基質金屬蛋白酶基因多型性及酵素活性
在兒童心室中膈缺損的相關性

Genetic Variations and Circulating Levels of Matrix Metalloproteinases in the Children with Ventricular Septal Defect

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博 士 論 文

A Thesis

Submitted to Institute of Bioinformatics and Systems Biology

College of Biological Science and Technology

National Chiao Tung University

for the Degree of Ph.D.

in February 2014

Hsinchu, Taiwan, Republic of China

中 華 民 國 一 百 零 三 年 二 月

Acknowledgement

口試完的當天，真的有鬆口氣的感覺。心裡想著終於不用抱電腦和無窮無盡的期刊資料坐在咖啡廳內奮鬥了。回想六年的時光都在不知不覺，在電腦螢幕和咖啡苦澀味道中度過。六年的時光並不算短而博士班學程的結束，得到除了是厚厚的論文和薄薄的一紙畢業證書以外，更重要的是留在心底的值得去細細品味的經歷與回憶。總結一句：如人飲水，冷暖自知。

學位口試的完成，要感謝**陳銘仁** 醫師、**吳介信** 老師、**呂衍達** 醫師、以及**曲在雯** 老師於百忙之中抽空為我的博士論文進行指導，並給予寶貴的建議。由其是**陳銘仁** 醫師不僅是在實驗題材給予我極大的幫忙，也在我做人處事方面給我非常珍貴的指導。而博士學位的取得，則要感謝在我攻讀博士學位時，盡心指導我的指導教授**林志生** 老師。**林志生** 老師是一位嚴以律己且肯為學生著想的老師，他以嚴格的言教與身教指導實驗室學生。即使一開始是門外漢如我，老師也肯耐著性子對我諄諄善誘。老師可怕的效率讓我不禁懷疑他是否平時真有好好休息（PS.半夜寄給他的信總是一下就有回信了），不論如何老師作事的嚴謹和認真對我後來作事的態度應會起了很大的模範作用。

在攻讀博士學位的期間，因為在職身份的我，並非能一直在實驗室中，還好 lab 中一直都有一群認真聰明的學弟妹們。感謝認真的**棠青**學弟，他算是我的救星，實驗有問題問他就沒錯了！再加上默默工作不需人操心的**睦元**，對於我整個實驗的流程幫助很大。另外，聰明的**千雅**、**燕秋**、**意涵**、及**芳沅**等等學弟妹們全力的支持，及**謝文郁** 醫師跟我相互打氣支持，我才能如此順利地完成此份論文。

感謝交大有這麼許許多多極富教學熱忱的老師如: 帥氣有型**黃憲達** 老師、高雅美麗的**王唯菁** 老師以及年輕有為的**林勇欣** 老師等等;每個老師認真上課的態度讓我覺得能在交大上課真的很幸福。

最後，要感謝的是一直守護我、支持我的父母和妻兒。感謝父母全心全意的支持和從小以來的教誨，讓我有勇氣繼續走下去;感謝老婆大人支持我取得博士學位的心意，讓我無窮盡在咖啡廳的日子裡無後顧之憂,令我相當窩心。感謝兩位胖兒子可愛的陪伴，

讓我在深夜著手論文時不曾喪失了信心而放棄。造就了此本論文需要感謝的人太多，雖未能一一點名列出，但是都會記在我的心中。

鄭崑山 謹誌

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中華民國 一百零三年 二月

基質金屬蛋白酶基因多型性及酵素活性在兒童心室中膈缺損的相關性

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中 文 摘 要

先天性心臟病佔了總出生人口比例約 1－3%，而其中心室中膈缺損(ventricular septal defect ,VSD)又是最為常見的先天性心臟病之一。比率可以佔所有先天性心臟病的40%，部分病童的中膈缺損會隨著年紀增長而逐漸變小或完全閉合，其餘在臨床追蹤時則依然保持缺陷。目前有許多因素被認為與 VSD 形成有關，其中特定基因的單核苷酸基因多型性(single nucleotide polymorphisms, SNPs)被指出可能是造成 VSD 疾病主要原因之一。

基質金屬蛋白酶 (matrix metalloproteinases, MMPs)是一類酵素, 其活性依賴於 Zn^{2+}等金屬離子, 參與降解細胞外基質(extracellular matrix, ECM)，並精密調控著組織中 ECM 的代謝平衡。MMPs 已被報導在胚胎發育、心肌分化，以及心臟組織的重塑扮演著重要的角色,文獻探討中可以發現許多心臟疾病也與MMPs的基因多型性有關。據此，我們檢測 VSD 的病患的 MMPs 基因 SNPs 與其血漿中 MMPs 活性，來探討 MMPs 基因 SNPs 和血液中 MMPs 活性與 VSD 之嚴重程度。

自 2010 年 9 月起，我們收集了 95 位 VSD 病童的血液樣品，使用聚合酶連鎖反應-限制酶片段長度多型性(polymerase chain reaction- restriction fragment length polymorphism, PCR-RFLP)來檢測這些病童的 MMP 基因 SNPs 包括：MMP -2 -735C>T，以及 MMP-9 -1562C>T、R279Q 及 R574。並使用明膠酶電泳(gelatin zymography)用以測定其血漿中 MMP-2 和 MMP-9 活性。

在所檢測的四個 SNPs 中，結果顯示 MMP-9 R279Q 基因頻率在控制組與病例組分

布比例有統計上的顯著差異性 ($p < 0.05$)。而檢測的 MMP-2/-9 的不同單核苷酸基因多型性基因型別中的 MMP-2 及 MMP-9 活性並無不同。另一方面，我們依病患超音波檢查之 VSD 直徑/主動脈主動脈直徑 (aortic root)比值分類病童 VSD 程度，將之分成輕微缺損(VSD/Ao ≤ 0.2)、輕度缺損(0.2 < VSD/Ao ≤ 0.3)及中度缺損(0.3 < VSD/Ao ≤ 0.66) 三組。明膠酶電泳結果顯示，特別在中度以上缺損病童分組中，皆可測得較高活性之 MMP-2 與 MMP-9 並顯著高於控制組。而分析結果也指出，隨著 VSD 趨於嚴重時，其 MMP-2 與 MMP-9 的活性皆有增加的趨勢。經過一年的追蹤，我們發現 VSD 的病患癒合與其血液中的 MMP-9 活性有顯著相關 ($p < 0.01$)。

另外我們也研究了其血液中第三型金屬蛋白酶抑制因子(TIMP-3)以及 B 型利鈉肽(BNP)的濃度和 VSD 病童血液中 MMP-2/-9 活性的關聯性。分析結果發現，TIMP-3 在 MMP-2 -735 C>T 的 CC 以及 MMP-9 R279Q 的 QQ 對偶基因型病人的濃度是較高，並與其他對偶基因有統計的顯著性差異; 並且 TIMP-3 和 MMP-9 活性、BNP 血中濃度都有正相關。此外，我們也發現 MMP-9 活性、TIMP-3 及 BNP 血中濃度都與 VSD 的嚴重度呈現正相關。

據本研究之結果，我們推測 MMPs 的活性可能對於 VSD 病童其患病與否、及嚴重程度、或其癒合比率上有一定的角色，因此值得未來進一步探討 MMPs 與 VSD 病理機轉之間的關係。

關鍵詞：基質金屬蛋白酶、金屬蛋白酶抑制因子、心室中膈缺損、單核苷酸基因多型性

Genetic variations and circulating levels of matrix metalloproteinases in the children with ventricular septal defect

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Abstract

Congenital heart disease (CHD) account for 1 – 3% population, and ventricular septal defect (VSD) is the most common form of CHD that accounts for approximately 40% of total patients. Some of children with VSD can have spontaneous closure with aging, but the others don't. Single nucleotide polymorphism (SNP) in specific gene is considered as one of the main factors of VSD formation.

Matrix metalloproteinases (MMPs) are a family of zinc-dependent endopeptidases involved in breakdown, and physiological homeostasis of extracellular matrix. MMPs play important roles in embryonic development, cardiomyocyte differentiation and causing cardiac tissue remodeling. The literature review shows many cardiac diseases are related to the SNPs of MMPs gene. Therefore, we aim to examine the SNPs of MMPs gene in patients with VSD and their plasma MMPs activities, in order to find out the relationship among gene SNPs, plasma MMPs activities and the severity of VSD.

Since September 2010, 95 children with perimembranous VSD were enrolled in this study and blood samples were collected. The SNPs of MMP-2 (-735C>T) and MMP-9 (-1562C>T, R279Q, and R574P) were determined using polymerase chain reaction followed by restriction fragment length polymorphism (PCR-RFLP) analysis. Plasma MMP-2 and MMP-9 activities were also confirmed by gelatin zymography.

From the results of four SNPs assay in this study, there is a significant difference of MMP-9 R279Q SNP frequency between VSD patients and control group ($p < 0.05$). There is no difference between MMP-2 or MMP-9 genotypes among all the four polymorphisms. Children with VSD according to the ratio of VSD diameter/diameter of aortic root (Ao), were categorized into different groups: Trivial group (VSD/Ao ratio ≤ 0.2), Mild group (0.2 < VSD/Ao ≤ 0.3), and Median group (0.3< VSD/Ao ≤ 0.66). According to the results of gelatin zymography, there is significant higher MMP-2/-9 activity in each groups compared with control group. After one year follow-up, we also found that there are significant differences of MMP-9 activities between VSD spontaneous closure group and non-closure group ($p < 0.01$).

The concentrations of plasma tissue inhibitor of metalloproteinase-3 (TIMP-3) and B-type natriuretic peptide (BNP) in VSD children, and the correlations with MMP-2/-9 activities in VSD patients with different severities were also investigated. The data showed that the TIMP-3 concentration is significantly higher in carriers of the CC genotype of MMP-2 -735 C>T and QQ genotype of the MMP-9 R279Q polymorphisms than other genotypes. We also found that TIMP-3 correlated with MMP-9 activity and BNP concentration. Overall, MMP-9, TIMP-3, and BNP all show a similar positive trend with regard to the severity of VSD.

In conclusion, we found the relation between the severity of VSD and activities of MMPs. Our data might hint that MMPs expression may play a role in the pathogenesis of VSD. It is worth to further investigate the correlation between MMPs and the mechanism of VSD formation or closure.

Keywords: Matrix metalloproteinase (MMP), Tissue inhibitors of metalloproteinase (TIMP), Ventricular septal defect (VSD), Single nucleotide polymorphisms (SNPs)

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I. Literature Review

1-1. Single-nucleotide polymorphisms

Polymorphisms represent natural sequence variants (alleles), which may appear in at least 1% of a population and are considered biologically normal. Approximately 90% of DNA polymorphisms are single-nucleotide polymorphisms (SNPs) due to a single base exchange (Collins et al., 1998). A SNP represents an alternative nucleotide in a given and defined genetic location. This definition does not include other types of genetic variability like insertions and deletions, and variability in copy number of repeated sequences. They occur one in every 300 nucleotides on average. Although the majority of DNA polymorphisms are probably functionally neutral, a proportion of them can exert allele-specific effects on the regulation of gene expression or function of the coded protein, then cause individual differences in various biological processes or in susceptibility to diseases (Brookes, 1999).

A SNP in which both alleles produce the same polypeptide sequence is called a synonymous polymorphism (sometimes called a silent mutation); which produce a different polypeptide sequence is called a non-synonymous polymorphism (replacement polymorphism). Mutations found in known diseases over half of all come from non-synonymous polymorphisms (Stenson et al., 2009). When gene expression is affected by a SNP, this SNP type is referred to as an eSNP (expression SNP) and might be upstream or downstream from the gene.

Analytical methods to discover novel SNPs and detect known SNPs include DNA sequencing, restriction fragment length polymorphism (RFLP), capillary electrophoresis, mass spectrometry, single-strand conformation polymorphism (SSCP), electrochemical analysis, denaturing HPLC, gel electrophoresis and hybridization analysis (Kwok and Chen,

2003). All of the background reference and information of SNPs in this study were obtained from National Center for Biotechnology Information (NCBI)-SNP database (http://www.ncbi.nlm.nih.gov/snp/).

1-2. Ventricular septal defect

Ventricular septal defect (VSD) is the most common form of congenital heart diseases which account for 40% of the patients having congenital heart diseases (Hoffman, 1995). The defect can be in any portion of the ventricular septum, and the physiologic consequences can range from trivial to severe. Although historically the incidence of VSD is cited as approximately 1.5 to 3.5 per 1,000 term infants and 4.5 to 7 per 1,000 premature infants (Moe and Guntheroth, 1987), recent studies demonstrated an incidence of VSD in newborns to be 5 to 50 per 1,000 (Tikanoja, 1995). Sometimes, VSD might be not only an isolated cardiac malformation but also an intrinsic component of several complex malformations such as tetralogy of Fallot. However, patients whose cardiac malformation is predominately caused by a VSD will be focus on in this proposal.

There are many systems to classify the different VSD types. One of the systems can classify VSD according to their location, either within the muscular septum (muscular defects) or at its margins. VSD at the margins of the muscular septum can be related to hinge-points of the leaflets of the atrioventricular valves (perimembranous type), those of the arterial valves (juxta-arterial or subarterial type), or both (**Fig. 1-1**) (Penny and Vick, 2011). Symptoms, natural history and management of VSD depend on size, anatomical characteristics, and patients' variances.

Rates of spontaneous closure for membranous and muscular VSD in infant and children were 37% and 50%, respectively during a mean follow-up of 12 months (Moe and Guntheroth,

1987). The others have no such a fortunate clinical course and their VSD persist. The development of echocardiography had provided a good tool to observe the natural processes of spontaneous VSD closure (Murphy et al., 1986); Perimembranous defects often close by the development of a saccular pouch or aneurysm derived from tissue from the septal leaflet of the tricuspid valve. Muscular defects appear to close by progressive growth of tissue from the right ventricular side of the circumference of the defect. Neither the detail molecular insight of spontaneous VSD closure nor the study of association between extracellular matrix (ECM) and spontaneous VSD closure has been clarified.

1-3. Matrix metalloproteinase

1-3-1. Structures and functions of matrix metalloproteinases

Matrix metalloproteinases (MMPs) is a family of extracellular zinc-dependent neutral endopeptidases (Lombard et al., 2005), capable of degrading essentially all ECM components including fibrillar and non-fibrillar collagens, fibronectin, laminin and basement membrane glycoproteins (Fedarko et al., 2004). MMPs usually play an important role in ECM remodeling in physiologic situations, such as embryonal development, tissue regeneration, and wound repair. In addition, there are more and more researchers turn their focus on relationships of MMP proteins function and different pathological conditions, because of their potent degradative capacities. Some articles indicate the change of nucleotides and the SNPs of MMPs and tissue inhibitors of matrix metalloproteinases (TIMPs) correlated with many different diseases like coronary artery disease (Renko et al., 2004), Kawasaki disease (Ikeda et al., 2008), and cancer (Liu et al., 2011) (**Table 1-1**).

The others show serum levels of MMPs are altered in various diseases, and have been

considered as potential clinical markers of disease activity. For example, serum MMP-2 level has been reported to be increased in the patients with liver cirrhosis (El-Gindy et al., 2003), and endometriosis (Huang et al., 2004); Serum MMP-9 level appears to increase in patients with congestive heart failure (Abou-Raya et al., 2004), stroke (Lynch et al., 2004; Reynolds et al., 2003), or myocardial infarction (Renko et al., 2004).

The MMP family currently consists of 28 enzymes with somewhat different capacities. The members are generally divided into si× groups according to either structure or preferred substrates and referred to as interstitial collagenases (MMP-1, -8, and -13), gelatinases (MMP-2 and MMP-9), stromelysins (MMP-3, -10, -11, and -12), matrilysins (MMP-7 and MMP-26), membrane-type MMPs (MMP-14, -15, -16, -17, -24, and -25) and others (Bode, 2003; Maskos and Bode, 2003; Visse and Nagase, 2003). Although the groupings of MMPs are based on their abilities to degrade various proteins of the ECM, they also play other important roles such as the activation of cell surface receptors and chemokines (Stefanidakis and Koivunen, 2006). In addition, MMP-2 has proteolytic activity to specific targets within the cell to cause acute, reversible contractile dysfunction in cardiac disease (Schulz, 2007).

The basic structures of MMPs can be approximately divided into three structurally well-preserved domain motifs, including a catalytic domain, an N-terminal domain and a C-terminal domain (**Fig. 1-2A**). Zinc-dependent catalytic domain of MMP is similar with subtle structural differences among the substrate specific groups (Nagase and Woessner, 1999). The N-terminal domain (propeptide domain) contains a unique PRCG(V/N)PD sequence in which the cysteine residue interacts with the catalytic zinc atom in the active site, prohibiting activities of the MMPs. Thus, the interaction has to be disrupted to “open” the cysteine switch in the process of MMPs activation (Van Wart and Birkedal-Hansen, 1990), which is a critical step that leads to ECM breakdown (Carmeli et al., 2004). The C-terminal hemopexin domain of metalloproteinases has a four-bladed propeller structure and contributes to substrate

specificity **(Fig. 1-2B)** (Wallon and Overall, 1997). In membrane-type MMPs, the hemopexin domain contains a transmembrane domain for anchoring the protein in the membrane; besides, the hemopexin domain in MMP-2 also has a function in the activation of the enzyme (Morgunova et al., 1999).

The regulations of MMPs occur at many levels, including transcription (the major one), post-transcriptional modulation of mRNA stability, secretion, localization, zymogen (proenzyme) activation and inhibition of activity by natural inhibitors of MMPs, TIMPs (Brew et al., 2000).

Cell migration and tissue remodeling are two fundamental processes of embryogenesis. One group of MMPs in particular, gelatinases, are implicated in tissue remodeling and in enabling cell migration and invasiveness. Two forms of gelatinases have been extensively studied in cells, and both can degrade type IV collagen and interstitial collagens as well as many other ECM molecules.

1-3-2. Gelatinase A (MMP-2, Type II collagenase)

In 1978, Sellers et al. was the first team to separate a gelatinase activity from collagenase and stromelysin in culture medium from rabbit bone (Sellers et al., 1978). Next year, a similar enzyme, acting on basement membrane type IV collagen, was reported by Liotta et al (1979). Gelatinase was purified from human skin, mouse tumor cells, rabbit bone, and human gingiva. Human and mouse MMP-2 are secreted as 72 kDa proenzymes of 631 and 662 amino acids, respectively. The removal of the pro-domain can be initiated by serine proteases such as thrombin and activated protein C. The resulting mature and active enzyme consists of a catalytic domain which is interrupted by three contiguous fibronectin type II-like domains, participating in binding to the gelatin substrates of the enzyme, and a C-terminal, hemopexin-like domain (Lee et al., 1997) **(Fig. 1-2B)**. The fibronectin type II-like domain is a

feature of gelatinase. The complete sequence of the human MMP-2 was published by Collier et al. (2001)

1-3-3. Gelatinase B (MMP-9, Type V collagenase)

Krane (1994) detected a gelatinase activity in rheumatoid synovial fluid. Sopata and Wize (1979) described a gelatinase from human polymorphonuclear leukocytes . Purification was achieved in 1983 and sequencing of the cDNA in 1989. Human neutrophil MMP-9 commonly occurs as a complex with lipocalin, and this complex protects this extracellular matrix remodeling enzyme from autodegradation (Fernandez et al., 2005).

MMP-9 is secreted as a 92 kDa zymogen. Cleavage of pro-MMP-9 at near residue 87 results in activation of enzyme with a mass of approximately 82 kDa. Pro-MMP-9 is secreted by monocytes, macrophages, neutrophils, keratinocytes, fibroblasts, osteoclasts, chondrocytes, skeletal muscle satellite cells, endothelial cells, and various tumor cells. Pro-MMP-9 can be activated by MMP-3 or by certain bacterial proteinases. MMP-9 has prodomain, three fibronectin type II motifs in catalytic domain, and a hemopexin-like domain just like MMP-2.

