การตรวจหาทรานสฟอร์มมิงโกรทแฟคเตอร์เบตา 1 ในสุนัขปกติและสุนัขที่เป็นโรคลิ้นหัวใจไมทรัลเสื่อม

นางสาววิยะดา วิญญูชนเจริญ

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DETECTION OF TRANSFORMING GROWTH FACTOR BETA 1 OF NORMAL DOGS AND DOGS WITH DEGENERATIVE MITRAL VALVE DISEASE

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A Thesis Submitted in Partial Fulfillment of the Requirements
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วิยะดา วิญญูชนเจริญ : การตรวจหาทรานสฟอร์มมิงโกรทแฟคเตอร์เบตา 1 ในสุนัขปกติและสุนัขที่เป็นโรคลิ้นหัวใจไมทรัลเสื่อม (DETECTION OF TRANSFORMING GROWTH FACTOR BETA 1 OF NORMAL DOGS AND DOGS WITH DEGENERATIVE MITRAL VALVE DISEASE) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ผศ. สพ.ญ. ดร.สิริลักษณ์ สุรเชษฐพงษ์, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: รศ. สพ.ญ. ดร.สมพร เตชะงามสุวรรณ, 52 หน้า.

โรคลิ้นหัวใจไมทรัลเสื่อม (DMVD) เป็นโรคหัวใจภายหลังกำเนิดที่พบได้บ่อยที่สุดในสุนัขโตเต็มวัยพันธุ์ขนาดเล็กและพันธุ์ขนาดกลาง ในลิ้นหัวใจไมทรัลเสื่อมพบว่ามีการสะสมของมิวโคพอลิแซ็กคาไรด์ปริมาณมากและชั้นคอลลาเจนถูกทำลายไป ทรานสฟอร์มมิงโกรทแฟคเตอร์เบตา 1 (TGF-β1) เป็นไซโตคายน์ตัวหนึ่งที่ควบคุมเกี่ยวกับการรักษาสมดุลของเมทริกซ์นอกเซลล์ภายในลิ้นหัวใจและคาดว่ามีความเกี่ยวข้องกับกระบวนการเสื่อมของลิ้นหัวใจ โดยมีการศึกษาพบว่ามีการปรากฏของโปรตีน TGF-β1 เพิ่มขึ้นอย่างมากในลิ้นหัวใจไมทรัลเสื่อม วัตถุประสงค์ของการศึกษาครั้งนี้คือตรวจวัดความเข้มข้นของโปรตีน TGF-β1 ในพลาสมาของสุนัขปกติเปรียบเทียบกับสุนัขที่เป็นโรค DMVD โดยใช้เทคนิคอีไลซ่า และเปรียบเทียบการปรากฏของ TGF-β1 mRNA จากตัวอย่างลิ้นหัวใจไมทรัลของสุนัขปกติและสุนัขที่เป็นโรค DMVD โดย RT-PCR ผลการศึกษาการวัดระดับความเข้มข้นของโปรตีน TGF-β1 ในพลาสมา เปรียบเทียบด้วยสถิติแมน-วิทนีย์ ยู ค่า *p*-value น้อยกว่า 0.05 ถือว่ามีนัยสำคัญทางสถิติ พบว่าความเข้มข้นของ TGF-β1 ในพลาสมาระหว่างสุนัขปกติ 22 ตัว (1.14 (0.94-1.33) ng/mL) และสุนัข DMVD 27 ตัว (1.21 (0.92-1.32) ng/mL) นั้นไม่มีความแตกต่างกันอย่างมีนัยสำคัญทางสถิติ สำหรับการศึกษาการปรากฏของ TGF-β1 mRNA ในลิ้นหัวใจไมทรัลปกติ (n=5) และลิ้นหัวใจไมทรัลที่เป็นโรค DMVD (n=5) พบว่ามีการปรากฏของ TGF-β1 mRNA จากลิ้นหัวใจไมทรัลทั้งหมด จากนั้นทำการเปรียบเทียบค่าเฉลี่ย ± ค่าเบี่ยงเบนมาตรฐานของอัตราส่วนระหว่างการแสดงออกของ TGF-β1 และการแสดงออกของ GAPDH ในตัวอย่างที่ปรากฏ positive band ระหว่างกลุ่มปกติและกลุ่ม DMVD ด้วยสถิติ Independent t-test พบว่าค่าเฉลี่ยของ ratio ของ positive band จากสุนัขกลุ่มกลุ่มปกติ (0.55 ± 0.07) และ DMVD (0.95 ± 0.06) แตกต่างกันอย่างมีนัยสำคัญ ($p < 0.05$) โดยสรุปการศึกษานี้พบระดับโปรตีน TGF-β1 น้อยมากในกระแสเลือด เป็นไปได้ว่าเซลล์ในลิ้นหัวใจไมทรัลน่าจะเป็นแหล่งหลักของ TGF-β1 ในสุนัขที่เป็นโรคลิ้นหัวใจไมทรัลเสื่อม

ภาควิชา อายุรศาสตร์ ลายมือชื่อนิสิต .....

สาขาวิชา อายุรศาสตร์สัตวแพทย์ ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

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WIYADA WINYUCHONJAROEN: DETECTION OF TRANSFORMING GROWTH FACTOR BETA 1 OF NORMAL DOGS AND DOGS WITH DEGENERATIVE MITRAL VALVE DISEASE. ADVISOR: ASST. PROF. SIRILAK SURACHETPONG, D.V.M., M.S., Ph.D., D.T.B.V.M., CO-ADVISOR: ASSOC. PROF. SOMPORN TECHANGAMSUWAN, D.V.M., M.Sc., Ph.D., D.T.B.V.P., 52 pp.

Degenerative mitral valve disease (DMVD) is the most common cause of acquired cardiac diseases in adult small and medium sized dogs. Transforming growth factor beta 1 (TGF-β1), a cytokine controlling extracellular matrix homeostasis within valves is thought to be involved in valve degenerative process. The expression of TGF-β1 protein was reported markedly increase in the DMVD valves. The first objective of this study was to measure the concentration of TGF-β1 in plasma of normal and DMVD dogs by ELISA technique. The second objective was to compare TGF-β1 mRNA expression between normal and DMVD dogs from mitral valve (MV) samples by RT-PCR. Comparison of plasma TGF-β1 concentrations was performed with Mann Whitney U test. *P*-value less than 0.05 was considered statistically significant. The result showed no statistical difference in median of plasma TGF-β1 concentrations between 22 normal (1.14(0.94-1.33) ng/mL) and 27 DMVD (1.21(0.92-1.32) ng/mL) dogs. The expression of TGF-β1 mRNA from 10 MV (normal = 5, DMVD = 5) samples showed positive band. For positive bands, the mean ± SD of TGF-β1 and GAPDH band intensity ratio was compared between normal and DMVD dogs assessed by independent t-test. The result between normal (0.55 ± 0.07) and DMVD (0.95 ± 0.06) valves were significantly different ($p < 0.05$). In conclusion, the concentration of TGF-β1 protein is very low in circulation. It is possible that cells within mitral valves are the major source of TGF-β1 in dogs affected with DMVD.

Department:	Veterinary Medicine	Student's Signature	.....
Field of Study:	Veterinary Medicine	Advisor's Signature	.....
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CHAPTER I

INTRODUCTION

Degenerative mitral valve disease (DMVD) is also known as myxomatous degenerative mitral valve disease, mitral valve endocardiosis or chronic valvular disease (Whitney, 1974; Borgarelli and Buchanan, 2012). DMVD is the most common cause of acquired cardiac diseases (Häggström et al., 2004). About 75 % of heart disease cases in small animal hospitals are mitral valve regurgitation caused by DMVD (Das and Tashjian, 1965; Detweiler and Patterson, 1965; Buchanan, 1977). The small and medium sized dogs such as Cavalier King Charles spaniels (CKCS), Miniature poodles, Cocker spaniels, Miniature schnauzers, Dachshunds, Pomeranians, Chihuahuas, Pekingese, Fox terriers, Boston terriers and mixed breed dogs are predisposing breed to DMVD (Disatian, 2010). The degenerated mitral valves affected by increasing of mucopolysaccharide accumulation within the valves lead to the valve thickening or nodular formation and elongation or chordae tendineae rupture in severe DMVD, resulting in abnormality of the mitral valves functions such as valves prolapse or regurgitation (Häggström et al., 2004). In case of severe mitral valve regurgitation, signs of left sided congestive heart failure will be shown including cough, exercise intolerance, dyspnea, and syncope. The histology of normal canine mitral valves are four well-defined layers composed of small amount of mucopolysaccharides, elastic, and collagen fibers as well as valve interstitial cells. In contrast, diseased mitral valves are presented with tissue layer disorganization and the large amount of mucopolysaccharide accumulation. Transforming growth factor beta (TGF- β) is a member of polypeptide growth factors family. It is a cytokine that controls both physiological and pathological role such as embryonic development, cell growth and differentiation, processing of inflammation, homeostasis of most tissue, regulation of immune responses, and wound healing (Sporn and Roberts, 1993; Massagué, 1998). Three distinct isoforms of TGF-β are synthesized from specific tissue. TGF-β1 is expressed by endothelial cells (Millan et al., 1991), haemopoietic cells, connective tissue cells (Border and Noble, 1994), and the myocardium (Eghbali,

1989). TGF-β2 is expressed by epithelial and neuronal cells, and TGF-β3 is expressed by the fibrocytes of the embryonic and mature myocardium (Millan et al., 1991; Epstein et al., 2000). The expression of TGF-β1 and TGF-β3 were reported markedly increased in the DMVD valves (Aupperle et al., 2008). Plasma or serum of TGF-β concentration in dogs and human is measured in several studies (Sharma et al., 1997; Wang et al., 1997; Syrris et al., 1998; Tashiro et al., 2002; Zois et al., 2012; Moesgaard et al., 2014). In coronary artery disease (CAD) patients, the level of plasma TGF-β is useful as a tool for prognostic CAD severity (Wang et al., 1997; Tashiro et al., 2002). Jian et al. (2002) demonstrated that TGF-β1 could increase collagen and mucopolysaccharide synthesis and regulate cultured valve interstitial cell proliferation and apoptosis. Khan and Sheppard (2006) showed that TGF-β1 was one of the main factors that caused cardiac fibrosis and excessive production of extracellular proteins within human heart valves. Consequently, TGF-β1 is feasible to have an important role in pathogenesis of DMVD in dogs. Few studies have been measured the concentration of TGF-β1 in circulation of DMVD dogs. In addition, there is no study to determine the relationship of TGF-β1 in circulation and mitral valve tissues of DMVD dogs. The study evaluating the expression of TGF-β1 in circulation and mitral valve tissues in DMVD dogs will elucidate the role of TGF-β1 in pathogenesis of DMVD.

