upper limit of normal range (Webster, 2010), kidney diseases: creatinine greater than 1.8 mg%; BUN greater than 27 mg% (Douglass et al., 2005) and neoplasia were excluded.

- **Control group:** consisted of 19 healthy dogs.

  Inclusion criteria

  To be included in this group, each dog had to have normal physical examination, normal blood profiles for CBC and blood chemistry.

  Exclusion criteria

  Dogs were excluded, if they had evidence of heart murmur, cardiovascular diseases or other systemic diseases such as hepatic diseases, kidney diseases and neoplasia.

Clinical Procedures

All data of history taking, physical examination, blood profiles, thoracic radiography and echocardiography were collected as baseline data

1. History taking consisted of signalment, clinical signs, drugs in use, and related disease.
2. Complete physical examination was performed and recorded including color of mucous membrane, capillary refilling time, hydration status, heart sound, lung sound, pulse quality, temperature, heart rate, respiratory rate and body condition score.
3. Blood collection for complete blood count, blood chemistry profiles and blood parasite were evaluated to rule out other systemic diseases such as liver and kidney diseases by in-house Veterinary Laboratory of Small Animal Hospital, Faculty of Veterinary Science, Chulalongkorn University.
4. Thoracic radiography was performed to evaluate heart size and shape, severity of pulmonary edema and rule out primary respiratory diseases. Two view radiographs including dorsoventral and lateral views were performed.

The radiography was interpreted by clinician at radiology unit of Small Animal Hospital, Faculty of Veterinary Science, Chulalongkorn University.

5. Echocardiography was performed to confirm the diagnosis of DMVD and determined the cardiac structural changes by using ultrasound machine (Logic™ 5 Pro) with 6-10 multifrequency and 5-6 MHz phrase array transducer microconvex probe. All dogs were not sedated and restrained in right lateral recumbency.

    5.1 Two-dimensional echocardiography was used to determine mitral valve lesions including thickening of valve leaflet and chordae tendineae, valve prolapse or chordal rupture (Fig. 6). All of these procedures were determined from right parasternal four chamber view by one sonologist.

    5.2 M-mode echocardiography was used to determine evidence of cardiac remodeling. Echocardiographic indices including left ventricular end diastolic diameter (LVEDd), left ventricular end systolic diameter (LVESd), wall thickness of left ventricular free wall during diastole (LVWd) and systole (LVWs), interventricular septal thickness during diastole (VSd) and systole (VSs) and the ratio of left atrium to aorta dimension (LA/AO) were determined. All of these parameters were measured from right parasternal view. The fractional shortening was calculated by (LVEDd-LVESd)/LVEDd x100.

5.3 Color flow Doppler on two-dimensional echocardiography was performed to identify mitral regurgitation and estimate severity of valve regurgitation (Fig. 7) by determining the ratio of regurgitant jet area to left atrium area during systole. The severity of regurgitation divided into mild ($<$ 20-30%), moderate ($\geq$ 20-30% but $\leq$ 70%) and severe ($>$ 70%) (Chetboul and Tissier, 2012).

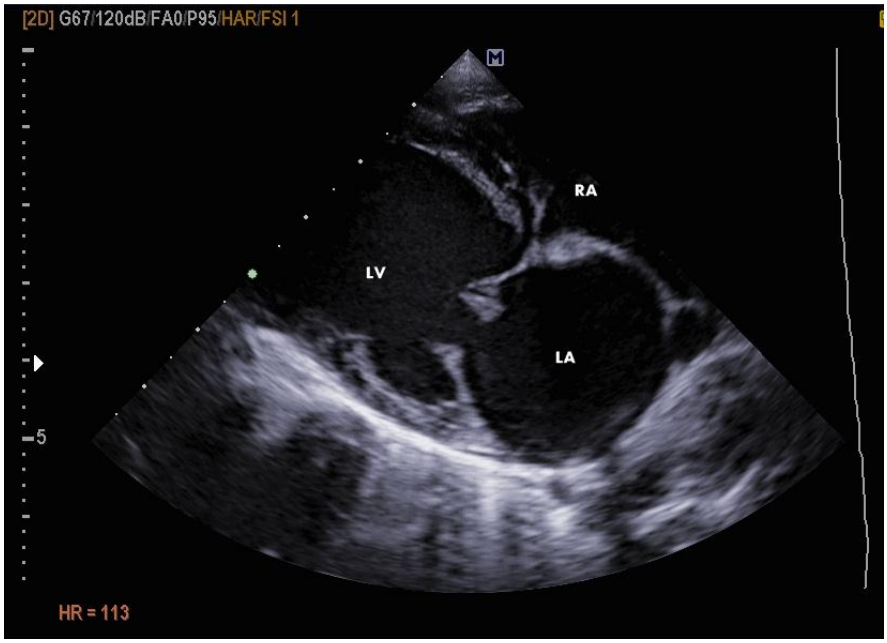

Figure 6: Two-dimensional right parasternal four chamber view of echocardiogram shows thickening of mitral valve leaflets. LV: left ventricle, LA: left atrium, RA: right atrium (Picture courtesy of Dr.Sirilak Surachetpong).

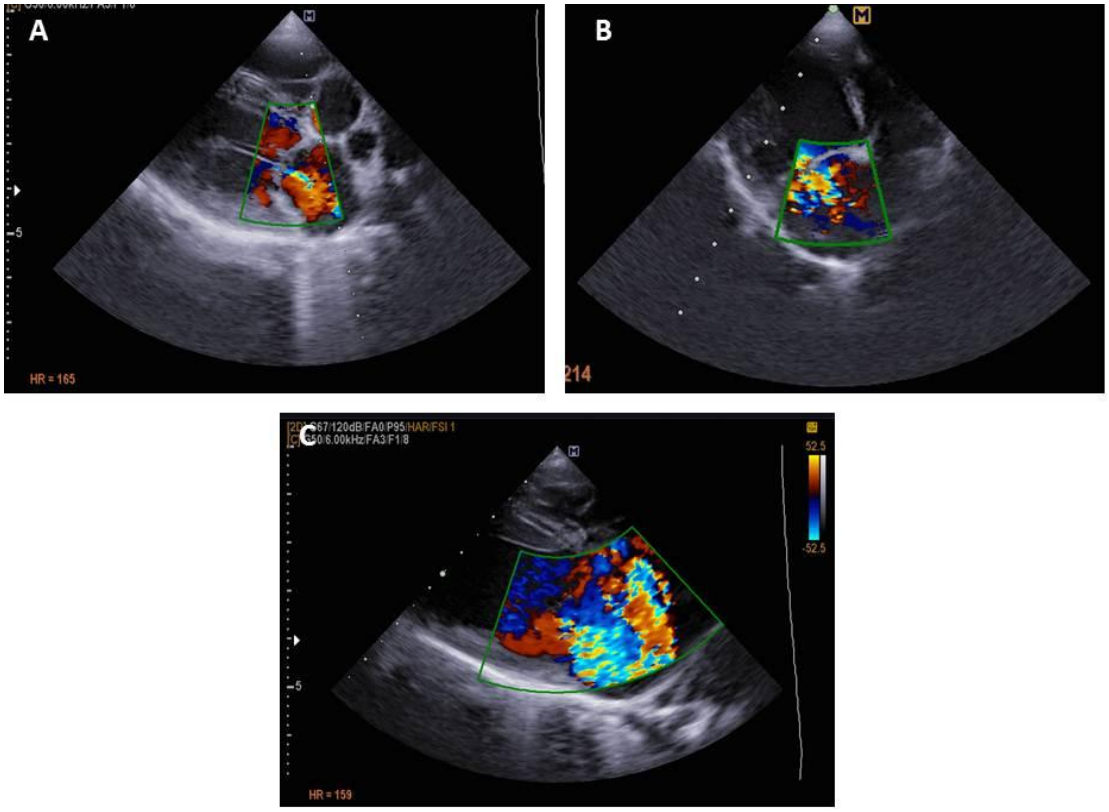

Figure 7: Color-flow Doppler echocardiogram shows severity of mitral valve regurgitation. Fig. 7A: mild (jet area $<$ 20-30%) and Fig. 7B: moderate (jet area $\geq$ 20-30% but $\leq$ 70%) and Fig. 7C: severe (jet area $>$ 70%) (Picture courtesy of Dr.Sirilak Surachetpong).

Sample collection and preparation

To measure the plasma levels of Gal-3, blood samples were collected in both groups of dogs. One milliliter of blood was collected from cephalic or saphenous vein. Blood was contained in eppendorf with EDTA as an anticoagulant. The blood sample was centrifuged at approximately 1000xg (or 3000 rpm) for 15 minutes. Then, plasma was placed into a new plain eppendorf and stored at -20 $^{\circ}$C until assay. All of these procedures were done by one person and same condition.

Measurement of Gal-3 concentration

All samples were batched for analysis and done in duplicate. Gal-3 concentration was analyzed by canine Galectin-3 (GAL3) ELISA kit (BlueGene Biotech, Shanghai, China). All procedures were done according to the test kit direction. Briefly, samples and buffer were incubated with GAL3-HRP conjugate in microplate. After 1 hour incubation period, wells were poured and washed to remove non-specific binding then incubated with substrate for HRP enzyme. Finally, the reaction was stopped by added stop solution into microplate. Spectrophotometry at 450 nm was used to measure intensity of color. Quantitative measurement of Gal-3 concentration can be done by comparing their absorbance with standard curve plotted from intensity of color and concentration from standard solution (BlueGene Biotech, Shanghai, China).

Validation of this ELISA assay was performed by using one of plasma samples from control dogs mixed with 5 different manufacturer standard concentrations of Gal-3 as a spike samples. Concentrations of manufacturer standards and spiked samples were plotted and evaluated for linearity. Recovery was calculated as percentage of recovered Gal-3 spiked samples to expect Gal-3 at 5 different concentrations (Arndt et al., 2009; Mangklabruks and Surachetpong, 2014).

Statistical analysis

Statistical analysis was performed by the computer-based software, SPSS program. All data were tested for normality by Shapiro-Wilk test before run statistical testing.