MMP-9 is inhibited by α2-macroglobulin or by TIMP-1. An interesting phenomenon, still not fully understood, is the binding of TIMP-1 to proMMP-9 to form a complex before secretion out of the cell (Roderfeld et al., 2007).

1-3-4. Roles of MMPs in cardiac development

MMP-2 is the earliest MMP known to be expressed during heart development. The process of cardiac looping converts the single, straight tubular heart into a S-shaped tube and transforms the primitive heart chambers into their adult anatomical positions before cardiac septation is complete. During the process of building a single heart tube, MMP-2 is expressed in the endocardium, early differentiating cardiomyocytes, and dorsal mesocardium but is soon lost within the myocardium (Cai et al., 2000).

Cell proliferation is more pronounced within the left splanchnic mesoderm and left dorsal mesocardium. Blocking MMP-2 activity not only disrupts this asymmetric pattern of proliferation, it also randomizes the direction of cardiac looping and increases the incidence of dextrocardia (i.e., right-sided heart). Blocking MMP-2 activity also prevents midline fusion of the primitive heart tubes leading to cardiac bifida (Linask et al., 2005).

Some experiments support the postulate that MMP-2 has an important functional role in early cardiogenesis, cardiac cushion migration, and remodeling of the direction of cardiac looping (Cai et al., 2000; Linask et al., 2005).

Defects in cardiogenesis during the first three weeks of gestation are usually fatal; however, embryo could survive with anomalous developmental events occurring in later stage. These anomalies often manifest as great vessels or cardiac septal defects in neonates. The septation of the atria and ventricles and division of the cardiac outflow tract into the aorta and pulmonary artery requires the migration, proliferation, and differentiation of two distinct mesenchymal populations, i.e. endocardial-derived cushion cells and cardiac neural crest (NC) cells (Creazzo et al., 1998).

MMPs have been implicated in regulating epithelial-to-mesenchymal transitions (EMTs) which responsible for forming both populations of cells. MMP-2 is expressed by endocardial cells prior to and during the EMT of the endocardium and the neural crest cells detach from the neural epithelium. Perturbed MMP-2 expressions in these studies disturb the migration and tissue remodeling of cushion cells and NC cells. Both of them are important and participate in formation of atrioventricular and outflow tract regions of the developing heart (Alexander et al., 1997; Cai et al., 2000)..

Hyaluronan is abundant in the ECM of the pre-mesenchymal heart (**Fig. 1-3A**). Hyaluronan is an essential mediator of cell migration and invasion for proper heart

development. Hyaluronan relates to regulate the expression of multiple MMPs in several cell types and the transformation of endothelial cells to mesenchymal cells. Therefore, MMPs might play pivotal roles and involve in the endocardial and cardiac septation, and cardiac developing (Camenisch et al., 2002).

1-4. Tissue inhibitor of metalloproteinases

1-4-1. Structures and functions of tissue inhibitor of metalloproteinases

As the variable and complex functions of MMPs, one of the important mechanism for the regulation of the activity of MMPs is via binding to a family of homologous proteins referred to as the tissue inhibitors of metalloproteinases (TIMP-1 toTIMP-4). TIMPs can form 1:1 enzyme-inhibitor complexes to inhibit matrixins, and each member of TIMPs can inhibit almost every member of the MMP family. The two-domain TIMPs are of relatively small size, yet have been found to exhibit several biochemical and physiological/biological functions, including inhibition of active MMPs, proMMP activation, cell growth promotion, matrix binding, inhibition of angiogenesis and the induction of apoptosis (Woessner, 2001).

The TIMPs have the shape of an elongated contiguous wedge consisting of an N-terminal segment, an all-β-structure left-hand part, an all-helical center, and a β-turn structure to the right. The N- and the C-terminal halves of the polypeptide chain form two opposing subdomains, each domain being stabilized by three disulfide bonds (Bode et al., 1999; Brew et al., 2000) (**Fig. 1-3B**).

The N-terminal subdomain exhibits a so-called OB-fold, known for a number of oligosaccharide/oligonucleotide binding proteins. This region consists of a five-stranded β-pleated sheet of Greek-key topology rolled into a closed β-barrel of elliptical cross-section.

The narrower opening of this barrel is bounded by the sB-sC loop, while its wider exit is (in contrast to other OB-fold proteins) covered by an extended segment connecting strands sC and sD, designated as "connector." After leaving the barrel, the polypeptide chain passes two helices, forms a two-stranded β-sheet, runs through a wide multiple-turn loop, and terminates in a β-hairpin sheet. The last C-terminal residues do not exhibit a defined conformation and presumably form a flexible tail on the TIMP surface (**Fig. 1-3B**)

Although the TIMPs are similar to each other to the extent of 35-40% amino acids identity, these key similarities suggest a significant structural conservation. For example, all TIMPs contain 12 cysteine residues at conserved locations, and in the case of TIMP-1, it has been shown that these participate in the formation of six intrachain disulfide bonds and then stabilize the whole structure. The N-terminal halves of the TIMPs share the most amino acid identity, suggesting that this region may underlie the common property of MMP binding and inhibition; It is possible that the more variant C-terminal half of the TIMPs may subserve distinctive properties of each TIMP and in the case of TIMP-3 the C-terminal half contains the ECM-binding domain (Anand-Apte et al., 1996).

1-4-2. Tissue inhibitor of metalloproteinase-3

In 1983, TIMP-3 was first isolated as a transiently expressed 21 kDa protein in the ECM of transforming chick fibroblast cultures (Blenis and Hawkes, 1983). Peptide sequencing subsequently demonstrated homology to the TIMPs and its identity as a novel member of the family was confirmed by cloning of the chicken cDNA and demonstration of MMP inhibition (Staskus et al., 1991). Subsequently, cDNAs of human TIMP-3 had been isolated (Apte et al., 1994).

TIMP-3 is an effective inhibitor of MMPS, proved by gelatin reverse zymography assays (Staskus et al., 1991), and in assays using radiolabeled ECM substrates (Apte et al., 1995).

These assays have shown that TIMP-3 and TIMP-1 are equipotent in their ability to inhibit MMP-1, MMP-2, MMP-3, and MMP-9. Otherwise, TIMP-3 has poor aqueous solubility and a specific localization in the ECM in contrast to TIMP-1,2,and 4 (Pavloff et al., 1992). The ECM ligand of TIMP-3 has been suggested being the hyaluronic acid (Yang and Hawkes, 1992).The affinity of TIMP-3 for ECM and its expression in a number of epithelia led some researchers to propose that TIMP-3 might be a component of basement membrane.

TIMP-3 also has unique function of playing an role in inhibiting the endothelial cell responses to physiologically relevant antigenic agents such as VEGF and bFGF, inhibiting migration and preventing invasion and tube formation by endothelial cells in a collagen matrix (Qi et al., 2003). TIMP-3 is the only TIMP known to be related to a disease: mutation of certain cysteine residues to serine results in early blindness, a condition known as Sorsby's fundus dystrophy (Felbor et al., 1995).

1-5. B-type natriuretic peptide

Over the recent decades, one group of neurohormonal markers, including atrial natriuretic peptide (ANP), N-terminal proBNP (NT-proBNP), and B-type natriuretic peptide (BNP), has generated much interest in the evaluation and management of cardiovascular disease. C-type natriuretic peptide is produced by endothelial cells and macrophages, whereas ANP and BNP are derived from cardiac muscle. ANP and BNP act through the natriuretic A-type receptor. Each of the natriuretic peptides is cleared by the natriuretic C-type receptor and degraded by neutral endopeptidases. ANP is produced from a 126 amino acid precursor (pro-ANP) whilst BNP is synthesized as a 134 amino acid pre-pro form that is secreted as a 108 amino acid pro-BNP precursor. Proteolytic cleavage of these pro-natriuretic peptide precursors produces active ANP and BNP (**Fig 1-4**) as well as other fragments. The N-terminal end of pro-BNP (NT-pro BNP), which comprises residues 1–76, lacks biological

activity, but the cleavage products of pro-ANP are active (Goetze, 2012).

Both the ANP and BNP have beneficial compensatory actions including vasodilation, natriuresis, growth suppression and inhibition of both the sympathetic nervous system and the renin-angiotensin-aldosterone axis (Ruskoaho, 2003). ANP is derived from atrial as well as ventricular myocytes, and significant amounts can be released from cytoplasmic granules in response to relatively minor stimuli; on the other hand, nearly all circulating BNP is derived from ventricular myocytes, and its pulsatile synthesis and release is predominantly in response to ventricular volume, pressure and wall tension (Sullivan et al., 2005). Besides the above advantage, BNP also has longer half-life than ANP and B-type natriuretic peptide (BNP) has better diagnostic, prognostic, and therapeutic values in heart failure and other heart diseases (Ruskoaho, 2003).

As it is successfully applied in adult heart diseases, BNP has attracted increasing interests as a biomarker of VSD in the field of pediatric cardiology (Ozhan et al., 2007; Rademaker and Richards, 2005). Some researchers have stated that MMP-9 serves as potential biomarker of heart remodeling (Halade et al., 2013; Lopez et al., 2010). These findings also drew our attention to the correlations among levels of plasma BNP in VSD patients.

Table 1-1. Outlines of the information of MMPs and TIMPs SNPs defined and relationship with particular diseases

Targeting gene (position, dbSNP rs# cluster ID)	Genotypes	Relationship with particular diseases	References
MMP-1 (-1607, rs1799750)	G GG	Patients who had the MMP-1 2G/2G genotype had a 1.71-fold increased risk of lung cancer (95% confidence interval, 1.22-fold to 2.41-fold increased risk) compared with patients who had the 1G/1G genotype.	(Liu et al., 2011)
MMP-2 (-1059, rs17859821)	GG AG AA	MMP-2 rs17859821 A allele carriers had lower all cause death rate, cardiac death rate and MACE rate than did GG genotype carriers (OR = 0.655, 0.580, 0.705; p = 0.030, 0.008, 0.011).	(Hua et al., 2009b)
MMP-13 (-77, rs2252070)	AA AG GG	The allele and genotype frequencies of MMP-13-77A>G showed significant differences between Kawasaki disease patients with coronary artery lesions and without coronary artery lesions (p = 0.00989 and p = 0.00551, respectively).	(Ikeda et al., 2008)
MMP-9 (-1562, rs3918242)	CC CT TT	The presence of MMP-9 1562C>T allele was found to be associated with early-onset coronary artery disease (OR = 3.2, p = 0.001). The ECAD patients with MMP-9 1562C>T allele had higher MMP-9 activity (p = 0.001).	(Saedi et al., 2012)

MMP-9 (exon 10, rs2250889)	CC CG GG	There are significant differences in the genotype distributions for rs2250889 between diffferant groups, suggest that the G allele of MMP-9 polymorphism rs2250889 is overrepresented in patients with histologically confirmed Giant cell arteritis.	(Rodriguez-Pla et al., 2008)
MMP-9 (-1562, rs3918242)	CC CT TT	The -1562C>T polymorphism of MMP-9 gene is significantly associated with atrial fibrillation risk in Chinese Han patients with hypertensive heart disease. The -1562T allele which is associated with increased expression of MMP-9 might be a genetic risk for the development of AF in this cohort.	(Gai et al., 2009)
MMP-9 (exon 6, rs17576)	GG AG AA	The MMP-9 rs17576 genotype AG and GG appeared to be significant 'at-risk' genotypes for Pelvic organ prolapse (OR: 5.41, 95% CI: 1.17– 25.04, p = 0.031; OR: 5.77, 95% CI: 1.29–25.86, p = 0.0219).	(Chen et al., 2010)

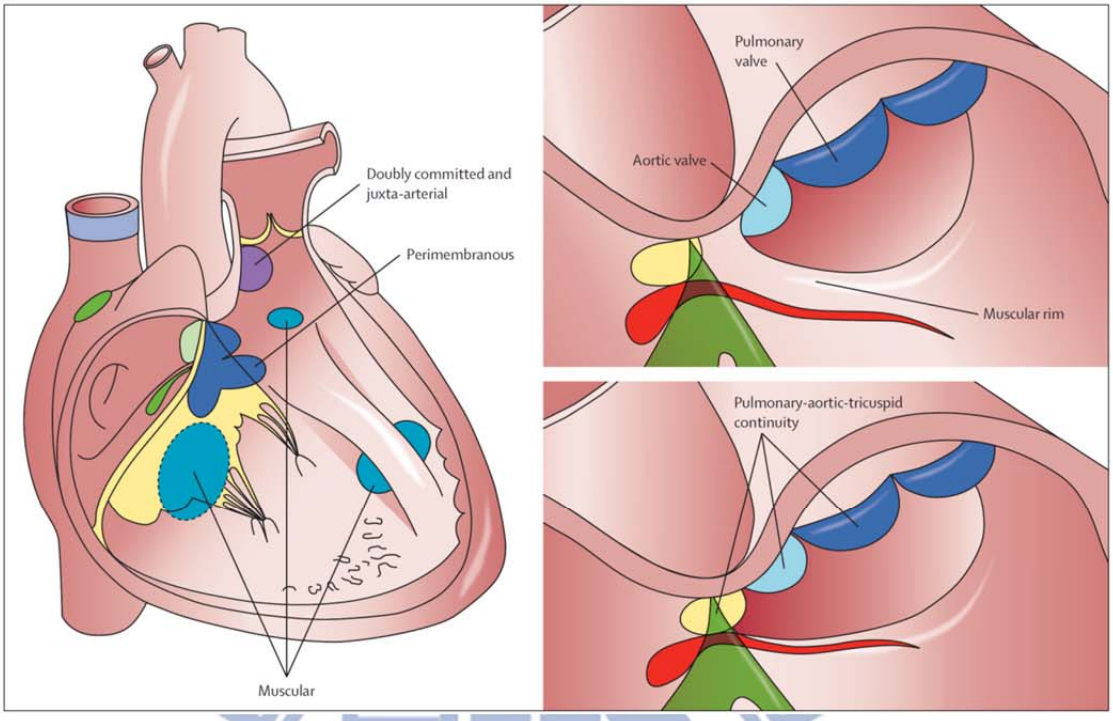
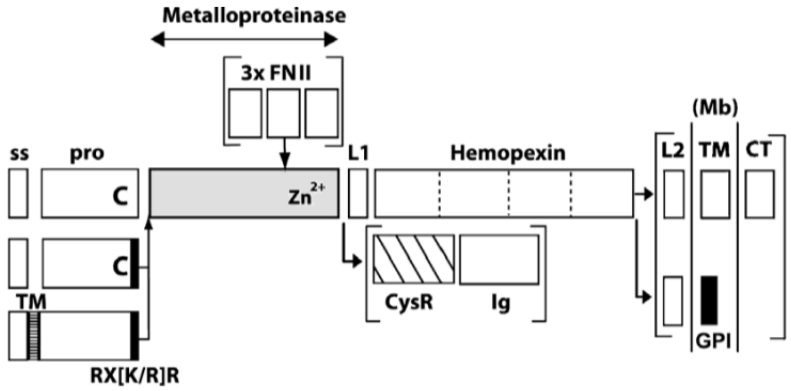


Figure 1-1. Location of different VSDs. Location of defects viewed from the right ventricle (left). Typical doubly committed and juxta-arterial defect (right upper). Doubly committed, juxta-arterial, and perimembranous defect (right lower) (Penny and Vick, 2011).

A.



B.

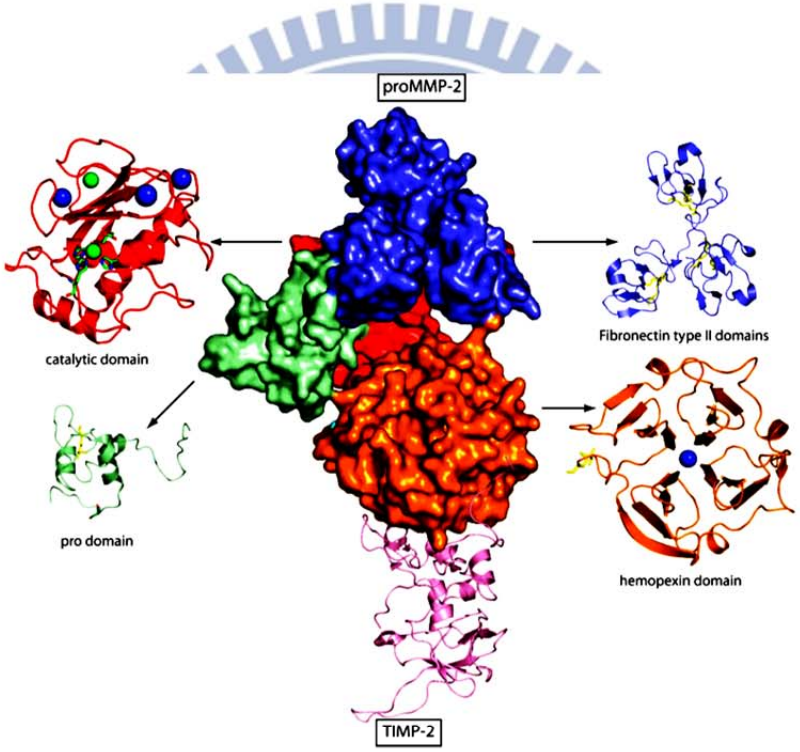
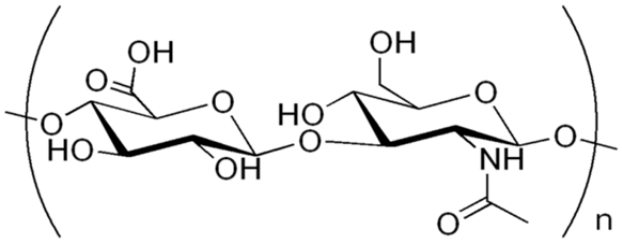


Figure 1-2. Domain structures of proMMP-2 are introduced here to stand for the general structures of MMP family. (A) A diagram of MMP family's scaffold. ss, signal sequence; pro, pro-domain, FNII, fibronectin type II motif; L1, linker1; L2, linker2; Mb, plasma membrane; TM, transmembrane domain; CT, cytoplasmic tail; CysR, cysteine rich; Ig, immunoglobulin domain; GPI, glycosylphosphatidylinositol anchor; C, cysteine; (B) The three-dimensional diagram of human proMMP-2. The pro-domain is shown in green, catalytic domain in red, fibronectin type II domain is blue, hemopexin domain in orange, and TIMP-2 in pink. Zinc ion is in green sphere, calcium ion in blue sphere and disulfide bonds in yellow (Murphy and Nagase, 2008).

A.



B.

