การตายของเซลล์และการแสดงออกของเมสเซนเจอร์อาร์เอ็นเอและโปรตีนในตระกูลบีซีแอลสอง (บีซีแอลสองและแบกซ์) และโปรตีนทรานสฟอร์มมิ่ง โกรว์ท แฟคเตอร์ เบตาร์1 ในโรคลิ้นหัวใจไมทรัลเสื่อมในสุนัข

นางสาว ตรีเนตร จิรนันทศักดิ์

APOPTOSIS AND EXPRESSION OF BCL-2 FAMILY (*bcl-2* and *bax*) messenger RNA

AND PROTEIN ASSOCIATED WITH TRANSFORMING GROWTH FACTOR-β1

(TGF-β1) SIGNALING PROTEIN IN CANINE MYXOMATOUS

MITRAL VALVE DISEASE

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A Thesis Submitted in Partial Fulfillment of the Requirements

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Thesis Title	APOPTOSIS AND EXPRESSION OF BCL-2 FAMILY (*bcl-2* and *bax*) messenger RNA AND PROTEIN ASSOCIATED WITH TRANSFORMING GROWTH FACTOR-β1 (TGF-β1) SIGNALING PROTEIN IN CANINE MYXOMATOUS MITRAL VALVE DISEASE
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โรคลิ้นหัวใจไมทรัลเสื่อมจัดเป็นสาเหตุหลักที่ทำให้เกิดโรคหัวใจล้มเหลวในสุนัข พยาธิกำเนิดพบการ เพิ่มจำนวนของเซลล์ร่วมกับมีการสะสมของสารมิวโคโพลีแซคคาไรด์ภายในลิ้นหัวใจไมทรัลเป็นจำนวนมาก จาก รายงานพบว่าเซลล์ในลิ้นหัวใจไมทรัลเสื่อมมีการหลั่งของโปรตีน ทรานสฟอร์มมิ่ง โกรว์ท แฟคเตอร์ เบตาร์1 (TGF-β1) เพิ่มขึ้นอย่างชัดเจน และการที่เซลล์ภายในลิ้นหัวใจมีความหนาแน่นมากขึ้น ไม่ได้เป็นสาเหตุมาจาก ความผิดปกติของการเพิ่มจำนวนของเซลล์ ดังนั้นการวิจัยครั้งนี้มีวัตถุประสงค์ เพื่อศึกษาหาความสัมพันธ์และ เปรียบเทียบการเปลี่ยนแปลงทางพยาธิวิทยาการตายของเซลล์และการแสดงออกของเมสเซนเจอร์อาร์เอ็นเอ และโปรตีนในตระกูลบีซีแอลสอง (Bax และ Bcl-2) และโปรตีนที่ใช้ในการส่งสัญญาณ TGF-β1 (p-Smad2/3) ในโรคลิ้นหัวใจไมทรัลเสื่อมในสุนัข ผลการศึกษาครั้งนี้พบว่า อายุและความหนาของลิ้นหัวใจไมทรัลปกติมีความ แตกต่างจากลิ้นหัวใจไมทรัลเสื่อมอย่างมีนัยสำคัญ ในลิ้นหัวใจไมทรัลเสื่อม พบร้อยละการเสียหายของดีเอ็นเอ (TUNEL) ต่ำกว่าลิ้นหัวใจไมทรัลปกติอย่างมีนัยสำคัญ นอกจากนี้ยังพบว่าในลิ้นหัวใจไมทรัลเสื่อมมีการ แสดงออกของโปรตีนที่เหนี่ยวนำให้เกิดอะพอพโทซิส (Bax) สูงขึ้น แต่มีการแสดงออกโปรตีนที่ยับยั้งการเกิด กระบวนการอะพอพโทซิส (Bcl-2) และโปรตีน p-Smad2/3 ต่ำลงอย่างมีนัยสำคัญเมื่อเทียบกับลิ้นหัวใจไมทรัล ปกติ ทั้งนี้โปรตีนแคสเปสที่ใช้บ่งชี้การตายของเซลล์แบบอะพอพโทซิส (cleaved caspase-3) ไม่มีการ เปลี่ยนแปลงอย่างชัดเจน จากการศึกษาหาความสัมพันธ์พบว่า การแสดงออกของ TUNEL, Bax, Bcl-2 และ p-Smad2/3 มีความสัมพันธ์กับความหนาของลิ้นหัวใจที่เพิ่มมากขึ้นอย่างมีนัยสำคัญ แต่ไม่มีความสัมพันธ์กับ อายุของสุนัขปกติ ในโรคลิ้นหัวใจไมทรัลเสื่อมมีการแสดงออกของเมสเซนเจอร์อาร์เอ็นเอของยีน *bcl-2* มากกว่า ยีน *bax* ส่งผลให้อัตราส่วนของ *bax/bcl-2* มีแนวโน้มลดลงเมื่อเทียบกับลิ้นหัวใจไมทรัลปกติ ดังนั้นจาก การศึกษาสรุปว่าโรคลิ้นหัวใจไมทรัลเสื่อมในสุนัข มีการส่งสัญญาณของเซลล์ผ่านทาง TGF-β1-Smad ลดลง ร่วมกับพบว่ามีการทำลายดีเอ็นเอของเซลล์ภายในลิ้นหัวใจลดลง นอกจากนี้ระดับที่ไม่สมดุลย์ของ Bax และ Bcl-2 อาจมีผลต่อการตายของเซลล์ในโรคลิ้นหัวใจไมทรัลเสื่อม เนื่องจากการศึกษาครั้งนี้มีการแสดงออกของ โปรตีน cleaved caspase-3 คงที่ร่วมกับไม่พบอะพอพโทติกบอดีบ่งชี้ว่าเซลล์ภายในลิ้นหัวใจไมทรัลเสื่อมอาจ ไม่เกิดการตายแบบอะพอพโทซิส อย่างไรก็ตามควรมีการศึกษาหากลไกที่ชัดเจนของการตายของเซลล์แบบอะ พอพโทซิส เพื่อจะได้นำข้อมูลมาประยุกต์ใช้ในการป้องกันและรักษาโรคลิ้นหัวใจไมทรัลเสื่อมในสุนัขต่อไป

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KEYWORDS: APOPTOSIS, BAX, BCL-2, P-SMAD2/3, TGF- β1

TREENATE JIRANANTASAK: APOPTOSIS AND EXPRESSION OF BCL-2 FAMILY (*bcl-2* and *bax*) mRNA AND PROTEIN ASSOCIATED WITH TRANSFORMING GROWTH FACTOR-β1 (TGF-β1) SIGNALING PROTEIN IN CANINE MYXOMATOUS MITRAL VALVE DISEASE. THESIS ADVISOR: ASSOC. PROF. ANUDEP RUNGSIPIPAT, D. V. M., Ph. D., THESIS CO-ADVISOR: SIRILAK DISATIAN, D. V. M., M.S. Ph. D., 66pp.

Canine myxomatous mitral valve disease (MMVD) causes severe congestive heart failure. The pathogenesis mainly involves with atypical proliferation of valvular interstitial cells (VICs) and accumulation of mucopolysaccharides. There is a strong evidence of TGF-β1 signaling in naturally-occurring MMVD. Additionally, the cell proliferation is unlikely a major mechanism of increased cell density in canine MMVD. Thereby, monitoring of TGF- β1 signaling related to anti-apoptotic and apoptotic responses of VICs in canine MMVD may play a beneficial role in determining the pathogenesis. This study presented that age and valve thickness of dogs affected MMVD were statistically significant difference when compare with normal dogs. TUNEL and immunohistochemical assays were applied to detect apoptotic marker (TUNEL), TGF- β1 signaling protein (p-Smad2/3), cleaved caspase-3, pro-apoptotic (Bax) and anti-apoptotic protein (Bcl-2) in VICs of normal and MMVD valves. This study showed that MMVD valves had down-regulated level of p-Smad2/3 and Bcl-2, whereas the level of Bax was up-regulated compared to normal valves. Cleaved caspase-3 remained unchanged. There were statistically significant correlation between valve thickness and expression of TUNEL, p-Smad2/3, Bcl-2 and Bax. Furthermore, mRNA expression of *bax* and *bcl-2* was used to determine the fold change and ratio of *bax* to *bcl-2*. There was not statistically significant different between *bax* and *bcl-2* mRNA expression and stage of MMVD. The ratio of *bax* to *bcl-2* tends to decrease with the late staged MMVD. In conclusion, the TGF β1-Smad signaling pathway is down-regulated in canine MMVD, which related to decrease in DNA damage in VICs. An imbalance of Bax and Bcl-2 may reflect the shift of pro-apoptotic and anti-apoptotic responses in MMVD. The VICs seem not to undergo apoptosis based on the constant expression of cleaved caspase-3 and the absence of apoptotic bodies in normal and MMVD valves. Further studies are warranted to specify the exact roles of apoptosis and anti-apoptosis involved in MMVD. This may lead to new strategies in the management of canine MMVD to prevent or stop the disease progression.

Department: ……….Veterinary Pathology…………..	Student's Signature……………………………..

Field of Study: ……Veterinary Pathobiology………...	Advisor's Signature……………………………...

Academic Year: …………2011………………….....	Co-advisor's Signature....................................

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°C	degree Celcius
α-SMA	alpha-smooth muscle actin
μ	micron
μg	microgram
μl	microliter
μM	micromolar
18s rRNA	18s ribosomal ribonucleic acid
ActR-II	serine/threonine kinases receptor type II
ActR-IIB	serine/threonine kinases receptor type II
AIF	apoptotic-inducing factor
ALK	serine/threonine kinases receptor type I
APAF1	apoptotic protease activating factor1
Bax	apoptotic regulatory gene and protein
Bcl-2	anti-apoptotic regulatory gene and protein
Bcl-XL	anti-apoptotic regulatory gene and protein

BMPR-II	serine/threonine kinases receptor type II
bp	base pair
caspases	cysteine aspartic acid-specific proteases
cDNA	complementary deoxyribonucleic acid
Ct	threshold cycles
DAB	3,3'-diaminobenzidine tetrahydrochloride
DNA	deoxyribonucleic acid
DNA-PK	DNA-dependent protein kinase
DNase I	deoxyribonuclease I
ECM	extracellular matrix
H&E	hematoxylin and eosin
Ki-67	Kiel-67; proliferative marker index
LAP	latent associated peptide
LTBP	latent TGF-β binding protein
min	minute
ml	milliliter
mM	millimolar

MMP	metalloproteinase
MMVD	myxomatous mitral valve disease
mRNA	messenger RNA
MyoFb	myofibroblast
nm	nanometer
OD	optical density
PARP	poly-ADP-ribose polymerase
PAS	Periodic Acid-Schiff reaction
PBS	phosphate buffer saline
PCR	polymerase chain reaction
p-Smad2/3	phosphorylated Smad2/3; activated TGF-β1 signaling protein
Rn	normalized reporter
RNA	ribonucleic acid
RT-PCR	reverse transcriptase-polymerase chain reaction
SBE	Smad-binding elements
SD	standard deviation
TβRII	serine/threonine kinases receptor type II

TGF-β1	transforming growth factor-β1
TIMP	tissue inhibitors of matrix metalloproteinase
Tm	melting temperature
TMA	tissue microarray
TUNEL	terminal deoxynucleotidyltransferase-mediated deoxyuridine triphosphate nick-end labeling
UV	ultraviolet
VIC	valvular interstitial cell

CHAPTER I

INTRODUCTION AND AIMS

Myxomatous mitral valve disease (MMVD) is the most common acquired cardiac disease of dogs characterized by thickening of the left atrioventricular valves or mitral valves, elongated and occasionally ruptured of the associated chordae tendineae. MMVD accounts for approximately 75% of dogs affected congestive heart failure (Kvart and Häggström, 2000). The etiology of canine MMVD remains unclear, but various factors such as hemodynamic, endogenous and genetics have been discussed (Aupperle et al., 2009). The pathogenesis mainly involves with atypical proliferative, metabolic and enzymatic activity of endothelial and valvular interstitial cells (VICs) resulting in an accumulation of acid mucopolysaccharides, fragmentation of collagen and elastic fibers, and transdifferentiation of stromal cells into myofibroblasts (Kogure, 1980; Black et al., 2005).

MMVD causes mitral valve regurgitation and leads to left-sided congestive heart failure (Braunwald, 1997). MMVD has breeds and age associations, such that certain breeds are overrepresented including Cavarliar King Charles Spaniel, Poodles, Shih-Tzu, Chihuahua, and Miniature pinscher. Middle- to old-aged dogs have varying degrees of disease (Kvart and Häggström, 2000). It is also comparatively interesting that it bears close similarity to mitral valve prolapse in human beings. The conditions, in both dogs and humans, are typified by the loss of mechanical integrity of the valve leaflets, failure of proper coaptation of the leaflet edges during ventricular systole, and mitral valve regurgitation. This loss of mechanical integrity is a consequence of the destruction of the fibrosa layer, expansion of the loose connective tissue in the spongiosa layer, excessive accumulation of glycosaminoglycans, exhibited features of

activated myofibroblasts and expressed elevated levels of proteolytic enzymes. These findngs suggest that dysregulation of matrix metabolism modulates the abnormalities in collagen and other extracellular matrix (ECM) components in this pathological condition (Rabkin et al., 2001). The definitive treatment for mitral degeneration in humans is surgical repair or replacement of damaged valves (Braunwald, 1997). However, secondary to the expense of cardiac bypass surgery, mitral valve surgery has been performed in just a few hospital case dogs (Griffiths et al., 2004; Orton et al., 2005). No drugs or treatments can slow the progression of myxomatous degeneration. Thus, the current treatment goal of dogs affected by MMVD is to reduce the effect of circulatory disturbance rather than to treat the actual lesions. It has been evident that the cell density was increased significantly in canine myxomatous mitral valves compared to normal valves. However, roles that cell proliferation, migration, or anti apoptosis plays in the increased cell density of myxomatous valves remain unclear. The cell proliferation is unlikely a major mechanism of increased cell density in canine myxomatous valves (Disatian et al., 2008). Rather that decreasing in cell death or anti-apoptosis mechanism may play an important role implicating persistence of VICs in diseased valves. Apoptosis is a gene regulated occurrence affected by several factors, mainly Bcl-2 family proteins including apoptotic (Bax) and anti-apoptotic (Bcl-2) proteins. It has been demonstrated that an increasing in transforming growth factor-β1 (TGF-β1) expression can regulate the transcription of the Bcl-2 gene family in many cell types (Sánchez-Capelo, 2005). Furthermore, there is a strong evidence of autocrine-paracrine TGF-β1 signaling in naturally-occurring MMVD in dogs (Disatian and Orton, 2009). Nevertheless, roles of TGF-β1 signaling and apoptosis in canine MMVD have been unexplored. Thereby, monitoring such factors may play a beneficial role in determining the pathogenesis and creating a new therapeutic intervention associated with apoptosis in canine MMVD in order to slow down disease progression.