Expression of Gal-3 in cardiac muscle

Descriptive statistic was applied for signalment including breeds and sex in normal and DMVD group. Mean $\pm$ SD was applied for signalment including age and weight in normal and DMVD group. Differences of age and weight between normal and DMVD group were determined by independent T - test. The data of percentage of fibrosis area and percentage of Gal-3 expression were presented as Mean $\pm$ SD. Independent T - test was used to compare percentage of fibrosis area and percentage of Gal-3 expression in cardiac muscles between normal and DMVD groups. Paired T - test was used to compare percentage of fibrosis area and percentage of Gal-3 expression between papillary muscles and LV walls of normal and DMVD groups. The correlations between percentage of fibrosis area and age and weight of normal group were evaluated by Pearson's correlation. The correlations between percentage of Gal-3 expression and age and weight of normal group were evaluated by Pearson's correlation. The correlation between percentage of fibrosis area and Gal-3 expression was evaluated by Pearson's correlation. P value < 0.05 is considered statistical significant.

Determination of plasma Gal-3

Descriptive statistic was applied for signalment including breeds and sex in normal and DMVD group. Mean $\pm$ SD was applied for signalment including age and weight in normal and DMVD group. Differences of age and weight between normal

and DMVD group were determined by independent T - test. Information about history taking, physical examination and thoracic radiography were represented as descriptive statistic. Mean $\pm$ SD was applied for CBC, blood chemistry and echocardiographic index. Independent T - test was used to compare the CBC, blood chemistry and echocardiographic indices between control and DMVD group. Plasma Gal-3 concentration was non-parametric and represented as median; 25^{th} and 75^{th} percentile. Mann-Whitney U test was used to compare plasma Gal-3 concentration between control and DMVD group. Correlations between plasma Gal-3 concentration and age and weight of normal group were evaluated by Spearman's rank correlation. The correlation between plasma Gal-3 concentration and echocardiographic indices were evaluated by Spearman's rank correlation. P value < 0.05 is considered statistical significant.

CHAPTER IV

RESULTS

Part I: Histopathology

Signalment

Cardiac muscles were collected from 22 necropsy dogs. Normal control group consisted of 10 dogs including one male and nine females (Female = 6; Female spay = 3). Breeds of dogs were Poodle (n = 4), Shih-Tzu (n = 2), French bulldog (n = 1), Chihuahua (n = 1), Beagle (n = 1) and Pomeranian (n = 1). DMVD group consisted of 12 dogs including 4 males (Male = 3; Male castrate = 1) and eight females (Female = 5; Female spay = 3). Breeds of dogs were Poodle (n = 7), Shih-Tzu (n = 3), Schnauzer (n = 1) and mixed breed (n = 1). Age of DMVD group was statistically significant older than normal group ($p < 0.05$). Weight between normal group and DMVD group was not statistically significant different. The major cause of death in DMVD group was associated with cardiorespiratory failure (n = 9). The others were died from other diseases (Septicemia = 2; Hepatic failure = 1).

Table 1: Signalment data of normal and DMVD groups (mean ± SD)

Group	N	Male	Female	Age (years)	Weight (kg)
Normal	10	1	9	9.45 ± 3.18	5.54 ± 2.00
DMVD	12	4	8	12.83 ± 4.03*	6.61 ± 2.01

The significant difference was assessed by independent - T test at $p < 0.05$.

* Indicate statistically difference at $p < 0.05$ between normal and DMVD groups.

Cardiac fibrosis evaluation by Masson trichrome staining

Cardiac muscles were collected from normal and DMVD dogs for investigating cardiac fibrosis macroscopically and microscopically. Gross appearance of DMVD hearts showed nonspecific lesion. DMVD dogs had round with left ventricular chamber dilatation (Fig. 8). Microscopically, large area of cardiac fibrosis was found in DMVD dogs, especially in sub-endocardium of papillary muscles (Fig. 9). Masson trichrome staining showed an increased collagen deposition in sub-endocardium area of DMVD hearts (Fig. 10). Percentage of fibrosis area was significantly higher in DMVD than normal group in both papillary muscles ($p < 0.01$) and LV walls ($p < 0.01$). The area of fibrosis was higher in the papillary muscles than in LV walls in both normal ($p < 0.05$) and DMVD ($p < 0.01$) groups.

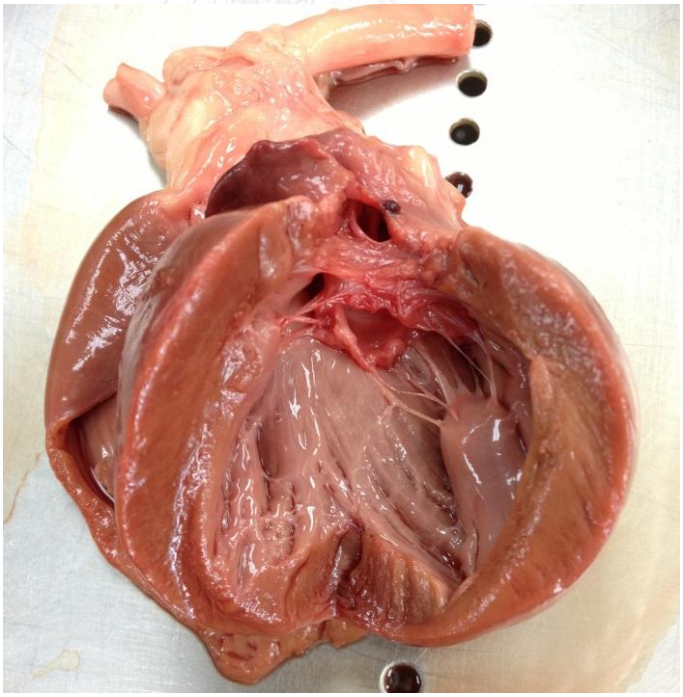

Figure 8: Gross appearance of DMVD heart was round with left ventricular chamber dilatation.

Table 2: Percentage of fibrosis area of papillary muscles and LV walls in normal and DMVD groups (mean ± SD)

Group	% Fibrosis area	
	Papillary muscles	LV walls
Normal	35.402 ± 8.457[a]	27.405 ± 7.911[b]
DMVD	66.125 ± 5.579[*, c]	52.975 ± 8.448[*, d]

The significant difference between normal and DMVD groups was assessed by independent - T test.

The significant difference between papillary muscles and LV walls was assessed by paired - T test.

[*] Indicate statistically difference at $p < 0.01$ between normal and DMVD groups.

[a,b] Indicate statistically difference at $p < 0.05$ between papillary muscles and LV walls of normal group.

[c,d] Indicate statistically difference at $p < 0.01$ between papillary muscles and LV walls of DMVD group.

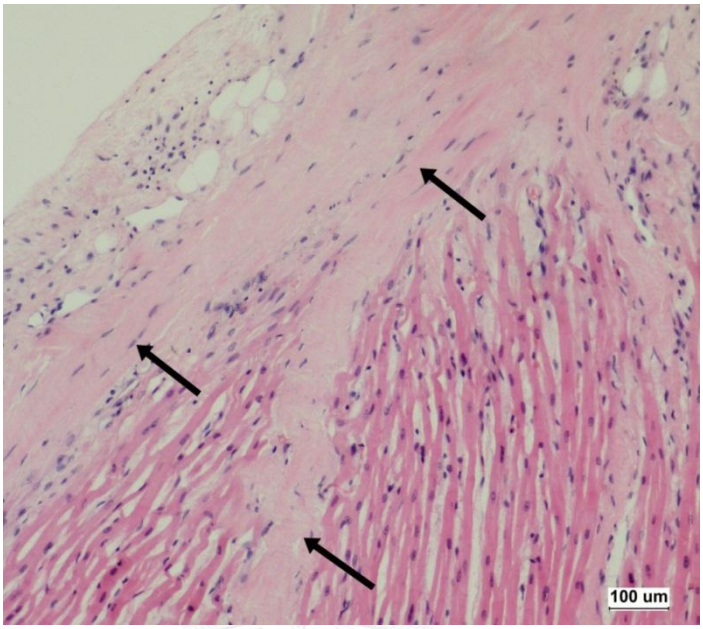

Figure 9: Sub-endocardial fibrosis in papillary muscle of DMVD dog (arrows) (H&E stain, 10x magnification).

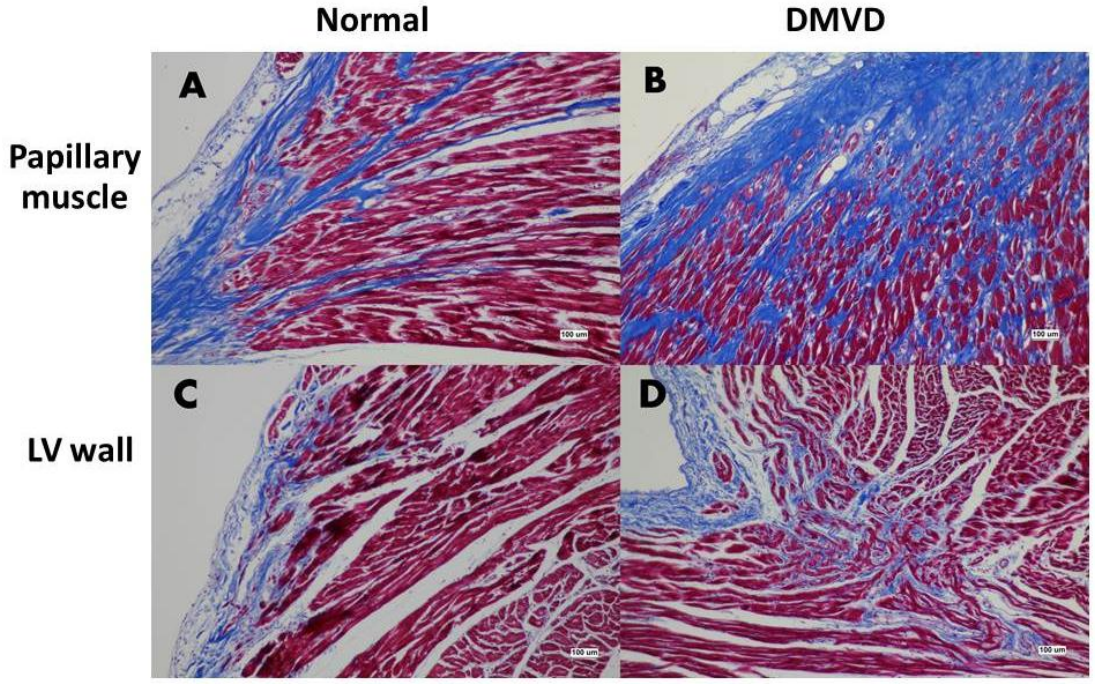

Figure 10: Sub-endocardial fibrosis (blue) in papillary muscle (A,B) and LV wall (C,D) of normal and DMVD groups respectively. The collagen deposition was mainly found in sub-endocardium (Masson trichrome stain, 10x magnification).