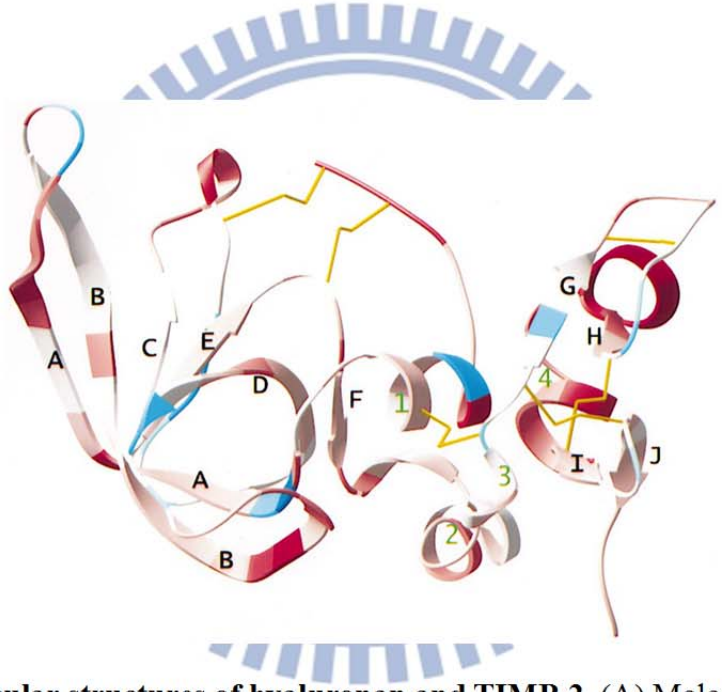


Figure 1-3. Molecular structures of hyaluronan and TIMP-2. (A) Molecular formula of hyaluronan, referring to all physiological forms of HA, the most common of which is the sodium salt. Hyaluronic acid (HA) is the simplest glycosaminoglycan (a class of negatively charged polysaccharides) and a major constituent of the extracellular matrix. It also has been suggested to being the ligand of TIMP-3 (Camenisch et al., 2002). (B) Ribbons depiction of the secondary structure of TIMP-2 based on 1BR9.pdb. Disulfide bonds are shown in yellow.

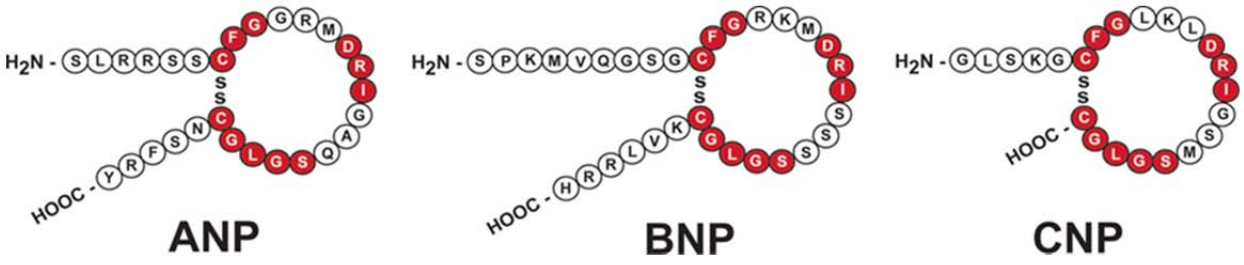


Figure 1-4. Schematic presentation of human atrial, B-type, and C-type natriuretic peptides. Homolog amino acid residues between the natriuretic peptides are highlighted with red circles (Goetze, 2012).

II. Research Strategy

Many researches documented the well-characterized MMP family intimately involved in normal tissue growth and ECM remodeling. Heart morphogenesis involves the characteristic mechanisms of cell proliferation, cell rearrangements, cell to cell, cell to matrix interactions, and tissue remodeling. MMPs family might regulate the coordination of these important processes, although little is known about the production, secretion, and clearance of these important proteinases throughout normal growth and development in human heart. VSD is the most popular and important congenital heart disease in pediatric cardiologic field. Morphogenesis and tissue growth, remodeling, and repair are sentinel features of childhood and adolescence. The hypothesis derived from literature research, in this study is that the regulation of MMPs and TIMP in ECM might participate the mechanism of VSD occurrence, and MMPs and TIMP might have important roles in mediating events associated with heart remodeling and recovering. To the best of our knowledge, this is the first study to ascertain the relationships between MMPs family and VSD. The aim of this study is investigating the association among different polymorphic genotypes of MMP-2, and MMP-9 with VSD occurrence. VSD patients were followed up for one year to figure out if these genomic polymorphisms related to the VSD spontaneous closure or not. Second, the VSD patients also were grouped according to different severities and tried to delineate the interaction of plasma MMP-2, MMP-9 and TIMP-3 activities involved in the occurrence of different VSD severities. Third, BNP was analyzed, which is the well-known cardiac biomarker, and was tested its relationship with MMP-2, MMP-9, and TIMP-3. This conducted study allowed us to realize the molecular interaction between ECM and congenital VSD, the most important pediatric cardiological problem. We also expected that this study could give us the insight of the role of MMPs and TIMPs in the heart morphological remodeling. The flowchart of reach strategies was present in **Fig. 2-1**.

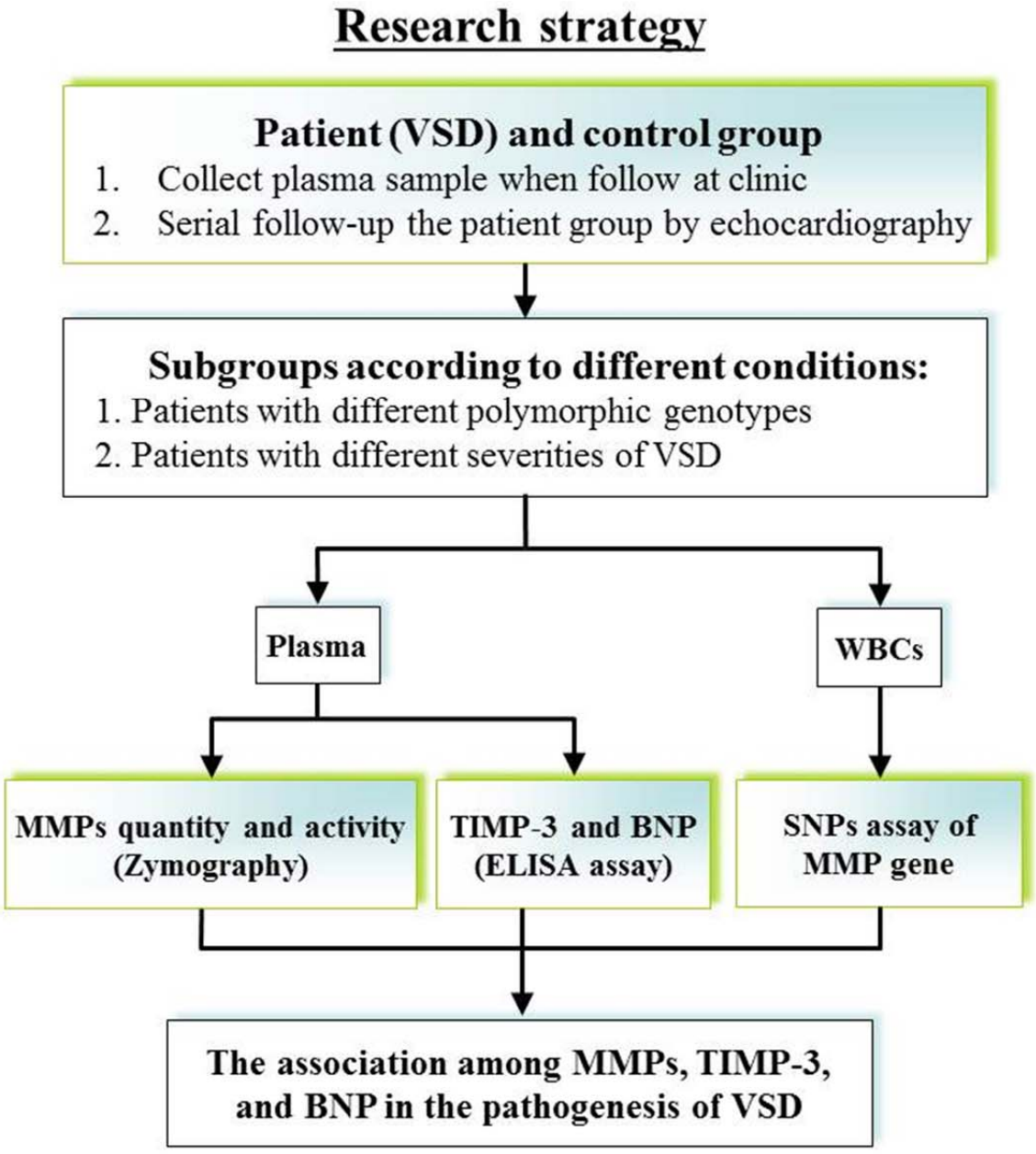


Figure 2-1. The flowchart of research strategies. The purpose of this study is to identify the association among different polymorphic genotypes of MMP-2, MMP-9 with VSD occurrence. Second, we also grouped the VSD patients according to different severities and tried to delineate the interaction of plasma MMPs and TIMP-3 activities involved in the occurrence of different VSD severities. Third, we also analyzed the BNP, the well-known cardiac biomarker, and tested its relationship with MMP-2, MMP-9, and TIMP-3.

III. Materials and Methods

3-1. Patients and sample collection

A total of 95 children (age range: 4.23 ± 2.51 years old) with perimembranous VSD were recruited from the outpatient base of the Taipei and Hsinchu Mackay Memorial Hospitals. There were 120 healthy individuals (age range: 5.53 ± 2.87 years old) were enrolled for gene polymorphism analysis. Age and gender distribution had no statistical difference between patient and control group. The study was performed with the approval of the Institutional Review Board of Mackay Memorial Hospital. Informed consent was obtained from the parents of all patients. The defects of all patients enrolled in this study were documented by 2-D echocardiography using a Phillips HP 4500 echocardiography machine with 5-8-MHz probe. Each patient received twice echocardiographys by two pediatric cardiologists for preventing interobserver variability and mean values of VSD sizes and aortic root diameters of patients were extracted. All the patients with defects enrolled in this study were documented by 2-D echocardiography. The plasma of 12 healthy children donated blood samples as a control group in the MMPs activity assays.

Blood samples were collected by needle aspiration from the antecubital vein. Plasma was isolated by centrifuge 3,000 × g for 10 min, and stored at −80°C for further zymography assay.

3-2. Patient grouping by VSD diameter/diameter of aortic root (Ao) ratio

Clinically, there is a wide range of parameters of a child's heart that depend on the patient's body size and age. Therefore, the VSD size can be normalized by the aortic root (Ao) diameter and the VSD/Ao ratio usually correlated with patients' clinical severities better than

VSD sizes did (Eroglu et al., 2003; Hornberger et al., 1989). Usually, VSD can be classified into three defect sizes: large, median and small sizes. The large defect is defined when the size is larger than two-thirds of the Ao; the median size is between two-thirds and one-third of the Ao; and small if the size is less than one-third of the Ao.

In our experimental design, we followed up the VSD patients regularly but very large VSD patients with poor medical control mandatorily undergo surgery. Therefore, patients in our study were exclusively those without extremely large defects. According to the definition of VSD/ Ao ratio, the patients were classified into three groups: VSD/Ao ratio $\leqq$ 0.2 as Trivial group; 0.2 < VSD/Ao $\leqq$ 0.3 as Small group; and 0.3 < VSD/Ao was considered as Median group.

3-3. Genomic DNA extraction

Genomic DNA for PCR-RFLP genotyping was isolated from the blood of 95 children by using the genomic DNA Mini kit. Up to 200 μL of whole blood was added with 30 μL of proteinase K (10 mg/mL) and mix briefly. After that, the mixture was incubated at 60°C for 15 min. Following then, 200 μL GB buffer was added and mixed by shaking vigorously, and incubated the mixture in a 60°C water bath for 15 min. A volume of 200 μL absolute ethanol was added to the sample lysate and immediately mixed by shaking vigorously. And then, the entire mixture (including any precipitate) was transferred to the GD Column and followed with centrifugation (at 14,000 × g for 5 min). The collections were added with 400 μL of W1 Buffer to the GD Column and centrifuged at 14,000 × g for 1 min. Then added 600 μL of Wash Buffer (ethanol added) to the GD Column and centrifuged at 14,000 × g for 1 min. The supernatant was displaced into new collection tube and centrifuged again at 14,000 × g for 3 min to dry the column matrix. Finally, 30 μL of pre-heated elution buffer (10 mM Tris-HCl,

pH 8.5) was added to the matrix and last for 5 min; after that, centrifuged at 14,000 × g for 1 min to elute the purified DNA. The maximum yield was up to 50 μg. Purified genomic DNA was stored in the −80°C.

3-4. Genotyping

3-4-1. MMP-2 -735C>T polymorphism

To analyze the -735C>T polymorphism, we amplified a region of the MMP-2 gene with the primers pair were shown in **Table 3-1**. The target sequence was amplified in a 50 μL reaction volume containing 1 μL of genomic DNA, 1 μL each primer (10 μM), 5 μL of 10X PCR buffer, 4 μL of 2.5 mM dNTPs, 0.5 μL of Taq Polymerase (Promega, Madison, WI, USA), and 37.5 μL distilled water. The PCR started with an initial activation step of 94°C for 2 min followed by 40 cycles of denaturation at 94°C for 30 sec, annealing at 60°C for 30 sec, and extension at 72°C for 30 sec, and a final step at 72°C for 7 min. The 297-bp fragment was purified with a purification kit (Invitrogen, Grand Island, NY, USA). Purified PCR products were then digested with *Sau* 96 I (New England BioLabs, Ipswich, MA, USA) at 37°C overnight and separated on a 2% agarose gel. *Sau*96 I does not digest the T allele but generates 202-bp and 95-bp fragments for the C allele.

3-4-2. MMP-9 -1562C>T polymorphism

The primers used to detect this polymorphism were shown in **Table 3-**1, which generated a 435-bp fragment containing the -1562C>T site in a 50 μL reaction volume. The annealing temperature was also shown in this table. The PCR product was then digested with *Sph* I (New England BioLabs) at 37°C overnight and separated on a 2% agarose gel. *Sph* I does not digest the C allele but generates 247-bp and 188-bp fragments for the T allele.

3-4-3. MMP-9 R279Q polymorphism

The primers used to detect this polymorphism were sense 5'-GGT GGG TGC TTC CTT TAA CA-3' and antisense 5' -GCG TTA GAG ACG TTG GAA CC-3' (Ikeda et al., 2008), which generated a 467-bp fragment containing the R279Q site in a 50 μL reaction volume, and the annealing temperature was shown in **Table 3-1**. The PCR product was then digested with *Sma* I (New England BioLabs) at 25°C overnight and separated on a 2% agarose gel. *Sma* I does not digest the A allele but generates 296-bp and 171-bp fragments for the G allele.

3-4-4. MMP-9 R574P polymorphism

The primers used to detect this polymorphism were shown in **Table 3-1**, which generated a 246-bp fragment containing the R574P site in a 50 μL reaction volume, and the annealing temperature was also shown in this table. The PCR product was then digested with *Nla* IV (New England BioLabs) at 25°C overnight and separated on a 2% agarose gel. Three genotypes could be distinguished: GG (246-bp), GC (246, 182 and 65-bp), and CC (182 and 65-bp).

3-5. Gelatin zymography assay

The activities of MMP-2 and MMP-9 in the plasma were measured by gelatin gel zymography as previously had described (Chen et al., 2008). Plasma was mixed with 2 X zymography sample buffer [0.125 M Tris-HCl, pH 6.8, 20% (v/v) glycerol, 4% (w/v) SDS, 0.005% bromophenol blue] incubated for 10 min at room temperature, and then loaded into SDS-PAGE which was performed in 7% acrylamide gels containing 0.1% (w/v) gelatin (Sigma-Aldrich, St. Louis, MO, USA). After electrophoresis under power supply of 100 V,

the gel was washed twice for 30 min in zymography renaturing buffer (2.5% Triton X-100) with gentle shake at room temperature to remove SDS, then incubated 18 h at 37°C in reaction buffer (50 mM Tris-HCl, pH 7.4, 200 mM NaCl, and 5 mM $CaCl_2$). The gels were then stained with coomassie blue for 30 min prior to destain with destain buffer (50% methanol, 10% acetic acid, and 40% ddH_2O). The presence of enzyme activity was evident by clear or unstained zones, indicating the action of the enzyme on the gelatin substrate (Stawowy et al., 2004). Gelatinase activity in the gel slab was quantified by Scion Image software (NIH, Bethesda, MD, USA), which quantifies the area of bands hydrolyzed by gelatinase. A MMP-2 or MMP-9 positive control (Chemicon, Temecula, CA, USA) was contained in each gel as a standard intensity value to normalize intensity of samples.

3-6. Determination of the TIMP-3 and BNP concentrations by enzyme-linked immunosorbent assay (ELISA)

TIMP-3 concentrations were measured by using an ELISA kit (cat# MTM100 R&D Systems, Minneapolis, MN, USA). According to the manufacturer's instructions, 100 μg of protein per sample was used. The plasma BNP concentration was determined by a commercially available immunoassay kit (Shionoria BNP assay kit; Shionogi Ltd, Osaka, Japan). Results obtained via spectrophotometry were compared against serial dilutions of known concentrations of the respective standards.

3-7. Statistical analysis

The levels of various markers in the VSD samples are expressed as the mean ± standard deviation (SD) or shown as the median (interquartile range; IQR). Data were analyzed by using SPSS for Windows (SPSS statistics package, Chicago, IL). All polymorphisms were

tested separately for confirmation with Hardy-Weinberg expectations in both groups. Differences between VSD patients and healthy control children were examined with the χ^2 test for categorical variables. To compare the quantitative MMP activities between two groups, Student's t-test was used for presumably normally distributed variables. One-way analysis of variance (ANOVA) method was used to test the differences in the MMPs enzyme activities among groups when the group numbers were more than two. A *p* value less than 0.05 was considered significant.

Table 3-1. Sequences of the primers used for MMP-2/-9 genotyping by PCR-RFLP

SNPs	Primers sequence (5→3)	PCR product size (bp)	Annealing temperature (°C)	Restriction enzyme	Length of restriction fragments (bp)
MMP-2 -735C>T	F-GGT GGG TGC TTC CTT TAA CA R-GCG TTA GAG ACG TTG GAA CC	297	60	*Sau96* I	CC (95 and 202 bp) CT (95, 202 and 297 bp) TT (297 bp)
MMP-9 -1562C>T	F-GCC TGG CAC ATA GTA GGC CC R-CTT CCT AGC CAG CCG GCA TC	435	62	*Sph* I	CC (435 bp) CT (435, 247 and 188 bp) TT (247 bp and 188 bp)
MMP-9 R279Q	F-TTC ACC CTC CCG CAC TCT GG R-GGT GGG CGG AGT CAC GGT C	467	65	*Sma* I	GG (171 and 296 bp) AG (171, 296 and 467 bp) AA (467 bp)
MMP-9 R574P	F-GCC CCT TCC TTA TCG CCG AC R-ACT CAG CCG CCC CTA CGT TTG	246	65	*Nla* IV	CC (65 and 182 bp) CG (65, 182 and 246 bp) GG (247 bp)

IV. Results

4-1. The information of patients and Patient grouping by VSD diameter/diameter of aortic root (Ao) ratio

Age and gender distribution of the 95 patients and 120 control children having MMP-2/-9 genetic polymorphisms analysis in this study were presented in **Table 2-1**. The mean age of all patients is 4.23 ± 2.51 (mean ± SD) years old, with no significant difference from the control group (5.53 ± 2.87 years) (**Table 4-1**).

All patients enrolled in this study were examined by two cardiologists using 2-D and Doppler echocardiography and got the average. The diameter of VSD was defined as VSD size, and ratio of VSD diameter and diameter of aortic root (Ao) was measured. According to the VSD/ Ao ratio, patients were classified into three groups, Trivial (VSD/Ao ratio = 0.14 ± 0.03), Small (0.25 ± 0.04) and Median (0.43 ± 0.10). Twelve control children having plasma MMP-2/-9 activities analysis and each subgroup (Trivial, Small and Median) are comprised of 12, 47, 29 and 19 patients, respectively (**Table 4-2**).