Objectives of Study

1. To measure the plasma concentration of TGF-β1 of normal and degenerative mitral valve dogs by canine-specific enzyme-linked immunosorbent assay technique.
2. To compare the mRNA expression of TGF-β1 gene in mitral valves of normal and degenerative mitral valve dogs by conventional PCR method.

Hypothesis

1. The plasma TGF-β1 concentration increases in DMVD dogs compare to normal dogs.

2. The expression of TGF-β1 increases in DMVD valves compare to normal valves.

Keywords

Canine, Degenerative mitral valve disease, Mitral valve, plasma, Transforming growth factor beta 1

สุนัข, โรคลิ้นหัวใจไมทรัลเสื่อม, ลิ้นหัวใจไมทรัล, พลาสมา, ทรานสฟอร์มมิง โกรท แฟคเตอร์ เบตา 1

Advantages of Study

To understand the relationship between TGF-β1 and pathogenesis of DMVD.

CHAPTER II

LITERATURE REVIEWS

Degenerative mitral valve disease

Degenerative mitral valve disease (DMVD) is the most common cause of acquired cardiac diseases in adult small and medium sized dogs (Häggström et al., 2004). DMVD is also known as myxomatous degenerative mitral valve disease, mitral valve endocardiosis, or chronic valvular disease (Whitney, 1974; Borgarelli and Buchanan, 2012). About 75 % of heart disease cases in small animal hospitals are mitral valve regurgitation caused by DMVD (Das and Tashjian, 1965; Detweiler and Patterson, 1965; Buchanan, 1977).

The mitral valves locate between the left atrium and left ventricle. They compose of two leaflets, anterior or septal and posterior or mural leaflets (Disatian, 2010). The degenerated atrioventricular valves or mitral valves affects by myxomatous degradation and increases extracellular matrix accumulation within the valves (Häggström et al., 2004; Disatian, 2010). Underlying cause and pathogenesis of DMVD are not yet clear. However, several studies have discussed about abnormalities of extracellular matrix components such as collagen (Häggström et al., 2004) or genetic factors (Olsen et al., 1999). Families of CKCS and Dachshunds contain the genetic factor that is able to be the etiology of DMVD (Swenson et al., 1996; Olsen et al., 1999). Small breeds such as Poodles, Miniature Schnauzers, Chihuahuas, Fox terriers, Cocker spaniels, Boston terriers, and mixed breed dogs are the predisposing breeds of DMVD (Disatian, 2010). Males have more prone to DMVD than females (Ware, 2003).

Normal mitral leaflets are thin and transparent in macroscopic appearance (Disatian, 2010). Normal canine mitral valves are composed of small amount of mucopolysaccharides, elastic fibers, elastic collagen, and valvular interstitial stromal cells. The structure of mitral valves is four well-differentiated layers; atrialis, spongiosa, fibrosa and ventricularis layer.

Degenerative mitral valves are thick, retracted, turbid with nodular formation and rolling up of free leaflet edges (Figure. 1). Chordae tendineae could be thickening or rupture (Buchanan, 1977; Häggström et al., 2004; Disatian, 2010). Degenerated valves are disorganized by an increase of mucopolysaccharide accumulation inside the valves (Aupperle et al., 2008; Disatian, 2010; Aupperle and Disatian, 2012).

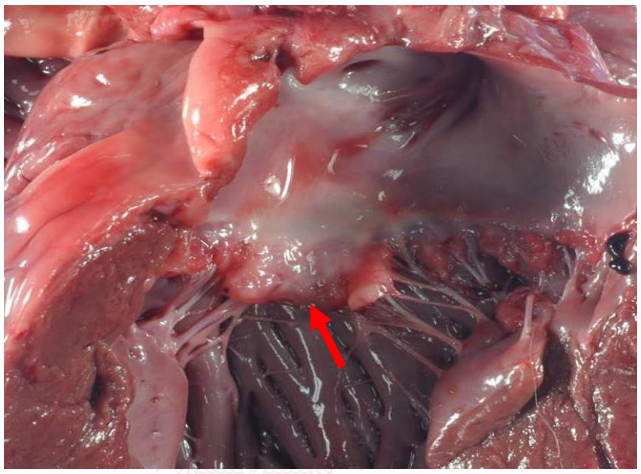

Figure 1. DMVD valve with increased thickness and nodulation formation in the valve (arrow) (Courtesy by Department of Pathology, Faculty of Veterinary Science, Chulalongkorn University).

The degenerated valve is dysfunction and cannot close properly causing valve regurgitation. Volume overload of left ventricle and left atrium along with reduction of forward stroke volume will occur. The signs of left sided congestive heart failure will appear secondarily to an increase of pressure in left atrium leading to pulmonary congestion and pulmonary edema (Dell'italia et al., 1997; Häggström et al., 2004). Cardiac ventricular remodeling (enlarged cardiac chambers and wall thinning) could be taken place (Dell'italia et al., 1997).

American College of Veterinary Internal Medicine (ACVIM) has updated categorization of DMVD into 4 stages; A, B, C, and D (Atkins et al., 2009).

Stage A is the dogs without any cardiac problems; however they have high risk to develop DMVD such as CKCS, Dachshunds, small and medium sized dogs.

Stage B is DMVD dogs without clinical signs. This stage is subdivided into B1 and B2.

- Stage B1 refers to asymptomatic DMVD dogs without cardiac remodeling such as cardiomegaly that is examined by thoracic radiograph or echocardiogram.
- Stage B2 refers to asymptomatic DMVD dogs that have cardiomegaly i.e. left sided heart enlargement.

Stage C is DMVD dogs that have clinical signs of congestive heart failure (CHF) (e.g. cough, exercise intolerance, dyspnea, or occasional syncope) with cardiac structural remodeling.

Stage D is end-stage DMVD with refractory heart failure even on standard therapy.

DMVD in dogs are not complicated to diagnose. The clinical signs of left sided congestive heart failure are obvious to the disease. However, it is sometimes difficult to distinguish from the respiratory disease secondary to the similar clinical signs of both diseases including cough, exercise intolerance, and dyspnea (Häggström et al., 2004). History taking, physical examination, blood pressure measurement, blood profiles (e.g. complete blood count (CBC) and blood chemistry of kidney and liver function), thoracic radiography, electrocardiogram (ECG) and echocardiogram should be performed to categorize degree of the disease (Disatian, 2010).

- **Signalment**: Dogs age more than 9 years old have high risk to DMVD (Whitney, 1974; Disatian, 2010). Males have more risk to this disease than females (Ware, 2003). Predisposing breeds to DMVD are CKCS and Dachshunds (Swenson et al., 1996; Olsen et al., 1999). Small breeds such as Poodles, Miniature Schnauzers, Chihuahuas, Fox terriers, Cocker spaniels, Boston terriers, and mixed breed dogs also have high prevalence to DMVD (Disatian, 2010). Prevalence of small breed dogs (< 20 kg) is higher than that of large breed dogs (Atkins et al., 2009).

- **History**: Clinical signs of left sided congestive heart failure such as exercise intolerance, cough, dyspnea, and syncope should be inquired. Some DMVD dogs especially dogs with early-stage of the disease could be asymptomatic.
- **Physical examination:** DMVD dogs have systolic murmur loudest at left apical area where the mitral valves are located. Early stage DMVD dogs may present with normal or soft murmur heart sound. Other diseases that cause mild degree murmur heart sound such as severe anemia or low grade aortic or pulmonic stenosis should be ruled out (Häggström et al., 2004). Precordial thrill can be palpated in severe stage DMVD dogs (Disatian, 2010).
- **Thoracic radiography:** Left sided heart enlargement, pulmonary edema lung pattern particularly at perihilar area, or pulmonary vein congestion are commonly found with DMVD; however, they are not specific to the disease. Some findings are similar to the chronic airway disease (Häggström et al., 2004; Disatian, 2010).
- **Electrocardiography (ECG):** ECG is not specific to DMVD. P-mitrale (widen P-wave) and/or widen QRS complex secondary to left heart enlargement are usually found**.**
    - **Echocardiogram:** Echocardiogram is currently a recommended method to evaluate DMVD in dogs by determining morphology and function of the valves. However, it is a time-consuming and expensive method that needs an experienced expert to perform. Thus, it is not suitable to use as a screening method in a large population (Häggström et al., 2004). Mitral regurgitation can be seen by using color-flow Doppler method. MR severity can be evaluated by the ratio of regurgitate jet area to left atrium area (ARJ/LAA ratio) as mild (<20-30 %), moderate (≥ 30 % but ≤70 %), or severe (>70 %) (Chetboul and Tissier, 2012).

Transforming growth factor beta

TGF-β is a part of a dimeric polypeptide growth factor family (Massagué, 1998) that is composed of TGF- β1-3, bone morphogenic proteins (BMPs), inhibins and activins (Hyytiäinen et al., 2004). TGF-β acts in two different ways. Firstly, it plays an important role in normal physiologic condition such as cell growth, differentiation, lineage determination, motility, adhesion and death, homeostasis, and embryonic development (Massagué, 1998; Nakajima et al., 2000). Secondly, it has a pathological role such as inflammation, fibrosis, angiogenesis, and oncogenesis (Dabek et al., 2006; Lim and Zhu, 2006; Bujak and Frangogiannis, 2007). In mammals, there are three isoforms of TGF-β: TGF-β1, TGF-β2, and TGF-β3. Each isoform is encoded by a distinct gene and expressed in a specific tissue. TGF-β1 is expressed by endothelial cells (Millan et al., 1991), haemopoietic cells, connective tissue cells (Border and Noble, 1994), and the myocardium (Eghbali, 1989). TGF-β2 is expressed by epithelial and neuronal cells (Millan et al., 1991), and TGF-β3 is expressed by the fibrocytes of the embryonic and mature myocardium (Millan et al., 1991; Epstein et al., 2000).