There was no statistically correlation between percentage of fibrosis area and age as well as weight of normal dogs.

Table 3: The correlations between percentage of fibrosis area and age and weight of normal dogs

Parameters	r	P-value
Age	-0.097	0.789
Weight	0.382	0.276

The significant correlation was assessed by Pearson's correlation at $p < 0.05$.

r = Pearson's correlation coefficient

Galectin-3 expression by immunohistochemistry

Immunohistochemistry was performed to detect Gal-3 in cardiac muscles. Expression of Gal-3 in 12 DMVD and 10 normal heart samples was evaluated quantitatively by Image – ProPlus software. Gal-3 expression was observed in cytoplasm of cardiomyocytes and larger cells beneath endocardium which were suspected to be Purkinje cells and mainly found in sub-endocardium in DMVD group (Fig. 11 and 12). All DMVD hearts (100 percent; 12/12) were positive for Gal-3, while 30% of normal hearts (3/10) were positive for Gal-3. Percentage of Gal-3 expression was statistically increased in DMVD group compared to normal group in papillary muscles ($p < 0.01$) and LV walls ($p < 0.01$). Papillary muscles had statistically higher percentage of Gal-3 expression than LV walls in normal ($p < 0.05$) and DMVD groups ($p < 0.01$).

Table 4: Percentage of Gal-3 expression of papillary muscles and LV walls in normal and DMVD groups (mean ± SD)

Group	**% Gal-3 expression**	
	Papillary muscles	**LV walls**
Normal	1.073 ± 0.668^{a}	0.517 ± 0.421^{b}
DMVD	$27.945 \pm 6.944^{*, c}$	$17.247 \pm 8.758^{*, d}$

The significant difference between normal and DMVD groups was assessed by independent - T test.

The significant difference between papillary muscles and LV walls was assessed by paired - T test.

* Indicate statistically difference at $p < 0.01$ between normal and DMVD groups.

a,b Indicate statistically difference at $p < 0.01$ between papillary muscles and LV walls of normal group.

c,d Indicate statistically difference at $p < 0.01$ between papillary muscles and LV walls of DMVD group.

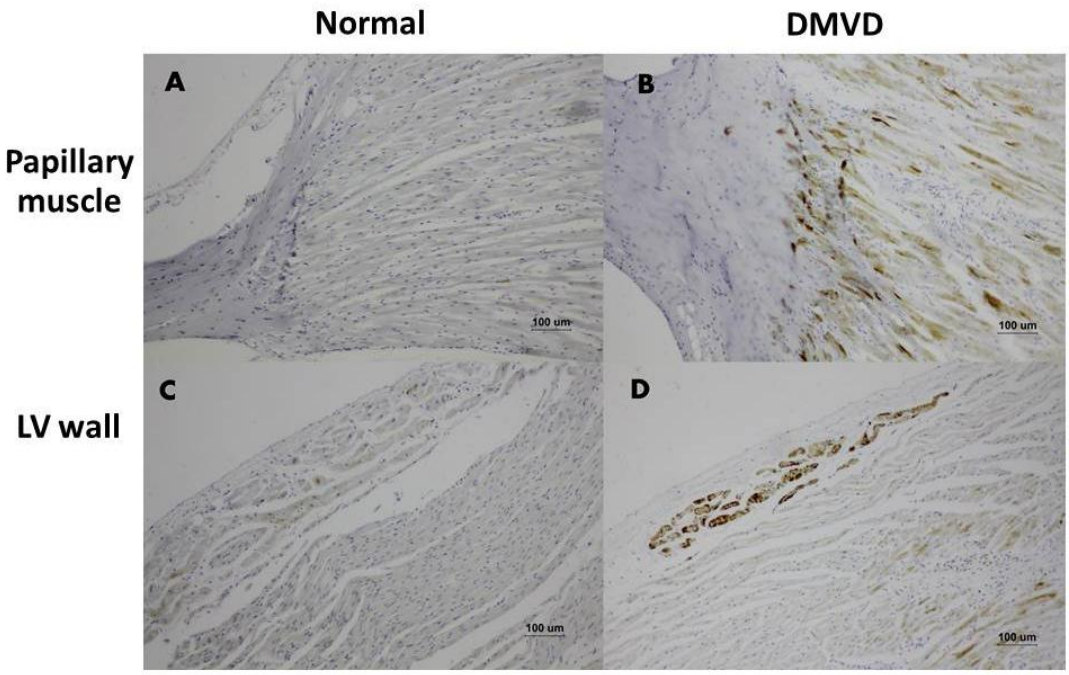

Figure 11: Gal-3 expression in papillary muscles (A,B) and LV muscles (C,D) of normal and DMVD groups respectively. Gal-3 expression was mainly found in papillary muscles and sub-endocardium. (LSAB, IHC, Mayer's Hematoxylin counterstained, 10x magnification).

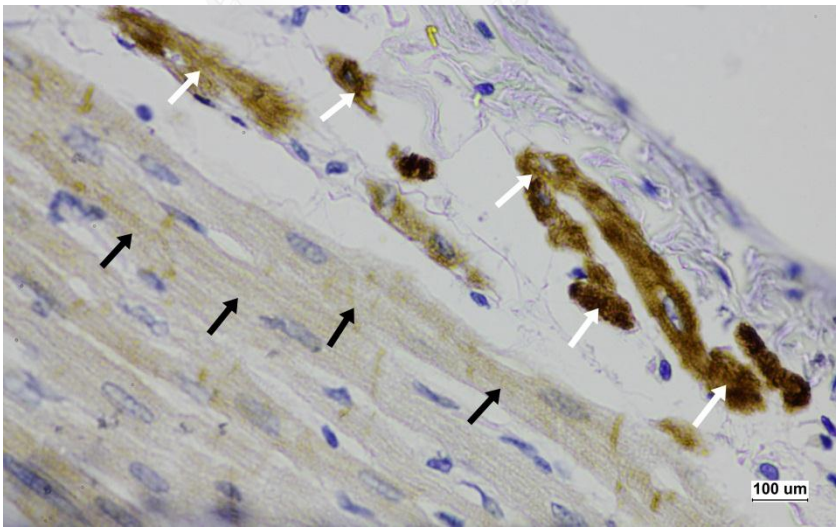

Figure 12: Gal-3 expression in cytoplasm of Purkinje like cells (white arrows) and cardiomyocytes (black arrows) (LSAB, IHC, Mayer's Hematoxylin counterstained, 40x magnification).

There was no correlation between percentage of Gal-3 expression and age as well as weight of normal dogs.

Table 5: The correlations between percentage of Gal-3 expression and age and weight of normal dogs

Parameters	r	P-value
Age	-0.179	0.621
Weight	0.186	0.606

The significant correlation was assessed by Pearson's correlation at $p < 0.05$.

r = Pearson's correlation coefficient

There was statistically significant positive correlation (r = 0.821) between percentage of fibrosis area and percentage of Gal-3 expression of entire population ($p < 0.01$) (Fig. 13).

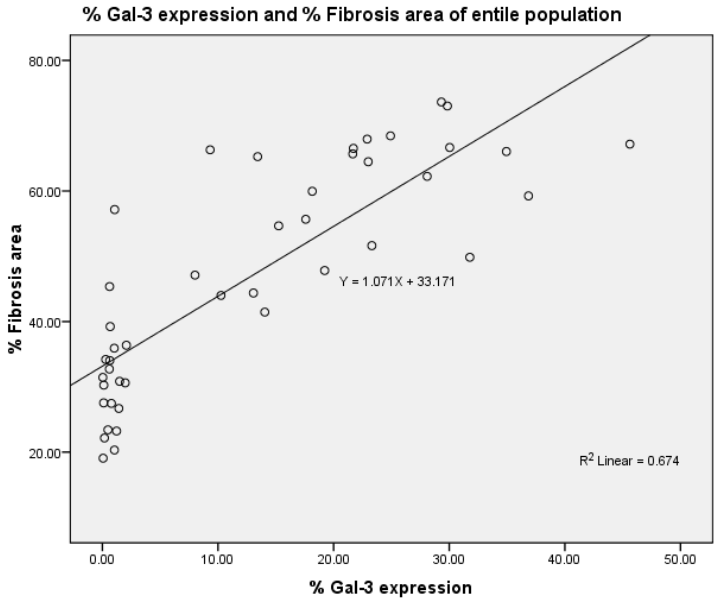

Figure 13: The correlation between percentage of fibrosis area and percentage of Gal-3 expression of entire population.

Part II: Determination of plasma Gal-3

Signalment

Forty six client owned dogs presented at the Small Animal Hospital, Faculty of Veterinary Science, Chulalongkorn University were selected into this study and divided into normal (n = 19) and DMVD groups (n = 27). Nineteen dogs in normal group included thirteen females (Female = 5; Female spay = 8) and six males (Male = 2; Male castrate = 4). Breeds of dogs were Poodle (n = 7), Shih-Tzu (n = 2), Miniature pincher (n = 2), Yorkshire terrier (n = 2), Chihuahua (n = 1), Pekingese (n = 1) and mixed breed (n = 4). Twenty seven dogs in DMVD group included fourteen females (Female = 5; Female spay = 9) and thirteen males (Male = 6; Male castrate = 7). 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), Miniature pincher (n = 1) and mixed breed (n = 4). All dogs had no other systemic disease such as hepatic diseases, kidney diseases or neoplasia. Dogs in DMVD group were newly diagnosed and had not been received cardiovascular medicine. Both groups did not receive any medicine at least one month before study.

Age of DMVD group was significantly older than normal group ($p < 0.01$). Weight between normal and DMVD groups was not statistically different.