4-2. Genotype distribution and allele frequency of MMP-2 -735C>T

The genomic organization of the human MMP-2 gene and localization of our target SNPs were depicted in **Fig. A1A**. In this study, -735C>T polymorphism of MMP-2 was analyzed by PCR-RFLP. The sequence of the human MMP-2 gene and the position of the PCR primers that were used to detect MMP-2 -735C>T was shown in **Fig. A1B**. Restriction enzyme digestion using the restriction enzyme *Sau96* I generated three different length bands among different genotypes (**Fig. A1A**). We also used direct sequencing to detect MMP-2 polymorphism in some patients, and there are three genotypes of MMP-2 -735C>T shown in

Fig. A2B.

The genotypes and allele frequencies of MMP-2 -735C>T were recorded in **Table 4-3**. The frequencies of MMP-2 -735C>T genotypes and allele frequency in the VSD patients and control subjects should not conform to the Hardy-Weinberg equilibrium if there exist differences. As showed in **Table 4-3**, the allele frequencies of C and T allele are 66.3% and 33.7%; however, the results were similar to control group (p = 0.919). The genotype frequencies of MMP-2 -735C>T were CC: 47.4%, CT 37.9%, TT 14.7%. No significant difference was found in the patient and control groups (p = 0.143).

4-3. Genotype distribution and allele frequency of MMP-9 -1562C>T, R279Q and R574P

Localizations of the studied SNPs of human MMP-9 gene were shown in **Fig. A3, A5, A7**. MMP-9 gene contains 13 exons. MMP-9 -1562C>T, R279Q and R574P polymorphisms of MMP-9 are located on promoter region, exon 6 and exon 10 of MMP-9 gene, respectively. PCR-restriction fragment length polymorphism analysis was used for the genotyping of the MMP-9 -1562C>T, R279Q and R574P polymorphism in this study

4-3-1. MMP-9 -1562C>T polymorphism

The sequence of the human MMP-9 gene and positions of the PCR primers that were used to detect MMP-9 -1562C>T was shown in **Fig. A3B**. PCR products were digested by restriction enzyme *Sph* I and generated two different length bands between different genotypes (**Fig. A4A**). There are two genotypes of direct sequencing maps for MMP-9 -1562C>T shown in **Fig. A4B**.

The distribution of the MMP-9 -1562C>T genotypes were described in **Table 4-4**. Only 2 genotypes, CC and CT, of the MMP-9 polymorphism -1562C>T were detected in both the

VSD and control groups. Genotype distribution and allele frequency were analyzed by using the chi-square test and the results showed that there was no significant difference in the genotypes and allele frequencies between the VSD and control groups.

4-3-2. MMP-9 R279Q polymorphism

The sequence of the human MMP-9 gene and positions of the PCR primers for MMP-9 R279Q were shown in **Fig. A5B**. PCR products were digested by restriction enzyme *Sma* I and generated three different bands (AA genotype: 467-bp, AG genotype: 467-bp, 296-bp, and 171-bp, GG genotype: 296-bp and 171-bp) (**Fig. A6A**). **Fig. A6B** shows the direct sequencing map of three genotypes of MMP-9 R279Q.

The genotypes and allele frequencies of MMP-9 R279Q between VSD and control group were summarized in **Table 4-5**. With regard to the genotype distribution and allele frequency of the MMP-9 polymorphism R279Q, the genotypes G/G, G/A, and A/A were detected in 41.1%, 53.7%, and 5.3% of subjects in the VSD group, respectively, and in 57.5%, 35%, and 7.5% of subjects in the control group, respectively. The genotype distribution of the MMP-9 polymorphism R279Q in the VSD group was significantly different compared to the control group (p =0.023); however, there was no significant difference in the allele frequency between the VSD (G: 67.9% and A: 32.1%) and the control (G: 75% and A: 25%) groups.

4-3-3. MMP-9 R574P polymorphism

The sequence of the human MMP-9 gene and positions of the PCR primers that were used to detect MMP-9 R574P are shown in **Fig. A7B**. PCR products were digested by restriction enzyme *Nla* IV and generated three different length bands among different genotypes (**Fig. A8A**). There are three genotypes of MMP-9 R574Q in the direct sequencing maps (**Fig. A8B**).

The genotypes and allele frequencies of MMP-9 R574P are reported in **Table 4-6**. The

results show that the homozygous C/C, heterozygous C/G and homozygous G/G genotypes of MMP-9 R574P are 52.5%, 38.3% and 9.2% in the control group. Otherwise, the patient groups are 48.4 %, 47.4 % and 4.2 %, respectively. There are no significant differences in genotypes and allele frequencies between two conditions.

4-4. Plasma MMPs activity in the VSD children with different MMP polymorphisms

In order to investigate the differences of MMPs activities in VSD patients of different MMP polymorphisms, we used the gelatin zymography to detect MMP-2 and MMP-9 activity in plasma.

4-4-1. MMP-2 activity in genotypes of -735C>T

The activities of MMP-2 enzyme in the different genotypes of MMP-2 -735C>T polymorphism were compared with the Control group in **Fig. 4-1** [CC, CT, TT genotypes *vs.* Control group: 808 (642–980), 806(617–996), 869 (581–1044) ng/mL *vs.* 554 (407–689) ng/mL]. CC; CT; and TT genotypes of MMP-2 -735 C>T alleles contain 45, 36, and 14 samples, respectively. We used standard MMP-2 as positive control (Chemicon, Temecula, CA, USA) to calibrate MMP-2 activity of each group. MMP-2 activity has no significant difference in three genotypes of MMP-2 -735 C>T polymorphism. All three genotypes have higher activities level than control group. (p = 0.01)

4-4-2. MMP-9 activity in genotypes of -1562C>T, R279Q and R574P

We also investigated the MMP-9 activities of VSD patients in different MMP-9 SNPs, including MMP-9 -1562C>T, MMP-9 R279Q and MMP-9 R574P.

The activities of MMP-9 enzyme in the different genotypes of MMP-9 -1562 C>T polymorphism were compared with the Control group in **Fig. 4-2A** [CC, CT genotypes *vs.*

Control group: 31.8 (24.6–38.2), 33.9(24.6–42.8) ng/mL *vs.* 29 (25–32) ng/mL]. These two genotypes of MMP-9 -1562 C>T include 72 and 23 patients, respectively. We test the data through Student's t-test and there is no significant difference of MMP-9 enzyme activities between different MMP-9 -1562C>T genotypes.

The results of MMP-9 polymorphism R279Q were shown in **Fig. 4-2B**. Each group of GG, GA, and AA has 40, 50, 5 samples and activities of them are 32.5 (25.8 – 39.0), 31.7 (24.1 - 39.1), 37.5(31.7– 45.6) ng/mL. **Fig. 4-2C** shows the part of MMP-9 R574P. Three groups of CC, CG, GG contain 47, 44, 4 samples and the results are 32.5 (24.6 – 41.5), 31.7 (24.2 – 38.2), 37.2(27.4 – 46.0) ng/mL, respectively. We used standard MMP-9 as positive control (Chemicon, Temecula, CA, USA) to calibrate MMP-9 activity of each group. As the data shown, MMP-9 activity had no significant difference in these MMP-9 genotypes of these two polymorphisms.

4-5. Relationship between plasma MMPs activity and VSD severity

Fig. 4-3 shows the echocardiograms of a normal people and a perimembranous VSD patient. In clinically, large VSD patients with poor medical control are mandatory to surgery. So, our patients conducted in this study were brought into focus on the VSD patients without extreme large defects. First, we used VSD and VSD/Ao ration as the independent variables and determined the relationships between circulating MMP-2 and MMP-9 activities and VSD defects defined by VSD size and VSD/Ao ratio. According to the definition of VSD/aortic root (Ao) ratio, the patients were further classified into three groups: VSD/Ao ratio $\leqq$ 0.2 is trivial group; 0.2 < VSD/Ao $\leqq$ 0.3 is small group; and 0.3 < VSD/Ao $\leqq$ 0.66 is considered to median group. Control group and each subgroup contain 12, 47, 29 and 19 patients, respectively. The MMPs activity was detected by zymography, and the results are shown in

next two paragraphs.

4-5-1. Relationships between MMPs activities and VSD defects

For the plasma MMP-2 activity, it did not show significant correlations with VSD size and VSD/Ao ratio (**Fig. 4-4A** and **4-4B**). Whereas, positive correlations between MMP-9 activity and VSD size ($r^2 = 0.073$, $p < 0.05$) as well as VSD/Ao ratio ($r^2 = 0.170$, $p < 0.05$) were evaluated (**Fig. 4-4C** and **4-4D**).

4-5-2. Relationships between MMPs activities and VSD defects grouping

MMP-2 activity in different VSD size groups

The MMP-2 activities in children with normal and different VSD groups were determined by gelatin zymography. The activities of MMP-2 enzyme in the VSD groups were compared with the Control group in Figure 13A (Median, Small, Trivial groups vs. Control group: 845 (710 – 945), 840 (618 – 1,050), 787 (604 – 935) ng/mL vs. 554 (407 – 689) ng/mL). The results revealed that the circulating MMP-2 activity was significantly increased in the VSD patients. Compared with the Control group, the MMP-2 activity of Trivial, Small and Median groups were enhanced by 1.4 ($p < 0.01$), 1.5 ($p < 0.01$) and 1.5 ($p < 0.01$) folds, respectively (**Fig. 4-5A**). However, differences in the plasma MMP-2 activity among the VSD groups were insignificantly.

4-5-3. MMP-9 activity in different size groups

The activities of plasma MMP-9 was also determined by gelatin zymography and shown in **Fig. 4-5B** (Median, Small, Trivial groups vs. Control group: 38 (32 – 43), 35 (26 – 43), 28 (19 – 35) ng/mL vs. 29 (25 – 32) ng/mL). A trend of increased MMP-9 activity in the VSD groups was found. However, only the MMP-9 activity in Median group was significantly higher than that in Control group ($p < 0.01$).

4-6. Correlations between plasma MMP-2, MMP-9 activity, concentrations of TIMP-3 and BNP in VSD patients

4-6-1. TIMP-3 in different VSD patients and MMPs polymorphisms

The concentrations of plasma TIMP-3 were also determined by ELISA, and similar to MMP-9 (**Fig. 4-6A**), there seemed to be a trend for a correlation between TIMP-3 concentration and VSD/Ao ratio; however, the result did not reach the significance threshold ($r^2 = 0.04$, $p = 0.054$, shown in **Fig. 4-6C**). The TIMP-3 concentrations are no significant difference among VSD grouped by VSD/Ao ratio. [Median, Small, Trivial groups vs. Control group: 1853 (1222 – 2019), 1748 (1092 – 1827), 1800 (1135 – 1859) pg/mL vs. 1230 (1045 – 1462) pg/mL].

The TIMP-3 ELISA assay showed that the concentration of circulating TIMP-3 was higher concentration in subjects of the CC genotypes of MMP-2 -735 C>T and the QQ genotype than in those with other genotypes of the MMP-9 R279Q polymorphism (**Fig. 4-7**)

4-6-2. BNP in different VSD patients and MMPs polymorphisms

The plasma BNP concentration was determined by an immunoassay. There was a positive trend between plasma BNP concentration and VSD/Ao ratio ($r^2 = 0.36$, $p < 0.001$ shown in **Fig. 4-6B**), similar to MMP-9. BNP concentrations were not significantly different among the different MMP-2, and MMP-9 polymorphisms investigated in this study, as shown in **Fig. 4-8B**, although each genotypes of MMP-2/-9 had higher BNP concentration than control group.

4-7. Pearson's correlation between MMP-2/-9, TIMP-3, and BNP

Pearson's correlation coefficient showed that there was correlation between plasma activity of MMP-9 and TIMP-3 concentration (r = 0.567, $p < 0.001$) (**Fig. 4-9A**) and also of MMP-9 and BNP (r = 0.463, $p < 0.001$) (**Fig. 4-9B**). In the contrast, there was no correlation between MMP-2 and TIMP-3 (r = 0.178, $p = 0.139$) (**Fig. 4-9C**); MMP-2 and BNP (r = 0.185, $p = 0.07$) (**Fig. 4-9D**). As shown in **Fig.4-9E**, there was no correlation between TIMP-3 and BNP concentration (r = 0.151, $p = 0.130$).

4-8. Relationship between MMPs activity and spontaneous closure rate of VSD

Patient groups were followed one year later by echocardiography. Totally 82 patients returned to the outpatient department for the programmed echocardiographic examinations. After one-year follow-up, a total of 14 spontaneous closures occurred (17%) and 68 patients remained their defects. The plasma MMP-2 activity of VSD patients in the spontaneous closure group was compared with non-closure group, and no significant difference was noted [791 (617 – 945) ng/mL vs. 808 (624 – 990) ng/mL, $p = 0.825$] (**Fig. 4-10A**). In contrast, the plasma MMP-9 activity of VSD patients in the spontaneous closure group was significantly higher than non-closure group [42 (32 – 48) ng/mL vs. 31 (25 – 38) ng/mL, $p < 0.01$] (**Fig. 4-10B**).

Table 4-1. VSD patients' age and control group' age, gender, VSD sizes, and VSD/Ao ratio

	VSD group (n = 95)	Control group (n = 120)
Male: Female	42 : 53	62 : 58
Age (years)	4.23 ± 2.51	5.53 ± 2.87
BNP (pg/mL)	35.5 ± 21.7	15.7 ± 9.5
VSD size (mm)	3.98 ± 1.40	–
Ao (mm)	18.92 ± 5.29	–
VSD/Ao ratio	0.23 ± 0.12	–

Data are expressed as mean ± SD; BNP, B-type natriuretic peptide; VSD, ventricular septal defect; VSD size, diameter of the VSD; Ao, diameter of the aortic root; VSD/Ao ratio, ratio of the diameter of the VSD and the diameter of the aortic root

Table 4-2. Patient grouping by VSD diameter/diameter of aortic root (Ao) ratio

	Control group (n=12)	Trivial group (n=47)	Small group (n=29)	Median group (n=19)
Male: Female	6:6	21:26	14:15	7:12
Age (years)	4.76 ± 2.74	4.06 ± 2.26	4.16 ± 2.66	4.84 ± 2.96
VSD size (mm)	-	3.06 ± 0.88	4.30 ± 0.85	5.75 ± 1.21
VSD/Ao ratio	-	0.14 ± 0.03	0.25 ± 0.04	0.43 ± 0.10

Data were expressed as mean ± SD; VSD, ventricular septal defect; Ao, aortic root diameter

Table 4-3. Genotype distribution and allele frequency of MMP-2 -735C>T polymorphism

SNP	Control (n = 120)	VSD (n = 95)	OR (95% CI)	p value
	n (%)	n (%)		
MMP-2 -735C>T				
CC	48 (40.0)	45 (47.4)	Reference	
CT	60 (50.0)	36 (37.9)	0.6 (0.16-1.14)	0.143
TT	12 (10.0)	14 (14.7)	1.24 (0.52-2.58)	0.662
C allele	156 (65.0)	126 (66.3)	0.96 (0.64–1.43)	0.919
T allele	84 (35.0)	64 (33.7)		

VSD, ventricular septal defect; SNP, single-nucleotide polymorphism; OR, odds ratio

Table 4-4. Genotype distribution and allele frequency of MMP-9 -1562C>T polymorphism

SNP	Control (n = 120)	VSD (n = 95)	OR (95% CI)	p value
	n (%)	n (%)		
MMP-9 -1562C>T				
CC	94 (78.3)	73 (76.8)	1.09 (0.57–2.08)	0.79
CT	26 (21.7)	22 (23.2)		
TT	0	0		
C allele	214 (89.2)	166 (88.3)	1.09 (0.60–1.99)	0.78
T allele	26 (10.8)	22 (11.7)		

VSD, ventricular septal defect; SNP, single-nucleotide polymorphism; OR, odds ratio

Table 4-5. Genotype distribution and allele frequency of MMP-9 R279Q polymorphism

SNP	Control (n = 120) n (%)	VSD (n = 95) n (%)	OR (95% CI)	p value
MMP-9 R279Q				
GG	69 (57.5)	39 (41.1)	Reference	
GA	42 (35)	51 (53.7)	0.47 (0.26–0.82)	0.023
AA	9 (7.5)	5 (5.3)	0.98 (0.31–3.14)	0.98
A allele	60 (25)	61 (32.1)	1.41 (0.92–2.15)	0.11
G allele	180 (75)	129 (67.9)		

VSD, ventricular septal defect; SNP, single-nucleotide polymorphism; OR, odds ratio

Table 4-6. Genotype distribution and allele frequency of MMP-9 R574P polymorphism

SNP	Control (n = 120) n (%)	VSD (n = 95) n (%)	OR (95% CI)	p value
MMP-9 R574P				
CC	63 (52.5)	46 (48.4)	Reference	
CG	46 (38.3)	45 (47.4)	1.34 (0.77–2.35)	0.31
GG	11 (9.2)	4 (4.2)	0.50 (0.15–1.66)	0.25
C allele	172 (71.7)	136 (72.3)	0.97 (0.63–1.48)	0.89
G allele	68 (28.3)	52 (27.7)		

VSD, ventricular septal defect; SNP, single-nucleotide polymorphism; OR, odds ratio

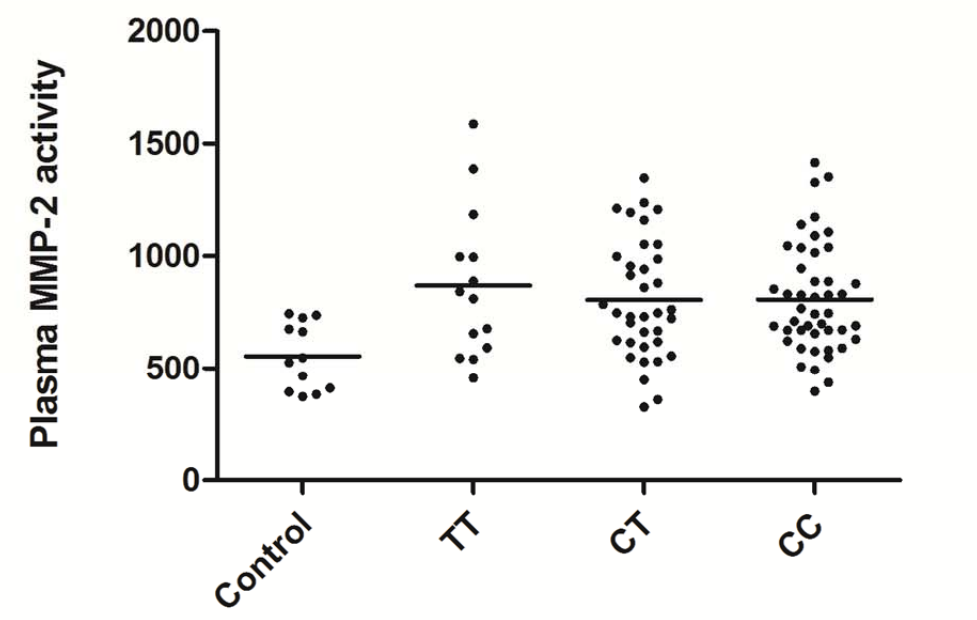


Figure 4-1. Plasma MMP-2 activities in patients of different MMP-2 SNP alleles. The plasma MMP-2 activities of three different genotypes of MMP-2 -735C>T, were detected by gelatin zymography. As the data shown above, there is no significant difference among MMP-2 SNP genotypes, although TT allele has the highest MMP-2 activity in MMP-2 -735C>T group.