TGF-β in human is one of an interesting pro-inflammatory cytokine that has been studied. It plays an important role in several diseases especially the cardiovascular disease (Waltenberger et al., 1993; Jian et al., 2003; Dabek et al., 2006; Khan and Sheppard, 2006; Lim and Zhu, 2006; Khan et al., 2007; Kim et al., 2008), cancer, and hereditary hemorrhagic telangiectasia (Epstein et al., 2000).

Degenerative mitral valve and transforming growth factor beta

The pathogenesis of DMVD valves remains unknown. Many studies have been reported the possible signaling pathways that may implicate in heart valve disease including Notch, nitric oxide, and angiotensin II. An involvement of serotonin and TGF-β in DVMD has been suggested. TGF-β signaling pathway could be triggered by serotonin, a monoamine neurotransmitter. Both mechanical and chemical stimulations may be associated with signaling pathways (Orton et al., 2012). The mechanical stimulation on pathogenesis of DMVD valves are tension, shear, compression and flexure (Butcher et al., 2008). Circulation substances that are

chemical stimulators releasing within plasma from circulating cells nearby heart valves such as high plasma serotonin could also be involved in signaling pathway (Oyama and Levy, 2010). Orton et al. (2012) reported a strong relationship between serotonin signaling, TGF-β signaling, and expression of myxomatous effector genes and proteins resulting in valvular interstitial cell proliferative, proteoglycan (PG) or glycosaminoglycan (GAG) deposition, extracellular matrix (ECM) degradation or collagen changing in chondrogenesis in degenerated valves (Figure. 2).

Mucopolysaccharide production in DMVD valves could be stimulated by TGF-β1-3 (Orton et al., 2012). TGF-β1 is one of the main factors that cause cardiac fibrosis and an increase production of extracellular matrix proteins within human heart valves (Khan and Sheppard, 2006; Khan et al., 2007). Jian et al. (2002) showed that TGF-β1 could increase collagen and glycosaminoglycan synthesis and regulate cultured valve interstitial cell proliferation and apoptosis. Normal mitral valves have few amount of TGF-β1, but it is significantly increased in DMVD valves (Aupperle et al., 2008).

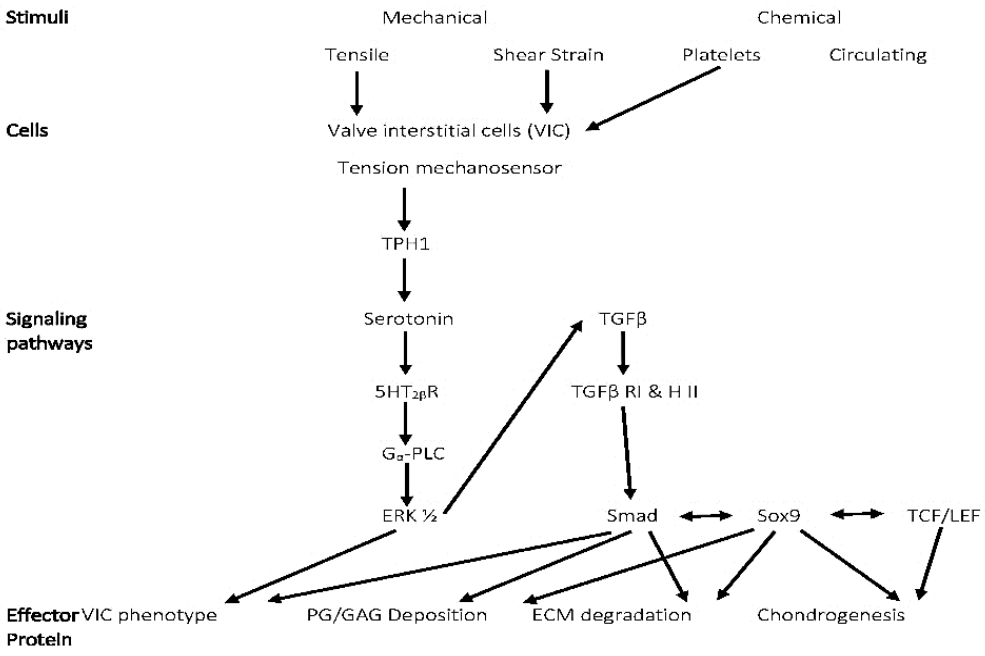

Figure 2. Signaling pathways in mitral valve degeneration (modified from Orton, et al. 2012).

An up-regulation of TGF-β1 is related to the pathogenesis of many diseases in humans such as an increase matrix production in carcinoid heart disease (Waltenberger et al., 1993; Jian et al., 2002), periodontal inflammation (Skalerič et al., 1997), calcific aortic stenosis (Jian et al., 2003), rheumatic heart disease (Kim et al., 2008), diabetic kidney disease (Sharma et al., 1997), and CAD (Wang et al., 1997). The own-regulation of circulating TGF- β1 can be used as a prognostic tool for the severity of several diseases. The study of plasma concentration of TGF-β1 in CAD patients showed that patients with low TGF- β1 plasma concentrations had a significantly poorer prognosis when compared with patients with high TGF- β1 plasma concentrations (Tashiro et al., 2002). In dogs, the overexpression of TGF-β isoform was found in several diseases such as tubulopapillary carcinomas (Andaluz et al., 2016), DMVD (Aupperle et al., 2008; Disatian and Orton, 2009; Obayashi et al., 2011), and German shepherd dogs with enteropathies (German et al., 2000).

Detection of transforming growth factor beta

In humans, TGF-β is one of the most potent regulators effecting on cell-cycle regulation and proliferation, metastasis, angiogenesis, immunosuppressive, and tumor suppression. In humans, TGF-β has roles in cancer (Epstein et al., 2000) and other diseases such as carcinoid heart disease (Waltenberger et al., 1993; Jian et al., 2002), inflammation (Skalerič et al., 1997), calcific aortic stenosis (Jian et al., 2003), rheumatic heart disease (Kim et al., 2008), diabetic kidney disease (Sharma et al., 1997), and CAD (Wang et al., 1997). In dogs with heart diseases, TGF-β in circulation has not been widely studied. In part of detection of TGF-β1 mRNA expression, German et al. (2000) studied TGF-β expression in duodenal mucosal tissue samples conducted by using semi-quantification of polymerase chain reaction (PCR) technique. Furthermore, the expression of TGF-β mRNA in circulation of dogs was examined by real-time PCR technique (Fonfara et al., 2012).

CHAPTER III

MATERIALS AND METHODS

Part I: Measurement of plasma transforming growth factor beta 1

Study Animals

Plasma samples were collected from forty-nine dogs presented with variable ages and breeds in Small Animal Teaching Hospital, Faculty of Veterinary Sciences, Chulalongkorn University with the owner permission. The protocols used for sample collection in this study were approved by the animal use and care committee, Faculty of Veterinary Science, Chulalongkorn University with Animal Use Protocol No. 1431027. All dogs were performed physical examination, thoracic radiography, echocardiogram, and blood collection for CBC and chemistry profile. Dogs were then separated into two groups: 22 control and 27 DMVD groups.

Inclusion criteria

The dogs older than 6 years old, small to medium sized breed weighing lower than 15 kilograms were recruited into the project. All dogs had normal blood profile values.

Exclusion criteria

The dogs with pregnancy, lactation, periodontal disease, mass and/or cancer disease, bone and/or joint disease, and other systemic or cardiovascular diseases such as hyperthyroidism, endocarditis and myocardial disease were excluded from the study.

The control group

This group comprised of healthy 22 dogs with normal physical examination, normal heart and lung sound, normal blood profile results, unremarkable abnormalities on radiography and echocardiogram (Table 1).

Table 1: Description of normal clinical procedures in the control group.

Clinical procedures	Findings
History	Signalment: Age > 6 years, female or male, small or medium breed, weight < 15 kilograms. Healthy dogs without history of any medical problems were included.
Physical examination	Dogs with normal physical examination finding including bright, alert and responsive, pink mucous membrane, normal hydration status, normal heart and respiratory rate, normal heart and lung sound, strong pulse, and normal temperature were recruited.
Blood collection	Dogs had normal blood profile results.
Thoracic radiograph	All dogs were performed two-dimensional radiograph in ventrodorsal and lateral views. Dogs with vertebral heart score (VHS) not over than 10.7 were recruited (Saunders et al., 2013). The thoracic radiographs were interpreted by clinicians at radiograph unit of Small Animal Teaching Hospital, Faculty of Veterinary Sciences, Chulalongkorn University. Only dogs with normal heart shape without abnormality of lung fields were included to the study.
Echocardiography	Echocardiography was performed by using an ultrasound machine (LOGIC™ 5 Pro) with 6-10 multifrequency and 5-6 MHz phrased array transducers. Sedation was not done in any dogs. Dogs were restrained in right lateral recumbent position. - **Two-dimensional echocardiography:** Right

	parasternal four chamber view was performed to assess mitral valve appearance including mitral valve thickening and chordae tendineae features. The motion of mitral valve was likewise determined. Dogs without thickened mitral valve leaflets were recruited. - **M-mode echocardiography:** M-mode echocardiography was performed to determine cardiac structure on right parasternal long and short axis views. The echocardiographic parameters included 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). Fractional shortening (FS) was calculated by (LVEDd-LVESd)/LVEDd x100**.** Dogs with M-mode echocardiographic values within normal limits were included. - **Color flow Doppler on two-dimensional echocardiography:** Mitral valve regurgitation was determined on right parasternal four chamber views. Dogs without mosaic color in left atrium during systole, i.e. mitral valve regurgitation were recruited.

The DMVD group

Twenty-seven DMVD dogs included into this group. The description of the DMVD group is explained in Table 2.

Table 2: Description of clinical procedures in the DMVD group.