Table 6: Signalment data of normal and DMVD groups (mean ± SD)

Group	N	Male	Female	Age (years)	Weight (kg)
Normal	19	6	13	8.42 ± 2.06	5.64 ± 2.73
DMVD	27	13	14	11.41 ± 2.63*	5.17 ± 2.01

The significant difference was assessed by independent T test.

* Indicate statistically different at $p < 0.01$ between normal and DMVD group.

History taking

History data was noted by owner interview, 48.15% (14/27) of dogs in DMVD group had clinical signs of CHF defined as stage C and the other 51.85% of dogs (13/27) had no clinical sign of CHF defined as stage B2. Clinical signs were noted by include cough (13/13; 100%), exercise intolerance (10/13; 76.92%) and dyspnea (1/13; 7.69%).

Table 7: History taking data of DMVD group

Clinical signs	**DMVD group (n = 27)**	
	Stage B2 (n = 14)	Stage C (n = 13)
Cough	-	13/13 (100%)
Exercise intolerance	-	10/13 (76.92%)
Dyspnea	-	1/13 (7.69%)

Physical examination

Abnormalities from physical examination in DMVD group at the first day of disease diagnosis included systolic heart murmur (27/27; 100%), crackle lung sound (5/27; 18.52%), increased lung sound (12/27; 44.44%), pale pink mucous membrane (5/27; 18.52%), tachypnea (6/27; 22.22%), dyspnea (2/27; 7.41%) and abdominal distention (2/27; 7.41%).

Table 8: Physical examination data of DMVD group

PE	DMVD group (n = 27)
Systolic heart murmur	27/27 (100%)
Increased lung sound	12/27 (44.44%)
Tachypnea	6/27 (22.22%)
Pale pink mucous membrane	5/27 (18.52%)
Crackle lung sound	5/27 (18.52%)
Dyspnea	2/27 (7.41%)
Abdominal distention	2/27 (7.41%)

Complete blood count and blood chemistry profiles

Complete blood count (CBC) profiles

The data of complete blood count profiles of normal and DMVD groups were presented as mean ± SD (Table 9). Red blood cell count, hematocrit and hemoglobin in control group were significantly higher than DMVD group ($p < 0.05$). Platelets count in normal group was not significantly different compared to DMVD group. There were no significant difference in white blood cell count, neutrophil, eosinophil, basophil, lymphocyte and monocyte number between normal and DMVD groups. All means of CBC value in normal and DMVD groups were within normal limit.

Table 9: Complete blood count profiles of normal and DMVD groups (mean ± SD)

Parameter	Unit	Normal value[a]	Normal group (n=19)	DMVD group (n=27)	P-value
RBC	$x10^6$ cell/μl	5.5-8.5	7.37 ± 0.78	6.46 ± 1.28	0.006*
Hematocrit	%	37.0-55.0	51.74 ± 5.82	46.67 ± 8.08	0.024*
Hemoglobin	g/dL	12.0-18.0	16.54 ± 1.83	14.61 ± 2.68	0.009*
Platelet	$x10^3$ cell/μl	200-500	384.58 ± 174.56	359.56 ± 151.55	0.596
WBC	$x10^3$ cell/μl	6.0-17.0	10,066.32 ± 3,278.40	12,031.48 ± 6,456.45	0.230
Neutrophil	cell/μl	3,000-11,500	7,471.45 ± 2,773.04	8,941 ± 6,254.67	0.345
Eosinophil	cell/μl	1,000-1,250	495.92 ± 397.23	297.30 ± 189.29	0.093
Basophil	cell/μl	0-100	80.7 ± 13.50	65.98 ± 22.67	0.369
Lymphocyte	cell/μl	1,000-4,800	1,409.15 ± 423.76	1,682.33 ± 852.54	0.172
Monocyte	cell/μl	180-1,350	714.15 ± 374.80	847.01 ± 469.37	0.317

The significant difference was assessed by independent T test.

[a] Normal reference value from Manual of Small Animal Emergency and Critical Care Medicine (Douglass et al., 2005).

* Indicate statistically difference at $p < 0.05$ between normal and DMVD groups.

Blood chemistry profiles

The data of blood chemistry profiles of normal and DMVD groups were presented as mean ± SD (Table 10). There were no significant difference in plasma ALT, ALP and creatinine between normal and DMVD group. However, plasma BUN in DMVD group was significantly higher than normal group ($p < 0.05$). All means of blood chemistry value in normal and DMVD groups were within normal limit.

Table 10: Blood chemistry profiles of normal and DMVD group (mean ± SD)

Parameter	Unit	Normal value[a]	Normal group (n=19)	DMVD group (n=27)	P-value
ALT	IU/L	5-60	53.26 ± 31.22	46.44 ± 28.67	0.436
ALP	IU/L	10-150	77.63 ± 61.25	74.79 ± 51.79	0.865
BUN	mg/dL	7-27	13.72 ± 3.72	19.22 ± 9.87	0.025*
Creatinine	mg/dL	0.4-1.8	0.77 ± 0.17	0.81 ± 0.23	0.536

The significant difference was assessed by independent T test.

[a] Normal reference value from Manual of Small Animal Emergency and Critical Care Medicine (Douglass et al., 2005).

* indicate statistically significant difference at $p < 0.05$ between normal and DMVD groups.

Thoracic radiography

Twenty two of 27 dogs in DMVD group (81.48%) were diagnosed cardiomegaly (VHS > 10.7) (Saunders et al., 2013) with left atrium enlargement. From 22 dogs with cardiomegaly, 11 dogs (50%) had pulmonary edema. All dogs in normal group had no abnormality on thoracic radiograph.

Echocardiography

Severity of mitral valve regurgitation (MR) in DMVD dogs was mild (37.04%; 10/27), moderate (29.63%; 8/27) and severe (33.33%; 9/27). Echocardiographic data of normal and DMVD groups were presented as mean ± SD showed in table 11. Percentage of fractional shortening in normal group was significantly lower than DMVD group ($p < 0.01$). Dogs in DMVD group had left atrium larger than dogs in normal group ($p < 0.05$). Moreover, ratio of left atrium to aorta of dogs in DMVD group was significantly higher than normal group ($p < 0.01$). Other echocardiographic indices included interventricular septal thickness during diastole and systole, left ventricular end diastolic diameter, left ventricular end systolic diameter, wall thickness of left ventricular free wall during diastole and systole and aorta dimension were not statistically different between normal and DMVD groups.

Table 11: Echocardiographic data of normal and DMVD group (Mean ± SD)

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**
Aorta index	2.25 ± 0.80	2.51 ± 0.97	0.335
LA index	3.31 ± 1.23	4.11 ± 1.53	0.030*
LA/Ao	1.39 ± 0.21	1.62 ± 0.25	0.002**

The significant difference was assessed by independent T test.

** Indicate statistically difference at $p < 0.01$ between normal and DMVD groups.

* Indicate statistically difference at $p < 0.05$ between normal and DMVD groups.

Plasma Gal-3 concentration

Validation of ELISA test kit was performed by in-house laboratory. Linearity and parallelism were determined using spiked plasma samples and 5 different manufacturer standard concentrations. Average recovery for canine Gal-3 ELISA test was 92.50%. Intra-assay coefficient of variance was 2.54 % for canine Gal-3 ELISA test. Plasma Gal-3 concentration was non-parametric data and expressed as ng/ml in table 10. The median of plasma Gal-3 concentration in DMVD group was significantly higher than normal group ($p < 0.01$). Cut-off value of plasma Gal-3 concentration in canine estimated by receiver operating characteristics curve (ROC) was 0.75 ng/ml (Fig. 16). 88.89% of DMVD dogs (24/27) had plasma Gal-3 level above normal cut-off value and 84.21% of normal dogs (16/19) had plasma Gal-3 level under normal cut-off value. Grey zone of plasma Gal-3 concentration was between 0.62-1.05 ng/ml. Dogs that had plasma Gal-3 concentration lower 0.62 ng/ml considered as negative for cardiac fibrosis while dogs that had plasma Gal-3 concentration over 1.05 ng/ml considered as positive for cardiac fibrosis.

Table 12: Plasma Gal-3 concentration of normal and DMVD groups (median; 25th and 75th percentile)

Group	Gal-3 (ng/ml)
Normal (n=19)	0.420; 0.271-0.630
DMVD (n=27)	1.498; 0.874-2.360*

The significant difference was assessed by Mann-Whitney U test.

* Indicate statistically different at $p < 0.01$ between normal and DMVD groups.

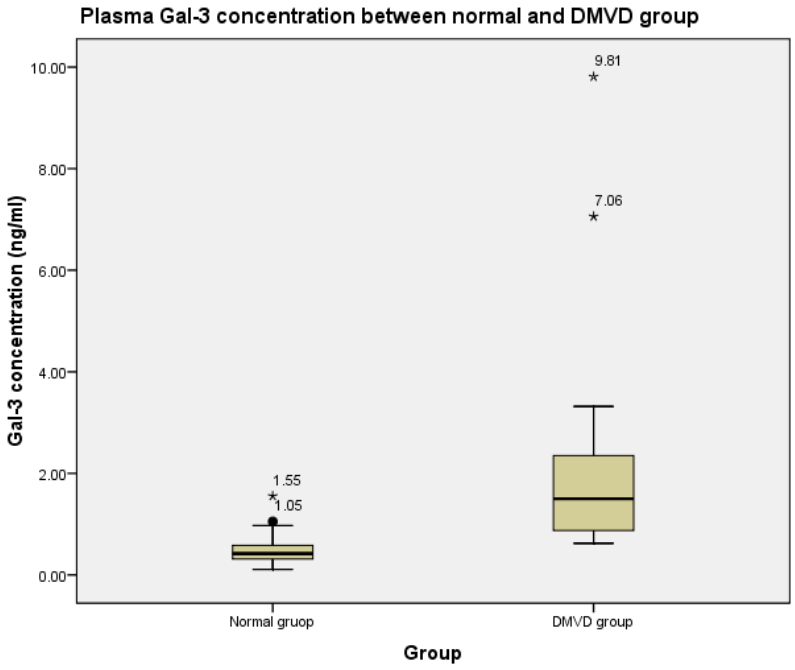

Figure 14: Boxplot of plasma Gal-3 concentration in normal and DMVD groups. Median value was represented as line within box. The 25th and 75th percentile value was represented as the limits of box. The outlier value (value that > 1.5 interquartile) were showed as stars.