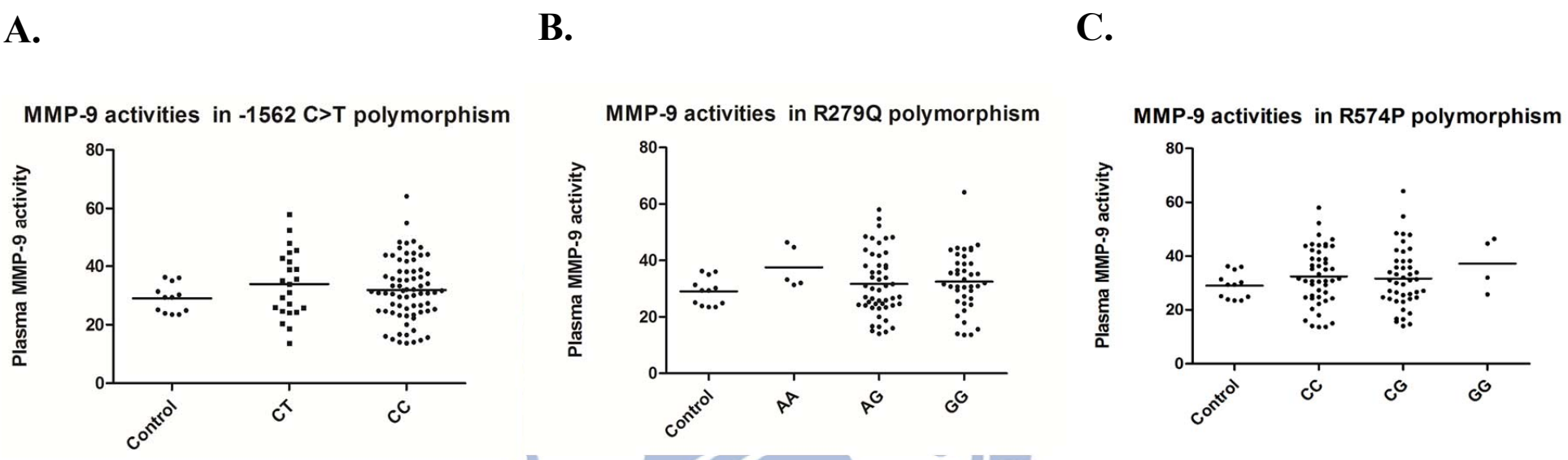
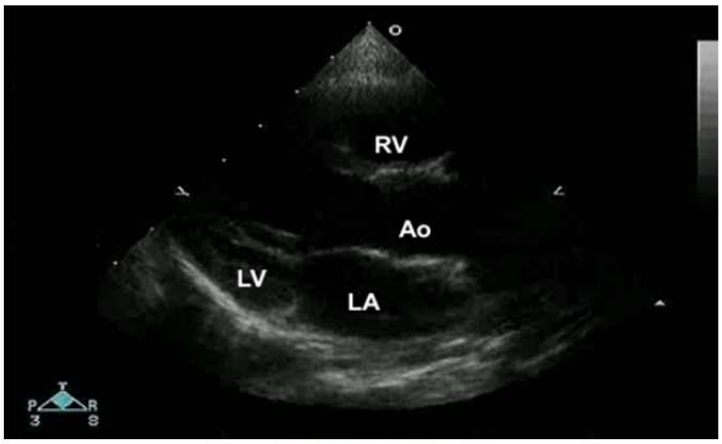


Figure 4-2. Plasma MMP-9 activities in the VSD children of -1562C>T, R279Q and R574P. (A) MMP-9 -1562C>T; (B) MMP-9 R279Q; (C) MMP-9 574P. As data shown, MMP-9 activities have no significant difference between VSD patients in these three subgroups of MMP-9 polymorphisms.

A.



B.

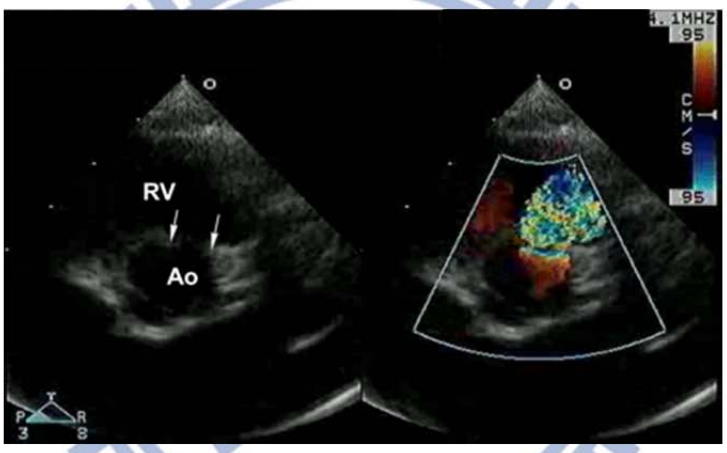


Figure 4-3. Echocardiography discerns normal and a patient of VSD. (A) A normal child; (B) A patient of medium size of perimembranous VSD. The white arrows indicate the site of VSD. LA, left atrium; LV, left ventricle; RV, right ventricle; Ao, aortic valve.

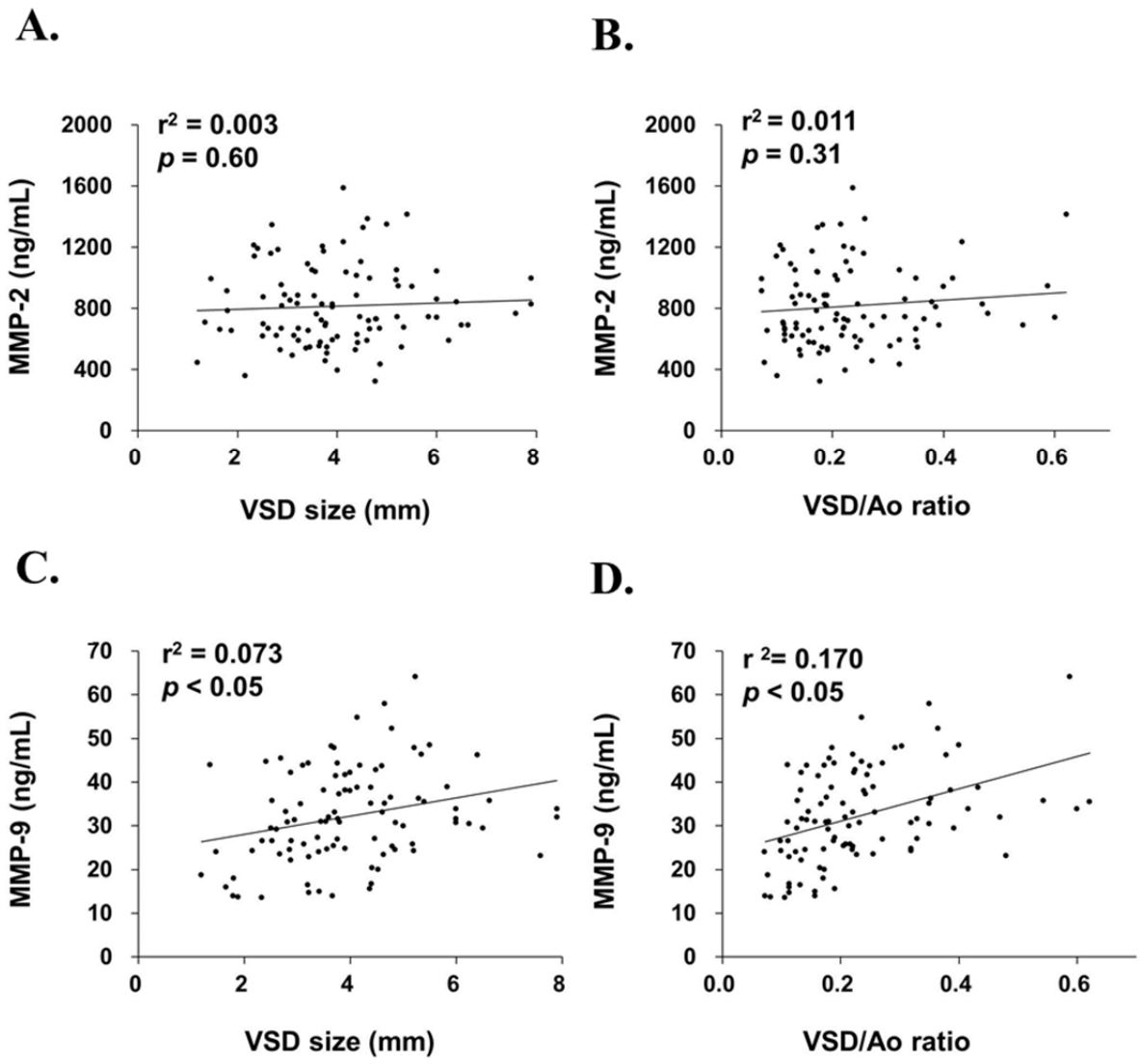


Figure 4-4. Correlations between circulating MMPs activities and VSD size or VSD/Ao ratio in the children patients. Pearson's correlation analysis (SPSS statistics package, Chicago, IL) was applied. Plasma MMP-2 activity in the VSD patients was insignificantly correlated with the (A) VSD size and (B) VSD/Ao. Whereas, MMP-9 activity was significantly and positively correlated with the VSD defects size (C) and VSD/Ao (D) ($p < 0.05$).

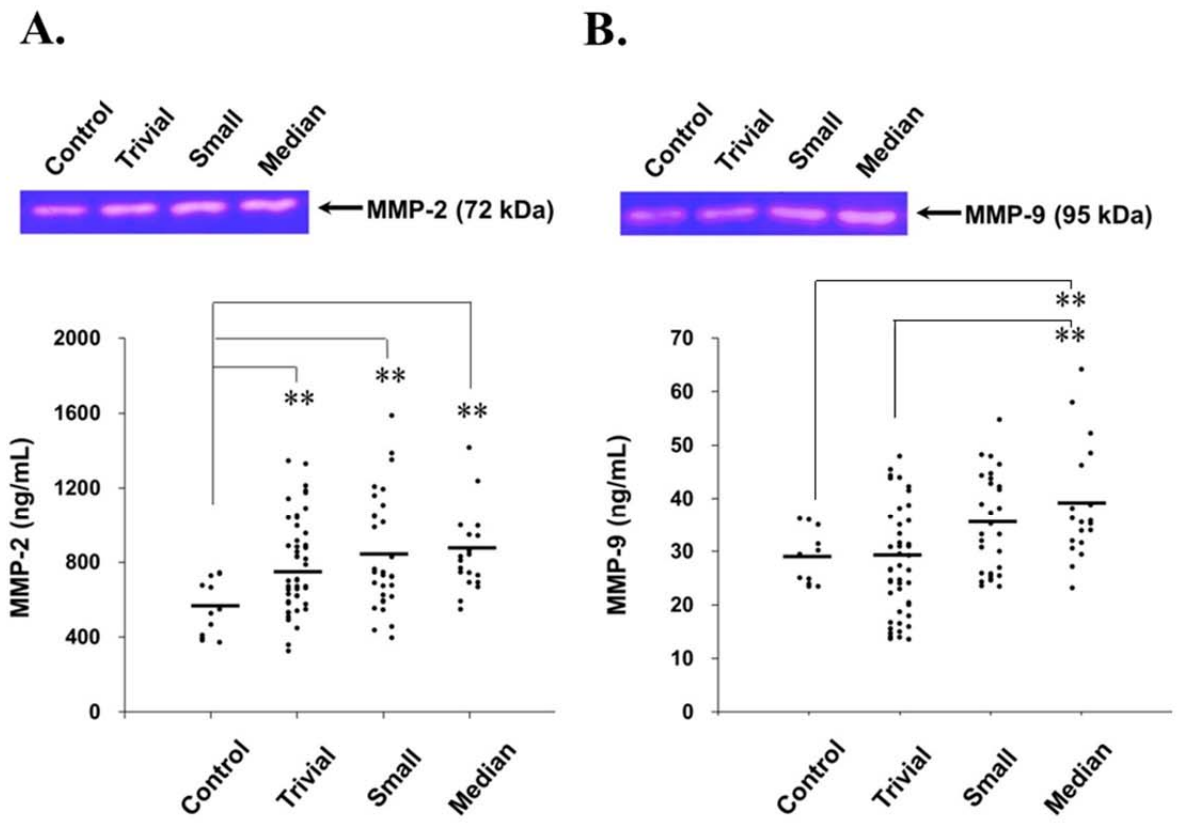


Figure 4-5. Circulating MMP-2 and MMP-9 activities in the patients with different levels of VSD severity. The MMP-2 (A) and MMP-9 (B) activities in the patients with different VSD severity were determined by gelatin zymography. The gelatinase activities detected in this study were based on pro-MMP-2 (72 kDa) or pro-MMP-9 (95 kDa). Each symbol represents one individual, and horizontal bars represent mean value in each group. The compared results of the MMP-2 or MMP-9 activity in the Control (n = 12), Trivial (n = 47), Small (n = 30) and Median (n = 19) groups with one way ANOVA analysis were performed. ** indicate $p < 0.01$, compared to the Control group.

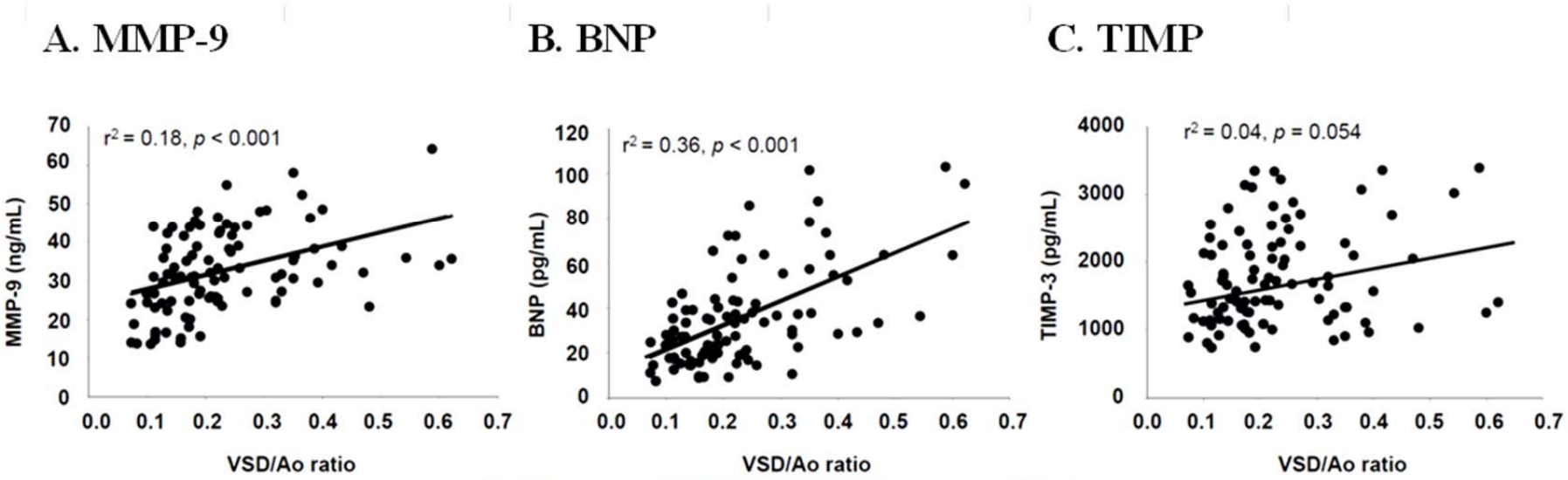


Figure 4-6. Plasma levels of MMP-9 (A); BNP (B); and TIMP-3 (C) in VSD patients with different VSD/Ao ratio. There is apparent positive trend between plasma MMP-9 activity and VSD sizes calibrated by Ao root diameter. There is also similar trend noted in the group of plasma BNP concentration and VSD/Ao ratio; TIMP-3 seemed to have a positive trend for a correlation VSD/Ao ratio similar to MMP-9; however, the results did not reach the significance threshold.

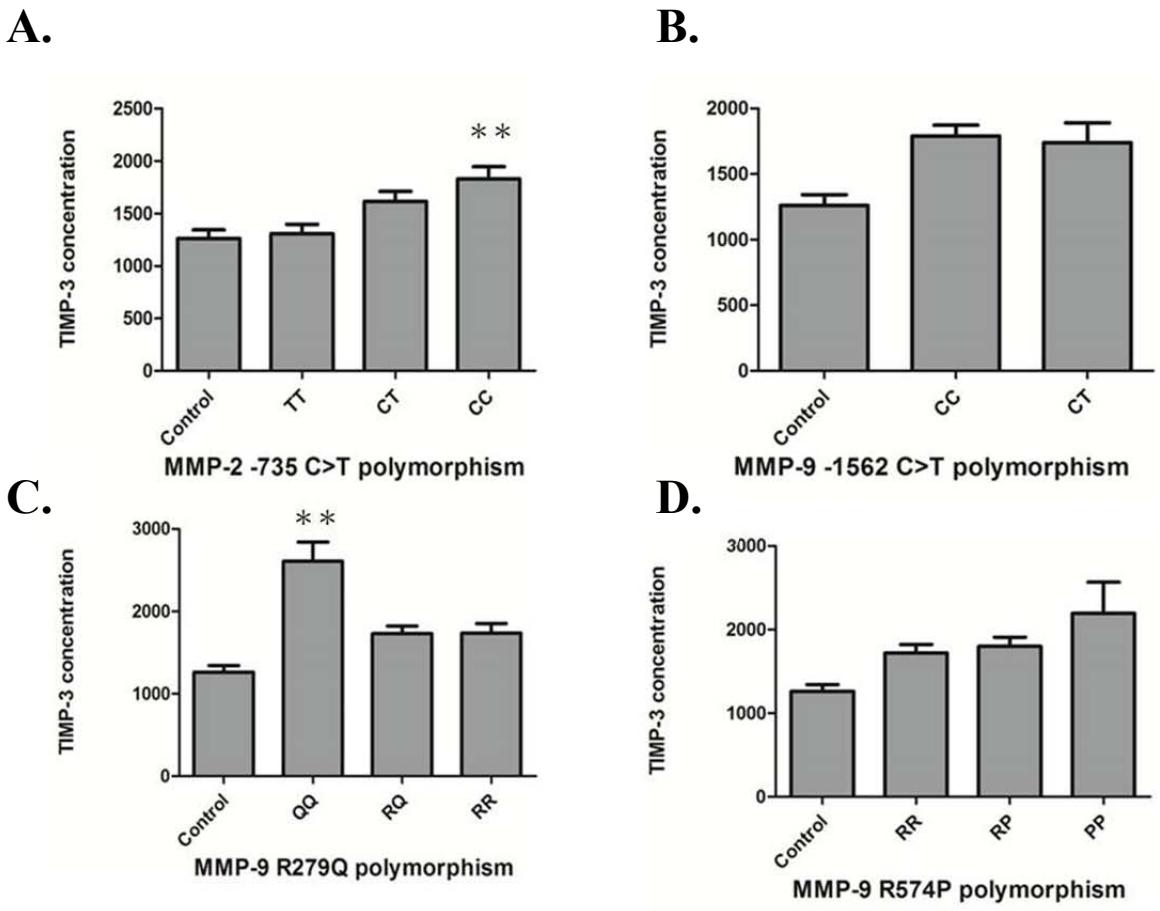


Figure 4-7. Plasma TIMP-3 concentrations in MMP-2/-9 polymorphisms. (A)TIMP-3 concentration in genotypes of MMP-2 -735 C>T polymorphism. (B-D) TIMP-3 concentration in genotypes of MMP-9 -1562C>T, R279Q and R574P polymorphisms. TIMP-3 has higher concentration in CC genotypes of MMP-2 -735 C>T and in the QQ genotype of MMP-9 R279Q polymorphism..