In this regard, I propose to assess the expression of Bcl-2 family members, both messenger RNA (mRNA) and protein of Bcl-2 and Bax as well as the immunoreactivity of cleaved caspase-3 and phosphorylated Smad 2/3 (activated TGF-β1 signaling proteins, p-Smad 2/3), in order to investigate the *bax/bcl-2* ratio associated with TGF-β1 signaling protein in dogs affected by MMVD.

The objectives of the present study were therefore: firstly, to determine apoptotic cells in spontaneous canine myxomatous mitral valves; second, to determine the relationship among temporal expression of pro-apoptotic protein (Bax), anti-apoptotic protein (Bcl-2) and phosphorylated-Smad2/3 (activated TGF-β1 signaling protein).

CHAPTER II

LITERATURE REVIEW

Myxomatous mitral valve disease in dogs

Normally, the mitral or left atrioventricular valve of the heart lies between the left atrium and the left ventricle preventing backward flow to the left atrium during ventricular systole. The mitral valve has two leaflets, namely the mural (posterior) and the septal (anterior) leaflets. Normal leaflets are thin, translucent, and soft. Tendinous cords attach the leaflets to two closely arranged groups of papillary muscles (Pedersen and Häggström, 2000). Microscopically, normal mitral valves had 3 well-defined tissue layers, each containing cells, characteristic of extracellular matrix (ECM) composition and configuration: (1) the fibrosa layer, which is composed predominantly of collagen fibers arranged parallel to the free edge of the leaflet and densely packed; (2) the centrally located spongiosa layer, which is composed of loosely arranged collagen and glycosaminoglycans; and (3) the atrialis layer, which is composed of elastic fibers (Aupperle et al., 2009). In contrast, myxomatous mitral valves have significant thickening of leaflets (Rabkin et al., 2001). Histopathologically, myxomatous mitral valves were graded by modified Kogure (1980), mild lesions are characterized by proliferation of the endothelium and the underlying fibroelastic tissue of the atrialis layer, fragmentation of elastic fibers of the atrialis layer and nodular thickening mainly due to the deposition of glycosaminoglycans. In mild disease the collagen bundles of the fibrosa layer are still intact. Moderate lesions are characterized by a moderate increase in glycosaminoglycans within the distal half of the valvular spongiosa layer and mild fragmentation of collagen bundles in the fibrosa layer. The proximal half of the valve

remains histologically normal. Marked lesions are characterized by a marked increase in glycosaminoglycans, disruption of collagen bundles in the distal half of the valve, complete displacement of the fibrosa layer by glycosaminoglycans and fibroelastic proliferation in the atrialis and fibrosa layers (Aupperle et al., 2009). Generally, TGF-β1 is recognized as a mediator of ECM remodeling and glycosaminoglycans synthesis in a variety of cells. In canine myxomatous mitral valves, it has been discovered that there is an up-regulation of latent TGF-β1 and TGF-β1 receptor I and II proteins when compared to normal mitral valves. These implicate that there are an autocrine TGF-β1 mechanism in the pathogenesis of canine naturally-occurring MMVD (Disatian and Orton, 2009).

Valvular interstitial cells in mitral valves

Valvular interstitial cells (VICs) are the most predominant cells type in cardiac valves but to date they have not been extensively studied in the dogs (Durbin and Gotlieb, 2002). VICs are capable of synthesizing collagen, elastin, proteoglycans, fibronectin, growth factors, cytokines, chemokines as well as matrix metalloproteinases (MMPs) and tissue inhibitors of matrix metalloproteinases (TIMPs) (Taylor et al., 2003) that are responsible for mediating the ongoing repair and remodeling which provides valvular durability (Rabkin et al., 2001; Heaney et al., 2009). In general, VICs are regulated by both environmental matrix stimuli and soluble mediators such as TGF-β1, and may appear as one of three different subtypes including myofibroblasts, secretory active cells and smooth muscle cells (Taylor et al., 2003; Aupperle et al., 2009). *In vitro* studied of myxomatous degeneration, TGF-β1 can mediate cellular processes by transformation of cell phenotype including expression of alpha-smooth muscle actin (α-SMA) (Disatian and Orton, 2009). VICs in normal mitral valves do not express α-SMA. Multiple activated and α-SMA-positive cells in myxomatous mitral valves indicate

myofibroblast-like differentiation (Walker et al., 2004). A significant increased number of VICs have been reported in human myxomatous compared to normal mitral valves (Rabkin et al, 2001). Disatian et al. (2008) reported that cell proliferation is unlikely a major mechanism of increased VIC density in canine myxomatous mitral valves. Other mechanisms such as decreased cell apoptosis may mediate an increase of VICs in diseased valves. Additionally, apoptotic events have not been studied in canine MMVD. As previously mentioned, there is an up-regulation of TGF-β1 signaling protein in canine myxomatous mitral valves (Disatian and Orton, 2009), and also TGF-β1-dependent apoptosis have been discussed in many cell types (Schuster and Krieglstein, 2002). Therefore, studying of VIC apoptosis in association with TGF-β1 signaling proteins may provide clues to the pathogenesis of canine MMVD.

TGF-β1 in association with MMVD

TGF-β1 is a cytokine characterized by diverse and often contradictory functions. It plays an important role in physiological (embryonic development, cell growth and differentiation) and pathological processes (inflammation, fibrosis, angiogenesis and oncogenesis) (Lim and Zhu, 2006). Normally, TGF-β1 is expressed and secreted as an inactive complex, consisting of the latent associated peptide (LAP) and the mature TGF-β1 peptide. LAP–TGF-β1 is often linked to LTBP glycoproteins (latent TGF-β binding protein 1-4), resulting in the large latent form of TGF-β1. The LTBPs serve to enhance the stability of the cytokine, facilitate its secretion and help in targeting it to the ECM. Latent TGF-β1 seems to be stored in the ECM where it can be released and activated, both locally and rapidly (Sánchez-Capelo, 2005). Intracellular signaling of TGF-β occurs via two receptor serine/threonine kinases, type I (ALK 1–7) and type II receptors (TβRII, ActR-II, ActR-IIB, and BMPR-II) (Sánchez-Capelo, 2005). The active

form of TGF-β binds to type II receptor, which is followed by the recruitment of the type I receptors to form tetrameric complexes with the type II receptors. After receptor activation, the signal is propagated downstream through the recently identified Smad protein family result in phosphorylation of Smad 2 and Smad 3 (Lim and Zhu, 2006). Smad 2 and Smad 3 then form a complex with Smad 4 and promote their translocation to the nucleus where they bind to Smad-binding elements (SBE) lead to the transcription of target genes. In the other hand, Smad7 inhibits TGF-β1 signaling by two mechanisms: it can prevent the interaction between Smad2/3 and the ALK receptor; and it can interact with ubiquitin ligases of the Smurf family inducing the ubiquitination and proteasomal degradation of ALK (DaCostaByfield and Roberts, 2004).

Since, TGF-β1 is a molecule that is fundamental for the maintenance of the homeostasis between cell growth and apoptosis. Pro-apoptotic responses have been observed in cells from multipotent hematopoietic cells (Francis et al., 2000) and lens epithelium (Lee et al., 2002). On the other hand, TGF-β1 supports the survival of a variety cells in other systems including chondrocytes (Lires-Deán et al., 2008) and mammary epithelial cells (Shin et al., 2001). The complexity of these signaling systems can be observed in the vascular system where TGF-β1 induces apoptosis in vascular endothelial cells but may inhibit apoptosis in vascular smooth muscle cells. A balance between cell proliferation, survival and cell death is central to many physiological processes and its dysregulation may induce disease. Abnormal levels of TGF-β1 have been associated with numerous disorders in human, including fibrotic diseases of the kidney and lung (Blobe et al., 2000) and Marfan syndrome (Mizuguchi et al., 2004). In fibrotic disease, TGF-β1 induces ECM expression but inhibits TIMPs synthesis, resulting in down-regulation of ECM degradation in association with Smad-3 dependent mechanisms (Flanders, 2004).

The association between TGF-β1 and apoptosis

In the heart, TGF-β stimulates genes responsible for controlling fibrosis, angiogenesis, cell proliferation, differentiation, migration and apoptosis (Blobe et al., 2000; Walker et al., 2004) by modulating both ECM and TIMPs (Dabek et al., 2006; Aupperle et al., 2009) via Smad-3 dependent process (Flanders, 2004).Over-expression of TGF-β1 has been demonstrated in several human heart valve diseases including carcinoid heart disease (Jian et al., 2002), calcified aortic stenosis (Jian et al., 2003), rheumatic heart disease (Kim et al., 2008). Also, an up-regulation of TGF-β1 signaling proteins has been reported in naturally-occurring canine MMVD suggesting the association between TGF-β1 signaling and this disease (Disatian and Orton, 2009). Since, Smad proteins are key signal transducer in apoptosis associated with TGF-β signaling pathway. It has been recently discovered that Bcl-xL, apoptotic regulating gene, is target of Smads that are activated by TGF-β (Jablonski et al., 2005). In addition, TGF-β dependent apoptosis in canine MMVD pathogenesis due to increasing in VIC density in disease valves is still question.

Apoptosis mechanisms

Apoptosis is a programmed form of cell death, which plays an essential role in the development, regulation and maintenance of multicellular organisms under physiological and pathological events (Mbazima et al., 2008). Apoptotic cells are characterized by rounding-up of the cell, reduction of cellular volume (pyknosis), membrane blebbing, chromatin condensation, nuclear fragmentation (karyorrhexis), followed by formation of apoptotic bodies with little or no ultrastructural modifications of cytoplasmic organelles (Sánchez-Capelo, 2005; JafariAnarkooli et al., 2008). Two major

apoptotic pathways in mammalian cells are via the death receptor-mediated external signal pathway-extrinsic pathway, and mitochondrial-mediated internal signal pathway-intrinsic pathway. The most important specific molecular mediators of apoptosis are cysteine aspartic acid-specific proteases (caspases) which are pro-apoptotic proteins participating in endonucleolytic and proteolytic cascades (Del Puerto et al., 2010). The intrinsic caspase–dependent pathway relies on the release of cytochrome *c* from mitochondria to induce formation of the apoptosome comprised of cytochrome *c*, pro-caspase-9, apoptotic protease activating factor1 (APAF1) and deoxyribonucleic ATP (Blobe et al., 2000). This homodimerization allows pro-caspase-9 to become an initiator caspase which in turn cleaves and activates effector caspases including caspase-3, caspase-6 and caspase-7, thus driving cells to apoptosis. The extrinsic caspase-dependent pathway begins via cell-death receptors on the cell surface. After activated by ligand binding, receptor triggers intracellular events resulting in activation of initiator caspase-8 and caspase-10 promoting the cleavage and activation of effector caspases (Neptune et al., 2003). Thus, caspase-3 is the most important enzymes in both signaling pathway, which can amplify and speeding up the apoptotic process (Kumaraguruparan et al., 2006). Substrates of the effector caspases include poly-ADP-ribose polymerase (PARP), DNA-PK (DNA-dependent protein kinase) and other regulatory and structural proteins that maintain cellular and genomic integrity. Therefore, the cleavage of these substrates results in fragmentation of protein and DNA breakdown (Mbazima et al., 2008). Furthermore, extracellular signals like TGF-β1 can modulate both extrinsic and intrinsic pathways (Sánchez-Capelo, 2005).

The Bcl-2 family

In many cell types, apoptosis is a gene-regulated occurrence affected by various factors, but the key elements are categorized in two main families of proteins including caspase enzymes and Bcl-2 family (JafariAnarkooli et al., 2008). The Bcl-2 family is a set of cytoplasmic proteins that play a central role in the control of apoptosis by regulating mitochondrial membrane permeability (Mbazima et al., 2008). The two main groups of this family including proteins that exert a pro-apoptotic and anti-apoptotic responses in association with caspases such as Bax and Bcl-2, respectively (Sánchez-Capelo, 2005; Kumaraguruparan et al., 2006). Bax, a pro-apoptotic member of the Bcl-2 family which is normally secreted in an inactive form within the cytosol of mitochondria, and acts as a functional antagonism to Bcl-2 by sharing amino acid homology. After receipt apoptotic stimuli, activated Bax inserts into the endoplasmic reticulum and mitochondrial outer membrane for pore formation. Calcium ions then release from endoplasmic reticulum facilitating apoptotic-inducing factors (AIFs) and cytochrome *c* to release from mitochondrial intermembrane spaces into the cytosol, which results in caspase activation and apoptosis (Sánchez-Capelo, 2005; Del Puerto et al., 2010). Bcl-2 are members of anti-apoptotic proteins localized in the outer mitochondrial membrane, and mediates its anti-apoptotic effects by maintain the integrity of the mitochondrial membrane. The stabilization of mitochondrial membrane facilitates to inhibition of pore forming and subsequently cytochrome *c* releasing (Del Puerto et al., 2010). Furthermore, Bcl-2 can promote cell survival by binding to pro-apoptotic molecules resulting in neutralization of apoptotic activity and activation of caspases (Mbazima et al., 2008).

Indeed, the ratio of pro-/anti-apoptotic molecules like Bax/Bcl-2 appears to be the best variable in assessing the susceptibility of cell to survival or undergo apoptosis.

Dysregulation of apoptosis due to an imbalance in Bax/Bcl-2 ratio has been suggested to play a central role in the pathogenesis of many degenerative diseases. Overexpression of pro-apoptotic proteins can eventually lead to the releasing of cytochrome c and activation of caspases, resulting in cleavage of vital protein and cell death subsequently (Kumaraguruparan et al., 2006). On the other hand, overexpression of Bcl-2 prevents the activation of caspases in apoptosis suggesting that Bcl-2 prevents cell death by upstream activation of caspases (Mbazima et al., 2008). Additionally, Bax/Bcl-2 ratio can be altered by TGF-β1 either to promote or inhibit apoptosis in both physiological and pathological conditions (Sánchez-Capelo, 2005). In several cell types including lymphocytes, liver cells, prostate epithelial cells and hematoma cell lines, TGF-β can mediate apoptotic cell death by activating through caspase-family (caspases 2, 3, 7, and 8) which is accompanied by down regulation of Bcl-xL expression and a low Bcl-xL/Bax ratio. TGF-β also acts as a death-inducing agent by itself in apoptotic pathway by sending it signal via Smad proteins to the nucleus. It is still unclear that TGF-β-dependent apoptosis is regulated whether through decreasing of anti-apoptotic protein (Bcl-2 and Bcl-xL) levels or caspase activation (Schuster and Krieglstein, 2002). TGF-β1 inhibits apoptosis in some cell types. It has been reported that TGF-β1 can protect cultured neurons from apoptosis by increasing Bcl-2 expression (Prehn et al., 1994). TGF-β-dependent apoptosis in association with apoptotic regulatory genes and proteins have not been studied in canine MMVD. Therefore, study of this association can help to explain the pathogenesis of canine MMVD particularly about an increase in cell density of VICs which may be responsible from anti-apoptotic genes and TGF-β1.