Clinical procedures	Findings
History	Signalment: Age > 6 years, female or male, small or medium breed, weight < 15 kilograms. Clinical signs: Signs of DMVD including cough, exercise intolerance, dyspnea, and/or syncope could be mentioned from owner. DMVD dogs with stage B (asymptomatic) and C (symptomatic) were recruited. Both newly diagnosed DMVD dogs and DMVD dogs treated with cardiovascular medicine were included.
Physical examination	DMVD dogs both with and without clinical signs of congestive heart failure including cyanotic membrane, cough, exercise intolerance, dyspnea, and/or syncope were included. All DMVD dogs had to have systolic murmur heart sound heard loudest at the mitral area.
Blood collection	Dogs had normal blood profile values were recruited.
Thoracic radiograph	Dogs were performed two radiographs on ventrodorsal and lateral views. Dogs with vertebral heart score ≥ 10.7 were included. Dogs with abnormal radiographic findings including cardiomegaly, left atrial enlargement, pulmonary vascular enlargement, and/or pulmonary edema were recruited
Echocardiography	- **Two-dimensional echocardiography:** Dogs with valve leaflet abnormalities including thickened or prolapsed valve, and/or flailing valve leaflet were recruited. - **Color flow Doppler on two-dimensional**

	echocardiography: All DVMD dogs had to have regurgitant flow assessed by color flow Doppler echocardiography. The severity of MR was reported in mild, moderate or severe grade.

Blood sample collection and preparation

Two and a half milliliters (mL) of blood were collected from cephalic or saphenous vein of each dog by venipuncture and preserved in two EDTA and one heparinized microcentrifuge tubes. Each EDTA microcentrifuge tube contains 0.5 mL and 1 mL of blood. Heparinized microcentrifuge tube contained 1 mL of blood.

Half a milliliter of EDTA blood samples was prepared for CBC. Plasma was extracted from another 1 mL EDTA tube. Briefly, one mL EDTA blood was centrifuged at 1000 × g for 15 minutes at 2-8 ℃ within 30 minutes. Plasma was separated into a new plain eppendorf tube and immediately stored at -20 ℃ until analysis for TGF-β1. The heparinized blood was prepared for blood chemistry measurements. All of these procedures were done by one person in a same condition.

Enzyme-linked immunosorbent assay (ELISA) technique

All plasma samples and reagents were prepared before assay procedure following the canine TGF-β1 ELISA kits (BlueGene biotech, Shanghai, china). All processes were operated as manufacturer's instructions. Concisely, plasma samples and reagents were run in duplicate with TGF-β1-HRP conjugate reagent and incubated for 1 hour at 37 ˚C. The microtiter plates were manually washed to remove an incubation mixture five times. Then, incubated the wells with a substrate for HRP enzyme, which presented the blue color referred to the enzyme-substrate reaction complex. The stop solution was added into the wells to stop the reaction then the color was turned from blue to yellow color. To measure the concentration of TGF-β1, the Optical Density (O.D.) was measured at 450 nm using a microplate reader. TGF-β1 concentrations were calculated by comparing their absorbance with a

curve plotted from intensity of the O.D. color and concentration of standards (BlueGene Biotech, Shanghai, China).

Part II: Detection the expression of transforming growth factor beta 1 mRNA in mitral valves and plasma of normal and degenerative mitral valve dogs by PCR technique.

Mitral valves samples

Ten mitral valves were collected from routine necropsy examination. The samples were separated into two groups: normal and DMVD groups. Each group comprised of five samples.

Inclusion criteria

For mitral valves samples, the naturally moribund dogs (older than 6 years old, small breed weighing lower than 15 kilograms) submitted to Department of Pathology, Faculty of Veterinary Science, Chulalongkorn University for necropsy were included.

Exclusion criteria

Specimens affected with other cardiac diseases such as dilated cardiomyopathy, valve endocarditis or cardiac mass were excluded.

Mitral valve sample collection

The mitral valves were cut from left-sided heart and then measured with Vernier Caliper before kept in 1.5 mL microcentrifuge tube. Fresh tissue samples were immediately frozen at -80 °C for RNA extraction. Five normal mitral valves of the normal group were smooth, thin and transparent without an evidence of chordae tendineae abnormalities. Valve thickness was less than 2 millimeters (Moesgaard et al., 2014). Five DMVD valves were thicker

than 2 millimeters, nodular, and rolling up free edge with/without elongated or ruptured of chordae tendineae.

Nucleotide extraction and PCR technique

Twenty milligram frozen tissue samples were extracted for total RNA using NucleoSpin® RNA kit (MACHEREY-NAGEL, Düren, Germany) according to the manufacturer's protocol. In brief, each grinding frozen tissue samples were lysed by a pestle in 600 µl Lysis Buffer RA1 containing 3.5 µl β-mercaptoethanol. The broken-up tissue was filtrated with a NucleoSpin® Filter/ Filter Midi. To adjust RNA binding conditions, 350 µl of 70 % ethanol was added. Then, 350 µl MDB (Membrane Desalting Buffer) was added to desalt silica membrane. A 95 µl DNase reaction mixture was applied into the membrane in the tube to digest DNA and washed by 200 µl Buffer RAW2 to inactivate DNase reaction. Finally, RNA was eluted by 50 µl RNase-free water. The concentration of RNA was measured in duplicate by Nanodrop spectrophotometer (NanoDrop Technologies Inc., USA) before reverse-transcription (RT) step. ProtoScript® First Strand cDNA Synthesis Kit (New England Biolabs., USA) was used for cDNA synthesis following the user manual's protocol. Concisely, RNA, random primer (d(T)23VN), 10 micromolar (mM) deoxynucleotides (dNTPs), and RNase free water were mixed into RNase-free microfuge tubes. To denature RNA, the mixture was incubated at 65 ˚C for 5 minutes. Then, RNA was mixed shortly and put promptly on ice. The components including 5X ProtoScript II RT reaction buffer, 0.1 M dithiothreitol, RNase inhibitor and Protoscript II Reverse Transcriptase were added into the tube to synthesis cDNA. The tubes with mixture were put into the PCR machine that was set with the following program, 4 ˚C for 2 minutes, 25 ˚C for 10 minutes, 42 ˚C for 50 minutes, 80 ˚C for 5 minutes, and 4 ˚C for 2 minutes.

For PCR technique, all cDNA samples were then amplified using GoTaq® Green Master Mix (Promega, USA). The master mix is composed of Taq DNA polymerase, dNTPs, $MgCl_2$, dye, and reaction buffers. Primer sequences for canine TGF-β1 and GAPDH genes were selected from German et al. (2000) (Table 3). The PCR was performed using the following condition: initial incubation at 94°C for 75

seconds, denaturation of TGF-β1 and GAPDH for 35 and 40 cycles, respectively at 94 ℃ for 45 seconds, annealing at 55 ℃ (TGF-β1) or 60 ℃ (GAPDH) for 45 seconds, and extension at 72 ℃ for 45 seconds and hold temperature at 4 ℃ (German et al., 2000). The PCR products were analyzed by 1 % agarose gel electrophoresis in Tris-Acetate-EDTA (1X TAE) at 100 volt for 45 minutes. In the ladder well, 2 µl of ladder was loaded. For each sample well, 10 µl of PCR product was loaded. The specific band was visible by UV transillumination with 1 % ethidium bromide staining gel. The cDNA-omitted sample was used as a negative control. GAPDH was used as a house keeping gene. Densitometry measurements of positive bands were performed using AlphaView SA software (Cell Biosciences, Santa Clara, CA). PCR products from TAE agarose gels were purified following NucleoSpin® Extract II kit (MACHEREY-NAGEL, Düren, Germany) protocols. Purified cDNA were confirmed the target cDNA sequence identity by Biogenomed, Bangkok, Thailand.

Table 3: The 5'–3' sequence primer of each gene (German et al., 2000).

Gene	Sequence primer(5'–3')	Product size (basepair)
TGF-β1	Forward : AGTTAAAAGCGGAGCAGCATGTGG	435
	Reverse : GATCCTTGCGGAAGTCAATGTAGAGC	
GAPDH	Forward : CACGGCAAATTCCACGGCAC	400
	Reverse : TTTTGGGTGGCGGTGATGGC	

Statistical analysis

Statistical analysis was performed by the computer-based software, IBM SPSS statistics 22 program. The normality of data was tested by Shapiro-Wilk test before statistical analysis.

Measurement of plasma transforming growth factor beta 1

- Signalment including breeds and sex in normal and DMVD groups was reported as descriptive statistical analysis.

- Age and weight of normal and DMVD groups were reported as mean ± standard deviation (SD) and applied independent t-test to compare the differences between groups.
- Information of history taking, physical examination and thoracic radiography was displayed as descriptive statistical analysis.
- Complete blood count (CBC), blood chemistry, and echocardiogram values were presented as mean ± SD and, independent t-test was used to compare the differences between normal and DMVD groups.
- Plasma TGF-β1 concentrations were non-parametric data and presented as median; 25th and 75th percentile. The Mann-Whitney U test was used to compare plasma TGF-β1 concentrations between control and DMVD groups and compare between normal and mild, moderate and severe MR DMVD groups.
- Correlations of plasma TGF-β1 concentrations and age or weight in studied dogs were evaluated by Spearman's rank correlation.
- Correlations between plasma TGF-β1 concentration and echocardiographic values were evaluated by Spearman's rank correlation.
- P-value < 0.05 is considered statistically significant.

Expression of transforming growth factor beta 1 mRNA

- Signalment of normal and DMVD including breeds and sex was described as descriptive statitical analysis.
- Age and weight of normal and DMVD groups were reported as mean ± SD and applied independent t-test to compare the differences between groups.
- The results of PCR amplification were presented as negative band or positive band.
- Densitometry measurements of the positive bands were displayed as TGF-β1/GAPDH ratios. Independent t-test was used to compare average of TGF-β1/GAPDH ratios between normal and DMVD groups.
- P-value < 0.05 is considered statistically significant.

CHAPTER IV

RESULTS

Part I: Measurement of plasma transforming growth factor beta 1

Signalment

Forty-nine dogs presented at Small Animal Teaching Hospital, Faculty of Veterinary Sciences, Chulalongkorn University were recruited to the study with the owner permissions. The normal group contained 22 dogs and the DMVD group was composed of 27 dogs. Twenty-two normal dogs were 7 Poodles (31.81%), 5 Mixed (22.72%), 4 Shih-Tzus (18.18%), 2 Yorkshire Terriers (9.09%), 2 Pomeranians (9.09%), 1 Miniature Pinscher (4.54%), and 1 Chihuahua (4.54%). Sex of dogs in the normal group was 11 females (50%), 4 spayed females (18.18%), 2 males (9.09%) and 5 castrated males (22.72%). Twenty-seven DMVD dogs consisted of 10 Poodles (37.03%), 5 Mixed (18.52%), 4 Shih-Tzus (14.81%), 2 Pomeranians (7.4%), 2 Chihuahuas (7.4%), and one of Miniature Schnauzer (3.7%), CKCS (3.7%), Miniature Pinscher (3.7%), and Pekingese (3.7%). Sex of dogs in the DMVD group was 2 females (7.4%), 9 spayed females (33.33%), 8 males (29.63%), and 8 castrated males (29.63%) (Figure.3, 4).