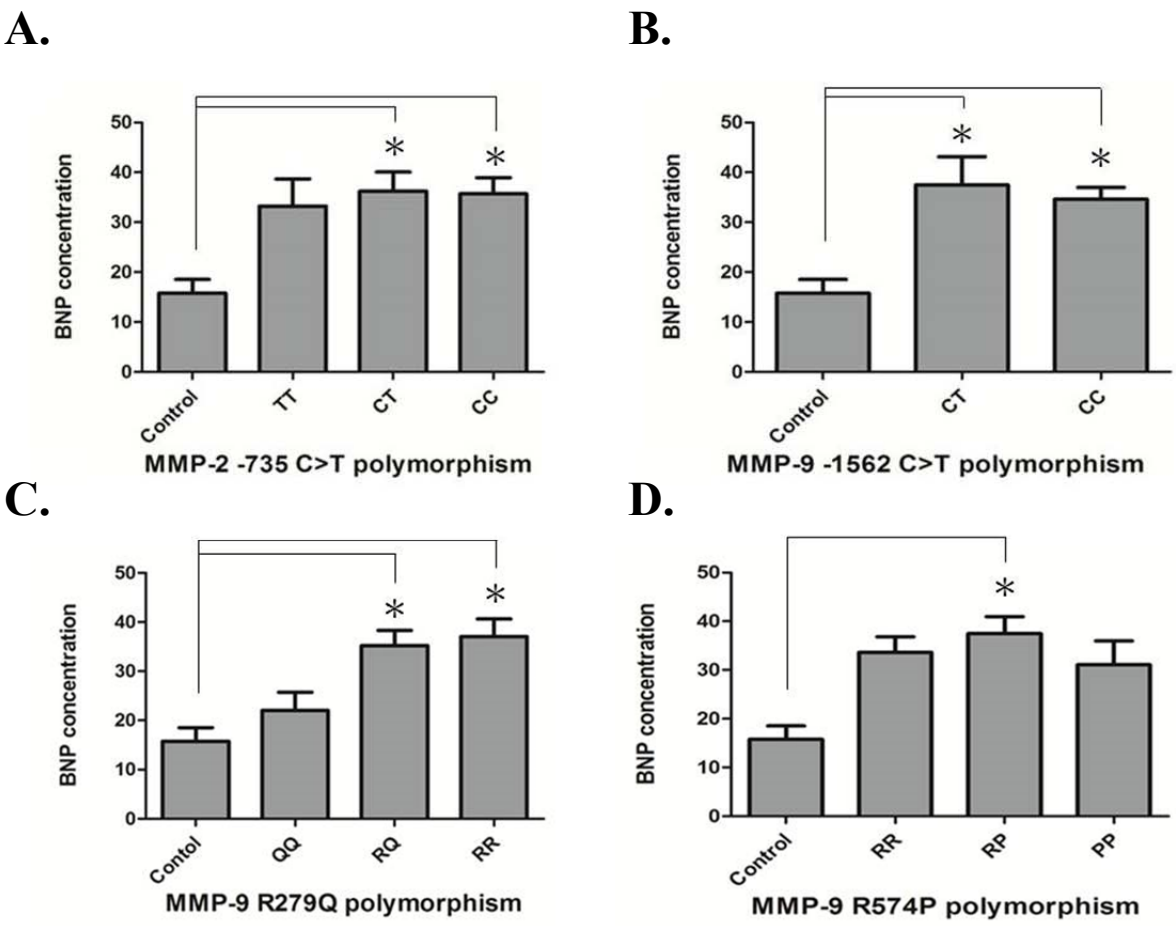


Figure 4-8. Plasma BNP concentrations in MMP-2, 9 polymorphisms. (A) BNP concentration in genotypes of MMP-2 -735 C>T polymorphism. (B-D) BNP concentration in genotypes of MMP-9 -1562C>T, R279Q and R574P polymorphisms. The concentration of circulating BNP had no significant difference in genotypes of MMP-2/-9 polymorphism although each genotype had higher BNP concentrations than control group.

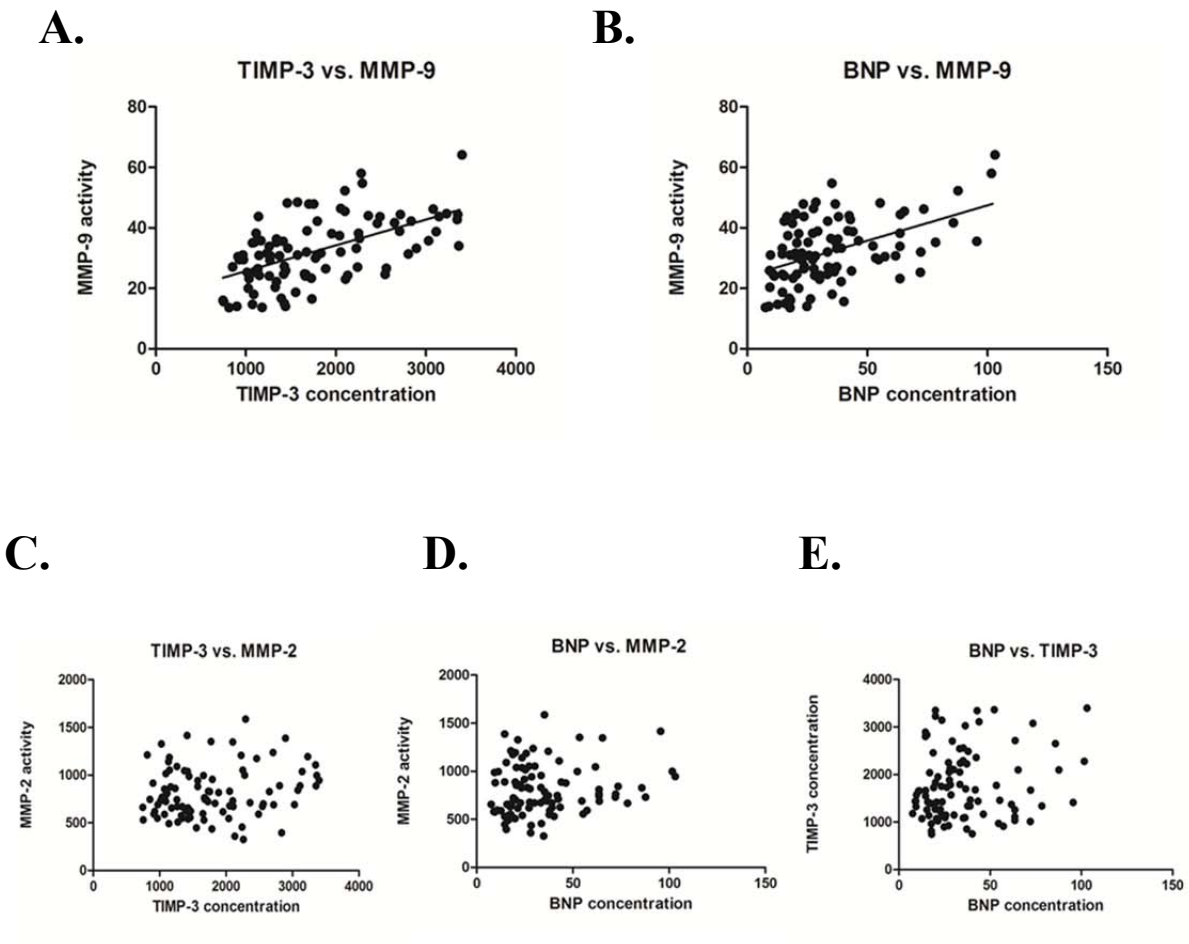


Figure 4-9. Pearson's correlation between MMP-2/-9, TIMP-3, and BNP. Pearson's correlation coefficient showed positive correlation between plasma activity of (A) MMP-9 and TIMP-3 concentration ($r = 0.567$, $p < 0.001$) and (B) MMP-9 and BNP ($r = 0.463$, $p < 0.001$); (C, D, E) there was no correlation between MMP-2 and TIMP-3, MMP-2 and BNP, and TIMP-3 and BNP concentration.

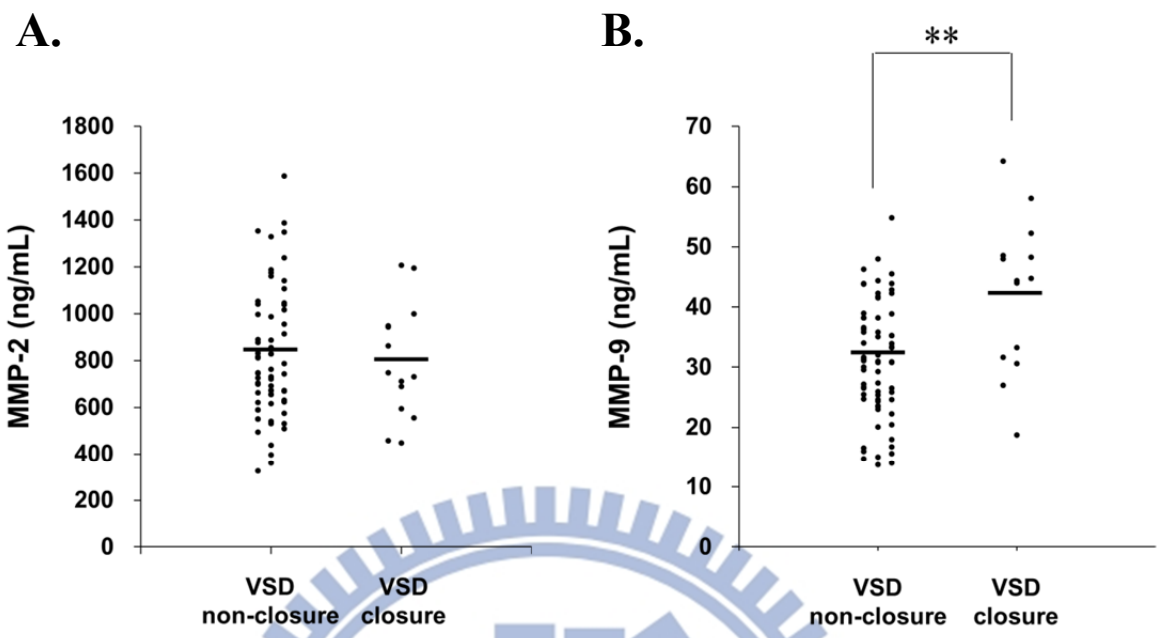


Figure 4-10. Circulating MMPs activities in the patients received serial echocardiographic follow-up examinations. The MMP-2 (A) and MMP-9 (B) activities in the plasma from each VSD closure and non-closure patient were indicated, with horizontal bars representing mean values in each group. ** indicates $p < 0.01$.

V. Discussion

As we know, the history of MMPs is more than 50 years from the beginning of first MMP member (MMP-1) purified from the tails of tadpoles by Gross and Lapiere in 1962 (Gross and Lapiere, 1962). Many researchers designed different experiments, cloned and sequenced MMP genes, in order to figure out the structures and try to understand the mechanisms of the big MMPs family contribute to normal physiology and disease pathology. It is generally accepted that optimal cardiac structure and function are maintained by a tightly regulated myocardial microenvironment. ECM assembles the structural framework and provides a dynamic microenvironment in which molecular signals, including growths factor and cytokines, converge to dictate the cell's behavior and conduct a well-coordinated heart function (Stefanidakis and Koivunen, 2006). Linask et al. (2005) documented MMP activity regulates the coordination of early heart organogenesis by affecting ventral closure of the heart and gut tubes, asymmetric cell proliferation in the dorsal mesocardium to drive looping direction, and ECM degradation within the dorsal mesocardium allowing looping to proceed toward completion. But nowadays, there is still little known about the production, secretion, and clearance of these important proteinases throughout normal growth and development in human heart.

Contreras-Ramos et al. (2008) presented that the interventricular septum has both mesenchymal and muscular components, and the mesenchymal element originates mainly from fusion of the conotruncal and atrioventricular endocardial cushions. There are at least two proposed mechanisms of the initiate development of the muscular septum. The first process is that the muscular septum forms from coalescence of the part of the ventricular wall is interposed between the enlarging free walls of the developing right and left ventricles; therefore, as the ventricular cavities become deeper the septum grows passively inward (Goor et al., 1970). The second process is an alternative hypothesis suggesting that the muscular

septum originates from a cluster of cells and the so-called primitive inter-ventricular septum, which expands actively towards the cushions of the atrioventricular canal (Srivastava and Baldwin, 2008). If some faults happened during these processes, the development of ventricular septum will be imperfection.

5-1. Gene Polymorphisms of MMP-2/-9 in VSD patients

Now, a recent PubMed search using the term ”MMP [ti]” lists more than two thousands articles within the last 5 years. To realize MMPs polymorphism, we also search the National Center for Biotechnology Information (NCBI)-SNP database, and found over 700 SNPs of MMP-2 and 300 SNPs of MMP-9 registed. The relatively important and correlative literatures were organized and shown in **Appendix 1** for MMP-2 and **Appendix 2** for MMP-9.

Gene polymorphism also is considered as an essential cause of VSD formation, especially the Nkx 2.5, TBX5 and GATA4 genes. SNPS of them are frequently discussed. TBX5 is a vital gene during embryonic differentiation that affects cardiac and upper limbs development (Basson et al., 1997). A TBX5 polymorphism is also associated with ventricular septal defect in the Chinese Han population (Liu et al., 2009). There are many researches point out that NKx2.5 and GATA4 genes mutations related to the occurrences of ASD (atrial septal defect) and VSD (Matsuoka, 2007; Rajagopal et al., 2007; Zhang et al., 2009). The expanding literatures also indicate the other genes like VEGF and NFATc1 also contribute to the process of VSD formation (Gu et al., 2011; Xie et al., 2007; Zhang et al., 2009). These genes’ expressions in the heart and their interactions are necessary for proper cardiac septation (**Fig. 5-1**) (Srivastava and Baldwin, 2008). To the best of our knowledge, this is the first research studying on the association among MMPs SNPs and MMPs activities to the mechanism of VSD formation and closure.

As we shown above, MMPs play important roles in many physiological functions and MMP polymorphisms have proven to be relative to many diseases. Among many ways to regulate the gene expression of different MMPs, a major mechanism is the sequence change in the promoter region, that is important for the transcription and causes effect in protein level and cell physiology (Vincenti and Brinckerhoff, 2007).

Therefore, two MMPs polymorphisms in promoter region were selected, MMP-2 -735C>T and MMP-9 -1562 C>T. The MMP-2 promoter polymorphism, located at nucleotide -735, destroys a Sp1-binding element and the T allele is associated with significantly diminished promoter activity (Yu et al., 2004). The polymorphism of promoter region of MMP-9 results in losing a nuclear repressor protein binding site and decreasing MMP-9 expression as the T allele is present, and thus increasing the enzyme expression compared to the C allele (Zhang et al., 1999).

As we know, non-synonymous SNPs in the coding region of a gene produce an amino acid change, which could also affect the protein tertiary structure and as a consequence, the biophysical and biochemical activity. Hence, we also examined SNPs in MMP-9/rs17576 (Q279R) and rs2250889 (R574P). SNP of MMP-9 rs17576 (Q279R) modifies an amino acid residue within the highly conserved gelatinase-specific fibronectin type II domain (FN2) which is the catalytic domain of the MMP-9 enzyme encoding the sequence required for binding of the enzyme to its substrate. An amino acid exchange in this region of the gene might affect the binding capacities of the protein to its substrate and have functional change (Allan et al., 1995).

SNP rs2250889 locates in the C-terminal hemopexin-like domain and represents a transition of C to G at nucleotide 1740 in exon 10 of the MMP-9 and is associated with the substitution of proline for arginine at 574 residue. This is a substantial change, because proline is a cyclic nonpolar amino acid, whereas arginine is basic and positively charged. The

hemopexin domain of MMP-9 is important for association with TIMP-1 and TIMP-3. In addition, the hemopexin domain of MMP-9 had a high affinity-binding site for gelatin. Although the specific functional consequences of the substitution of proline for arginine in MMP-9 have not been studied yet, the location of SNP rs2250889 in the C-terminal hemopexin-like domain might lead to an important protein structural modification. This structural modification could either decrease the affinity of MMP-9 for its specific inhibitors, resulting in a higher level of protein activity, or enhance the affinity binding for gelatin which might increase its gelatinase activity (Rodriguez-Pla et al., 2008).

According to the basis mentioned above, we selected these SNPs to try to underlie the relationship of MMP and VSD. The results showed that genotype distribution and allele frequencies of MMP-2 -735C>T between the VSD and the control group had no significant differences. This result suggests that C>T polymorphism located at nucleotide -735 may not influence the incidence of VSD in Taiwan individuals (**Table 4-3**).

The results of polymorphism in the promoter of MMP-9 -1562 C>T showed no correlation of this genotype to VSD. However, there was no VSD patient had TT genotype of MMP-9 -1562 C>T in our study. We speculate that the lack of the TT genotype of this MMP-9 polymorphism can be explained by racial differences, which is supported by other disease studies conducted in Asian populations. There are other reports where only few or even none of the participants were homozygous (TT) for the MMP-9 -1562 C>T polymorphism (Buss et al., 2009; Chen et al., 2010).

In the aspects of two non-synonymous MMP-9 substitutions, our data demonstrated that there is an association of the MMP-9 Q279R polymorphism and risk of VSD. There are other papers conclude the relationship of MMP-9 Q279R and many clinical diseases, such as melanoma, lumbar-disc herniation, type 2 diabetes, and pelvic organ prolapse (Ahluwalia et al., 2009; Chen et al., 2010; Cotignola et al., 2007). The importance of the R279Q

polymorphism is based on its ability to change the MMP-9 protein structure and its substrate binding affinity, which is related to many diseases mentioned above. The number of subjects with the GA genotype was significantly higher in the VSD group than in the control group. Although there were few subjects with the AA genotype in the VSD group, there was an obvious difference in the genotype distribution when compared to the control group. We found the interesting fact, and the correlation of MMP-9 Q279R non-synonymous substitution and VSD is worth to work out.

However, we didn't find significant differences in both the genotype and allele distribution between the VSD and control groups in Taiwanese population of MMP-9 R574P substitution, which Blankenberg et al. (2003) had presented that the G allele of MMP-9 R574P polymorphism was overrepresented in patients with histologically confirmed giant cell arteritis.

5-2. Plasma activities of MMP-2/-9 in VSD patients and their relation with spontaneous closure rate of VSD

Plasma MMP-2 activities of VSD patients were also examined by zymography, categorized by their different genotypes (CC, CT, and TT) of MMP-2 -735 polymorphism and conducted in statistic. As mentioned earlier, the MMP-2 C>T polymorphism located at nucleotide -735 should significantly diminish promoter activity. However, the statistical results in this study showed that there was no significant correlation between MMP-2 -735 genotypes and their activity. The result also showed that the T allele of MMP-2 -735 polymorphism has the highest MMP-2 activity. Our results were different from the results of Yu C et al. (2004) that they described C allele could enhance MMP-2 protein transcription and corresponded to higher MMP-2 activity.

The polymorphism at position -1562 was expected to change the promoter activity of MMP-9 (Van den Steen et al., 2002; Zhang et al., 1999); however, our results showed no relationship between the genotypes of this polymorphism and MMP-9 promoter activity. Our results are in agreement with those of Demacq et al. (2006) who obtained plasma samples from healthy subjects.

Surprisingly, despite the different genotypes of the Q279R or R574P polymorphism, there were no significant differences in MMP-9 activity in genotypes of MMP-9 R279Q and R574P polymorphisms, indicating that these SNPs may have effects on protein structure and substrate binding efficiency, but have no notable effect on enzyme activity.