CHAPTER III

MATERIALS AND METHODS

Tissue collection and preparations

Septal (anterior) mitral valve leaflets were obtained from necropsy cases at the Department of Veterinary Pathology, Faculty of Veterinary Science, Chulalongkorn University. Signalment and clinical history profiles had been collected. MMVD valves were enlarged and thickening with rolling up edges (Kvart and Häggström, 2000). Normal mitral valve leaflets characterized by thin and translucent in appearances (Kvart and Häggström, 2000) were served as control for this study. Valve thickness was measured by a vernier caliper (10 mm, 0.1 mm division) (Fisher Scientific, NY, USA) in the middle portion of each leaflet and expressed as a mean of 3 measurements (Rabkin et al., 2001). Heart valve tissues were categorized based on gross appearance and valve leaflet thickness as normal <1 millimeter (mm) (n=15), early stage disease 1-2 mm (n=20) and late stage disease >2 mm (n=20). Each valve leaflet was divided into 2 parts: one was preserved in 10% buffered formalin for histopathological examination, and another one was immediately collected and snapped frozen in liquid nitrogen before stored at -80°C for extraction of mRNA. Inclusion criteria were included age of dog affected MMVD, which was more than 1 year old, and valve thickness that was more than 1 mm. An exclusion criterion was endocarditis which represented as infiltration of inflammatory cells in valve tissue.

Construction of mitral valve-tissue microarray (modified from Rosen et al., 2004)

Tissue microarrays (TMA) are a method of collecting small pieces of tissues in donor paraffin blocks and placing them in an array on a single new recipient paraffin block so that multiple of tissue samples can be applied with various techniques including immunohistochemistry and fluorescence *in situ* hybridization (Rosen et al., 2004). In the present study, tissue microarray blocks were manually constructed with specimens by using "TMA Eazy", a modified module of Dr. Banchob Sripa at Pathology Research Unit, Department of Pathology, Faculty of Medicine, Khonkaen University. First, tissue samples were taken from the central area of the septal mitral valve leaflet including the free margin to the base and a portion of myocardium. Thereafter, mitral valve tissue samples were preserved in 10% buffered formalin for 24 hours and embedded in paraffin blocks. For TMA construction, paraffin-embedded tissue blocks from 55 consecutive cases of normal (n=15), early stage (n=20) and late stage (n=20) canine MMVD were selected. Four-micrometer-thickness serial sections were stained with routine Hematoxylin and Eosin (H&E) staining and special staining with Periodic Acid-Schiff reaction (PAS)-alcian blue for morphologic study and staging MMVD by using a modified classification system of Kogure (1980). Three representative areas of normal and MMVD valve tissues were identified for constructing the TMA blocks. All of tissue slides were reviewed by two individual pathologists at 200x magnification under the light microscope. A TMA block was constructed by taking three cylindrical core samples from the identified areas (which consisting of all three layers of mitral valves) of paraffin-embedded mitral valves and assembled those cores into a recipient paraffin block (modified from Rosen et al., 2004). For each case, three 1.5 -mm in diameters and 4-mm in depth cores, were collected and placed in the same recipient block. Each TMA block was contained 123 spots with samples spaced 1 mm apart (Figure 1-1).

Four-micrometer-thickness serial sections from the TMA blocks were stained with routine H&E staining and special staining with PAS-alcian blue in order to confirm the presence of histopathology of all three layers of MMVD. For both H&E and special stainings, TMA slides were examined individually twice by two pathologists. Each core was examined at 200x magnification under light microscope for confirmation of morphology in each study group.
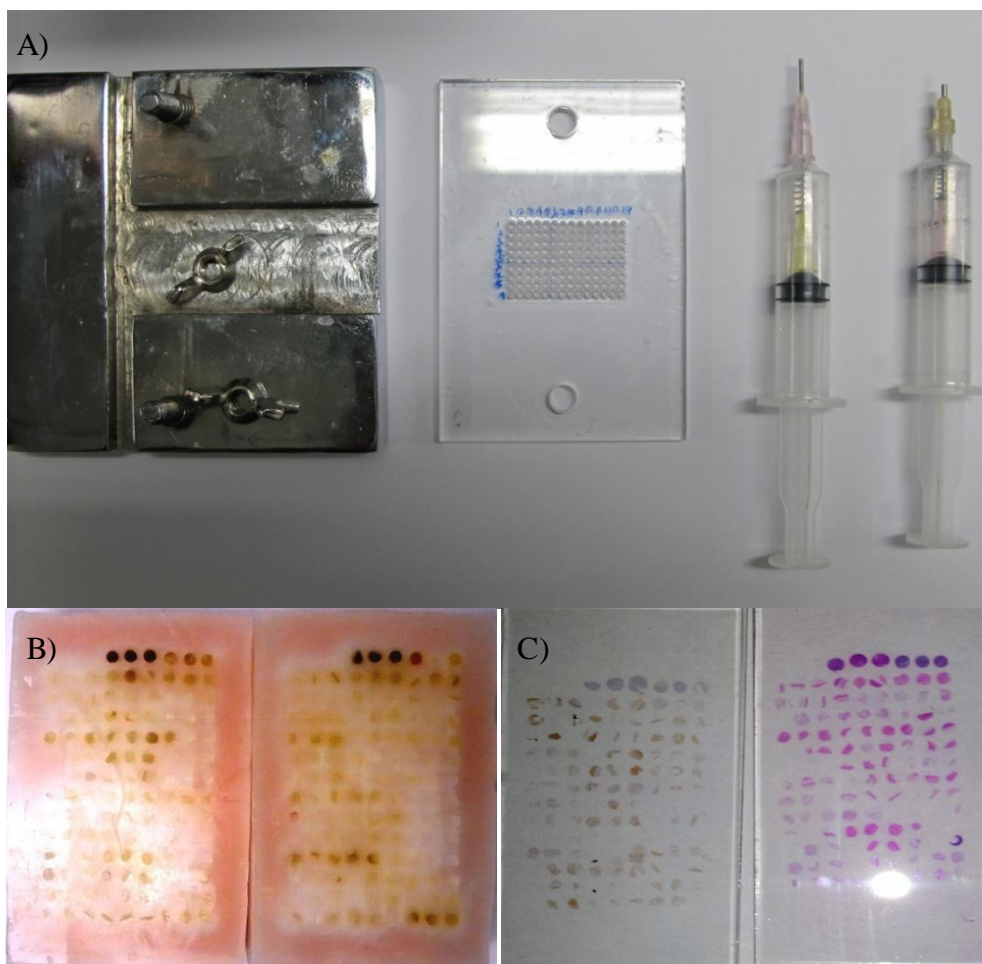
Figure 1-1: A) Tissue microarray instruments. B) Tissue microarray blocks. C) Tissue microarray slides.

Terminal deoxynucleotidyltransferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) staining

TUNEL reaction was performed as apoptotic marker detection by using ApopTag® Peroxidase in Situ Apoptosis Detection Kit (S7100, Chemicon, CA, USA) according to manufacturer's instructions. In brief, four-micrometer-thickness serial sections from the TMA blocks were deparaffinized in xylene, rehydrated in graded alcohol solutions and antigen unmasked with 0.01%proteinase K in phosphate buffered saline (PBS) for 15 minutes (min) at room temperature. Sections were incubated sequentially with 3% hydrogen peroxide in absolute methanol for 5 min at room temperature and Equilibration Buffer for 5 min at room temperature. Then, Working Strength TdT Enzyme was applied with plastic cover slip in a humidified chamber for 60 min at 37°C. The reaction was terminated by incubated slides in Stop/Wash Buffer for 10 min at room temperature. In addition, slides were rinsed with PBS after each incubation step. Labeled damaged DNA was detected with anti-digoxigenin conjugated solution for 30 min at room temperature. Peroxidase activity was visualized with 3,3' diaminobenzidine tetrahydrochloride in hydrogen peroxidase solution (0.05% 3,3' diaminobenzidine and 0.03% hydrogen peroxide in 0.1M Tris HCl buffer; DAB, Dako, Denmark). Finally, slides were counterstained with Mayer's Hematoxylin for 2 min and mounted with permount solution. TMA slides were examined twice by one pathologist. Five randomly selected areas in each core were examined at 400x magnification (high power field; HPF) under light microscope and captured photographs by a digital camera (Nikon, Japan). TUNEL results were presented as percentage of positive-stained cells per total cell counts. Positive control was TUNEL labeling against canine lymphoma tissue.

As TUNEL sometimes labeled nonspecific DNA degradation in necrotic cells and transient DNA strand breaks in mitotic cells, as well as DNA fragmentation in apoptotic cells, care was taken to exclude nuclei that had cellular appearance of necrosis or mitosis.

Protein expressions of cleaved caspase–3, activated TGF-β1 signaling protein (phosphorylated Smad2/3, p-Smad2/3), and Bcl-2 family protein (Bax and Bcl–2) by using immunohistochemistry method (modified from Zhang et al., 2006)

After deparaffinization with xylene and rehydration with graded ethanol, TMA slides were treated with 1M citrate buffer pH 6.0 for 20 min at 95°C by water bath to unmask antigen epitopes. Then, four-micrometer-thickness serial sections from the TMA blocks were preincubated with 3% hydrogen peroxide in methanol for 5 min to block endogenous peroxidase activity. The slides were incubated with 5% skim milk to block non-specific background staining. Primary antibodies, polyclonal rabbit antibody against cleaved caspase-3 (C8487, Sigma-Aldrich, St. Louis, MO, USA) in a dilution of 1:800, monoclonal mouse antibody against Bcl-2 (clone124, Dako, Carpenteria, CA, USA) in a dilution of 1:100, and monoclonal mouse antibody against Bax (clone1F5-1B7, Sigma-Aldrich, St. Louis, MO, USA) in a dilution of 1:200, and polyclonal rabbit antibody against p-Smad2/3 (Ser 423/425: sc-11769-R, Santa Cruz Biotechnology, CA, USA) in a dilution of 1:100 were incubated in humidified chamber for 60 min at 37°C. Slides were applied with anti-rabbit/mouse immunoglobulin-HRP conjugate (Envision™, Dako, Carpenteria, CA, USA) as secondary antibody for 45 min at room temperature. Slides were washed three times with PBS for 5 min between each incubation step. Peroxidase activity was visualized with freshly prepared 3,3' diaminobenzidine tetrahydrochloride in hydrogen peroxidase solution. Slides were counterstained with Mayer's Hematoxylin for

2 min and mounted with permount solution. Negative controls were created by omitting primary antibody to the specimens. Positive controls included p-Smad2/3 immunolabeling against canine kidney tissue; cleaved caspase-3, Bcl-2 and Bax immunolabeling against canine lymphoma. TMA slides were examined twice by one pathologist. Five randomly selected areas in each core were examined at HPF under light microscope and captured photographs by a digital camera (Nikon, Japan). Positive intracytoplasmic and intranuclear stainings against cleaved caspase-3 and p-Smad2/3 antibodies were counted and analyzed. For Bax and Bcl-2 antibodies, positive intranuclear staining was counted. All immunolabeling results were presented as percentage of positive-stained cells per total cell counts.

RNA extraction

Total RNA was extracted from randomly selected fresh 4 mitral valves in each group by using Aurum Total RNA Fatty and Fibrous Kit, according to the manufacturer's instructions (Bio-Rad, Hercules, CA, USA). Briefly, mitral valve tissue sample (up to 100 mg) was transferred into 1 ml of lysis solution (PureZOL), immediately disrupted by a mortar and pestle and homogenate by a homogenizer. Then, the lysate was incubated for 5 min at room temperature to allow complete dissociation of protein complexes. After the incubation, 0.2 ml of chloroform was added to the lysate and incubated for 5 min at room temperature. Then, the mixture was centrifuged at 12,000x g (Andreas Hettich universal 32R, Tuttlingen, Germany) for 15 min at 4°C to separate organic and aqueous phases. The supernatant (aqueous phase) containing RNA was immediately transferred to 2.0 ml microcentrifuge tube and equal volume of 70% ethanol was added. The sample was then passed through a silica membrane packed in the RNA binding mini column to allow the binding of nucleic acid and centrifuged at 12,000x g for 1 min to

discard the supernatant. 700 μl of low-stringency wash solution was added into RNA binding mini column and centrifuged at 12,000x g for 1 min to remove residual lysis solution. In order to remove contaminated DNA, 80 μl of DNase I (mixture of 5 μl of reconstituted DNase I with 75 μl of DNase dilution solution) was added to the membrane stack of each column and incubated for 15 min at room temperature. Then 700 μl of high stringency wash solution was added and followed by 700 μl of low stringency wash solution. Each step required centrifugation at 12,000x g for 1 min and discarded the filtrate from the wash tube. Finally, the total RNA was eluted with 70°C elution solution. Total RNA concentration and the purity were verified by determining optical density (OD) absorption ratio OD260/OD280 with spectrophotometer (Model U-2000, Hitachi Instrument Inc, Tokyo, Japan). The synthesized RNA was stored at -20°C until next step. The calculation of RNA was calculated by using the equation (Birren et al., 1997) as shown below.

$$\text{Total RNA concentration (μg/μl)} = [40 \times OD_{260} \times \text{dilution factor}]$$

OD_{260}: UV absorbance of total RNA was measured at a wavelength of 260 nm.

OD_{280}: UV absorbance of total protein was measured at a wavelength of 280 nm.

Reverse transcription

Full length complementary DNA (cDNA) of *bcl-2* and *bax* gene was produced and amplified by using iScript™ Reverse Transcription Supermix (Bio-Rad, CA, USA), according to the manufacturer's instructions. A 20 µl of reverse transcription reaction mixture containing 1 µg of total RNA, 10 mM random-primer, 4 µl of 5x iScript selected reaction mix (mixture composed of dNTPs, magnesium chloride, stabilizers and 1 µl of iScript reverse transcriptase enzyme) and nuclease-free water was incubated at 25°C for 5 min followed by 42°C for 30 min, and 85°C for 5 min (1 cycle) in a thermal cycler (Applied Biosystems, Foster City, CA, USA).Total cDNA concentration and the purity were verified by determining optical density (OD) absorption ratio OD260/OD280 with spectrophotometer (Model U-2000, Hitachi Instrument Inc, Tokyo, Japan). The synthesized cDNA was stored at -20°C until next step. The calculation of cDNA was calculated by using the equation (Birren et al., 1997) as shown below.

$$\text{Total cDNA concentration (µg/µl)} = [50 \times OD_{260} \times \text{dilution factor}]$$

OD_{260}: UV absorbance of total cDNA was measured at a wavelength of 260 nm.

OD_{280}: UV absorbance of total protein was measured at a wavelength of 280 nm.