Age and weight of both groups are described in Table. 4 as mean ± SD. Age of the DMVD group was significantly older than that in the normal group ($p < 0.05$). Mean of weight between normal and DMVD groups was not significantly different (Table. 4).

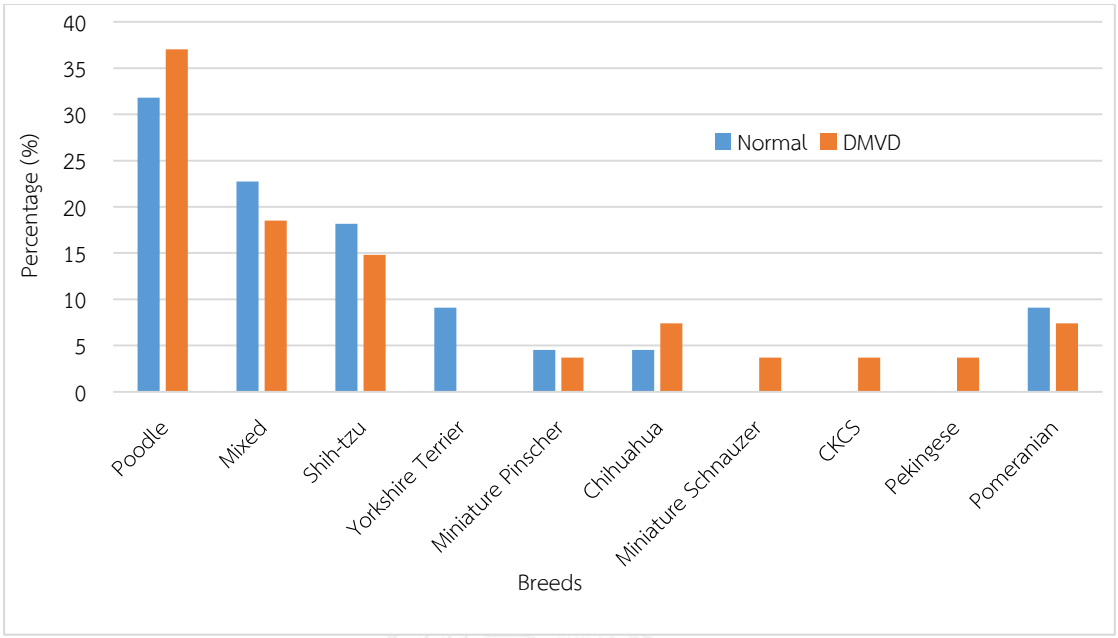

Figure 3. Breeds of normal and DMVD groups

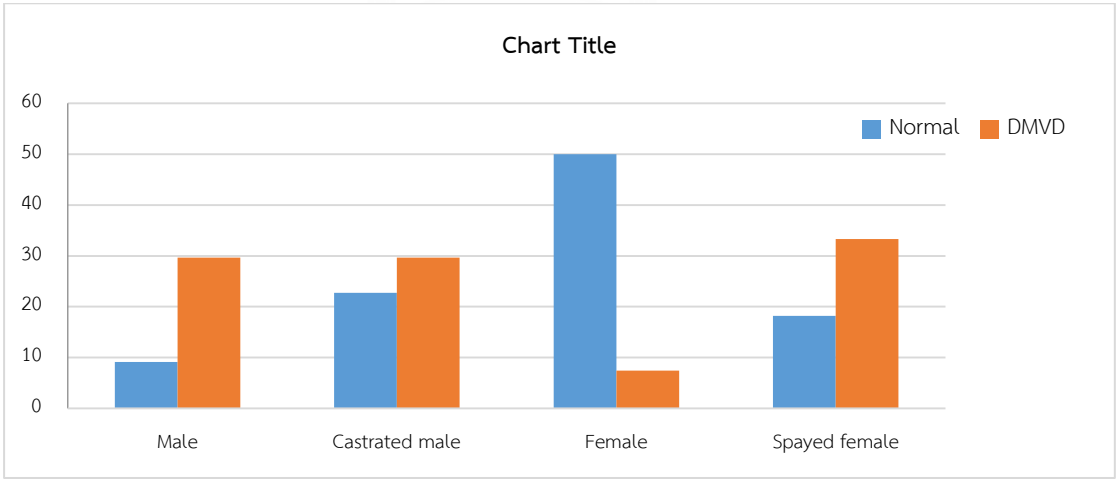

Figure 4: Sex of normal and DMVD groups

Table 4: Age and weight of normal and DMVD groups (mean ± SD)

Group	N	Age (years)	Weight (kg)
Normal	22	8.86 ± 2.05	5.35 ± 2.05
DMVD	27	11.85 ± 2.12*	5.88 ± 2.12

The significant difference was analyzed by independent - t test at $p < 0.05$.

* Represent age of the DMVD group statistically older than the normal group at $p < 0.05$

History taking

From the owner interview, 66.66 % (18/27) of dogs in the DMVD group had at least one of CHF signs. These dogs were classified as DMVD stage C. The clinical signs included exercise intolerance (100 %, 18/18), cough (77.77 %, 14/18), and dyspnea (11.11 %, 2/18). Thirty-three point three percent (9/27) of asymptomatic DMVD dogs (stage B) were defined as stage B2. History of medical treatment in the DMVD group was collected. Newly diagnosed DMVD dogs without medical treatment were 55.56 % (15/27) and DMVD dogs treated with cardiovascular medicine were 44.44 % (12/27). The twelve DMVD dogs were on medication including furosemide, pimobendan, angiotensin converting enzyme inhibitors (enalapril or ramipril) and spironolactone. All treated dogs received ACE inhibitors and diuretics. Seven dogs from twelve treated DMVD dogs received inotropic drugs. All dogs did not have any abnormalities.

Physical examination

All DMVD dogs had systolic heart murmur (100 %, 27/27). Other abnormalities detected by physical examination included crackle lung sound (18.52 %, 5/27) increased lung sound (44.44 %, 12/27), decreased lung sound (25.93 %, 7/27), pale

pink mucous membrane (33.33 %, 9/27), and dyspnea (3.70 %, 1/27) (Table. 5). All dogs had no other systemic disease.

Table 5: Physical examination abnormalities in the DMVD group

Physical examination	DMVD group (n = 27)
Systolic heart murmur	27/27 (100 %)
Crackle lung sound	5/27 (18.52 %)
Increased lung sound	12/27 (44.44 %)
Decreased lung sound	7/27 (25.93 %)
Pale pink mucous membrane	9/27 (33.33 %)
Dyspnea	1/27 (3.70 %)

Complete blood count and blood chemistry profiles

CBC profiles

All studied dogs had normal CBC results. Normal range, CBC data of studied dogs (mean ± SD) are presented in Table. 6. Hematocrit in the normal group was significantly higher than that in the DMVD group ($p < 0.01$). There were no significant difference in red blood cell count, hemoglobin, platelets count, white blood cell count, neutrophil, eosinophil, basophil, lymphocyte, and monocyte values between normal and DMVD groups. All means of CBC parameters in normal and DMVD dogs were in normal range.

Table 6: CBC results of normal and DMVD groups (mean ± SD)

Parameter	Unit	Normal value[a]	Normal group (n=22)	DMVD group (n=27)	P-value
RBC	$x10^6$ cell/μl	5.5-8.5	6.51 ± 0.79	6.10 ± 0.67	0.055
Hematocrit	%	37.0-55.0	51.38 ± 4.22	46.40 ± 6.44	0.004*
Hemoglobin	g/dL	12.0-18.0	16.32 ± 1.61	15.52 ± 1.39	0.064
Platelet	$x10^3$ cell/μl	200-500	349.91 ± 20.38	338.11 ± 24.27	0.182
WBC	$x10^3$ cell/μl	6.0-17.0	10,149.05 ± 3,021.35	9,452.41 ± 3,469.59	0.463
Neutrophil	cell/μl	3,000-11,500	6,693.18 ± 1,168.82	6,837.03 ± 1,029.52	0.649
Eosinophil	cell/μl	1,000-1,250	1,095.00 ± 85.98	1,088.18 ± 107.27	0.810
Basophil	cell/μl	0-100	13.55 ± 18.14	17.77 ± 25.01	0.510
Lymphocyte	cell/μl	1,000-4,800	2,072.72 ± 1,145.66	2,451.48 ± 1,142.10	0.255
Monocyte	cell/μl	180-1,350	660 ± 267.86	1,036.00 ± 1,614.37	0.286

The significant difference was analyzed 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 are presented as mean ± SD (Table 7). Means of ALT, ALP, and BUN of the DMVD group were higher than those of the normal group but not significantly different. Creatinine in the DMVD group was significantly higher than that in the normal group ($p < 0.01$). All means of ALT, ALP, BUN and creatinine were in normal limit.

Table 7: Blood chemistry profiles of normal and DMVD groups (mean ± SD)

Parameter	Unit	Normal value[a]	Normal group (n=19)	DMVD group (n=27)	P-value
ALT	IU/L	5-60	36.63 ± 13.70	41.62 ± 9.84	0.161
ALP	IU/L	10-150	89.45 ± 22.36	93.00 ± 24.08	0.599
BUN	mg/dL	7-27	17.86 ± 3.15	19.59 ± 4.22	0.118
Creatinine	mg/dL	0.4-1.8	0.75 ± 0.38	1.04 ± 0.34	0.008*

The significant difference was analyzed by independent t test.

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

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

Thoracic radiography

Dogs in the DMVD group with cardiomegaly (VHS>10.7) assessed by thoracic radiography were 74.07 % (20/27). From 20 dogs with cardiomegaly, 12 dogs (60 %) had pulmonary edema. All dogs in the normal group had no abnormality on thoracic radiographs.