VSD and VSD/Ao were used as the independent variables and tried to disclosure the relationship between MMP-2/-9 and different VSD severities. There is a notable finding that MMP-9 but not MMP-2 activities show the increasing tendency with different VSD defect levels. The VSD patients were further categorized into three levels, Trivial (VSD/Ao ratio ≤ 0.2), Small (0.2 < VSD/Ao ≤ 0.3 and Median (VSD/Ao > 0.3) groups, according to the examinations of 2-D echocardiography. The patients with large VSD were excluded since surgery is mandatory for such patients with poor medical control. There is a notable finding that circulating MMP-2 and MMP-9 activities, but not circulating TIMP-3 concentrations bear a positive association with the levels of VSD severity. This is the first study to specifically identify the relationships between circulating MMP-2 as well as MMP-9 and severity of VSD in children patients. In the literature search, the elevated level of amniotic fluid MMP-9 has been reported in ASD cases (Abdallah et al., 2012). However, no investigation addressing the changes of circulating MMPs in the children patients either with ASD or VSD has ever been documented.

The rate of spontaneous closure of VSD has been reported to be between 11% and 70.8% in various researches (Ahunbay et al., 1999; Kidd et al., 1993; Mehta and Chidambaram,

1992). In our study, spontaneous closure rate was detected in 17 % of perimembranous defect. This variation may be due to study population, criteria for diagnosis, and methods of investigation, follow-up period, and the percentage of different types of VSD.

In several studies, it was proposed that ventricular septal aneurysm is an important mechanism of closure and shows a more favorable prognosis in perimembranous defects. (Freedom et al., 1974; Ramaciotti et al., 1995). We had investigated on the circulating MMP-2 and MMP-9 activities in the VSD patients who have spontaneous closure and non-closure in this study. Although MMP-2 activity has no significant difference in the patients with different levels of VSD severity, MMP-9 activity showed significant difference related to the VSD closure. These data showed that spontaneous closure group has a higher level of circulating MMP-9 activity. Therefore, we proposed that *in vivo* proteases might play a role related to the spontaneous closure of VSD. It seemed to infringe the instincts; but with the expanding and growing data, some authors also suggest that MMPs may act as good candidates in helping physicians resolve some pathologic conditions (Hettiaratchi et al., 2007; Vincenti and Brinckerhoff, 2007).

We expect great difficulties in striving to realize the interaction between MMP family and mechanism of VSD because of their intricate and complicated roles in physiology and pathogenesis. There remains an urgent need to study further on this matter.

5-3. Correlations between plasma MMP-2/-9 activities, concentrations of TIMP-3 and BNP

For the purpose of exporting the relationships between MMP-2/-9 and TIMP; therefore, the activity of circulating MMP-2/-9 and the TIMP-3 concentrations were measured in VSD patients. Although there were no differences in the activity of circulating MMP-2/ -9 among the genotypes of each polymorphism, TIMP-3 concentrations were higher in subjects with the

CC genotype of MMP-2 -735 C>T, and QQ genotype of the MMP-9 R279Q polymorphism. We used Pearson's correlation for further inquiries. Plasma MMP-9 activity had positive correlation with TIMP-3 concentration.

TIMPs regulate the activity of zinc metalloproteases, and that TIMP-3 can form a tight complex with proMMP-2 and proMMP-9 to regulate gelatinase activities *in vivo* TIMP-3 also has unique characteristics of ECM-binding; it is highly expressed in the heart and involved in maintaining cardiac structure and function (Nagase and Woessner, 1999). In addition, MMP-9 and TIMP-3 are typical inducible ECM metabolic proteins, and the damage caused by ischemia-reperfusion injury may induce the synthesis of MMP-9 and TIMP-3 (Murphy et al., 1986). In heart diseases, TIMP-3 may contribute to the regulation of myocardial remodeling, it deficiency disrupts matrix homeostasis and causes spontaneous left ventricular dilation, cardiomyocyte hypertrophy and contractile dysfunction (Fedak et al., 2003). Our published paper proposed that the interaction between MMP-2, MMP-9 and TIMP-3 may contribute to atrial ECM remodeling of atrial fibrillation (Yong and Guoping, 2008). In addition, many studies also appear that the association of MMP-2, MMP-9 and TIMP-3 in heart diseases (Givvimani et al., 2010), heart remodeling (Lin et al., 2010; Yeh et al., 2013) and cardiocytes function properly (Manso et al., 2010). Although there is no former report investigating the relationship of circulating MMP-2, MMP-9 activity and TIMP-3 concentration in VSD patients, our report provides first insights into the roles of MMP-9 and its inhibitor in the complex network of ECM in VSD patients.

It is worth mentioning that BNP is secreted mainly in the ventricles in response to increased wall stress and plays important roles in maintaining cardiorenal homeostasis, which is primarily mediated by the second messenger cyclic guanosine monophosphate (Lee et al., 1997). Although the production of BNP and its role as local regulator of ventricular fibrosis are well established, the interaction of BNP in the regulation of the cardiac interstitium

remains unclear (Collier et al., 2001). Regarding the correlations observed in our work, we found not only that there is cross talk between MMP-9 and BNP but also both of them have a similar relationship with the severity of VSD. Interestingly, we can't find such a trend between MMP-2 and BNP. Furthermore, a previous study showed that gene expression and zymographic MMP-9 activity were significantly higher in BNP-transgenic mice than in non-transgenic mice (Sopata and Wize, 1979). These investigators suggested that transient MMP-9 expression induced by elevated levels of BNP during the earliest phase after myocardial infarction is a cardioprotective mechanism. On the basis of these findings and our results, we speculate that MMP-9 and BNP may act together in the pathogenesis of VSD.

Some limitations of our study merit considerations. First, our sample size is fairly modest, which would primarily affect the power of our analysis to detect significant associations. This is attributed to the difficulty in collecting children's blood samples. Second, patients were recruited from only one hospital system and may not be representative of the general population. However, we used incident cases to lessen the selection bias. Nevertheless, it would be important to confirm these findings in multicenter studies. Third, it is really a difficult task to disentangle the role of each gelatinase within a single study because of its complexity of regulation in biological network.

Last, the echocardiography studies of healthy controls and the patients enrolled were not carried out in this present study, which should be undertaken optimized pharmacological treatment and that may cause an effect on circulating MMP-9 activity and TIMP-3 concentration.

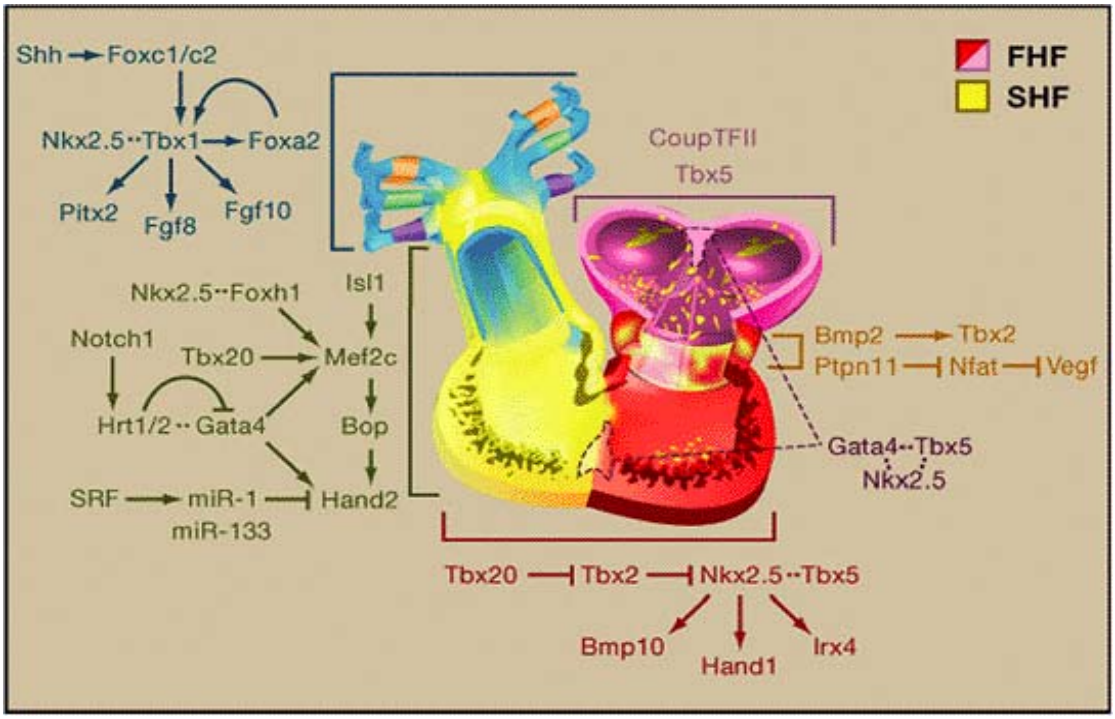


Figure 5-1. Pathways of regulating region specific cardiac morphogenesis. FhF, first heart field; SHF, second heart field (Srivastava and Baldwin, 2008).

VI. Conclusions

VSD is the most common congenital heart disease; however, the pathogenesis of VSD still remains vague. The potential role of MMP SNPs in regulation of ECM effector was proposed in this study. The results of our work indicate that the R279Q polymorphism of the MMP-9 gene is significantly associated with VSD risk in Taiwanese children. It is difficult to distinguish the role of each gelatinase because its complexity of regulation in biological network in a single study. However, this does not preclude that MMPs family could have its own pathophysiological significance in VSD patients. These findings supported the notion that connective tissue remodeling mediated by MMP-9 may play an important role in the mechanism of VSD formation, at least the MMP-9/R279Q polymorphism. To the best of our knowledge, this is the first evidence of any association between MMP-9 SNPs and VSD patients.

In the aspects of MMP-2/-9 activities, the results in this study demonstrate a trend between circulating MMP-2 and MMP-9 levels and different VSD defects. An interesting association that VSD patients with spontaneous closure had higher MMP-9 level than non-closure group also was observed. MMP-2 and MMP-9 activity might contribute to VSD severity, but more functional investigations still were needed to determinate the influences of MMP-2 and MMP-9 activities. Whether MMP-9 level provides a prediction in the ability of spontaneous closure of VSD will be assessed in additional studies.

It is intriguing that plasma MMP-9 activity and TIMP-3 concentration showed positive correlation and TIMP-3 concentration is higher in carriers of the QQ genotype of the MMP-9 polymorphism R279Q. MMP-9, and BNP also have positive correlation and both of them are positively correlated with the severity of VSD.

As more understanding of the genes responsible for pathogenesis of VSD, the more chance raised the prospects that the future of pediatric cardiology will involve more directed therapeutic and preventive measures.

VII. References

Abdallah MW, Pearce BD, Larsen N, Greaves Lord K, Nørgaard Pedersen B, Hougaard DM, Mortensen EL, Grove J. 2012. Amniotic fluid MMP-9 and neurotrophins in autism spectrum disorders: an exploratory study. Autism Res. 5:428-33.

Abou-Raya S, Naim A, Marzouk S. 2004. Cardiac matrix remodelling in congestive heart failure: the role of matrix metalloproteinases. Clin Invest Med. 27:93-100.

Ahluwalia TS, Khullar M, Ahuja M, Kohli HS, Bhansali A, Mohan V, Venkatesan R, Rai TS, Sud K, Singal PK. 2009. Common variants of inflammatory cytokine genes are associated with risk of nephropathy in type 2 diabetes among Asian Indians. PLoS ONE. 4:e5168.

Ahunbay G, Onat T, Celebi A, Batmaz G. 1999. Regression of right ventricular pressure in ventricular septal defect in infancy: a longitudinal color-flow Doppler echocardiographic study. Pediatr Cardiol. 20:336-42.

Alexander SM, Jackson KJ, Bushnell KM, McGuire PG. 1997. Spatial and temporal expression of the 72-kDa type IV collagenase (MMP-2) correlates with development and differentiation of valves in the embryonic avian heart. Dev Dyn. 209:261-8.

Allan JA, Docherty AJ, Barker PJ, Huskisson NS, Reynolds JJ, Murphy G. 1995. Binding of gelatinases A and B to type-I collagen and other matrix components. Biochem J. 309 (Pt 1):299-306.

Anand-Apte B, Bao L, Smith R, Zetter B, Iwata K, Olsen BR, Apte SS. 1996. A review of tissue inhibitor of metalloproteinases-3 (TIMP-3) and experimental analysis of its effect on primary tumor growth. Biochem Cell Biol. 74:853-62.

Apte SS, Mattei M-G, Olsen BR. 1994. Cloning of the cDNA encoding human tissue inhibitor of metalloproteinases-3 (TIMP-3) and mapping of the TIMP3 gene to chromosome 22. Genomics. 19:86-90.

Apte SS, Olsen BR, Murphy G. 1995. The gene structure of tissue inhibitor of metalloproteinases (TIMP)-3 and its inhibitory activities define the distinct TIMP gene family. J Biol Chem. 270:14313-8.

Ban HJ, Heo JY, Oh KS, Park KJ. 2010. Identification of type 2 diabetes-associated combination of snps using support vector machine. BMC genetics. 11:26.

Basson CT, Bachinsky DR, Lin RC, Levi T, Elkins JA, Soults J, Grayzel D, Kroumpouzou E, Traill TA, Leblanc-Straceski J, Renault B, Kucherlapati R, Seidman JG, Seidman CE. 1997. Mutations in human TBX5 [corrected] cause limb and cardiac malformation in Holt-Oram syndrome. Nat Genet. 15:30-5.

Beeghly-Fadiel A, Lu W, Shu XO, Long J, Cai Q, Xiang Y, Gao YT, Zheng W. 2011. MMP9 polymorphisms and breast cancer risk: a report from the Shanghai Breast Cancer Genetics Study. Breast Cancer Res Treat. 126:507-13.

Belo VA, Souza-Costa DC, Luizon MR, Lanna CMM, Carneiro PC, Izidoro-Toledo TC, Ferraz KC, Tanus-Santos JE. 2012. Matrix metalloproteinase-9 genetic variations affect MMP-9 levels in obese children. Int J Obes. 36:69-75.

Blankenberg S, Rupprecht HJ, Poirier O, Bickel C, Smieja M, Hafner G, Meyer J, Cambien F, Tiret L. 2003. Plasma concentrations and genetic variation of matrix metalloproteinase 9 and prognosis of patients with cardiovascular disease. Circulation. 107:1579-85.

Blenis J, Hawkes SP. 1983. Transformation-sensitive protein associated with the cell substratum of chicken embryo fibroblasts. Proc Natl Acad Sci USA. 80:770-4.

Bode W. 2003. Structural basis of matrix metalloproteinase function. Biochem Soc Symp:1-14.

Bode W, Fernandez-Catalan C, Grams F, Gomis-Ruth FX, Nagase H, Tschesche H, Maskos K. 1999. Insights into MMP-TIMP interactions. Ann N Y Acad Sci. 878:73-91.

Brew K, Dinakarpandian D, Nagase H. 2000. Tissue inhibitors of metalloproteinases: evolution, structure and function. Biochim Biophys Acta. 1477:267-83.

Brookes AJ. 1999. The essence of SNPs. Gene. 234:177-86.

Buss A, Pech K, Roelver S, Bloemeke B, Klotzsch C, Breuer S. 2009. Functional polymorphisms in matrix metalloproteinases -1, -3, -9 and -12 in relation to cervical artery dissection. BMC Neurol. 9:40.

Cai DH, Vollberg TM, Sr., Hahn-Dantona E, Quigley JP, Brauer PR. 2000. MMP-2 expression during early avian cardiac and neural crest morphogenesis. Anat Rec. 259:168-79.

Camenisch TD, Schroeder JA, Bradley J, Klewer SE, McDonald JA. 2002. Heart-valve mesenchyme formation is dependent on hyaluronan-augmented activation of ErbB2-ErbB3 receptors. Nat Med. 8:850-5.

Carmeli E, Moas M, Reznick AZ, Coleman R. 2004. Matrix metalloproteinases and skeletal muscle: a brief review. Muscle Nerve. 29:191-7.

Chen CL, Huang SK, Lin JL, Lai LP, Lai SC, Liu CW, Chen WC, Wen CH, Lin CS. 2008. Upregulation of matrix metalloproteinase-9 and tissue inhibitors of metalloproteinases in rapid atrial pacing-induced atrial fibrillation. J Mol Cell Cardiol. 45:742-53.

Chen HY, Lin WY, Chen YH, Chen WC, Tsai FJ, Tsai CH. 2010. Matrix metalloproteinase-9 polymorphism and risk of pelvic organ prolapse in Taiwanese women. Eur J Obstet Gynecol Reprod Biol. 149:222-4.

Cheong JY, Cho SW, Lee JA, Lee KJ, Wang HJ, Lee JE, Kim JH. 2008. Matrix metalloproteinase-3 genotypes influence recovery from hepatitis B virus infection. J Korean Med Sci. 23:61-5.

Collier IE, Saffarian S, Marmer BL, Elson EL, Goldberg G. 2001. Substrate recognition by gelatinase A: the C-terminal domain facilitates surface diffusion. Biophys J. 81:2370-7.

Collins FS, Brooks LD, Chakravarti A. 1998. A DNA polymorphism discovery resource for research on human genetic variation. Genome Res. 8:1229-31.

Contreras-Ramos A, Sanchez-Gomez C, Garcia-Romero HL, Cimarosti LO. 2008. Normal development of the muscular region of the interventricular septum--I. The significance of the ventricular trabeculations. Anat Histol Embryol. 37:344-51.

Cotignola J, Reva B, Mitra N, Ishill N, Chuai S, Patel A, Shah S, Vanderbeek G, Coit D, Busam K, Halpern A, Houghton A, Sander C, Berwick M, Orlow I. 2007. Matrix Metalloproteinase-9 (MMP-9) polymorphisms in patients with cutaneous malignant melanoma. BMC Med Genet. 8:10.

Creazzo TL, Godt RE, Leatherbury L, Conway SJ, Kirby ML. 1998. Role of cardiac neural crest cells in cardiovascular development. Annu Rev Physiol. 60:267-86.

Demacq C, de Souza AP, Machado AA, Gerlach RF, Tanus-Santos JE. 2006. Genetic polymorphism of matrix metalloproteinase (MMP)-9 does not affect plasma MMP-9 activity in healthy subjects. Clin Chim Acta. 365:183-7.

El-Gindy I, El Rahman AT, El-Alim MA, Zaki SS. 2003. Diagnostic potential of serum matrix metalloproteinase-2 and tissue inhibitor of metalloproteinase-1 as non-invasive markers of hepatic fibrosis in patients with HCV related chronic liver disease. Egypt J Immunol. 10:27-35.

Eroglu AG, Oztunc F, Saltik L, Bakari S, Dedeoglu S, Ahunbay G. 2003. Evolution of ventricular septal defect with special reference to spontaneous closure rate, subaortic ridge and aortic valve prolapse. Pediatr Cardiol. 24:31-5.

Fatar M, Stroick M, Steffens M, Senn E, Reuter B, Bukow S, Griebe M, Alonso A, Lichtner P, Bugert P. 2008. Single-nucleotide polymorphisms of MMP-2 gene in stroke subtypes. Cerebrovasc Dis. 26:113-9.

Fedak PW, Altamentova SM, Weisel RD, Nili N, Ohno N, Verma S, Lee TY, Kiani C, Mickle DA, Strauss BH, Li RK. 2003. Matrix remodeling in experimental and human heart failure: a possible regulatory role for TIMP-3. Am J Physiol Heart Circ Physiol. 284:H626-34.