Quantitative real-time RT-PCR analysis of apoptosis-regulatory genes

A quantitative real-time reverse transcriptase polymerase chain reaction (qRT-PCR) technique was carried out to determine the levels of *bcl-2* and *bax* mRNA expressions (modified from Del Puerto et al., 2010). For qRT-PCR, the final volume of 20 µl mixture containing 1 µg of cDNA, 10 µL of 2x qPCR/RTD-PCR Master mix E4 with UNG, nuclease-free water and 10 µmol of each complementary primer specified for *bcl-2* and *bax* sequences as well as for *18s rRNA* sequence as an internal control (housekeeping gene) to normalize target gene expression (Table1). Primer sequences for *bcl-2*, *bax*, *18s rRNA* were obtained from previously published sequences (Sano et al., 2005), and were based on the Genbank sequences, respectively. SYBR Green quencher dye was used for detection of product copy number. The real-time PCR conditions were initial denaturation at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 30 seconds, annealing and extension at 62°C for 1 min in a thermal cycler (ABI 7300 Real time PCR system, Applied Biosystems, Foster City, CA, USA). Control and target genes were amplified in duplicate under PCR conditions. Real-time quantitative RT-PCR system was performed to analyze the expression levels of *bax* and *bcl-2* relative to the housekeeping genes (*18s rRNA*). The specificity of PCR products was confirmed by the single peak dissociation curves (Schmittgen and Livak, 2008) and by 2% ethidium-bromide agarose gel (Bio-Rad, CA, USA) electrophoresis and visualized under the UV transluminator (Pharmacia Biotech, Uppsala, Sweden). The PCR amplification products had the predicted size approximately 290 bp, 206 bp and 399 bp for *bax*, *bcl-2* and *18S rRNA*, respectively. Fluorescent signals were detected at the end of extension stage in each cycle. The threshold of fluorescence detection was set at the number of the threshold cycles (Ct) corresponding to the inflection point of the fluorescence curve from the baseline to the exponential phase.

Relative quantitative measurement was measured as the fold changes in mRNA expression between groups by using the following formula (Schmittgen and L ivak, 2008):

$$\text{Fold change} = 2^{-\Delta\Delta_{Ct}}$$

$$\Delta Ct = Ct^{\text{target gene}} - \text{avg.}Ct^{\text{endogenous control gene}}$$

$$\Delta\Delta Ct = [\Delta Ct]_{\text{target group}} - \text{avg.}[\Delta Ct]_{\text{control group}}$$

Table1-1 The primers used for amplification and sequence-specific detection of target cDNA in the real-time quantitative RT-PCR.

Genes	Primer Sequence	Orientation	Position	Accession no.
bax	5'-CAT GGA GTT GCA GAG GAT GA-3'	Sense	273-293	AB080230
	5'-ACG TGG GTG TCC CAA AGT AG-3'	Antisense	543-563	
bcl-2	5'-TGG ATG ACT GAG TAG CTG AA-3'	Sense	270-289	AB116145
	5'-GGC CTA CTG ACT TCA CTT AT-3'	Antisense	457-476	
18S rRNA	5'-CCG CGG TTC TAT TTT GTT GGT TTT-3'	Sense	118-141	AF102857
	5'-CGG GCC GGG TGA GGT TTC-3'	Antisense	499-516	

Statistical analysis

Descriptive analysis was applied for histological changes in H&E and special stainings. Mean±SD was applied for expression of apoptotic marker and immunohistochemical analysis in each study group. Differences in immunohistochemical expression of anti-cleaved caspase 3, p-Smad2/3, Bcl-2, Bax and apoptotic cells between three study groups were determined by One-way ANOVA followed by Bonferroni's Multiple Comparison Test. The Ryan-Joiner test was used to test the normality of data. The correlation among valve thickness and the expression of apoptotic marker and immunolabeling against cleaved caspase-3, p-Smad2/3, Bcl-2 and Bax was evaluated by Pearson's correlation. The correlation among age of normal dogs and the expression of apoptotic marker and immunolabeling against cleaved caspase-3, p-Smad2/3, Bcl-2 and Bax was evaluated by Pearson's correlation. The *bax*/*bcl-2* ratio was calculated. Mean±SD was applied for expression of mRNA analysis in each study group. One-way ANOVA followed by Bonferroni's Multiple Comparison Test was stratified for comparing of mRNA expression between groups. The correlation between valve thickness and mRNA expression of *bax* and *bcl-2* was evaluated by Pearson's correlation. The correlation between age of normal dogs and mRNA expression of *bax* and *bcl-2* was evaluated by Pearson's correlation. $p < 0.05$ was considered statistically significant for all tests.

CHAPTER IV

RESULTS

The mitral valves of 55 dogs were examined during routine necropsy. Breeds included the Miniature pinscher (n=2), Shih-Tzu (n=3), Poodle (n=3), Bulldog (n=2), Thai (n=1), Rottweiler (n=1), Siberian Husky (n=2), Pomeranian (n=1), Labrador Retriever (n=5), Dalmatian (n=1), Dachshund (n=1), Cocker spaniel (n=3), Pekingese (n=1), Shar-Pei (n=2), Bull terrier (n=2), Boxer (n=1), Golden Retriever (n=3), Pug (n=2), Australian heeler (n=1), Bangkaew (n=1), Chihuahua (n=1), German Shepherd (n=2) and mixed breed dogs (n=14) (Table 2-1). Age of dogs affected MMVD was varied between 2-13 years old. Male affected more than female for approximately 1.5:1. Distribution and severity of valvular disease in dogs of this study are shown in Table 3-1. The cause of death was associated with mitral valve disease for 2 dogs, but other dogs were died with other diseases unrelated to cardiovascular diseases.

Table 2-1 Breed and number of samples.

Breed	Samples(n)						
	Normal		Early staged MMVD		Late staged MMVD		Total
	Male	Female	Male	Female	Male	Female	
Mixed	3	3	4		2	2	14
Duchshund		1					1
Miniature pinscher	1	1					2
Shih-Tzu		1	1	1			3
Bulldog	1		1				2
Thai				1			1
Rottweiler			1				1
Poodle			1		2		3
Siberian Husky			2				2
Pomeranian			1				1
Labrador retriever	1	1		2	1		5
Shar-Pei		1			1		2
Cocker spaniel						3	3
Dalmatian						1	1
Bull terrier			1		1		2
Pekingese					1		1
Boxer					1		1
Golden retriever					2	1	3
Australian heeler	1						1
Bangkaew			1				1
Pug				2			2
Chihuahua				1			1
German Shepherd					1	1	2
Total	7	8	13	7	12	8	55

Table 3-1 Stages, ages and valve thickness of samples.

	Valve thickness (mm)	Average thickness (mm)	Age (yrs)	Average age (yrs)
Normal Mitral Valve (n=15)	0.64-1.00	0.81±0.13^a	1-8	3.56±2.69$^{*,\#}$
Early Staged MMVD (n=20)	1.20-2.00	1.61±0.23^b	2-14	8.71±3.15*
Late Staged MMVD (n=20)	2.10-3.90	2.64±0.57^c	6-13	10.34±1.87$^{\#}$

Values are presented as mean±SD

[a, b, c] superscripts indicate statistically significant difference in column; [*, #] superscripts indicate statistically significant difference between groups. Significant difference was assessed by One-way ANOVA followed by Bonferroni's Multiple Comparison Test at $p<0.05$.

In the present study, valve thickness of normal mitral valves was statistically significant difference from early and late staged MMVD valves ($p<0.0001$) (Table 3-1). Thickness of valves affected with early staged MMVD also had statistically significant difference from those affected with late staged MMVD ($p<0.0001$) (Table 3-1). Moreover, age of normal dogs was statistically significant difference from dogs with early and late staged MMVD ($p<0.0001$) (Table 3-1). Age of dogs with early staged MMVD was not significant difference from that of late staged MMVD dogs ($p=0.0824$) (Table 3-1).

Macroscopic and microscopic findings

The septal leaflets of mitral valves were collected and investigated macroscopically and microscopically for gross appearance, routine histopathology examination, and the immunohistochemical study for expression of cleaved caspase–3, activated TGF-β1 signaling protein (phosphorylated Smad2/3, p-Smad2/3), and Bcl-2 family proteins (Bax and Bcl–2). Macroscopically, normal leaflets were thin, translucent, soft, and had thickness less than 1 mm. Microscopic examination of H&E and special stainings (PAS-alcian blue) showed 3 well-defined tissue layers of normal mitral valves each containing VICs and ECM: (1) the atrialis layer, which was composed of elastic fibers; (2) the centrally located spongiosa layer, which was composed of loosely arranged collagen and mild accumulation of mucopolysaccharides; and (3) the fibrosa layer, which was composed predominantly of collagen fibers arranged parallel to the free edge of the leaflet and densely packed. In contrast, the early staged MMVD valves became thickened (1–2 mm) with rolling up edge. In late staged MMVD, the entire cusp of septal mitral valve was progressively thickened (up to 2 mm) and deformed. Moreover, elongation of chordae was generally seen in both stages of MMVD. Late staged MMVD seldom accompanied with rupture of chordae. Histopathologically, myxomatous mitral valves were graded by modified Kogure (1980), early stage MMVD was characterized by the underlying fibroelastic tissues of the atrialis layer, moderate increase in VICs proliferation and accumulation of mucopolysaccharides within the distal half of the spongiosa layer and mild fragmentation of elastic fibers and collagen bundles in the atrialis and fibrosa layers, respectively. The proximal half of the valve remained histologically normal. Late stage MMVD was characterized by fibroelastic proliferation in the atrialis layer, marked increase mucopolysaccharide accumulation and VICs proliferation in the spongiosa layer, complete disruption and fragmentation of collagen

bundles in both distal and proximal halves of the valves, and fibroelastic proliferation in the fibrosa layer (Figure 2-1). Macrophages were sparsely observed in both normal and myxomatous mitral valves. In addition, there was focally calcification in the spongiosa and fibrosa layer in a valve affected late staged MMVD.
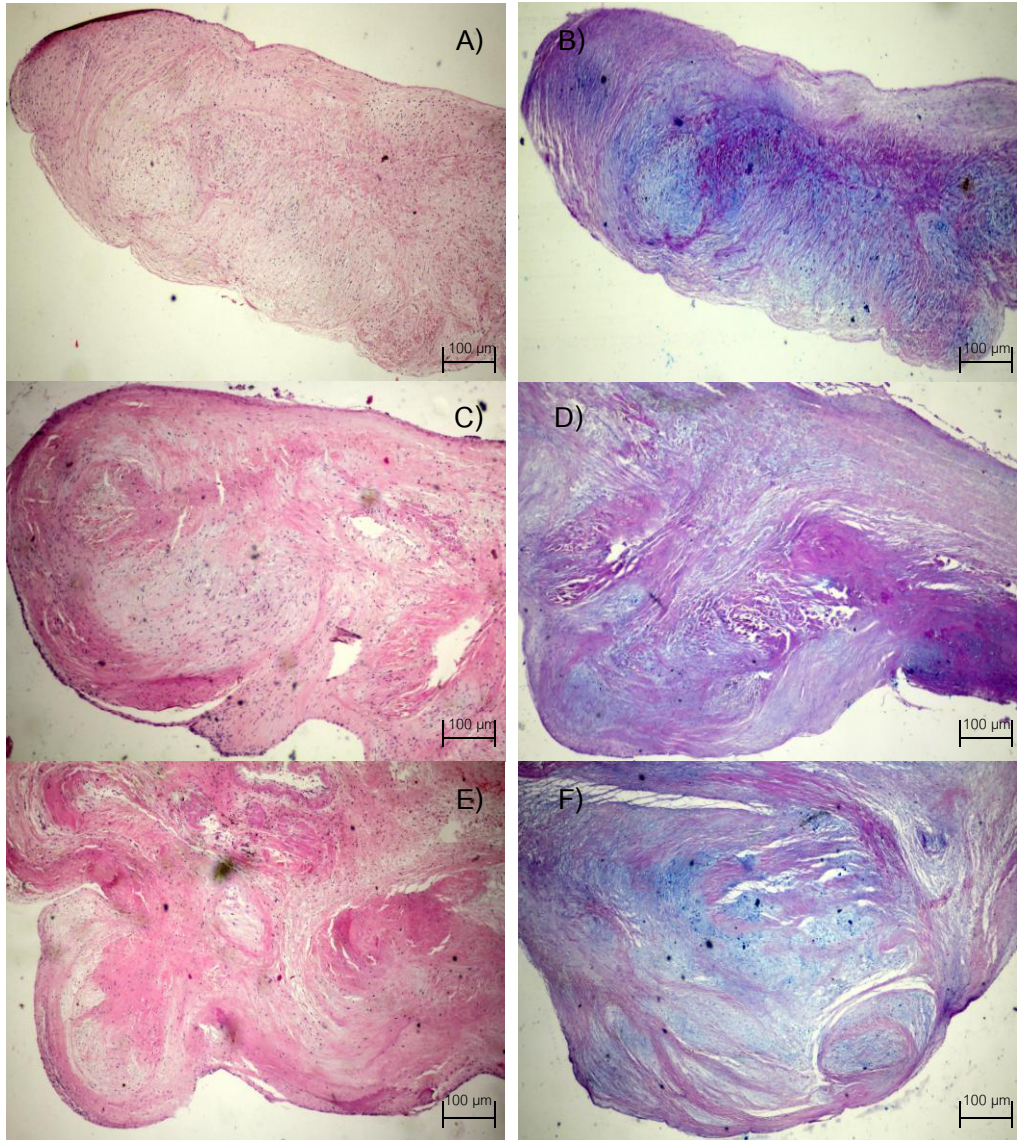
Figure2-1: Histopathological pictures of mitral valve tissues were stained with H&E (A, C, E) and PAS-alcian blue staining (B, D, F)   (40x magnification).

A)   and   B)   Normal   mitral   valve   shows   mild   accumulation   of mucopolysaccharides (blue). The collagen bundles (magenta) arrange parallel and intact.

C)   and   D)   Early   staged   MMVD   valve   tissue   shows   accumulation   of mucopolysaccharides (blue) mainly in the distal part of spongiosa layer and mild disruption of collagen bundles (magenta).