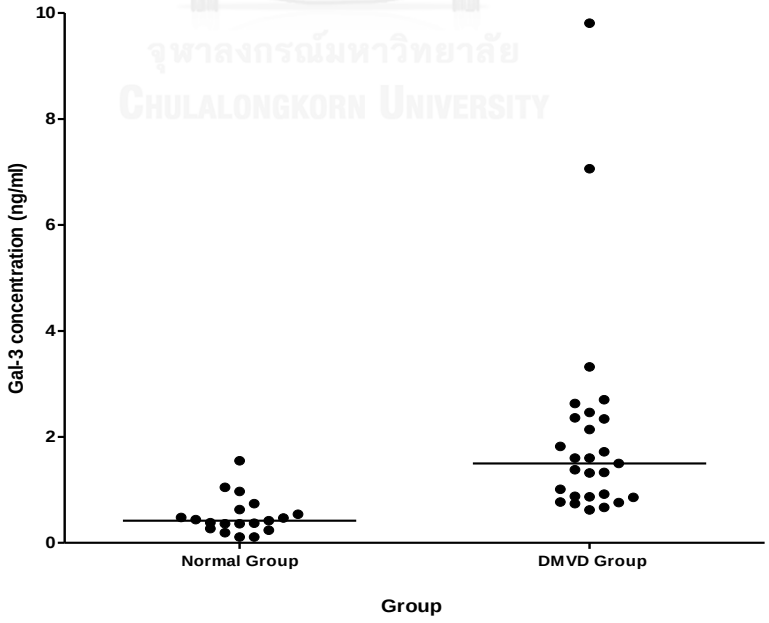

Figure 15: Scatter plot of plasma Gal-3 concentration in normal and DMVD groups. Median value was represented as line.

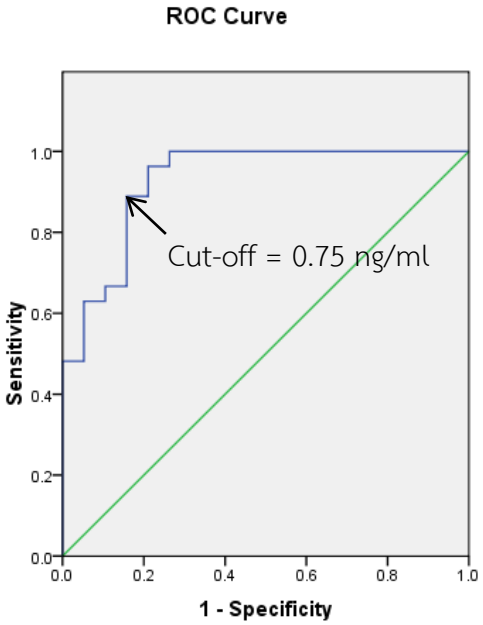

Figure 16: Receiver operating characteristic (ROC) curve. As seen from the ROC curves, the maximum value of sensitivity and specificity was 0.75 ng/ml (sensitivity 0.889, specificity 0.842). It was defined as the optimal cut-off value. Area under curve was 0.928.

There was no correlation between Gal-3 concentration and age as well as weight of normal dogs.

Table 13: The correlations between plasma Gal-3 concentration and age and weight of normal dogs

Parameters	r	P-value
Age	-0.188	0.442
Weight	0.128	0.601

The significant correlation was assessed by Spearman's rank correlation at $p < 0.05$.

r = Spearman's rho

There was no statistical correlation between plasma Gal-3 concentration and echocardiographic indices including interventricular septal thickness during diastole and systole, left ventricular end diastolic diameter, left ventricular end systolic diameter, wall thickness of left ventricular free wall during diastole and systole, aorta, left atrium, percentage of fractional shortening and ratio of left atrium to aorta of entire population.

Table 14: The correlations between plasma Gal-3 concentration and echocardiographic indices of entire population

Parameters	r	P-value
Septum-d index	-0.022	0.883
LV chamber-d index	0.109	0.472
LV wall-d index	0.108	0.474
Septum-s index	0.152	0.314
LV chamber-s index	-0.074	0.627
LV wall-s index	0.133	0.377
%FS	0.288	0.146
Aorta index	0.013	0.932
LA index	0.160	0.289
LA/Ao	0.202	0.312

The significant correlation was assessed by Spearman's rank correlation at $p < 0.05$.

r = Spearman's rho

CHAPTER V
DISCUSSION

Part I: Histopathology

Signalment

Degenerative mitral valve disease (DMVD) is the most common acquired cardiac disease in adult small to medium sized breed dogs. The prevalence of DMVD is age related (Borgarelli and Buchanan, 2012). To reduce effect of age and weight on cardiac fibrosis and Gal-3 expression in cardiac muscles, dogs included in 2 study groups were aged and sized match. Age of dogs affected with DMVD in the present study varied from 9-20 years old. Average age of DMVD dogs was statistically significant older than normal dogs. Although, age between normal and DMVD groups was statistically different, it was considered clinically insignificant. All dogs were categorized as aging dogs. Weight of normal and DMVD groups was not statistically different. All dogs were small breeds and weight less than 15 kg. Most dogs in DMVD group were died from cardiorespiratory failure resulting from DMVD.

Cardiac fibrosis

DMVD is a progressive disease which can cause valve regurgitation and cardiac remodeling secondary to volume overload (Haggstrom et al., 2009). Cardiac fibrosis is one of pathological changes resulting from cardiovascular disease including DMVD. This present study demonstrated cardiac fibrosis especially in sub-endocardium of DMVD dogs as same as previous study by Falk et al (2006). Percentage of fibrosis area in DMVD group was significantly higher than normal group in papillary muscles and LV walls similar to Falk et al. (2010). In addition, percentage of fibrosis area was

higher in papillary muscles than LV walls. This is probably because the inner part of myocardium including papillary muscles and sub-endocardium has lower blood flow. An impaired coronary flow secondary to cardiovascular disease can cause ischemia leading to cardiac fibrosis in these areas (Falk et al., 2006). Cardiac fibrosis has been found secondary to aging change or cardiovascular disease in human patients. Healthy aging animal models such as rats and rabbits have cardiac fibrosis. Rats had increased collagen deposition from 5.5% in young hearts to 12% in aging heart (Biernacka and Frangogiannis, 2011). In human, collagen deposition was increased approximately 50% in aging hearts (Biernacka and Frangogiannis, 2011). Fibrosis in aging hearts was called as reactive fibrosis defied as an increased collagen deposition without cardiomyocyte loss. The present study demonstrated that normal dogs also had cardiac fibrosis which could be related to aging change (Masson et al., 2005; Falk et al., 2013). However, the correlation between age and cardiac fibrosis was not found in the present study. This probably because all dogs in the study were in the same age range. The area of cardiac fibrosis related to aging is usually small because it is associated to the reduction of collagen degradation not an increase of synthesis (Masson et al., 2005). On the other hand, the area of cardiac fibrosis associated to cardiac damage or cardiovascular problem like DMVD is often large because of fibrosis replacement called reparative fibrosis from an increased collagen production (Biernacka and Frangogiannis, 2011; Falk et al., 2013). As in this study, the fibrosis area was markedly larger in DMVD dogs than in normal aging dogs.

Gal-3 expression in cardiac muscles

Histopathology is a useful method to determine location of cardiac fibrosis lesions. The expression of Gal-3 by immunohistochemistry, fibrosis marker could demonstrate specific area of cardiac fibrosis. Gal-3 expression was localized in

cytoplasm of the cardiomyocytes and larger cells beneath endocardium suspected as Purkinje cells. However, there was no study relationship between Gal-3 expression and Purkinje cells. Further study determining effects of cardiac fibrosis on conduction system like Purkinje cells and electrophysiology should be performed. Gal-3 expression was mainly found in sub-endocardium and fibrosis lesion confirmed by Masson trichome staining. This finding was similar to previous studies in mice (Yu et al., 2013) and rats (de Boer et al., 2009; de Boer et al., 2010). Percentage of Gal-3 expression was increased in DMVD group compared to normal group similar to previous study in mice (Yu et al., 2013). The Gal-3 expression was higher in papillary muscles than in LV walls. These results indicated role of Gal-3 protein involving pathophysiology of cardiac fibrosis as up-regulate.

In summary, DMVD dogs had more cardiac fibrosis lesion than normal aging dogs according to increasing area of collagen deposition and expression of fibrosis marker. Cardiac fibrosis and Gal-3 expression were found mainly in sub-endocardium. Moreover, Gal-3 expression was correlated well with cardiac fibrosis. The results of this study indicated that Gal-3 could be a potential marker for detecting fibrosis in cardiac muscles of DMVD dogs.

Part II: Determination of plasma Gal-3

Signalment

Both two study groups were age, weight and size matched. Unfortunately, average age of DMVD group was higher than normal group. However, dogs in both groups were in the same age range considered to be senior or aging dogs. Because DMVD is mostly found in small to medium breed dogs, to reduce the effect of breed variation to plasma Gal-3 concentration, this study selected only small breeds predisposition to DMVD into the study including Poodle, Pomeranian, Cavalier King

Charles spaniel, Chihuahua, Shih-Tzu, Schnauzer and Miniature pincher. Poodle was the major breed population in the DMVD group. This finding is different from other studies which found that CKCS and Dachshund were the most breeds affected by DMVD (Haggstrom et al., 2004; Borgarelli and Buchanan, 2012; Parker and Kilroy-Glynn, 2012). Most studies suggested that DMVD are more frequent in male than female dogs. Male are developed this disease earlier than female (Haggstrom et al., 2004; Borgarelli and Buchanan, 2012; Parker and Kilroy-Glynn, 2012). However, this study showed that number of males (n = 13) affected by DMVD were approximately the same to females (n = 14).