Echocardiography

The results of echocardiogram in normal and DMVD dogs are present as mean ± SD. Echocardiographic values were indexed by weight (kg) of each dog (Table. 8). All echocardiographic values were not statistically different between normal and DMVD groups. Severity of mitral valve regurgitation in DMVD dogs was mild (29.62 %; 8/27), moderate (37.03 %; 10/27) and severe (33.33 %; 9/27).

Table 8: Echocardiographic data of normal and DMVD groups (Mean ± SD)

Parameter	Normal	DMVD	P-value
Septum-d index	1.42 ± 0.53	1.41 ± 0.53	0.936
LV chamber-d index	4.23 ± 1.28	4.74 ± 1.82	0.277
LV wall-d index	1.27 ± 0.46	1.32 ± 0.52	0.736
Septum-s index	1.88 ± 0.80	2.05 ± 0.78	0.461
LV chamber-s index	2.53 ± 0.97	2.63 ± 0.89	0.697
LV wall-s index	1.93 ± 0.66	2.00 ± 0.93	0.749
% FS	36.92 ± 11.36	42.34 ± 10.17	0.085
Aorta index	2.37 ± 0.79	2.38 ± 0.78	0.949
LA index	3.15 ± 0.94	3.65 ± 1.23	0.130
LA/Ao	1.31 ± 0.20	1.72 ± 1.08	0.087

The significant difference was assessed by independent t test.

Plasma transforming growth factor beta 1 concentration

Concentrations of plasma TGF-β1 was measured by canine-specific ELISA. To validate the precision or repeatability of test results, inter and intra-assay coefficients

of variability (CV) were analyzed. Inter and intra-assay CV for canine TGF-β1 ELISA test were 4.4 % and 2.2 %, respectively. Plasma TGF-β1 concentrations were non-parametric data and expressed as median; 25^{th} and 75^{th} in Table. 9. No statistical difference of the median of plasma TGF-β1 concentration between normal (1.14; 0.94-1.33) and DMVD (1.21; 0.93-1.32) groups was found (Table. 9 and Fig.5). There was no correlation between TGF-β1 concentrations and age or weight in entire population of dogs (Table. 10). There was no statistical correlation between plasma TGF-β1 concentrations and echocardiographic values in the population of dogs in the present study (Table. 11). No statistical difference of the median of plasma TGF-β1 concentrations between normal and different stage of MR severity in the DMVD group (Table. 12).

Table 9: Plasma TGF-β1 concentration of normal and DMVD groups (median; 25th and 75th percentile)

Group	TGF-β1 **(ng/mL)**
Normal (n=22)	1.14; 0.94-1.33
DMVD (n=27)	1.21; 0.93-1.32

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

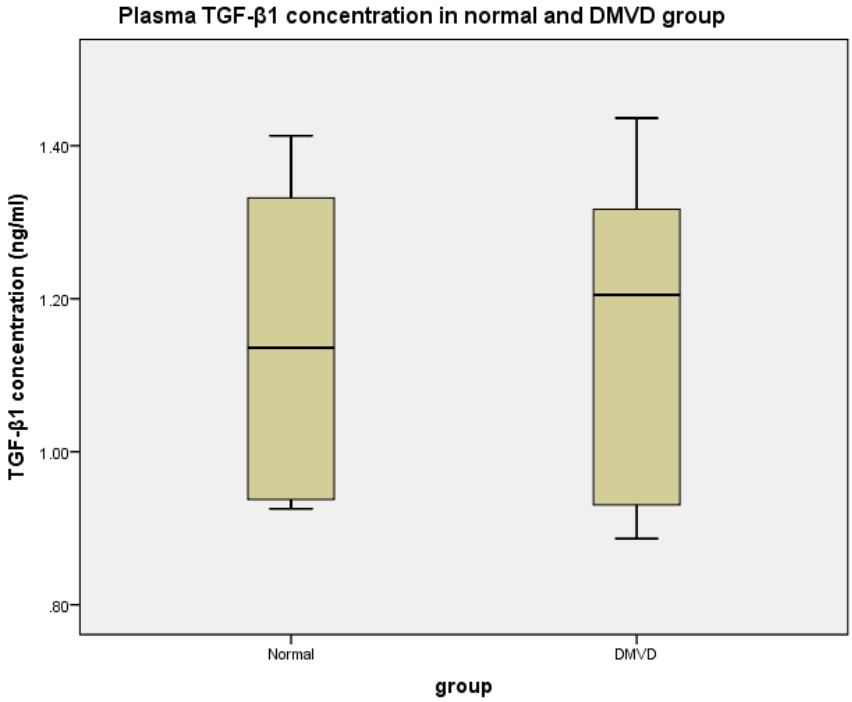

Figure 5. Boxplot of plasma TGF-β1 concentration in normal and DMVD dogs presented median values (lines within boxes), minimum values (lines at the bottom) and maximum values (lines at the top). The 25th and 75th percentile values are represented as the limits of box.

Table 10: The correlations between plasma TGF-β1 concentration and age and weight in studied dogs

Parameters	r	P-value
Age	-0.056	0.701
Weight	0.081	0.582

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

Table 11: The correlations between plasma TGF-β1 concentration and echocardiographic values of entire population

Parameters	r	P-value
Septum-d index	-0.021	0.884
LV chamber-d index	0.078	0.594
LV wall-d index	0.036	0.808
Septum-s index	-0.008	0.959
LV chamber-s index	0.039	0.790
LV wall-s index	-0.180	0.217
%FS	-0.031	0.832
Aorta index	-0.113	0.441
LA index	0.029	0.841
LA/Ao	0.222	0.126

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

r = Spearman rank correlation coefficient

Table 12: Plasma TGF-β1 concentrations of normal dogs compared with mild, moderate and severe MR DMVD dogs (median; 25th and 75th percentile)

Group	TGF-β1 plasma concentrations	P-value
Normal (n=22)	1.13 (0.93, 1.33)	
Mild DMVD (n=8)	1.28 (0.93, 1.34)	0.95
Moderate DMVD (n=10)	1.09 (0.93, 1.34)	0.88
Severe DMVD (n=9)	0.93 (0.91, 1.30)	0.21

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

Part II: Expression of transforming growth factor beta 1 mRNA in mitral valves and plasma of normal and degenerative mitral valve dogs by PCR technique.

Mitral valve samples

Signalment

Ten mitral valves were collected during necropsy each 5 mitral valves divided into normal and DMVD groups. Breeds of normal groups were Shih-Tzu (n=1), Mixed terrier (n=1), Miniature Schnauzer (n=1), Beagle (n=1), and French Bulldog (n=1). Sex dogs in the normal group were 3 females, 1 male and 1 spayed female. The DMVD group consisted of Shih-Tzu (n=2), Poodle (n=1), Miniature Schnauzer (n=1), and Miniature pinscher (n=1). Sex of DMVD dogs was 3 males, 1 castrated male and 1 female (Table. 13). Means ± SD of age and weight of normal and DMVD dogs are presented in Table. 14. Age and weight were not significantly between normal and DMVD dogs. Mean ± SD of valve thickness is presented in Table. 15.

Table 13: Signalment of dogs in the tissue study

Group	Name	Breed	Sex
Normal	Dog 1	Shih-Tzu	Spayed female
Normal	Dog 2	Mixed terrier	Female
Normal	Dog 3	Miniature Schnauzer	Male
Normal	Dog 4	Beagle	Female
Normal	Dog 5	French Bulldog	Female
DMVD	Dog 6	Shih-Tzu	Female
DMVD	Dog 7	Poodle	Castrated male
DMVD	Dog 8	Shih-Tzu	Male
DMVD	Dog 9	Miniature Schnauzer	Male
DMVD	Dog 10	Miniature pinscher	Male

Table 14: Age and weight of normal and DMVD groups (mean ± SD)

Group	N	Age (years)	Weight (kg)
Normal	5	9.20 ± 2.16	8.90 ± 3.68
DMVD	5	10.80 ± 1.92	7.95 ± 1.49

The significant difference was analyzed by independent - t test at $p < 0.05$.

* Represent age of the DMVD group statistically older than the normal group at $p < 0.05$

Table 15: Valve thickness of normal and DMVD groups (mean ± SD)

Group	N	Valve thickness (mm.)
Normal	5	1.38 ± 0.28
DMVD	5	3.08 ± 0.25

Transforming growth factor beta 1 mRNA expression results

Twenty samples (10 mitral valve samples and 10 plasma samples) were recruited for the study of TGF-β1 mRNA expression. The results reported as negative (-) or positive (+) (Fig 6). Amplification results from five normal and five DMVD valves were positive. GAPDH was used as a house keeping gene. The intensity of positive PCR bands was measured and reported as the TGF-β1/GAPDH ratio (Table. 16). The average of ratios between normal and DMVD valves were significantly different. The identity of TGF-β1 PCR product was 97 % (384/395) nucleotide identical to TGF-β1 GenBank accession numbers AF091135 which referred to Canis lupus familiaris TGF-β1.

.

Table 16: Average of TGF-β1/GAPDH ratios of PCR amplification products from MV samples (mean ± SD)

Group	TGF-β1/GAPDH ratio	P-value
Normal mitral valves	0.55 ± 0.07	0.006
DMVD valves	0.95 ± 0.06*	

* indicate significant difference at $p < 0.01$ between normal and DMVD groups. The significant difference was assessed by independent t-test.

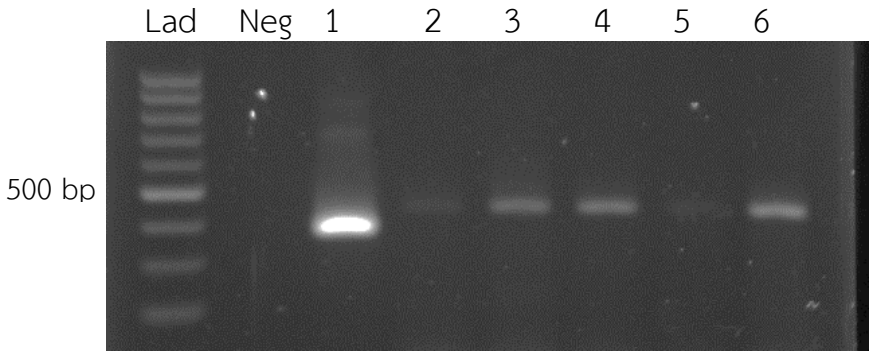

Figure 6. RT-PCR amplification products from canine mitral valves preparations. Lane 1 displayed GAPDH transcripts at 400 bp. Lane 2-6 presented TGF-β1 expression from DMVD mitral valves. The specific band was visible by UV transillumination with 1 % agarose gel. Lad = Ladder, Neg= negative sample.