E) and F) Late staged MMVD valve tissue shows thickening of the spongiosa layer marked accumulation of mucopolysaccharides (blue) in the spongiosa layer with fragmentation and disruption of collagen bundles (magenta).
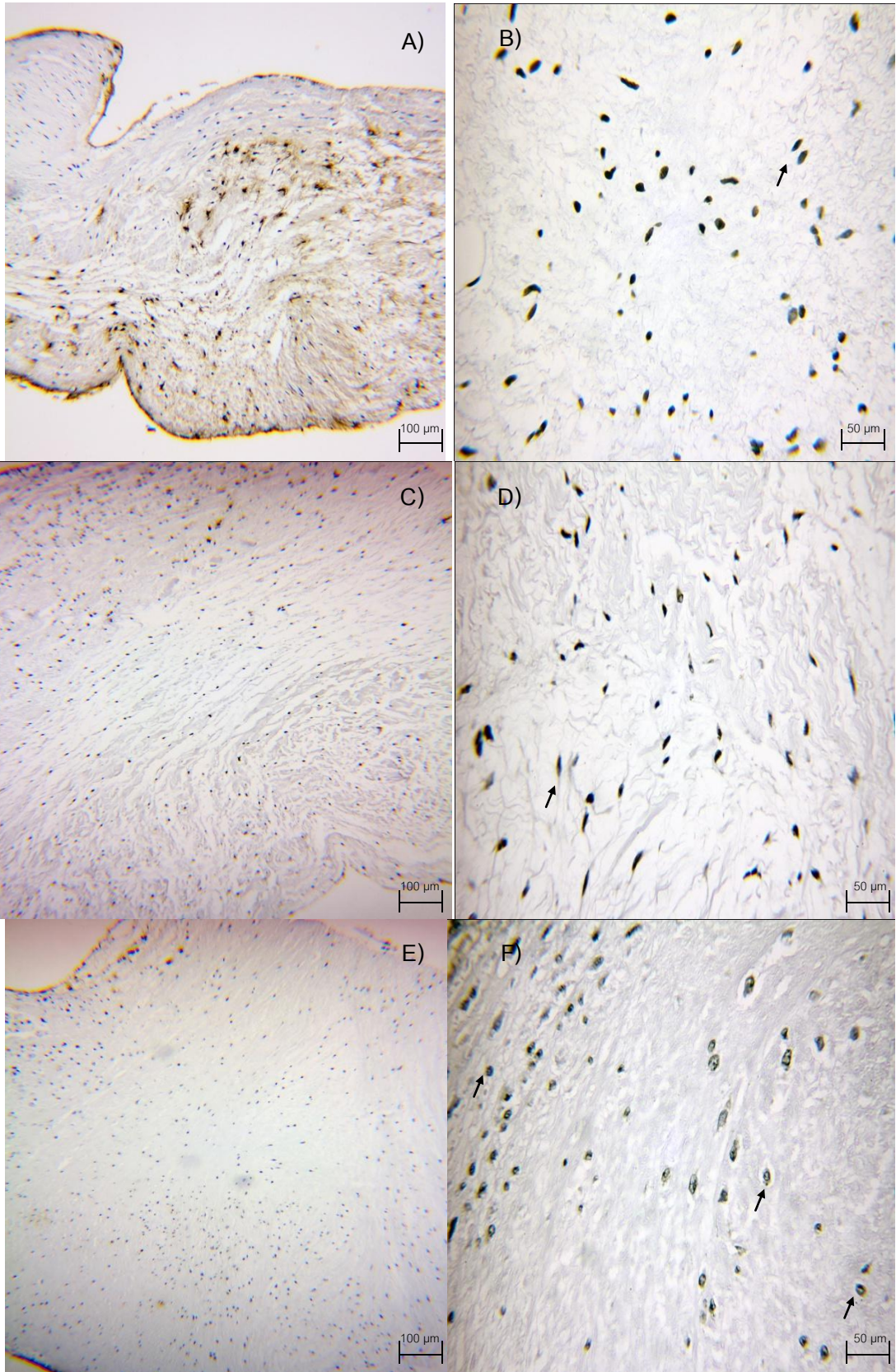
Figure3-1: Histopathological of mitral valve tissues with Apoptotic marker at 200x (A, C, E) and HPF (B, D, F) under light microscope.

A) and B) Normal mitral valve tissue shows positive intranuclear labeling in spindle-shaped VICs (arrow) without apoptotic bodies.

C) and D) Early staged MMVD valve tissue shows positive intranuclear labeling in spindle-shaped VICs (arrow) without apoptotic bodies.

E) and F) Late staged MMVD valve tissue shows positive intranuclear labeling in rounded VICs. These cells had space between nuclear membrane and nucleus (arrows) without apoptotic bodies.
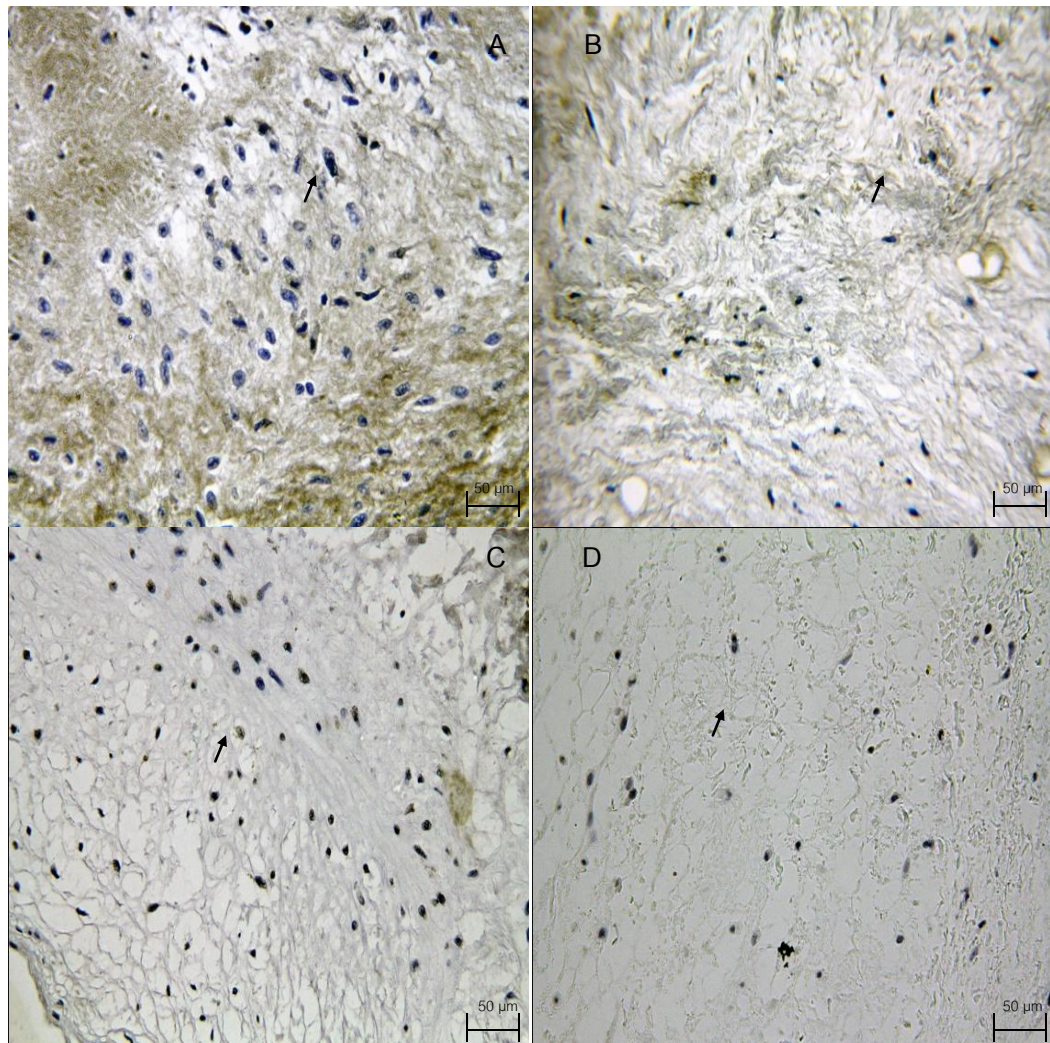
Figure4-1: Immunohistochemistry staining of valve tissues against cleaved caspase-3, p-Smad2/3, Bax and Bcl-2 at HPF under light microscope.

A) Late staged MMVD valve shows positive intranuclear for p-Smad2/3 staining in VICs (arrow) and ECM.

B) Late staged MMVD valve shows intranuclear staining for cleaved caspase-3 in VICs (arrow) and ECM.

C) Late staged MMVD valve shows intranuclear staining for Bax in VICs (arrow).

D) Late staged MMVD valve shows intranuclear staining for Bcl-2 in VICs (arrow).

Terminal deoxynucleotidyltransferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) assay

Apoptotic markers were performed to detect DNA damage in association with apoptotic bodies in normal and myxomatous mitral valves by using TUNEL assay. The result showed high expression of TUNEL staining in canine VICs in normal mitral valves. The positive intranuclear staining was found in spindle-shape cells without appearance of apoptotic bodies. In contrast to normal valves, the TUNEL staining was found positive in the nuclei of rounded VICs in myxomatous mitral valves. These cells had space between nuclear membrane and nucleus without apoptotic bodies (Figure 3-1). An expression of TUNEL of early and late staged MMVD valves was lower than that of normal mitral valves (Figure 5-1). TUNEL expression was statistically significant difference between normal mitral valves and early staged MMVD valves ($p<0.0001$) (Table 4-1). Also, TUNEL expression of normal mitral valves was statistically significant difference from late staged MMVD valves ($p=0.0004$) (Table 4-1). Moreover, TUNEL index of early staged MMVD valves was also statistically significant difference from that of late staged MMVD valves ($p=0.0002$) (Table 4-1).

Therefore, the present study showed down-regulation of TUNEL in early and late staged myxomatous mitral valves when compared to normal mitral valves. There was no correlation between TUNEL index and age of normal dogs *r=0.1066*, *p=0.7169*; whereas, the negative correlation between TUNEL index and valve thickness was revealed *r=-0.3889*, *p=0.0044* (Figure 6-1B).

Protein expressions of cleaved caspase−3, activated TGF-β1 signaling protein (phosphorylated Smad2/3, p-Smad2/3), and Bcl-2 family protein (Bax and Bcl−2).

Immunohistochemistry was performed to localize cleaved caspase−3, activated TGF-β1 signaling protein (phosphorylated Smad2/3, p-Smad2/3), and Bcl-2 family proteins (Bax and Bcl-2) within the mitral valve tissues by using antibodies against these proteins. The results showed expression of intranuclear and intracytoplasmic stainings against cleaved caspase-3, p-Smad2/3 in canine VICs (Figure 4-1). The positive staining was dispersed through the extracellular matrix (ECM) in normal, early staged and late staged MMVD valves (Figure 4-1). The intranuclear positive staining of Bcl-2 and Bax in VICs was revealed in both normal and MMVD valves. The expression of p-Smad2/3 and Bcl-2 in early and late staged MMVD valves was lower than that in normal mitral valves (Figure 5-1). In contrast, an expression of Bax in early and late staged MMVD valves was higher than that in normal mitral valves (Figure 5-1). Immunolabeling of p-Smad2/3 in normal mitral valves was statistically significant difference from that of early staged MMVD ($p<0.0001$) and late staged MMVD valves ($p<0.0001$) (Table 4-1); whereas no statistically significant difference was found between early and late staged MMVD valves ($p=0.8805$) (Table 4-1 and Figure 5-1). Immunolabeling of Bax in normal mitral valves was statistically significant difference from that of early staged MMVD ($p<0.0001$) and late staged MMVD valves ($p<0.0001$) (Table 4-1); whereas no statistically significant difference was found between early staged and late staged MMVD valves ($p=0.3476$) (Table 4-1). Immunolabeling of Bcl-2 in normal mitral valves was statistically significant difference from that of early staged MMVD ($p<0.0001$) and late staged MMVD valves ($p<0.0001$) (Table 4-1); whereas no statistically significant difference was found between early and late staged MMVD valves ($p=0.1053$) (Table 4-1). In addition, there was statistically significant difference of the average percentage positive cells to

cleaved caspase-3 between normal mitral valves and valve affected with early staged MMVD (p=0.0292) (Table 4-1). In contrast, there was no statistically significant difference between late staged MMVD and normal mitral valves (p=0.2845) (Table 4-1) as well as early staged MMVD and late staged MMVD valves (p=0.2003) (Table 4-1).
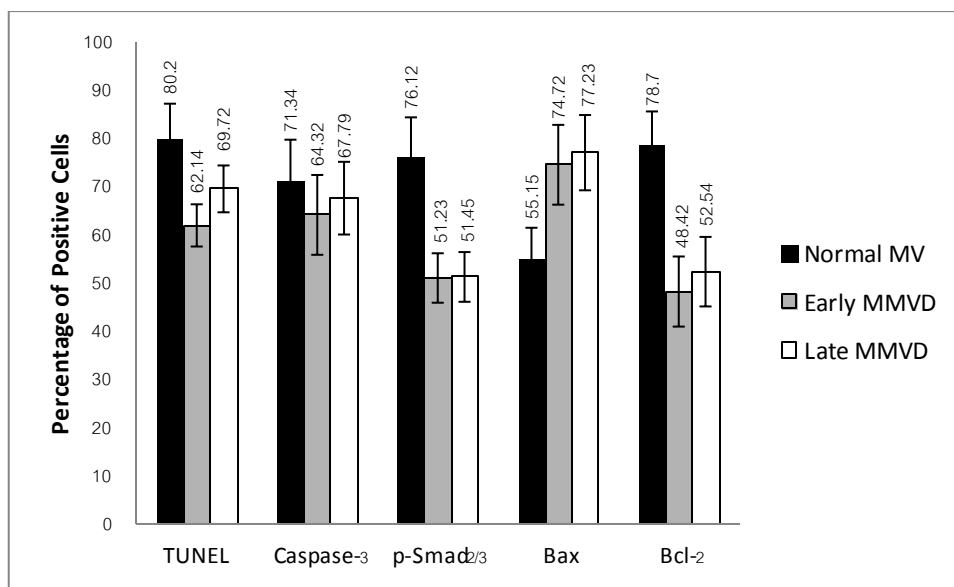

Figure 5-1: Histograms illustrate means±SD. of percentage of positive cells of normal mitral valves (n=15), early staged MMVD (n=20) and late staged MMVD (n=20).

Table 4-1: Percentage of immunolabeling of TUNEL, cleaved caspase-3, p-Smad2/3, Bax and Bcl-2 in normal, early and late staged MMVD valves.

	TUNEL	Cleaved caspase-3	p-Smad2/3	Bax	Bcl-2
Normal Mitral Valve (n=15)	80.20±7.19^a	71.34±8.58*	76.12±8.44^a	55.15±6.50^a	78.7±7.08^a
Early Staged MMVD (n=20)	62.14±4.38^b	64.32±8.28*	51.23±5.13	74.72±8.28	48.42±7.27
Late Staged MMVD (n=20)	69.72±4.86^c	67.79±7.54	51.45±5.16	77.23±7.81	52.54±7.21

Values are percentage of positive cells per total cells at HPF, and presented as mean±SD

[a, b, c] superscripts indicate statistically significant difference in column; [*] superscript indicate statistically difference between groups. Significant difference was assessed with One-way ANOVA followed by Bonferroni's Multiple Comparison Test at $p<0.05$.