History taking

According to American College of Veterinary Internal Medicine (ACVIM) consensus, 48.15% and 51.85% of dogs were defined as stage C and stage B2, respectively. Coughing was the most clinical signs presented in dogs affected with DMVD. Coughing in DMVD dogs may cause by dorsal compression and elevation of main stem bronchi secondary to left atrial enlargement and/or pulmonary edema (Disatian, 2010; Ferasin et al., 2013). Coughing in small breed dogs can cause by other diseases such as tracheal stenosis, bronchitis and other respiratory problems. Therefore, thoracic radiography should be done to differentiate cardiovascular from respiratory cough. Dogs with cough from respiratory diseases usually have normal cardiac size but coughing from cardiovascular diseases or a combination usually have cardiomegaly from thoracic radiograph (Guglielmini et al., 2009). In this study, coughing DMVD dogs were presented with cardiomegaly secondary to elevation of main stem bronchi and/or pulmonary edema.

Physical examination

All dogs in DMVD group showed systolic heart murmur from physical examination resulted from mitral valve regurgitation. Dogs in stage C had increased lung sound or crackle lung sound resulting from pulmonary edema which can be determined by thoracic radiography (Ploysongsang et al., 1989). DMVD dogs with murmur heart sound with abnormal lung sound should be performed thoracic radiography to rule out respiratory problems such as pulmonary fibrosis that may interfere plasma Gal-3 concentration (Koca et al., 2014) and confirm mitral valve regurgitation by echocardiography.

Complete blood count and blood chemistry profiles

Blood profiles including CBC and blood chemistry in this study were evaluated to rule out other systemic diseases such as blood parasite, hepatic disease or kidney disease. These diseases are common in aging dogs and may affect plasma Gal-3 concentration. Means of CBCs in normal and DMVD groups were in the normal limit. However, means of RBCs, hematocrit and hemoglobin of DMVD group were statistically lower than normal group. Reduction of RBCs, hematocrit and hemoglobin in DMVD can be caused by a reduction in erythropoietin (EPO) production resulted from CHF induced chronic renal insufficiency or an increase of cytokines such as TNF-α and IL-6 from damaged cardiac muscles interfering EPO activities (Silverberg et al., 2005). DMVD dogs in this study had normal renal indicator value. However, subclinical renal insufficiency secondary to chronic hemodynamic disturbance should be concerned. The EPO level and activity should be determined to confirm this hypothesis.

Blood chemistry profiles including BUN, creatinine, ALT and ALP were evaluated to rule out kidney disease and hepatic disease. Blood chemistry profiles in

normal and DMVD groups were in the normal limit. However, plasma BUN in DMVD group was statistically higher than normal group. This result suggests an altered renal function which is probably due to a decrease in cardiac output (Nicolle et al., 2007). This result is similar to study of Nicolle et al. (2007) which found an increase of BUN but not creatinine in dogs affected with DMVD.

Thoracic radiography

Thoracic radiography is a useful diagnostic method for evaluating cardiac size and shape, abnormality of lung and blood vessels. Most DMVD dogs in this study were presented with cardiomegaly defined as vertebral heart score (VHS) > 9.7 $\pm$ 0.5 (Jepsen-Grant et al., 2013) and left atrium enlargement. CHF dogs or DMVD dogs in stage C had cardiomegaly with pulmonary edema. These abnormalities resulted from cardiac remodeling and cardiac volume overload secondary to mitral valve regurgitation.

Echocardiography

Echocardiography is a useful method to detect DMVD dogs especially in early stage cases with no clinical sign of CHF (Haggstrom et al., 2004). Two-dimensional and M-mode echocardiography are valuable and non-invasive procedures provided information of mitral valve lesions, severity of mitral valve regurgitation, cardiac function and cardiac remodeling (Haggstrom et al., 2004; Boswood, 2008; Disatian, 2010). All dogs in DMVD group had mitral valve leaflet thickening with or without valve prolapse. The numbers of dogs with mild, moderate and severe MR were approximately same proportion. Some dogs in this study showed left atrium enlargement without left ventricular enlargement because the left atrial wall is thinner than the left ventricular wall so it can dilate easier than left ventricular wall.

Fractional shortening was used to evaluate systolic function of myocardium. The present study showed an increased percentage of fractional shortening in dogs with DMVD compared to normal dogs. This result is similar to previous human studies (Chetboul and Tissier, 2012; Suzuki et al., 2013). DMVD causes volume overload and increased wall stretch. To maintain normal stroke volume, increased stretch of left ventricular wall causes an increase of cardiac contraction according to Frank starling law (Nakamura et al., 2014).

Plasma Gal-3 concentration

To reduce factors affecting plasma Gal-3 concentration, all dogs included into the study were excluded from hepatic, renal diseases or neoplasia. No medicine was given at least a month prior blood collection.

Plasma Gal-3 concentration in DMVD group was statistically higher than normal group similar to studies in humans with congestive heart failure from cardiovascular disease (Milting et al., 2008; Lok et al., 2010; Beiras-Fernandez et al., 2013). Normal cut-off value of plasma Gal-3 concentration in this study was 0.75 ng/ml evaluated by receiver operating characteristics curve (ROC). While normal cut-off value of Gal-3 in humans is 17.7 ng/ml (Lok et al., 2010). This result indicated that dogs had lower level of plasma Gal-3 concentration than human. 88.89% of DMVD dogs (24/27) had plasma Gal-3 level above normal cut-off value and 84.21% of normal dogs (16/19) had plasma Gal-3 level under normal cut-off value. Area under curve of this study was 0.928 near 1 indicated that it can be used as best forecasting (Marzban, 2004). Therefore, 0.75 ng/ml can be used as a reliable cut-off value to differentiate dogs without cardiac fibrosis from DMVD dogs with cardiac fibrosis. However, 15.79% of normal dogs (3/19) had plasma Gal-3 level above cut-off value and 11.11% of DMVD dogs (3/27) had plasma Gal-3 level below cut-off value. Grey

zone of plasma Gal-3 concentration was between 0.62-1.05 ng/ml. All three DMVD dogs that had plasma Gal-3 concentration below the cut-off value were in stage B2 suggesting that these dogs may not develop cardiac fibrosis yet. Human studies indicated that Gal-3 level was increased along with cardiovascular disease progression and associated with diseased severity (Lopez-Andres et al., 2012; van der Velde et al., 2013). Moreover, Gal-3 concentration can be used as a predictor for an on-set of CHF in asymptomatic patients (Ho et al., 2012; van der Velde et al., 2013). Higher level of Gal-3 was related with high incidence of heart failure and mortality in human patients (Lok et al., 2010; de Boer et al., 2011; Ho et al., 2012). The study determining the relationship between Gal-3 level and disease progression should be performed to prove a role of Gal-3 as a predictor and a prognosis marker in DMVD dogs. Plasma Gal-3 concentration was not statistically correlated with echocardiographic indices similar to human beings studies (Milting et al., 2008; Hrynchyshyn et al., 2013). Because cardiac fibrosis can cause cardiac stiffness and diastolic dysfunction, an evaluation of cardiac diastolic function should be performed (Ichihara et al., 2001). The present study did not determine the diastolic function of the heart which might be the limitation. However, the echocardiography is not a sensitive and an effective tool for detecting cardiac fibrosis (Falk et al., 2010). On another word, cardiac structure assessed from echocardiography may not be changed, even though cardiac fibrosis has already developed. Therefore, biomarker such as Gal-3 might be potential indicator for detecting cardiac fibrosis in alive subjects.

The limitation of this study firstly was an application of monoclonal mouse antibody against human galectin-3 in canine cardiac tissue by immunohistochemistry. However, Gal-3 expression both in canine mammary adenoma and canine splenic hemangiosarcoma were previously reported (Choi et al., 2004; Johnson et al., 2007). Moreover, protein sequencing of canine Gal-3 is highly homologous to human Gal-3

in both structure and amino acid sequence (Dumic et al., 2006; Poland et al., 2011). Thus, it is reasonable to use this antibody in the present study. Secondary, an evaluation of plasma Gal-3 concentration was performed only one time for each dog. Nowadays, there is lack information of pharmacokinetic of Gal-3 in dogs. The study in mice indicated that half-life (T1/2) of Gal-3 was 3 hour in serum and 4.3 hour of blood cells (John et al., 2003). Serial measurement of plasma Gal-3 concentration in human is stable (Nilsson et al., 2014). Therefore, single test of plasma Gal-3 concentration would be sufficient for this study. Thirdly, the evaluation of Gal-3 expression in myocardium and Gal-3 concentration in circulation was not performed in the same dogs, so it was not possible to evaluate the relationship between Gal-3 expression in myocardium and Gal-3 concentration in circulation.

In conclusion, dogs with naturally occurring DMVD developed cardiac fibrosis more than the age and size matched normal dogs. An increase of Gal-3 expression was related to an increase area of cardiac fibrosis in DMVD dogs. Plasma Gal-3 concentration was higher in DMVD dogs than normal dogs in agreement with Gal-3 expression in cardiac muscles. Based on the result of this study, Gal-3 might be a potential candidate of cardiac fibrosis markers in dogs with DMVD.

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APPENDICES

Appendix A: Signalment in the normal dog specimens

No.	Name	Breed	Sex	Age (year)	Weight (kg)
1	คริสติน	French bulldog	F	7	8.5
2	วีออส	Poodle	F	13	4.5
3	จีจี้	Shih-Tzu	Fs	7	5.9
4	พริตตี้	Chihuahua	F	7	3.6
5	เงินดอลล่าร์	Beagle	F	11	8.8
6	ยูโร	Poodle	M	7	6
7	ซูซี่	Shih-Tzu	Fs	14	6.1
8	ดีดี้	Poodle	Fs	14	4.15
9	ไข่ไก่	Pomeranian	F	6.5	2.5
10	ลัคกี้	Poodle	F	8	5.3

Appendix B: Signalment in the DMVD dog specimens

No.	Name	Breed	Sex	Age (year)	Weight (kg)
1	หมูหวาน	Shih-Tzu	F	9	7.5
2	ลัคกี้	Poodle	F	11	5.6
3	มารวย	Poodle	M	20	3.8
4	ดาวเรือง	Poodle	Fs	11	8.2
5	Pepper	Schnauzer	M	10	9.1
6	ไวท์	Poodle	F	18	8
7	เป๋าฮื้อ	Poodle	M	13	8.6
8	จีจี้	Shih-Tzu	F	9	5
9	Woopie	Mixed	F	11	8.8
10	หมี	Poodle	Mc	11	4.4
11	แก้ว	Poodle	Fs	14	3.8
12	ถุงเงิน	Shih-Tzu	Fs	17	6.5