CHAPTER V

DISCUSSION

Degenerative mitral valve disease (DMVD) in dogs is age and breed related disease (Häggström et al., 2004; Disatian, 2010). Means of age between normal and DMVD groups in this study were in agreement with a previous study reported that dogs more than 9 years old have a high risk to DMVD (Whitney, 1974; Disatian, 2010). The mean age of the DMVD group was significantly older than that in the normal group. The range of age in this study was 8-14 years. All dogs in this study were small and medium sized dogs (weight not over 15 kg). This inclusion criteria was assigned based on the nature of DMVD which were prone to small and medium breed dogs (Häggström et al., 2004; Disatian, 2010). Poodle is the major population of dogs in this study. This result was different from a previous study showing that Dachshund and CKCS were the top lists of breeds affecting with DMVD (Swenson et al., 1996; Olsen et al., 1999). It is possibly because both two breeds are not popular in Thailand. Number of sex in the DMVD group was 16 males and 4 females. This result is in agreement with a prior study that reported males had a higher risk to develop degenerative mitral valves than females (Ware, 2003).

History was taken from the owners. Dogs with any abnormality besides DMVD were excluded from the study. DMVD dogs in this study were categorized according to American College of Veterinary Internal Medicine (ACVIM) consensus into stage B2 (33.33 %) and C (66.67 %). Exercise intolerance is the most common clinical sign that was found in this study. The chief complaint from the owners was the dogs frequently pant especially when they are excited. The second most clinical sign found in this study was cough. Cough in DMVD dogs may occur secondarily to an enlargement of the left atrium and/or pulmonary edema (Disatian, 2010). However, other diseases can also cause cough such as tracheal stenosis, bronchitis, and other respiratory problems. To rule out the other cause of cough, thoracic radiography was performed in this study. Coughing in DMVD dogs was related to cardiomegaly and pulmonary edema. Systolic murmur heart sound secondary to MR was detected from

physical examination from all DMVD dogs. Some DMVD dogs with presented with crackle lung sound or increased lung sound due to pulmonary edema.

The population of dogs in this study did not have kidney or liver abnormalities that may interfere the interpretation of plasma TGF-β1 concentration. The significantly elevated creatinine level in the DMVD group compared with the normal group but still in the normal limit can occur due to the fact that the DMVD group consisted of treated dogs with the diuretic drugs that may increase the renal work load (Nicolle et al., 2007; Peddle et al., 2012).

Echocardiogram is a useful tool for diagnosing DMVD because it can determine real-time morphology and function of cardiac muscle and the valves including mitral valve lesions, severity of mitral valve regurgitation, cardiac myocardial function and cardiac structural remodeling (Häggström et al., 2004; Disatian, 2010). In this study, all dogs in the DMVD group had mitral valve leaflet thickening. Cardiac remodeling was evaluated from echocardiogram. Some DMVD dogs had left ventricular and left atrial enlargement. An increase preload increases the wall stretch that subsequently augments cardiac contraction to maintain stroke volume according to Frank starling law (Nakamura et al., 2014). An increase of cardiac contraction was demonstrated by an increase of percent fractional shortening in the DMVD group compared to the normal group in this study. This result is similar to previous studies (Chetboul and Tissier, 2012; Suzuki et al., 2013).

A previous immunohistochemical study reported a significant increase of the expression of TGF-β1 in valves of DMVD dogs compared to normal dogs (Aupperle et al., 2008). In addition, TGF-β1 has been thought to be involved with mucopolysaccharide synthesis and collagen destruction in degenerative mitral valves (Aupperle et al., 2008; Orton et al., 2012). However, plasma TGF-β1 concentrations between normal and DMVD groups in this study were not different. This result was similar to a recent publication investigating plasma cytokine concentration including TGF-β1 in normal and DMVD dogs (Moesgaard et al., 2014). The result of that study showed no difference of plasma TGF-β1 concentrations between normal and DMVD dogs. The present study showed that there was no correlation between echocardiogram values and plasma TGF-β1 concentrations. This result was similar to

the result of a previous study (Moesgaard et al., 2014). The expression of TGF -β1 mRNA was shown in both normal and DMVD valves. The expression of TGF -β1 mRNA was increased in DMVD dogs compared to normal dogs. This result is in agreement with Aupperle et al. (2008) that demonstrated an up-regulation of the TGF -β1 protein in DMVD valves by an immunohistochemical technique. This result suggested that TGF-β1 possibly synthesize locally in the valve but not in the circulation. In human, the TGF-β1 half-life in circulation is very short because systemic clearance is rapid (Zhang et al., 2003). The systemic clearance of canine TGF-β1 is unknown. TGF-β1 half-life in dogs may also be short same as in humans. Thus, it is difficult to detect in TGF-β1 in blood circulation. This hypothesis needs further studies to clarify.

The limitation of this study was that no reference value of TGF-β1 in circulation has been reported in dogs. Secondly, this study was conducted with a small sample size. Thirdly, the duration of the mitral valve sample collection varied from 4 hours to less than 48 hours, which could affect to the quality of the samples possibly resulting in low level of the RNA yield. Another limitation is the possibility of TGF-β1 production from several organs. However, this problem was minimizing by an exclusion of dogs with other systemic diseases from the study. Using conventional PCR to detect TGF-β1 mRNA expression in this study was another limitation; thus, the result could be evaluated only as qualitative data. For further study, real-time PCR is recommended to use for comparing the amount of TGF-β1 mRNA expression between normal and DMVD dogs. In addition, the relationship between the local expressions of TGF-β1 in mitral valve tissues and plasma concentrations should be performed in a larger number of dogs.

In conclusion, TGF-β1 mRNA expression could be detected by conventional PCR from mitral valve tissues. The DMVD dogs have higher TGF-β1 mRNA expression than normal dogs. The concentration of plasma TGF-β1 is not different between normal and DMVD dogs. The local synthesis is likely to be a major source of TGF-β1 in DMVD dogs.

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APPENDIX

Appendix A: Signalment and plasma TGF-β1 concentration in the normal dogs

No.	Name	Breed	Sex	Age (year)	Weight (kg)	Plasma TGF-β1 concentration (ng/ml)
1	Dog 1	Shih-Tzu	Mc	11	5.5	1.33
2	Dog 2	Poodle	F	7	9.8	0.93
3	Dog 3	Mixed	F	9	9	0.93
4	Dog 4	Poodle	F	8	3.8	1.35
5	Dog 5	Shih-Tzu	M	10	6	1.30
6	Dog 6	Mixed	F	7	6.22	1.41
7	Dog 7	Mixed	Mc	7	4.6	1.41
8	Dog 8	Yorkshire Terrier	F	7	2.8	0.94
9	Dog 9	Poodle	Fs	10	3.5	0.95
10	Dog 10	Poodle	F	14	7.5	0.93
11	Dog 11	Pomeranian	F	6	3.8	0.98
12	Dog 12	Mixed	Fs	10	8	0.94
13	Dog 13	Poodle	Fs	8	4.5	0.94
14	Dog 14	Shih-Tzu	Mc	8	6.9	0.95
15	Dog 15	Miniature Pinscher	Fs	8	3.4	0.95
16	Dog 16	Mixed	F	7	5.6	0.93
17	Dog 17	Pomeranian	M	13	7	1.32
18	Dog 18	Shih-Tzu	F	10	5.6	1.34
19	Dog 19	Poodle	F	7	3.7	1.30
20	Dog 20	Poodle	Mc	12	5.46	1.39
21	Dog 21	Yorkshire Terrier	Mc	8	2.9	1.31
22	Dog 22	Chichahua	F	8	2.3	1.32

Appendix B: Signalment and plasma TGF-β1 concentration in the DMVD dogs

No.	Name	Breed	Sex	Age (year)	Weight (kg)	Plasma TGF-β1 concentration (ng/ml)
1	Dog 23	Pomeranian	M	11	2.2	0.96
2	Dog 24	Chihuahua	M	8	3.4	0.89
3	Dog 25	Schnauzer	M	11	7.6	1.41
4	Dog 26	Poodle	F	12	3.7	1.36
5	Dog 27	Poodle	Mc	13	6.5	0.93
6	Dog 28	Shih-Tzu	Fs	15	5.5	0.92
7	Dog 29	Poodle	Mc	13	5.3	0.92
8	Dog 30	Poodle	M	13	3.4	0.91
9	Dog 31	Mixed	F	17	6.4	0.96
10	Dog 32	Poodle	Fs	14	4.6	1.31
11	Dog 33	Shih-Tzu	M	12	4.9	1.29
12	Dog 34	CKCS	Fs	7	9	1.29
13	Dog 35	Mixed	Mc	9	8.06	1.32
14	Dog 36	Chihuahua	M	12	3.5	1.32
15	Dog 37	Miniature Pinscher	Mc	10	8	1.35
16	Dog 38	Mixed	Fs	14	7.4	1.33
17	Dog 39	Poodle	Fs	12	7.7	1.21
18	Dog 40	Pomeranian	Fs	11	2.84	0.95
19	Dog 41	Shih-Tzu	Mc	11	7.8	0.91
20	Dog 42	Peking	M	11	9.8	0.96
21	Dog 43	Poodle	Mc	11	5.5	0.94

No.	Name	Breed	Sex	Age (year)	Weight (kg)	Plasma TGF-β1 concentration (ng/ml)
22	Dog 44	Poodle	Mc	11	2.8	0.93
23	Dog 45	Mixed	Mc	14	9.5	1.36
24	Dog 46	Shih-Tzu	Fs	14	5.6	0.93
25	Dog 47	Mixed	M	12	7.8	1.44
26	Dog 48	Poodle	Fs	10	5.7	1.31
27	Dog 49	Poodle	Fs	12	4.5	1.27