Figure 6-1: A) The relationship between TUNEL index and valve thickness of normal (n=15), early staged MMVD (n=20) and late staged MMVD (n=20) valves. B) The correlation of TUNEL index and valve thickness of normal and myxomatous mitral valves.

Figure 7-1: A) The relationship between p-Smad2/3 and valve thickness of normal (n=15), early staged MMVD (n=20) and late staged MMVD (n=20) valves. B) The correlation of p-Smad2/3 and valve thickness of normal and myxomatous mitral valves.

Figure 8-1: A) The relationship between Bax and valve thickness of normal (n=15), early staged MMVD (n=20) and late staged MMVD (n=20) valves. B) The correlation of Bax and valve thickness of normal and myxomatous mitral valves.
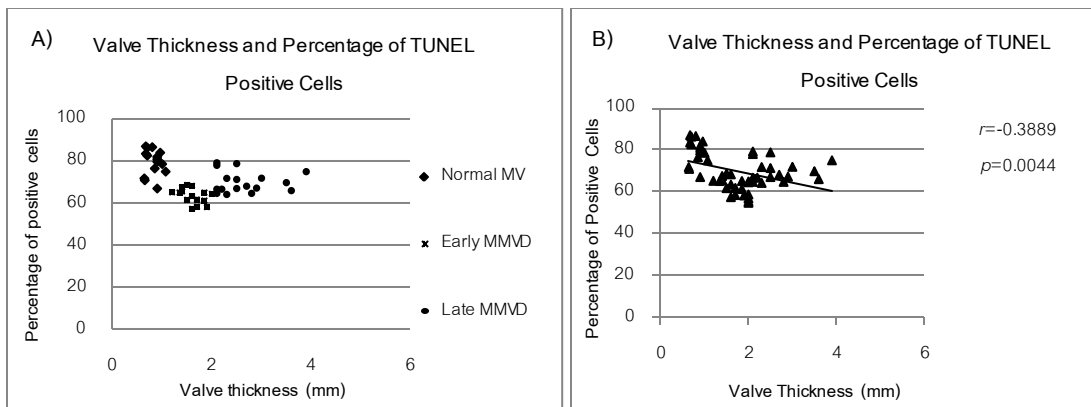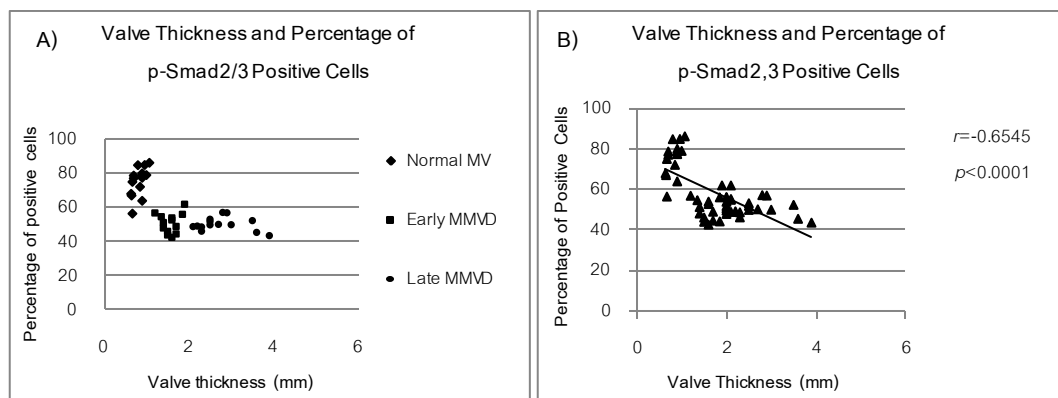

Figure 9-1: A) The relationship between Bcl-2 and valve thickness of normal (n=15), early staged MMVD (n=20) and late staged MMVD (n=20) valves. B) The correlation of Bcl-2 and valve thickness of normal and myxomatous mitral valves.
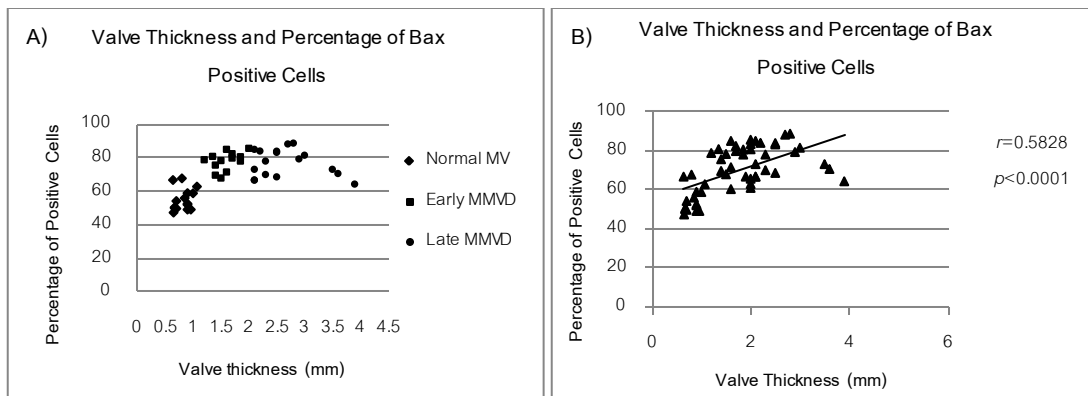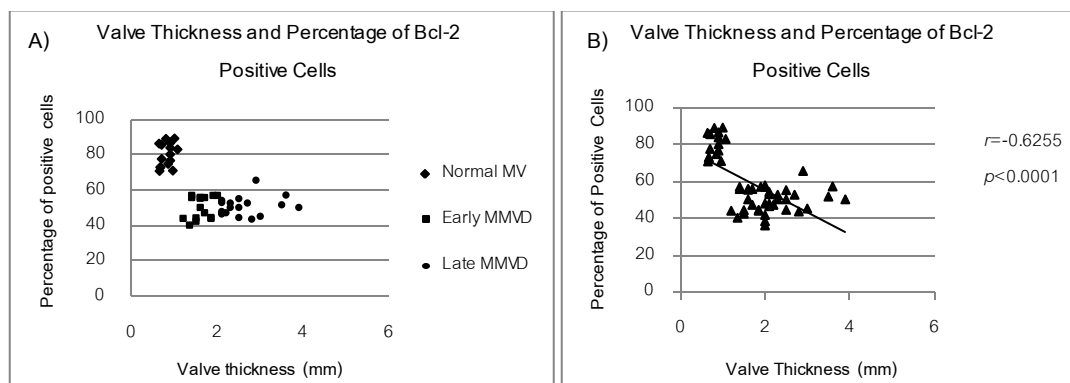
Based on immunohistochemical staining, the present study showed up-regulation of Bax in early and late staged MMVD valves when compared to normal mitral valves. In contrast, there was down-regulation of p-Smad2/3 and Bcl-2 in early and late staged MMVD valves when compared to normal mitral valves. There were statistically significant negative correlation between the valve thickness of normal and myxomatous mitral valves and the expression of p-Smad2/3 ($r=-0.6545$, $p<0.0001$) (Figure 7-1B) and Bcl-2 ($r=-0.6255$, $p<0.0001$) (Figure 9-1B). In addition, there was statistically significant correlation between the valve thickness of normal and myxomatous mitral valves and the expression of Bax ($r=0.5828$, $p<0.0001$) (Figure 8-1B). In contrast, there were no correlation between age of normal dogs and the expression of p-Smad2/3 *($r=-0.0959$, $p=0.7443$)*, Bax *($r=0.2815$, $p=0.3295$)*, Bcl-2 *($r=-0.3039$, $p=0.2908$)*. There was no correlation between the expression of cleaved caspase-3 and valve thickness of normal and MVD valves *($r=-0.1013$, $p=0.4747$)* and age of normal dogs *($r=-0.2778$, $p=0.3362$)*.

The fold change of gene expression of *bax* (apoptotic regulatory gene) and *bcl-2* (anti-apoptotic regulatory gene) relative to *18s rRNA* in normal mitral valve, early staged MMVD and late staged MMVD.

The amplification plots and the dissociation plots of *18s rRNA*, *bax* and *bcl-2* are demonstrated as representative of each gene depicted from normal mitral valves, early staged MMVD and late staged MMVD (Figure 10-1). The fold change of mRNA expression of *bax* and *bcl-2* relative to *18s rRNA* in normal mitral valves, early staged MMVD and late staged MMVD was reported (Figure 11-1). When compared between normal mitral valves and diseased valves, *bcl-2* mRNA expression was higher in valves affected with late staged MMVD than those with early staged MMVD and normal mitral

valve; whereas *bax* mRNA expression was slightly higher in early and late staged MMVD valves than normal mitral valves. There was no statistically significant difference between *bcl-2* mRNA expression in normal, early staged (p=0.5772) and late staged MMVD valves (p=0.2738). Also, there was no statistically significant difference between *bcl-2* mRNA expression in early staged and late staged MMVD valves (p=0.3769) Moreover, there was no statistically significant difference between *bax* mRNA expression in normal, early staged (p=0.4126) and late staged MMVD valves (p=0.8952). Also, there was no statistically significant difference between *bax* mRNA expression in early staged and late staged MMVD valves (p=0.502). The ratio of *bax*/*bcl-2* in normal valve, early staged and late staged MMVD valves was 0.43, 0.19 and 0.16, respectively.

Tm 87.27

Temperature (°C)

Tm 88.37

Temperature (°C)

Tm 88.19

Temperature (°C)
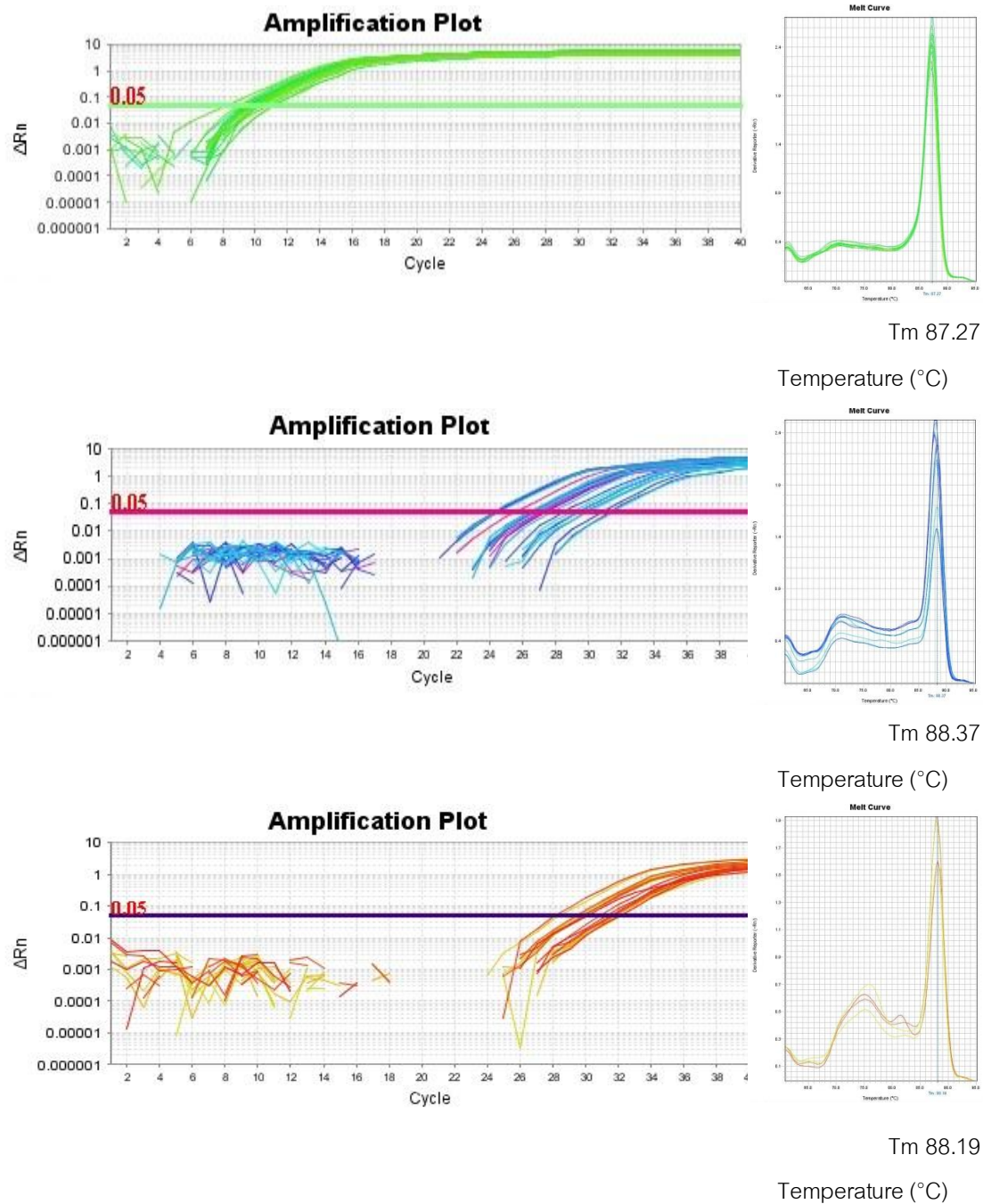
Figure 10-1: Amplification curves (left panel) and dissociation curves (right panel) of mRNA quantification of *bax* (apoptotic regulatory gene) and *bcl-2* (anti-apoptotic regulatory gene) showed homogeneity of PCR product in normal mitral valve (n=3), early staged MMVD (n=4) and late staged MMVD (n=4) as (A) *18s rRNA*, (B) *bcl-2* and (C) *bax*.
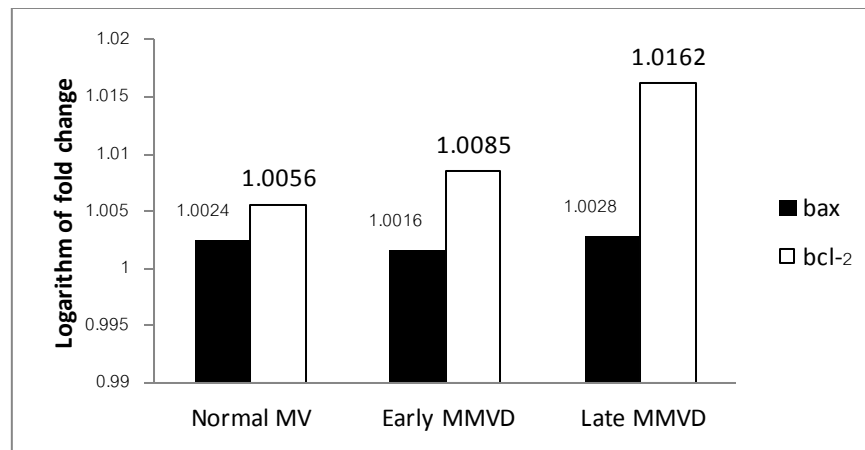
Figure 11-1: Histograms illustrate fold change of mRNA expression of *bax* (apoptotic regulatory gene) and *bcl-2* (anti-apoptotic regulatory gene)in normal mitral valve (n=3), early staged MMVD (n=4) and late staged MMVD (n=4) as (A) normal mitral valve, (B) early staged MMVD and (C) late staged MMVD.