Appendix C: Percentage of Fibrosis area and Gal-3 expression in the normal dog specimens

No.	Name	Fibrosis area (%)		Gal-3 expression (%)	
		Papillary muscles	LV walls	Papillary muscles	LV walls
1	คริสติน	57.16	45.36	1.06	0.62
2	วีออส	32.73	31.44	0.61	0.04
3	จีจี้	30.62	23.42	1.97	0.48
4	พริตตี้	36.37	34.04	2.07	0.63
5	เงินดอลล่าร์	35.92	27.46	1.02	0.78
6	ยูโร	26.69	23.23	1.42	1.23
7	ซูซี่	30.84	20.32	1.49	1.04
8	ดีดี้	39.23	22.17	0.68	0.18
9	ไข่ไก่	34.19	27.55	0.29	0.11
10	ลัคกี้	30.26	19.07	0.12	0.06

Appendix D: Percentage of Fibrosis area and Gal-3 expression in the DMVD dog specimens

No.	Name	Fibrosis area (%)		Gal-3 expression (%)	
		Papillary muscles	LV walls	Papillary muscles	LV walls
1	หมูหวาน	51.63	44.00	23.31	10.26
2	ลัคกี้	68.44	66.31	24.92	9.32
3	มารวย	67.95	55.66	22.90	17.58
4	ดาวเรือง	64.48	47.11	22.99	8.01
5	Pepper	73.63	41.45	29.32	14.05
6	ไวท์	62.24	44.37	28.09	13.06
7	เป้าฮื้อ	66.66	59.95	30.04	18.15
8	จีจี้	67.17	59.25	45.62	36.85
9	Woopie	73.02	65.26	29.84	13.43
10	หมี	66.05	49.85	34.94	31.78
11	แก้ว	65.68	47.82	21.65	19.23
12	ถุงเงิน	66.53	54.66	21.71	15.24

Appendix E: Signalment and plasma Gal-3 concentration in the normal dogs

No.	Name	Breed	Sex	Age (year)	Weight (kg)	Plasma Gal-3 concentration (ng/ml)
1	อั่งเปา	Poodle	F	6	3.8	0.48
2	จินนี่	Mixed	Fs	8	9	0.36
3	ไวว่า	Poodle	F	6	3.7	0.24
4	หลินจือ	Poodle	F	13	7.5	0.19
5	เฮงเฮง	Chihuahua	Mc	7	4.2	1.55
6	ผิงผิง	Shih-Tzu	F	9	5.8	0.44
7	ฮวนฮวน	Poodle	Mc	11	5.76	0.54
8	เบน	Miniature Pincher	M	8	5.7	0.36
9	โดเรม่อน	Shih-Tzu	Mc	7	6.9	0.63
10	อิจิ	Mixed	Mc	7	9	0.42
11	น้ำหวาน	Poodle	Fs	13	1.95	0.11
12	ริชชี่	Poodle	Fs	9	3.1	0.97
13	น้ำหอม	Yorkshire terrier	Fs	10	4.9	0.38
14	lucky	Poodle	Fs	9	4.5	0.37
15	ดอลล่าร์	Pekingese	Fs	8	6.1	0.47
16	แจ๊กกี้	Miniature Pincher	Fs	8	3.4	0.74
17	จินนี่	Mixed	Fs	7	13.5	1.05
18	ซูโย๊ะ	Mixed	M	7	5.6	0.11
19	เร็กซี่	Yorkshire terrier	F	7	2.8	0.27

Appendix F: Signalment and plasma Gal-3 concentration in the DMVD dogs

No.	Name	Breed	Sex	Age (year)	Weight (kg)	Plasma Gal-3 concentration (ng/ml)
1	น้ำตาล	Poodle	Mc	10	5.5	2.14
2	เข่ออ้าย	CKS	F	12	5.5	2.70
3	ลูกชิ้น	Chihuahua	Mc	8	2.7	0.77
4	ยักษ์	Schnauzer	M	9	7.6	2.46
5	โก้	Mixed	Mc	16	4.3	2.34
6	เดซี่	CKS	Fs	5	9	1.32
7	ม่อน	Poodle	Fs	12	4.5	2.36
8	คากิ	Poodle	M	12	6.5	0.88
9	กระป๋อง	Pomeranian	M	11	2.2	1.82
10	ก๋วยจั๊บ	Shih-Tzu	M	11	4.9	1.38
11	น้ำตาล	Poodle	F	14	3.7	1.33
12	จีจี้	Mixed	F	16	6.4	3.32
13	เต้าเจี้ยว	Poodle	M	15	4.1	0.62
14	มาร์ค	Miniature pincher	Mc	9	8	1.01
15	คาร์ฟู	Poodle	Fs	9	6.8	0.67
16	มันนี่	Mixed	Fs	13	9.6	1.50
17	กาก้า	Poodle	F	10	3.5	2.63
18	กระปุก	Poodle	Fs	13	4.6	0.76
19	น้อยหน่า	Shih-Tzu	Fs	14	5.5	0.92
20	ป๊อปแป๊ป	Poodle	M	12	5.5	0.86
21	คิม	Pomeranian	Fs	10	2.84	1.72
22	น้ำฝน	Poodle	Fs	14	5.4	1.60
23	น้ำเงิน	Poodle	Mc	10	2.5	0.74
24	มาร์ค	Pomeranian	Mc	10	4.72	7.06
25	เลดี้	Chihuahua	F	8	2.3	0.87
26	แก้ว	Poodle	Fs	14	3.8	1.60
27	บะหมี่	Mixed	Mc	11	7.5	9.81

Appendix G: Echocardiographic indices in the normal dogs

No.	Name	IVSd	LVIDd	LVPWd	IVSs	LVIDs	LVPWs	FS	Ao	LA	LA/Ao
1	อั่งเปา	1.66	5.16	1.71	2.03	3.13	2.50	39.23	2.55	4.34	1.50
2	จินนี่	0.82	2.41	0.86	1.11	1.67	1.02	26.96	1.80	2.06	1.14
3	ไวว่า	1.62	4.16	1.95	2.46	2.46	2.81	41.18	3.11	3.32	1.07
4	หลินจือ	0.91	3.49	0.91	1.23	2.27	1.44	35.25	1.99	2.55	1.28
5	เฮงเฮง	1.31	4.36	1.69	2.00	2.31	2.55	47.17	3.17	3.50	1.10
6	ผิงผิง	1.02	4.43	0.95	1.55	2.88	1.29	35.04	2.40	3.12	1.30
7	ฮวนฮวน	1.09	4.43	0.83	1.44	2.57	1.20	41.80	1.82	2.60	1.43
8	เบน	1.23	4.67	0.74	1.54	2.35	1.51	49.59	1.70	2.98	1.76
9	โดเรม่อน	0.72	3.06	0.87	0.91	2.36	1.23	22.60	1.91	2.64	1.38
10	อิจิ	0.81	2.70	0.76	1.06	2.23	0.83	17.52	1.36	2.23	1.65
11	น้ำหวาน	2.31	8.92	1.74	3.28	3.23	4.82	63.78	3.64	6.62	1.50
12	ริชชี่	2.00	6.10	1.42	2.81	3.16	2.77	48.26	2.58	4.39	1.71
13	น้ำหอม	1.45	4.43	1.20	1.80	3.08	1.63	30.38	2.20	3.65	1.66

No.	Name	IVSd	LVIDd	LVPWd	IVSs	LVIDs	LVPWs	FS	Ao	LA	LA/Ao
14	lucky	1.42	4.58	1.18	1.62	2.64	2.20	42.01	2.47	3.18	1.29
15	ดอลล่าร์	1.16	3.34	1.03	1.44	2.05	1.36	38.46	2.11	2.64	1.26
16	แจ๊กกี้	1.79	5.79	1.59	2.00	4.50	2.29	22.78	3.62	4.41	1.23
17	จินนี่	0.50	2.63	0.47	0.71	1.67	0.52	36.51	1.56	1.06	1.47
18	ซูโย๊ะ	1.16	3.61	1.04	1.84	1.57	2.07	56.52	2.36	2.73	1.16
19	เร็กซี่	2.36	5.93	2.11	2.68	3.86	3.07	34.66	0.32	4.93	1.53

IVSd, interventricular septal thickness at end-diastole; LVIDd, left ventricular end-diastolic dimension; LVPWd, left ventricular posterior wall thickness at end-diastole; IVSs, interventricular septal thickness at end-systole; LVIDs, left ventricular end-systole dimension; LVPWs, left ventricular posterior wall thickness at end-systole; FS, fractional shortening; Ao, aortic root dimension; LA, left atrium dimension; LA/Ao, left atrial to aortic root ratio

Appendix H: Echocardiographic indices in the DMVD dogs

No.	Name	IVSd	LVIDd	LVPWd	IVSs	LVIDs	LVPWs	FS	Ao	LA	LA/Ao	MR
1	น้ำตาล	1.44	3.44	1.55	2.78	2.05	2.27	40.00	2.27	3.78	1.67	mild
2	เข่ออ้าย	1.24	5.05	1.24	1.78	2.91	1.85	42.68	3.36	2.78	1.20	mild
3	ลูกชิ้น	2.11	7.70	2.07	3.48	3.59	3.37	53.62	3.70	6.33	1.71	moderate
4	ยักษ์	1.01	3.58	1.14	1.75	1.83	1.50	48.85	1.53	3.00	1.96	mild
5	โก้	1.33	7.72	1.30	2.47	4.00	2.37	48.30	2.72	6.00	2.21	severe
6	เดซี่	0.93	2.74	0.88	1.04	1.91	1.20	30.53	1.74	2.58	1.48	moderate - severe
7	ม่อน	1.71	4.42	1.58	3.87	1.91	2.22	56.82	2.62	3.73	1.42	moderate
8	คากิ	1.26	4.09	1.22	1.66	2.72	1.86	33.33	2.35	3.02	1.28	mild
9	กระป๋อง	2.73	6.32	3.00	4.18	2.00	5.32	68.29	4.18	5.82	1.40	moderate
10	ก๋วยจั๊บ	1.45	3.82	1.67	2.14	1.98	2.18	48.18	2.49	3.53	1.42	mild
11	น้ำตาล	1.84	6.32	1.70	2.54	3.62	2.78	42.58	2.81	5.22	1.86	mild
12	จีจี้	1.22	5.55	1.48	2.00	2.39	2.30	56.83	2.27	3.91	1.72	moderate - severe
13	เต้าเจี้ยว	1.46	6.41	1.29	1.83	3.34	2.05	47.92	2.80	5.33	1.90	moderate - severe
14	มาร์ค	1.01	3.30	0.86	1.14	1.76	1.43	46.41	2.20	2.46	1.70	moderate
15	คาร์ฟู	1.04	4.37	1.01	1.78	2.12	1.62	51.45	1.90	3.04	1.60	mild
16	มันนี่	0.99	2.99	0.81	1.19	1.66	1.35	44.59	1.93	2.89	1.50	moderate - severe