Appendix C: Echocardiographic indices in the normal dogs

No.	Name	IVSd	LVIDd	LVPWd	IVSs	LVIDs	LVPWs	FS	Ao	LA	LA/Ao
1	Dog 1	9	15.8	7	9.9	9.7	10.3	38.89	14.5	14.5	1
2	Dog 2	9.2	26.3	8	12.7	14.7	10.4	44.28	19.6	11.5	1.06
3	Dog 3	7.4	21.7	7.7	10	15.6	9.2	26.96	16.2	18.5	1.14
4	Dog 4	6.3	19.6	6.5	7.7	11.9	9.5	39.23	9.7	16.5	1.7
5	Dog 5	9	17.6	7.5	11.8	10.1	10.8	42.5	14.9	16.5	1.1
6	Dog 6	7.5	20.6	6.8	9.5	14	10.3	31.76	12.5	16.3	1.3
7	Dog 7	7.7	22.6	5.3	10.1	3.6	9	39.81	16.5	13.2	1.25
8	Dog 8	6.6	16.6	5.9	7.5	10.8	8.6	34.66	0.9	13.8	1.53
9	Dog 9	6.2	18.9	4.4	8.7	9.8	8.6	48.26	8	13.6	1.71
10	Dog 10	6.5	26.2	6.8	9.2	17	10.8	35.25	14.9	19.1	1.28
11	Dog 11	4.8	15.1	5.3	6.4	11.4	6.8	24.85	9.8	14.2	1.45
12	Dog 12	7.3	19.1	5.8	8.8	15.5	8.3	18.42	12.2	17.1	1.4
13	Dog 13	6.4	20.6	5.3	7.3	11.9	9.9	42.01	11.1	14.3	1.24
14	Dog 14	5	21.1	6	6.3	16.3	8.5	22.6	13.2	18.2	1.38
15	Dog 15	6.1	19.7	5.4	6.8	15.3	7.8	22.78	12.3	15	1.23

No.	Name	IVSd	LVIDd	LVPWd	IVSs	LVIDs	LVPWs	FS	Ao	LA	LA/Ao
16	Dog 16	6.5	20.2	5.8	10.3	8.8	11.6	56.52	13.2	15.3	1.16
17	Dog 17	5.8	29.5	5.4	5.8	17.3	12.2	37.44	10.8	18.3	1.7
18	Dog 18	5.9	25.7	5.5	9	16.7	7.5	35.04	13.9	18.1	1.3
19	Dog 19	6	15.4	7.2	9.1	9.1	10.4	41.18	11.5	12.3	1.07
20	Dog 20	6.3	25.5	4.8	8.3	14.8	6.9	41.8	10.5	15	1.43
21	Dog 21	7.2	18.7	5.2	9.4	14.4	7.2	22.94	9.7	12.1	1.25
22	Dog 22	5.7	15.1	5.5	9.8	5.3	7.8	65.22	8.4	10.9	1.3

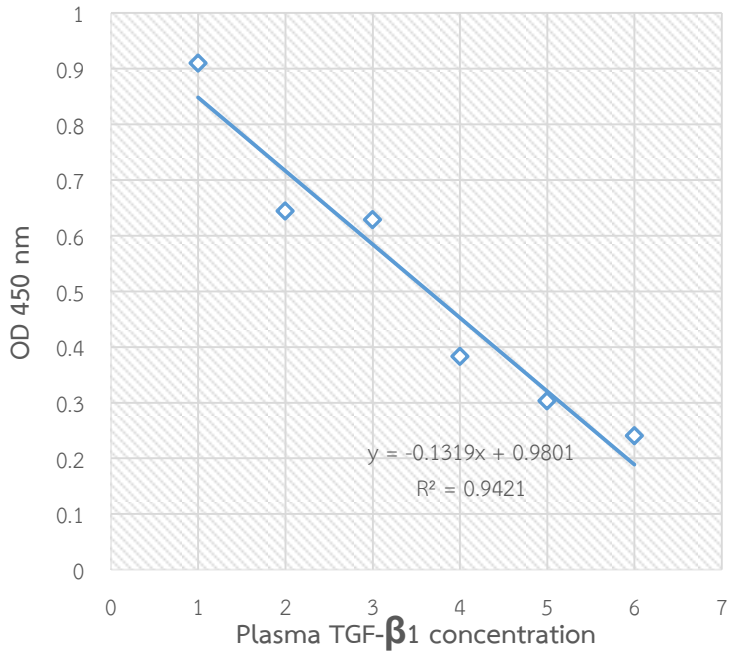
IVSd; Interventricular septal thickness at end-diastole, **LVIDd**; Left ventricular end-diastolic dimension, **LVPWd;** Left ventricular posterior wall thickness at end-diastole, **IVSs;** interventricular septal thickness at end-systole, **LVIDs**; left ventricular end-systole dimension, **LVPWs**; 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

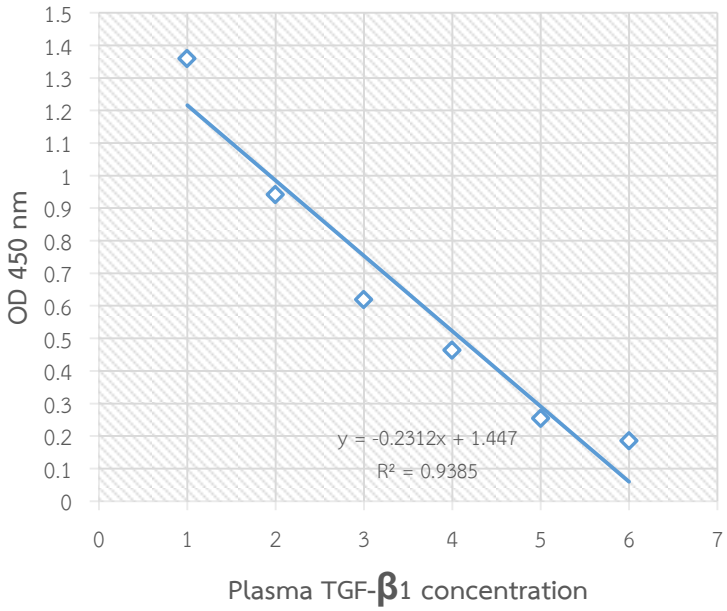
Appendix D: Echocardiographic indices in the DMVD dogs

No.	Name	IVSd	LVIDd	LVPWd	IVSs	LVIDs	LVPWs	FS	Ao	LA	LA/Ao
1	Dog 23	6	13.9	6.6	9.2	4.4	11.5	68.29	9.2	12.8	1.4
2	Dog 24	6.2	22.6	5.3	9.4	10.2	10.2	55	11.3	16.6	1.47
3	Dog 25	7.7	27.2	8.7	13.3	13.9	11.4	48.85	11.6	22.8	1.96
4	Dog 26	6.8	33.4	6.3	9.4	13.4	10.3	42.55	10.4	19.3	1.86
5	Dog 27	8.2	26.6	7.9	10.8	17.7	12.1	33.33	15.3	19.6	1.28
6	Dog 28	7.7	22.2	6.4	11.8	9.9	11.2	55.44	12.7	16.5	1.24
7	Dog 29	8.5	27.1	6.9	9.3	18.2	11.9	32.87	14	21.7	1.54
8	Dog 30	4.3	29.5	4.3	9.7	11.8	10.7	58.73	14.8	20	1.35
9	Dog 31	7.8	35.5	9.5	12.5	15.3	14.5	56.83	14.5	25	1.72
10	Dog 32	7.3	23.9	5	9	22.8	8.3	32.57	15.8	23.1	1.46
11	Dog 33	7.1	18.7	8.2	10.5	9.7	10.7	44.18	12.2	17.3	1.42
12	Dog 34	8.4	24.7	7.9	9.4	17.2	10.8	30.53	15.7	23.2	1.48
13	Dog 35	8.8	19.1	7.2	9.4	12.2	8.8	31.58	16.1	21	1.3
14	Dog 36	6.5	29.6	6.3	10.6	16	7.7	45.92	9.8	22.2	2.26
15	Dog 37	8.1	26.4	6.9	9.1	14.1	11.4	46.41	11.6	19.7	1.7

No.	Name	IVSd	LVIDd	LVPWd	IVSs	LVIDs	LVPWs	FS	Ao	LA	LA/Ao
16	Dog 38	7.9	31.3	6.6	14.3	18.5	9.1	40.96	14	21.1	1.51
17	Dog 39	9.4	31.5	7.4	13.8	19.7	10.8	39.44	14.1	24.9	1.77
18	Dog 40	4.4	15.6	3.9	6.6	8.6	6.4	45.04	8.8	12.9	1.48
19	Dog 41	7.8	24.9	6.6	12.4	12.4	12.1	50.32	16.9	20.1	1.19
20	Dog 42	7.5	32.1	6.3	9.4	22.4	6.9	30.24	14	24.3	1.74
21	Dog 43	7.9	18.9	8.5	15.3	11.3	12.5	40	12.5	20.8	1.67
22	Dog 44	8.4	14.7	6.8	10	9.1	9.9	38.06	3.48	6.02	1.73
23	Dog 45	4.4	18	17.3	10.9	11.5	12.4	35	14.6	13.9	0.94
24	Dog 46	8.2	23.6	5.5	9.1	16.5	8	30.23	13.2	18.7	1.42
25	Dog 47	8.3	39.2	6.2	10.6	22	9.3	43.92	16	32	7
26	Dog 48	7.6	22.9	6.3	10	15.5	9.8	32.59	12.4	16.8	1.36
27	Dog 49	7.5	22.4	5.5	11	14.6	7.4	34.5	12.1	17.1	1.42

Appendix E: Standard curves of ELISA tests plotted from intensity of the O.D. color and concentration of standards (BlueGene Biotech, Shanghai, China).





VITA

Miss Wiyada Winyuchonjaroen was born on May 27th, 1988 in Bangkok, Thailand. She finished her high school education from Triamudom Suksa School, Bangkok in 2006 and graduated with a Bachelor's degree from Faculty of Veterinary Science, Chulalongkorn University in 2012. She was interesting in cardiology and internal medicine in dogs. After that, she attended to the degree of Master of Science Program in Department of Veterinary Medicine, Faculty of Veterinary Science, Chulalongkorn University in 2012.