CHAPTER V

DISCUSSION

Tissue microarray technology is an efficient method on histopathologic and immunohistochemical studies on the MMVD research. Canine MMVD causes severe congestive heart failure with unfavorable remodeling of the mitral valves characterized by thickening of the leaflets with rolling up edges (Kvart and Häggström, 2000). The present study found that male affected more than female for approximately 1.5:1 similar to previous report (Aupperle et al., 2009). Age of dogs affected with MMVD varied between 2-13 years old. Dogs with normal mitral valves had an average age lower than dogs affected with MMVD. Age of normal dogs was statistically significant difference from dogs with early and late staged MMVD ($p<0.0001$). The average valve thickness of early staged and late staged MMVD valves was more than normal mitral valves. Valve thickness of normal mitral valves was statistically significant difference from early and late staged MMVD valves ($p<0.0001$). Moreover, thickness of valve affected with early staged MMVD also had statistically significant difference from valve affected with late staged MMVD ($p<0.0001$). Histopathologically, the early staged MMVD affected only in distal half of the valves characterized by moderate increase in VIC proliferation and accumulation of mucopolysaccharides within the spongiosa layer and mild fragmentation of elastic fibers and collagen bundles in the atrialis and fibrosa layers, respectively. In contrast, the advanced stage MMVD involved the entire leaflets characterized by marked increase mucopolysaccharide accumulation and VIC proliferation in the spongiosa layer, complete disruption and fragmentation of collagen bundles in both distal and proximal halves of the valves.

Extracellular matrix helps to support the architecture of the valve and also supplies the functional growth factors and signal such as TGF-β1 (Melinda et al., 2005). TGF-β1 is considered the effective factor that can induce the expression of α-SMA in VICs by up-regulation of extracellular matrix proteins and inhibition of metalloproteinases (Blobe et al., 2000). VICs are the predominant cell type in mitral valve leaflets. The regulation of VIC phenotype depends on the balance of signaling derived from both insoluble macromolecules in extracellular matrix and soluble cytokine signals which modulate cell growth and cellular density (Schmitt-Gräff et al., 1994). Activated VICs that express α-SMA (contractile protein in vascular smooth muscle cells) called myofibroblasts are responsible for collagen production. In normal valves, VICs express low level of α-SMA when compare to remodeling valves that commonly contained activated α-SMA-positive VICs (Rabkin-Aikawa et al., 2004). An enhancement of α-SMA positive VICs and cellular density was also reported in canine MMVD valves (Disatian et al., 2008).

The present study showed expression of intranuclear and intracytoplasmic stainings against p-Smad2/3 in canine VICs. Immunolabeling of p-Smad2/3 in normal mitral valves was statistically significant difference from that of early staged MMVD (p<0.0001) and late staged MMVD valves (p<0.0001). Moreover, there were statistically significant negative correlation between the thickness of mitral valves and the expression of p-Smad2/3. Therefore, there was down-regulation of p-Smad2/3 in MMVD valves when compared with normal mitral valves. Since, TGF-β1 has many biological functions. The most important functions are anti-proliferative activity participating in repair processes and induction of apoptosis. Anti-proliferative activity of TGF-β1 is exerted through the inhibition of DNA synthesis and cell proliferation (Piekarska et al., 2006; Petrov et al., 2008). Many reports indicated that the expression of TGF-β1 was

related to the severity of MMVD and being a potential mediator of cardiac remodeling (Aupperle et al., 2009; Disatian and Orton, 2009). Previously, the expression of TGF-β1 signaling proteins has been reported to up-regulate in MMVD. The statistically significant association was noted between expression of receptors as well as local production of TGF-β1 and severity of MMVD (Disatian et al., 2008). In contrast, the results of the present study found the down-regulation of p-Smad2/3, the TGF-β1 downstream signaling proteins, in MMVD valves suggesting that the activation of TGF-β1 associated Smad pathway was decreased in disease valves compared to normal valves. However, TGF-β1 signaling has several downstream pathways not only Smad pathway but also the mitogen-activated protein kinase, ρ-like Rho-GTPase, and PI3K-AKT pathways (Sánchez-Capelo, 2005). As the up-regulation of receptors and source of TGF-β1 has been reported (Disatian et al., 2008), it is reasonable to speculate that the activation of TGF-β1 signaling mediating valve remodeling may occur in MMVD by passing through the other pathways. In addition, Smad7 can prevent the interaction between Smad2/3 and the ALK receptor; and it can interact with ubiquitin ligases of the Smurf family resulting in proteasomal degradation of ALK receptor (DaCostaByfield and Roberts, 2004). These mechanisms may imply the reason for decreasing in p-Smad2/3 in MMVD valves. The present study showed that the expression of p-Smad2/3 was correlated reversely to the thickness of mitral valves suggesting the negative relationship between disease severity and p-Smad 2/3 expression. There was no correlation between age and the expression of p-Smad2/3 implying a non-effect of age on p-Smad2/3 expression in dogs.

Apoptosis, programmed cell death, is an active process of cell elimination. However, disturbances in this process may lead to improper damage control to normal cells (Piekarska et al., 2006). Apoptotic stimuli are modulated by reduction in the

mitochondrial membrane potential and subsequent activation of caspase-3 and mitochondrial death pathway. Caspase-3 cleavage and activation in apoptosis are common mechanism among different epithelial and mesenchymal cell lines (Cao et al., 2007). Previous study has shown that cardiomyocyte apoptosis can be detected by using immunohistochemistry and TUNEL assay to prove for early myocardial damage (Ceauşu et al., 2008). Using TUNEL assay and immunohistochemical analysis for cleaved caspase-3, this study has shown decrease TUNEL index expression in early and late staged MMVD valves when compare with normal mitral valves. TUNEL expression was statistically significant difference between normal mitral valves and early staged MMVD valves ($p<0.0001$). Also, TUNEL expression of normal mitral valves was statistically significant difference from late staged MMVD valves ($p=0.0004$). Moreover, TUNEL index of early staged MMVD valves was also statistically significant difference from that of late staged MMVD valves ($p=0.0002$). These findings suggested that the DNA damage was decrease in MMVD valves. The expression of TUNEL was correlated reversely to the thickness of mitral valves suggesting the negative relationship between disease severity and apoptotic marker. For cleaved caspase-3, the intranuclear and intracytoplasmic positive stainings were revealed in VICs of normal and MMVD valves. There was no statistically significant difference of the average percentage positive cells to cleaved caspase-3 between normal mitral valves and valve affected with late staged MMVD ($p=0.2845$). These findings suggested the cells might not undergo apoptosis through caspase pathway since the expression of cleaved caspase-3 was unchanged in normal and diseased valves. As previously mention, the p-Smad2/3 was down-regulated in early staged and late staged MMVD. Therefore, the decrease in TUNEL expression in the same manner as the expression of p-Smad2/3 may reflect the relationship between the down-regulation of TGF-β1associated Smad signaling and decrease of DNA damage within the disease valves.

The Bcl-2 family composes of apoptotic and anti-apoptotic proteins. Bcl-2, the anti-apoptotic protein, prevent apoptosis by inhibiting the reduction in the mitochondrial membrane potential and subsequent interactions to inhibit the activity of caspases 3, 7 and 9 and mitochondrial death pathway. The pro-apoptotic protein, Bax, is a key component for cellular induced apoptosis through mitochondrial stress. The migration and mitochondrial translocation of Bax increases the mitochondrial membrane permeability through interactions with pore proteins on the mitochondrial membrane which leads to activation of caspases and apoptosis (Kamdar et al., 2008). Expression of Bcl-2 and Bax in many tissues suggested a role for cell proliferation and maturation (Olivé and Ferrer, 2000).

The present study revealed intranuclear positive staining of Bcl-2 and Bax in VICs of normal and MMVD valves. Immunolabeling of Bax in normal mitral valves was statistically significant difference from that of early staged MMVD ($p<0.0001$) and late staged MMVD valves ($p<0.0001$). Immunolabeling of Bcl-2 in normal mitral valves was statistically significant difference from that of early staged MMVD ($p<0.0001$) and late staged MMVD valves ($p<0.0001$). Therefore, the results showed up-regulation of Bax in early and late staged MMVD valves when compared to normal mitral valves; whereas, there was down-regulation of Bcl-2 in early and late staged MMVD valves when compared to normal mitral valves. There were statistically significant correlation between the valve thickness of normal and myxomatous mitral valves and the expression of Bax. There were statistically significant correlation between the valve thickness of normal and myxomatous mitral valves and the expression of Bcl-2. Study of the fold change of *bcl-2* and *bax* mRNA expression revealed that *bcl-2* mRNA expression was higher in valves affected with late staged MMVD than those with early staged MMVD ($p=0.3769$) and normal mitral valve ($p=0.2738$); whereas *bax* mRNA expression was slightly higher in

late staged MMVD valves than those with early staged MMVD (*p*=0.502) and normal mitral valve (*p*=0.8952). The ratio of *bax*/*bcl-2* was decrease in MMVD valves when compared to normal mitral valves.

Bcl-2 protein regulates apoptosis by enhancing a survival advantage to rapidly proliferating cells. Bcl-2, a novel apoptotic inhibitor, is very important for the development and differentiation of many cell types (Tsamandas et al., 2003). Previously, Bax and Bcl-2 expressions were detected in cultured cardiomyocytes and myocardium from rats (Sumida et al., 2010) and humans (Ceauşu et al., 2008). In the current study, Bcl-2 protein was expressed in VICs in both normal mitral valves and MMVD valves. The decrease Bcl-2 protein expression of early and late staged MMVD was statistically significant difference from normal mitral valves. The *bcl-2* mRNA was tended to increase in late staged MMVD valves when compared to normal mitral valves, but not statistically significant (*p*=0.2738). Although, the slightly increase of *bcl-2* mRNA was seen, the Bcl-2 protein was down regulated in MMVD valves. The striking difference observed between *bcl-2* mRNA and protein expression in this study could be attributed to a disorder of posttranscriptional regulation of the *bcl-2* gene (Tsamandas et al., 2003) resulting in prevention of this mRNA from being translated into protein in cases of MMVD.

Bax protein promotes apoptosis by enhancing cell susceptibility to apoptotic stimuli. The action of Bax can be neutralized by its heterodimerization with Bcl-2 and some other suppressor of cell death, such as Bcl-xL and Mcl-1. It was previously thought that when half or more of the endogenous Bax was heterodimerized with either Bcl-2 or Bcl-xL, apoptosis was repressed (Tsamandas et al., 2003). In this study, Bax protein was expressed in VICs of both normal mitral and MMVD valves. The increase of Bax expression in VICs parallels to increase disease severity. Although, the Bax protein

expression was high in MMVD valves, but it was not related to DNA damage and VICs apoptosis detected by TUNEL technique and immunohistochemistry for cleaved caspases-3. Previous study reported that increased expression of Bax protein has been observed in regenerative tissues such as necrosis-regeneration of rat skeletal muscles (Olivé and Ferrer, 2000) and degenerating fibers from human myopathies (Prayon and Yu, 2001). Moreover, distribution of Bax had not always shown the limitation of tissues to high apoptotic death rates. Pro-apoptotic proteins are expressed with no relation to the cell death process (Olivé and Ferrer, 2000). Therefore, the increase expression of Bax implies that this protein may relate to the disease process. Overexpression of Bax in this study may not be necessarily linked to apoptosis.

This study demonstrates that Bcl-2 and Bax proteins were expressed within VICs of both canine normal mitral valves and MMVD valves. The expression of Bax and Bcl-2 proteins, as detected by immunohistochemistry, was produced within VICs, and the expressions were variably related to the severity of the MMVD. Using immunohistochemical analysis with pro-apoptotic and anti-apoptotic markers, this study has shown a decrease of Bcl-2 protein with an increase of Bax protein in early and late staged MMVD valves. Valve thickness was reversely correlated with the percentage of Bcl-2 expression and correlated directly with the percentage of Bax expression ($p<0.001$). Therefore, Bcl-2 and Bax immunoreactivities were not disease-specific but abnormalities in these proteins could be related with the severity of the disease (Olivé and Ferrer, 1999).

The co-expression of *bcl-2* mRNA and Bax protein in MMVD valves is somehow a paradox. Tsamandas et al. (2003) reported that *bcl-2* mRNA expression reflects the resistance of cells to apoptosis, whereas Bax expression in the same cells denotes an independent way of apoptosis. In this study, Bax protein co-expression with *bcl-2* mRNA

probably does not reflect an adaptive down-regulation but a net increase in Bax expression in VICs during the progress of MMVD. The ratio of *bax* to *bcl-2* determines death or survival after an apoptotic stimulus, and mRNA expression showed a decrease of that ratio in the MMVD valves. Interestingly, the decrease of the ratio was more significant in the advanced stage of MMVD. Although the expression of *bax* and *bcl-2* mRNA was not statistically significant difference, it did not mean that the level of these genes were not involved in ongoing processes such as anti-apoptosis and proliferation of VICs in MMVD. The observed level of *bax* and *bcl-2* mRNA in difference stage of MMVD may describe the dynamic of process taking place during the course of the disease.

In conclusion, the TGF beta associated Smad pathway is down-regulated in canine MMVD. The decrease TGFβ1-Smad signaling is related to decrease in DNA damage. An imbalance of Bcl-2 protein family, Bax and Bcl-2 reflects the shift of pro-apoptotic and anti-apoptotic balance in MMVD. Finally, the VICs seem not to undergo apoptosis based on the constant expression of cleaved caspase-3 and the absence of apoptotic bodies in normal and MMVD valves. Further studies are warranted to specify the exact roles of apoptosis and anti-apoptosis involved in MMVD. In addition, the underlying mechanisms and signaling regulated apoptosis and anti-apoptosis should be explored. This knowledge may lead to new strategies in the management of canine MMVD to prevent or stop the disease progression.

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APPENDICES

Appendix A: Hematoxylin and Eosin staining method (modified from Luna, 1968)

Four-micrometer-thickness serial sections from the paraffin-embedded tissue and TMA blocks were deparaffinized in xylene, rehydrated in graded alcohol solutions. Slides were placed in Harris' hematoxylin for 2 min. Then, slides were dip in ammonia water for three to five dips. After that slides were placed in Eosin for 1 min. Slides were washed in running tap water and rinsed in distilled water for each incubation step. Slides were dehydrated in 95% and absolute alcohols for two changes of 2 minutes each. Slides were placed in xylene for two changes of 2 minutes each. Finally, slides were mounted with Permount solution. The results were stained with blue in nuclei and various shades of pink in cytoplasm.