No.	Name	IVSd	LVIDd	LVPWd	IVSs	LVIDs	LVPWs	FS	Ao	LA	LA/Ao	MR
17	กาก้า	1.29	8.11	1.34	2.11	3.80	2.57	53.28	0.29	0.56	1.90	moderate - severe
18	กระปุก	1.59	7.37	1.09	1.96	4.96	1.80	32.59	4.09	5.02	1.46	severe
19	น้อยหน่า	1.40	4.04	1.16	2.15	1.80	2.04	55.44	2.31	3.00	1.29	moderate
20	ป๊อปแป๊ป	1.16	5.75	0.87	1.40	3.44	1.80	40.28	2.42	4.04	1.67	severe
21	คิม	1.55	5.49	1.37	2.32	3.03	2.25	45.07	3.10	4.54	1.48	mild
22	น้ำฝน	1.04	4.30	0.85	1.70	2.54	1.57	41.08	2.56	3.11	1.38	mild
23	น้ำเงิน	3.36	5.88	2.72	4.00	3.64	3.96	38.06	3.48	6.02	1.73	moderate
24	มาร์ค	1.17	4.75	1.08	1.69	2.33	2.12	50.84	0.19	0.40	2.00	moderate
25	เลดี้	2.48	6.57	2.39	4.26	2.30	3.39	65.22	3.65	4.74	1.30	mild
26	แก้ว	1.97	8.84	1.42	2.74	4.63	2.68	47.53	3.16	5.29	1.67	moderate
27	บะหมี	0.87	4.23	0.99	1.11	2.19	1.77	48.22	1.63	2.96	1.82	moderate - severe

IVSd, interventricular septal thickness at end-diastole; LVIDd, left ventricular end-diastolic dimension; LVPWd, left ventricular posterior wall thickness at end-diastole; IVSs, interventricular septal thickness at end-systole; LVIDs, left ventricular end-systole dimension; LVPWs, left ventricular posterior wall thickness at end-systole; FS, fractional shortening; Ao, aortic root dimension; LA, left atrium dimension; LA/Ao, left atrial to aortic root ratio; MR, degree of mitral valve regurgitation

Appendix I: Subgroup analysis of plasma Gal-3 concentration among stage of DMVD (Median; 25th and 75th percentile)

Stage	Number	Plasma Gal-3 concentration (ng/ml)
Normal	19	0.420; 0.271-0.630*
DMVD stage B2	13	1.378; 0.876-2.409
DMVD stage C	14	1.547; 0.834-2.416

The significant difference was assessed by Mann-Whitney U test.

* indicate statistically significant difference at $p < 0.01$ between normal and DMVD stage B2 and DMVD stage C.

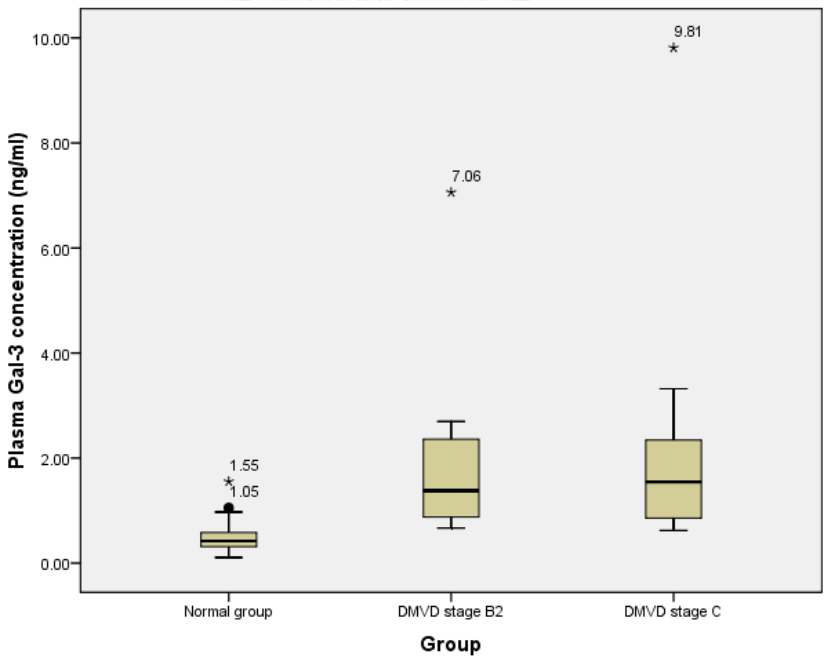

Figure 17: Boxplot of plasma Gal-3 concentration dividing by stage of DMVD. Median value was represented as line within box. The 25th and 75th percentile value was represented as the limits of box. The outlier value (value that > 1.5 interquartile) were showed as star.

Appendix J: Subgroup analysis of plasma Gal-3 concentration among disease severity (Median; 25th and 75th percentile)

Disease Severity	Number	Plasma Gal-3 concentration (ng/ml)
No MR	19	0.420; 0.271-0.630*
Mild	10	1.487; 0.877-2.220
Moderate - severe	17	1.498; 0.811-2.497

The significant difference was assessed by Mann-Whitney U test.

* indicate statistically significant difference at $p < 0.01$ between no MR and mild and moderate to severe MR.

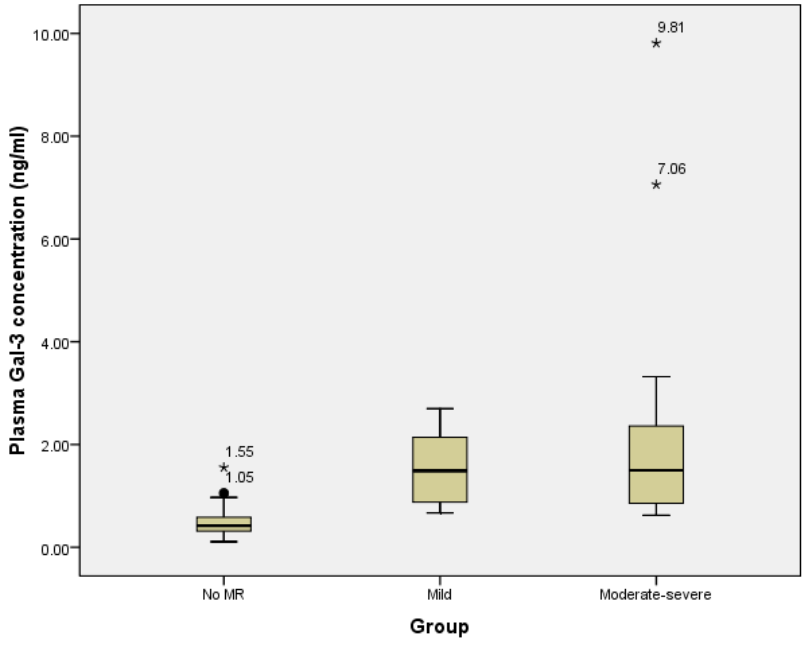

Figure 18: Boxplot of plasma Gal-3 concentration dividing by disease severity. Median value was represented as line within box. The 25th and 75th percentile value was represented as the limits of box. The outlier value (value that > 1.5 interquartile) were showed as star.

Appendix K: Receiver operating characteristic (ROC) curve

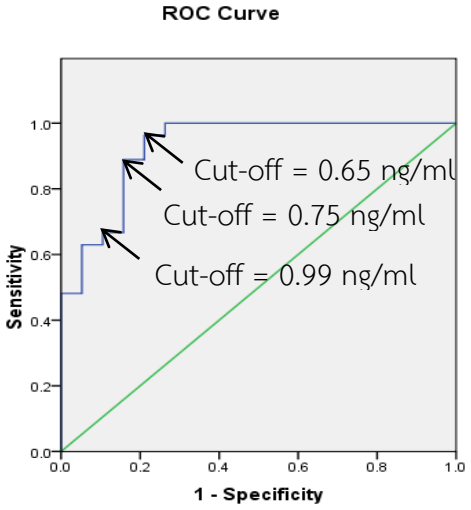

Figure 19: Receiver operating characteristic (ROC) curve dividing by sensitivity and specificity. As seen from the ROC curves, cut-off value was 0.75 ng/ml, when considered sensitivity equal to sensitivity (sensitivity 0.889, specificity 0.842). When considered high sensitivity (sensitivity 0.963, specificity 0.789), cut-off value was 0.65 ng/ml and cut-off value was 0.99 ng/ml, when considered high specificity (sensitivity 0.667, specificity 0.895).

Table 15: Cut-off value of plasma Gal-3 concentration

Cut-off value	DMVD (n = 27) [a]	Normal (n = 19) [b]
0.65 ng/ml	26	15
0.75 ng/ml	24	16
0.99 ng/ml	18	17

[a] Indicate number of dogs in DMVD group that had plasma Gal-3 concentration above cut-off value.

[b] Indicate number of dogs in normal group that had plasma Gal-3 concentration under cut-off value.

VITA

Miss Siriwan Sakarin was born on December 24, 1987 in Bangkok, Thailand. She finished her high school education from Samsen Wittayalai School, Bangkok, and graduated from Faculty of Veterinary Science, Chulalongkorn University, 2006-2012. She received her Bachelor degree of Veterinary Science with 1st class Honors in 2012. After that, she admitted to the degree of Master of Science Program in Veterinary Medicine. Department of Veterinary Medicine, Faculty of Veterinary Science, Chulalongkorn University in 2012 under Chulalongkorn University Graduate Commemorate the 72nd Anniversary of His Majesty King Bhumibol Adulyadej Scholarship.