Appendix B: Combined PAS-alcian blue staining method (modified from Kiernan, 2008)

Four-micrometer-thickness serial sections from the paraffin-embedded tissue and TMA blocks were deparaffinized in xylene, rehydrated in graded alcohol solutions. Slides were placed in 1%alcian blue solution pH 2.5 for 5 min. After that slides were placed in 1%periodic acid for 10 min. Then, Schiffs reagent was applied to the slides for 20 min. Slides were washed in running tap water and rinsed in distilled water for each incubation step. Finally, slides were counterstained with Mayer's Hematoxylin for 2 min and mounted with permount solution. The results were stained with turquoise-blue for alcian blue-positive materials (mucopolysaccharides and glycosaminoglycans) and magenta for PAS-positive materials (collagen fibers).
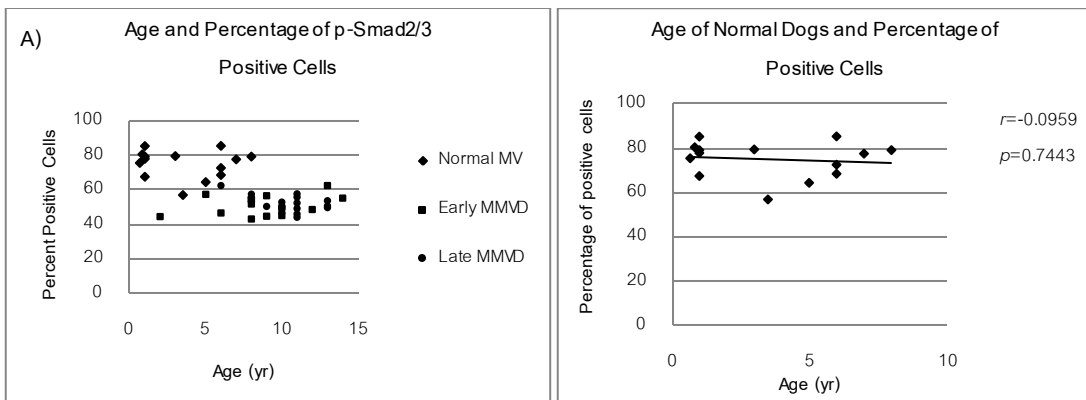
Appendix C: The relationship and correlation between TUNEL index, immunohistochemistry analysis against cleaved caspase-3, p-Smad2/3, Bax, Bcl-2 and age of normal, early staged MMVD and late staged MMVD dogs.
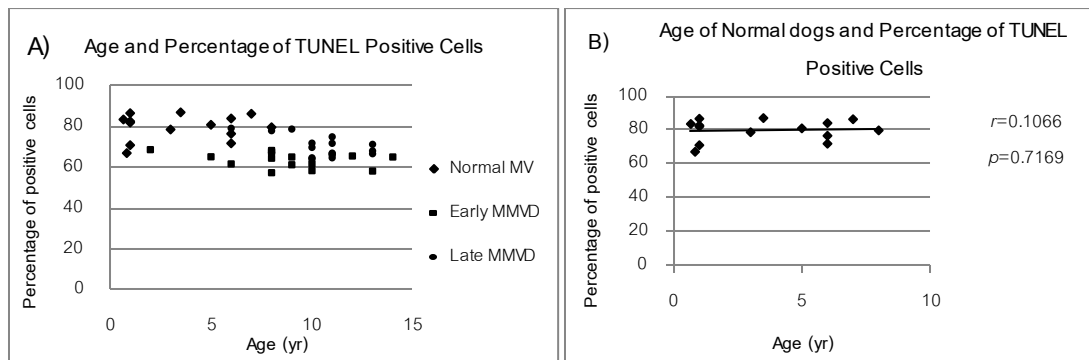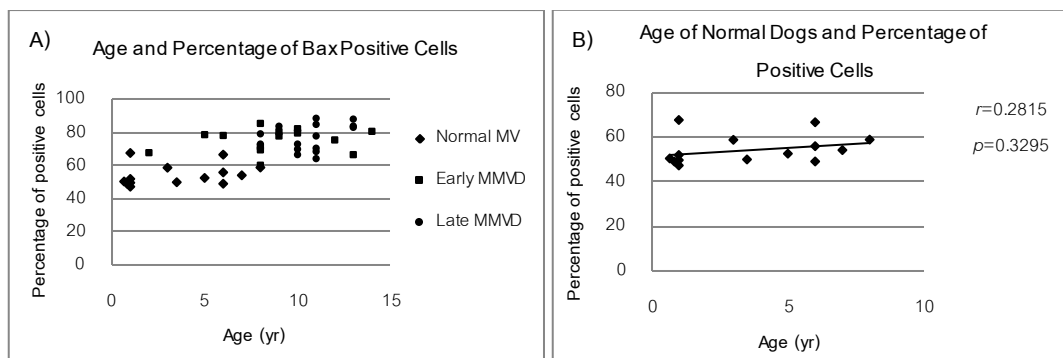

Appendix C-1: A) The relationship between TUNEL index and age of normal (n=15), early staged MMVD (n=20) and late staged MMVD (n=20) dogs. B) The correlation of TUNEL index and age of normal dogs.
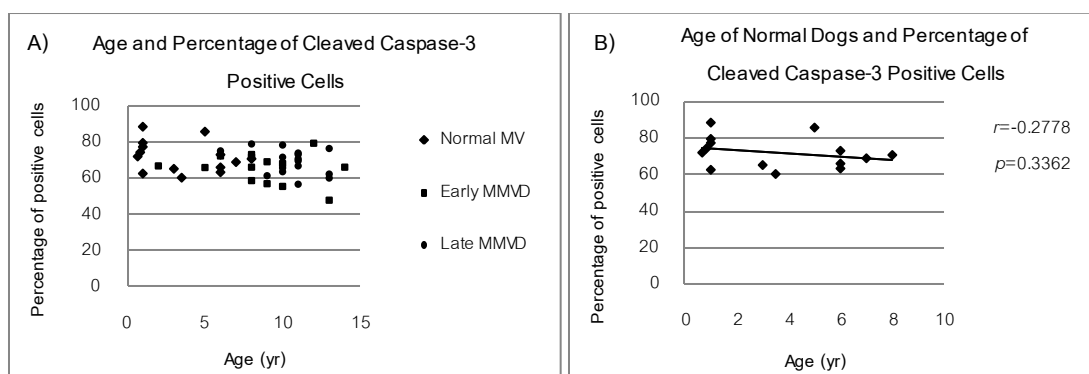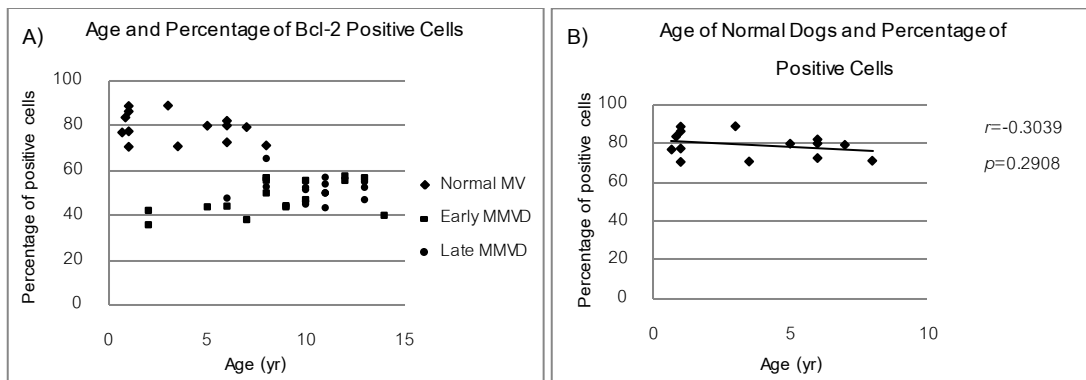

Appendix C-2: A) The relationship between cleaved caspase-3 and age of normal (n=15), early staged MMVD (n=20) and late staged MMVD (n=20) dogs. B) The correlation of cleaved caspase-3 and age of normal dogs.

Appendix C-3: A) The relationship between p-Smad2/3 and age of normal (n=15), early staged MMVD (n=20) and late staged MMVD (n=20). B) The correlation of p-Smad2/3 and age of normal dogs.
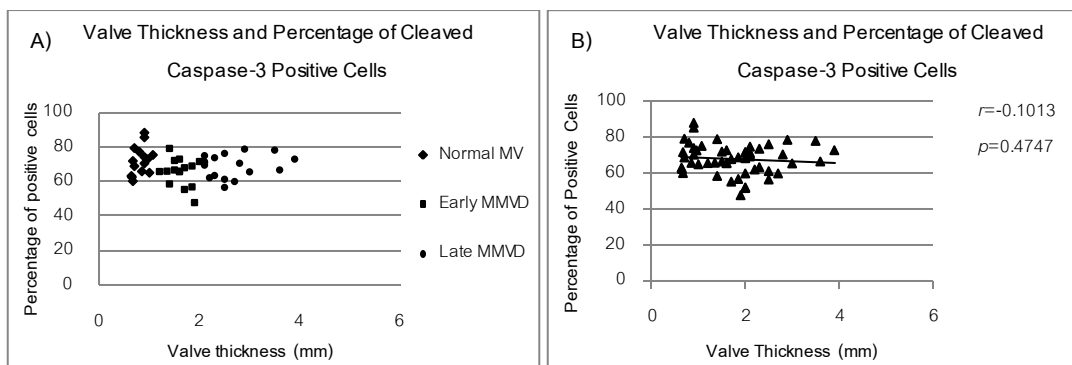

Appendix C-4: A) The relationship between Bax and age of normal (n=15), early staged MMVD (n=20) and late staged MMVD (n=20) dogs. B) The correlation of Bax and age of normal dogs.
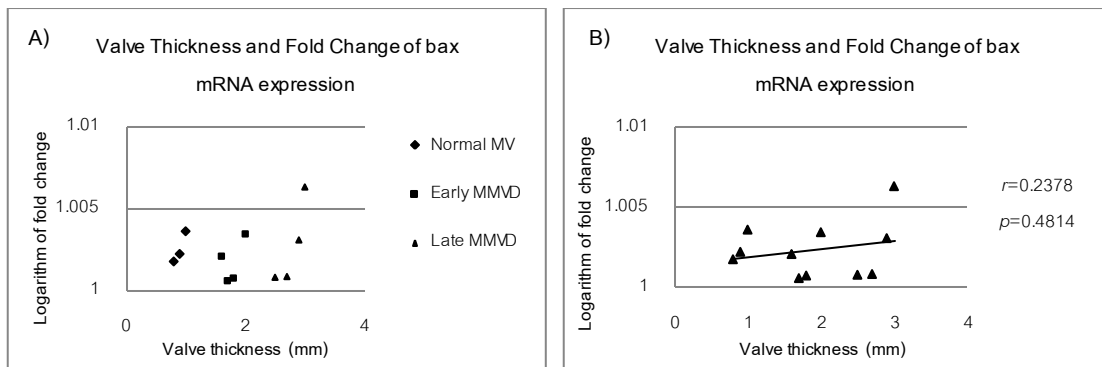
Appendix C-5: A) The relationship between Bcl-2 and age of normal (n=15), early staged MMVD (n=20) and late staged MMVD (n=20) dogs. B) The correlation of Bcl-2 and age of normal dogs.

Appendix D: The relationship and correlation between cleaved caspase-3 and valve thickness of normal, early staged MMVD and late staged MMVD valves.
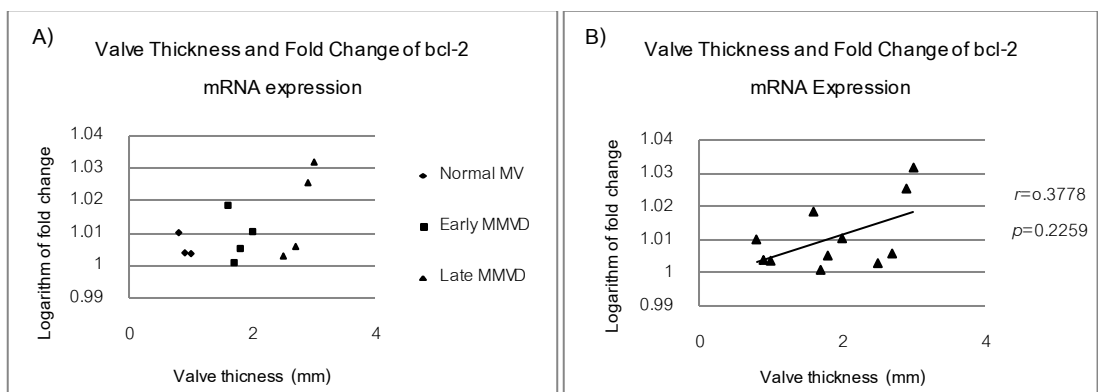

Appendix D-1: A) The relationship between cleaved caspase-3 and valve thickness of normal (n=15), early staged MMVD (n=20) and late staged MMVD (n=20) valves. B) The correlation of cleaved caspase-3 and valve thickness of normal and myxomatous mitral valves.

Appendix E: The relationship and correlation between mRNA abundance of *bax* (apoptotic regulatory gene), *bcl-2* (anti-apoptotic regulatory gene) and valve thickness of normal, early staged MMVD and late staged MMVD valves.

Appendix E-1: A) The relationship between fold change of *bax* mRNA expression and valve thickness of normal (n=3), early staged MMVD (n=4) and late staged MMVD (n=4) valves. B) The correlation of *bax* mRNA expression and valve thickness of normal and myxomatous mitral valves.

Appendix E-2: A) The relationship between fold change of *bcl-2* mRNA expression and valve thickness of normal (n=3), early staged MMVD (n=4) and late staged MMVD (n=4) valves. B) The correlation of *bcl-2* mRNA expression and valve thickness of normal and myxomatous mitral valves

BIOGRAPHY

Miss Treenate Jiranantasak was born on May 12th, 1984 in Bangkok, Thailand. She graduated from Faculty of Veterinary Science, Chulalongkorn University, 2003-2009. She received her Bachelor degree of Veterinary Science in 2006 with 2nd class of Honor. She admitted with the degree of Master of Science Program in Veterinary Pathobiology, Department of Veterinary Pathology, Faculty of Veterinary Science, Chulalongkorn University in 2009 under H. M. the King's 72nd Birthday Scholarship.

LIST OF PUBLICATIONS

1. **Treenate Jiranantasak**, Anudep Rungsipipat, Sirilak Disatian. 2011. Histopathological Changes and Apoptosis Detection in Canine Myxomatous Mitral Valve Disease Using Tissue Microarray Technique. Proceeding of the Joint Meeting of the 5th Conference and Congress of Asian Society of Veterinary Pathology (ASVP) 2011. November 22-24, 2011, Bogor, Indonesia.