Fedarko NS, Jain A, Karadag A, Fisher LW. 2004. Three small integrin binding ligand N-linked glycoproteins (SIBLINGs) bind and activate specific matrix metalloproteinases. FASEB J. 18:734-6.

Felbor U, Stohr H, Pruett RC, Vogt G, Weber BHF. 1995. A novel Ser156Cys mutation in the tissue inhibitor of metalloproteinases-3 (TIMP3) in Sorsby's fundus dystrophy with unusual clinical features. Hum Mol Genet. 4:2415-6.

Fernandez CA, Yan L, Louis G, Yang J, Kutok JL, Moses MA. 2005. The matrix metalloproteinase-9/neutrophil gelatinase-associated lipocalin complex plays a role in breast tumor growth and is present in the urine of breast cancer patients. Clin Cancer Res. 11:5390-5.

Freedom RM, White RD, Pieroni DR, Varghese PJ, Krovetz LJ, Rowe RD. 1974. The natural history of the so-called aneurysm of the membranous ventricular septum in childhood. Circulation. 49:375-84.

Gai X, Lan X, Luo Z, Wang F, Liang Y, Zhang H, Zhang W, Hou J, Huang M. 2009. Association of MMP-9 gene polymorphisms with atrial fibrillation in hypertensive heart disease patients. Clin Chim Acta. 408:105-9.

Givvimani S, Tyagi N, Sen U, Mishra PK, Qipshidze N, Munjal C, Vacek JC, Abe OA, Tyagi SC. 2010. MMP-2/TIMP-2/TIMP-4 versus MMP-9/TIMP-3 in transition from compensatory hypertrophy and angiogenesis to decompensatory heart failure. Arch Physiol Biochem. 116:63-72.

Goetze JP. 2012. B-type natriuretic peptide: from posttranslational processing to clinical measurement. Clin Chem. 58:83-91.

Goor DA, Lillehei CW, Rees R, Edwards JE. 1970. Isolated ventricular septal defect. Development basis for various types and presentation of classification. Chest. 58:468-82.

Gross J, Lapiere CM. 1962. Collagenolytic activity in amphibian tissues: a tissue culture assay. Proc Natl Acad Sci U S A. 48:1014-22.

Gu H, Gong J, Qiu W, Cao H, Xu J, Chen S, Chen Y. 2011. Association of a tandem repeat polymorphism in NFATc1 with increased risk of perimembranous ventricular septal defect in a Chinese population. Biochem Genet. 49:592-600.

Halade GV, Jin YF, Lindsey ML. 2013. Matrix metalloproteinase (MMP)-9: A proximal biomarker for cardiac remodeling and a distal biomarker for inflammation. Pharmacol Ther. 139:32-40.

Han DH, Kim SK, Kang S, Choe B-K, Kim KS, Chung J-H. 2008. Matrix metallopeptidase 2 gene polymorphism is associated with obesity in Korean population. The Korean Journal of Physiology and Pharmacology. 12:125-9.

Haq I, Chappell S, Johnson SR, Lotya J, Daly L, Morgan K, Guetta-Baranes T, Roca J, Rabinovich R, Millar AB, Donnelly SC, Keatings V, MacNee W, Stolk J, Hiemstra PS, Miniati M, Monti S, O'Connor CM, Kalsheker N. 2010. Association of MMP-2 polymorphisms with severe and very severe COPD: a case control study of MMPs-1, 9 and 12 in a European population. BMC medical genetics. 11:7.

Hettiaratchi A, Hawkins NJ, McKenzie G, Ward RL, Hunt JE, Wakefield D, Di Girolamo N. 2007. The collagenase-1 (MMP-1) gene promoter polymorphism - 1607/2G is associated with favourable prognosis in patients with colorectal cancer. Br J Cancer. 96:783-92.

Hoffman JI. 1995. Incidence of congenital heart disease: I. Postnatal incidence. Pediatr Cardiol. 16:103-13.

Hornberger LK, Sahn DJ, Krabill KA, Sherman FS, Swensson RE, Pesonen E, Hagen-Ansert S, Chung KJ. 1989. Elucidation of the natural history of ventricular septal defects by serial Doppler color flow mapping studies. J Am Coll Cardiol. 13:1111-8.

Hua Y, Song L, Wu N, Lu X, Meng X, Gu D, Yang Y. 2009a. Polymorphisms of MMP-2 gene are associated with systolic heart failure risk in Han Chinese. The American journal of the medical science. 337:344-8.

Hua Y, Song L, Wu N, Xie G, Lu X, Fan X, Meng X, Gu D, Yang Y. 2009b. Polymorphisms of MMP-2 gene are associated with systolic heart failure prognosis. Clin Chim Acta. 404:119-23.

Huang HF, Hong LH, Tan Y, Sheng JZ. 2004. Matrix metalloproteinase 2 is associated with changes in steroid hormones in the sera and peritoneal fluid of patients with endometriosis. Fertil Steril. 81:1235-9.

Ikeda K, Ihara K, Yamaguchi K, Muneuchi J, Ohno T, Mizuno Y, Hara T. 2008. Genetic analysis of MMP gene polymorphisms in patients with Kawasaki disease. Pediatr Res. 63:182-5.

Jacobs EJ, Hsing AW, Bain EB, Stevens VL, Wang Y, Chen J, Chanock SJ, Zheng SL, Xu J, Thun MJ. 2008. Polymorphisms in angiogenesis-related genes and prostate cancer. Cancer Epidemiology Biomarkers & Prevention. 17:972-7.

Katz J, Gong Y, Salmasinia D, Hou W, Burkley B, Ferreira P, Casanova O, Langaee TY, Moreb JS. 2011. Genetic polymorphisms and other risk factors associated with bisphosphonate induced osteonecrosis of the jaw. Int J Oral Maxillofac Surg. 40:605-11.

Kidd L, Driscoll DJ, Gersony WM, Hayes CJ, Keane JF, O'Fallon WM, Pieroni DR, Wolfe RR, Weidman WH. 1993. Second natural history study of congenital heart defects. Results of treatment of patients with ventricular septal defects. Circulation. 87:I38-51.

Kim JH, Pyun J-A, Lee KJ, Cho SW, Kwack KB. 2011. Study on association between single nucleotide polymorphisms of MMP7, MMP8, MMP9 genes and development of gastric cancer and lymph node metastasis. The Korean Journal of Gastroenterology. 58:245-51.

Krane SM. 1994. Clinical importance of metalloproteinases and their inhibitors. Ann N Y Acad Sci. 732:1-10.

Kwok PY, Chen X. 2003. Detection of single nucleotide polymorphisms. Curr Issues Mol Biol. 5:43-60.

Lacchini R, Jacob-Ferreira ALB, Luizon MR, Gasparini S, Ferreira-Sae MCS, Schreiber R, Nadruz W, Tanus-Santos JE. 2011. Common matrix metalloproteinase 2 gene haplotypes may modulate left ventricular remodelling in hypertensive patients. J Hum Hypertens. 26:171-7.

Lee AY, Akers KT, Collier M, Li L, Eisen AZ, Seltzer JL. 1997. Intracellular activation of gelatinase A (72-kDa type IV collagenase) by normal fibroblasts. Proc Natl Acad Sci U S A. 94:4424-9.

Lin JM, Lai LP, Lin CS, Chou NK, Chiu CY, Lin JL. 2010. Left Ventricular Extracellular Matrix Remodeling in Dogs with Right Ventricular Apical Pacing. J Cardiovasc Electrophysiol. 21:1142-9.

Linask KK, Han M, Cai DH, Brauer PR, Maisastry SM. 2005. Cardiac morphogenesis: matrix metalloproteinase coordination of cellular mechanisms underlying heart tube formation and directionality of looping. Dev Dyn. 233:739-53.

Liotta LA, Wicha MS, Foidart JM, Rennard SI, Garbisa S, Kidwell WR. 1979. Hormonal requirements for basement membrane collagen deposition by cultured rat mammary epithelium. Lab Invest. 41:511-8.

Liu CX, Shen AD, Li XF, Jiao WW, Bai S, Yuan F, Guan XL, Zhang XG, Zhang GR, Li ZZ. 2009. Association of TBX5 gene polymorphism with ventricular septal defect in the Chinese Han population. Chin Med J (Engl). 122:30-4.

Liu L, Wu J, Wu C, Wang Y, Zhong R, Zhang X, Tan W, Nie S, Miao X, Lin D. 2011. A functional polymorphism (-1607 1G-->2G) in the matrix metalloproteinase-1 promoter is associated with development and progression of lung cancer. Cancer. 117:5172-81.

Lombard C, Saulnier J, Wallach J. 2005. Assays of matrix metalloproteinases (MMPs) activities: a review. Biochim. 87:265-72.

Lopez B, Gonzalez A, Diez J. 2010. Circulating biomarkers of collagen metabolism in cardiac diseases. Circulation. 121:1645-54.

Low SK, Zembutsu H, Takahashi A, Kamatani N, Cha PC, Hosono N, Kubo M, Matsuda K, Nakamura Y. 2011. Impact of LIMK1, MMP2 and TNF-α variations for intracranial aneurysm in Japanese population. J Hum Genet. 56:211-6.

Lynch JR, Blessing R, White WD, Grocott HP, Newman MF, Laskowitz DT. 2004. Novel diagnostic test for acute stroke. Stroke. 35:57-63.

Manso H, Krug T, Sobral J, Albergaria I, Gaspar G, Ferro J, Oliveira S, Vicente A. 2010. Variants of the Matrix Metalloproteinase-2 but not the Matrix Metalloproteinase-9 genes significantly influence functional outcome after stroke. BMC medical genetics. 11:40.

Maskos K, Bode W. 2003. Structural basis of matrix metalloproteinases and tissue inhibitors of metalloproteinases. Mol Biotechnol. 25:241-66.

Matsuoka R. 2007. GATA4 mutation and congenital cardiovascular diseases: importance of phenotype and genetic background clarification. J Mol Cell Cardiol. 43:667-9.

Mehta AV, Chidambaram B. 1992. Ventricular septal defect in the first year of life. Am J Cardiol. 70:364-6.

Moe DG, Guntheroth WG. 1987. Spontaneous closure of uncomplicated ventricular septal defect. Am J Cardiol. 60:674-8.

Morgunova E, Tuuttila A, Bergmann U, Isupov M, Lindqvist Y, Schneider G, Tryggvason K. 1999. Structure of human pro-matrix metalloproteinase-2: activation mechanism revealed. Science. 284:1667-70.

Mossböck G, Weger M, Faschinger C, Zimmermann C, Schmut O, Renner W, El-Shabrawi Y. 2010. Role of functional single nucleotide polymorphisms of MMP1, MMP2, and MMP9 in open angle glaucomas. Mol Vis. 16:1764.

Murphy DJ, Jr., Ludomirsky A, Huhta JC. 1986. Continuous-wave Doppler in children with ventricular septal defect: noninvasive estimation of interventricular pressure gradient. Am J Cardiol. 57:428-32.

Murphy G, Nagase H. 2008. Progress in matrix metalloproteinase research. Mol Aspects Med. 29:290-308.

Nagase H, Woessner JF, Jr. 1999. Matrix metalloproteinases. J Biol Chem. 274:21491-4.

Nan H, Niu T, Hunter DJ, Han J. 2008. Missense polymorphisms in matrix metalloproteinase genes and skin cancer risk. Cancer Epidemiology Biomarkers & Prevention. 17:3551-7.

Ozhan H, Albayrak S, Uzun H, Ordu S, Kaya A, Yazici M. 2007. Correlation of plasma B-type natriuretic peptide with shunt severity in patients with atrial or ventricular septal defect. Pediatr Cardiol. 28:272-5.

Palacios R, Gazave E, Goñi J, Piedrafita G, Fernando O, Navarro A, Villoslada P. 2009. Allele-specific gene expression is widespread across the genome and biological processes. PLoS ONE. 4:e4150.

Pavloff N, Staskus PW, Kishnani NS, Hawkes SP. 1992. A new inhibitor of metalloproteinases from chicken: ChIMP-3. A third member of the TIMP family. J Biol Chem. 267:17321-6.

Penny DJ, Vick GW, 3rd. 2011. Ventricular septal defect. Lancet. 377:1103-12.

Pinto LA, Depner M, Klopp N, Illig T, Vogelberg C, von Mutius E, Kabesch M. 2010. MMP-9 gene variants increase the risk for non-atopic asthma in children. Respir Res. 11:23-32.

Qi JH, Ebrahem Q, Moore N, Murphy G, Claesson-Welsh L, Bond M, Baker A, Anand-Apte B. 2003. A novel function for tissue inhibitor of metalloproteinases-3 (TIMP3):

inhibition of angiogenesis by blockage of VEGF binding to VEGF receptor-2. Nat Med. 9:407-15.

Rademaker MT, Richards AM. 2005. Cardiac natriuretic peptides for cardiac health. Clin Sci. 108:23-36.

Rajagopal SK, Ma Q, Obler D, Shen J, Manichaikul A, Tomita-Mitchell A, Boardman K, Briggs C, Garg V, Srivastava D, Goldmuntz E, Broman KW, Benson DW, Smoot LB, Pu WT. 2007. Spectrum of heart disease associated with murine and human GATA4 mutation. J Mol Cell Cardiol. 43:677-85.

Ramaciotti C, Vetter JM, Bornemeier RA, Chin AJ. 1995. Prevalence, relation to spontaneous closure, and association of muscular ventricular septal defects with other cardiac defects. Am J Cardiol. 75:61-5.

Renko J, Kalela A, Jaakkola O, Laine S, Hoyhtya M, Alho H, Nikkari ST. 2004. Serum matrix metalloproteinase-9 is elevated in men with a history of myocardial infarction. Scand J Clin Lab Invest. 64:255-61.

Reynolds MA, Kirchick HJ, Dahlen JR, Anderberg JM, McPherson PH, Nakamura KK, Laskowitz DT, Valkirs GE, Buechler KF. 2003. Early biomarkers of stroke. Clin Chem. 49:1733-9.

Roderfeld M, Graf J, Giese B, Salguero-Palacios R, Tschuschner A, Muller-Newen G, Roeb E. 2007. Latent MMP-9 is bound to TIMP-1 before secretion. Biol Chem. 388:1227-34.

Rodriguez-Pla A, Beaty TH, Savino PJ, Eagle RC, Jr., Seo P, Soloski MJ. 2008. Association of a nonsynonymous single-nucleotide polymorphism of matrix metalloproteinase 9 with giant cell arteritis. Arthritis Rheum. 58:1849-53.

Ruskoaho H. 2003. Cardiac hormones as diagnostic tools in heart failure. Endocr Rev. 24:341-56.

Saedi M, Vaisi-Raygani A, Khaghani S, Shariftabrizi A, Rezaie M, Pasalar P, Rahimi Z, Pourmotabbed T. 2012. Matrix metalloproteinase-9 functional promoter polymorphism 1562C>T increased risk of early-onset coronary artery disease. Mol Biol Rep. 39:555-62.

Schulz R. 2007. Intracellular targets of matrix metalloproteinase-2 in cardiac disease: rationale and therapeutic approaches. Annu Rev Pharmacol Toxicol. 47:211-42.

Sellers A, Reynolds JJ, Meikle MC. 1978. Neutral metallo-proteinases of rabbit bone. Separation in latent forms of distinct enzymes that when activated degrade collagen,

gelatin and proteoglycans. Biochem J. 171:493-6.

Skarmoutsou E, D'Amico F, Marchini M, Stivala F, Malaponte G, Scorza R, Mazzarino MC. 2011. Analysis of matrix metalloproteinase-9 gene polymorphism-1562 C/T in patients suffering from systemic sclerosis with and without ulcers. Int J Mol Med. 27:873.

Sopata I, Wize J. 1979. A latent gelatin specific proteinase of human leucocytes and its activation. Biochim Biophys Acta. 571:305-12.

Srivastava D, Baldwin HS. 2008. Molecular Determinants of Cardiac Development and Disease. In Allen HD, Driscoll DJ, Shaddy RE, Feltes TF. Moss and Adams' heart disease in infants, children, and adolescents: including the fetus and young adults. Lippincott Williams & Wilkins, p 510-23.

Staskus PW, Masiarz FR, Pallanck LJ, Hawkes SP. 1991. The 21-kDa protein is a transformation-sensitive metalloproteinase inhibitor of chicken fibroblasts. J Biol Chem. 266:449-54.

Stawowy P, Margeta C, Kallisch H, Seidah NG, Chretien M, Fleck E, Graf K. 2004. Regulation of matrix metalloproteinase MT1-MMP/MMP-2 in cardiac fibroblasts by TGF-beta1 involves furin-convertase. Cardiovasc Res. 63:87-97.

Stefanidakis M, Koivunen E. 2006. Cell-surface association between matrix metalloproteinases and integrins: role of the complexes in leukocyte migration and cancer progression. Blood. 108:1441-50.

Stenson PD, Mort M, Ball EV, Howells K, Phillips AD, Thomas NS, Cooper DN. 2009. The Human Gene Mutation Database: 2008 update. Genome Med. 1:13.

Sullivan DR, West M, Jeremy R. 2005. Utility of brain natriuretic peptide (BNP) measurement in cardiovascular disease. Heart Lung Circ. 14:78-84.

Tikanoja T. 1995. Effect of technical development on the apparent incidence of congenital heart disease. Pediatr Cardiol. 16:100-1.

Van den Steen PE, Dubois B, Nelissen I, Rudd PM, Dwek RA, Opdenakker G. 2002. Biochemistry and molecular biology of gelatinase B or matrix metalloproteinase-9 (MMP-9). Crit Rev Biochem Mol Biol. 37:375-536.

Van Wart HE, Birkedal-Hansen H. 1990. The cysteine switch: a principle of regulation of metalloproteinase activity with potential applicability to the entire matrix metalloproteinase gene family. Proc Natl Acad Sci U S A. 87:5578-82.

Vincenti MP, Brinckerhoff CE. 2007. Signal transduction and cell-type specific regulation of matrix metalloproteinase gene expression: can MMPs be good for you? J Cell Physiol. 213:355-64.

Visse R, Nagase H. 2003. Matrix metalloproteinases and tissue inhibitors of metalloproteinases: structure, function, and biochemistry. Circ Res. 92:827-39.

Wallon UM, Overall CM. 1997. The hemopexin-like domain (C domain) of human gelatinase A (matrix metalloproteinase-2) requires Ca2+ for fibronectin and heparin binding. Binding properties of recombinant gelatinase A C domain to extracellular matrix and basement membrane components. J Biol Chem. 272:7473-81.

Wang LF, Chien CY, Kuo WR, Tai CF, Juo SHH. 2008. Matrix metalloproteinase-2 gene polymorphisms in nasal polyps. Arch Otolaryngol Head Neck Surg. 134:852.

Woessner JF. 2001. That impish TIMP: the tissue inhibitor of metalloproteinases-3. J Clin Invest. 108:799-800.

Wojciechowski R, Bailey-Wilson JE, Stambolian D. 2010. Association of matrix metalloproteinase gene polymorphisms with refractive error in Amish and Ashkenazi families. Invest Ophthalmol Vis Sci. 51:4989-95.

Wu S, Hsu LA, Teng MS, Lin JF, Chang HH, Chang PY, Hu CF, Ko YL. 2010. Association of matrix metalloproteinase 9 genotypes and cardiovascular disease risk factors with serum matrix metalloproteinase 9 concentrations in Taiwanese individuals. Clin Chem Lab Med. 48:543-9.

Xie J, Yi L, Xu ZF, Mo XM, Hu YL, Wang DJ, Ren HZ, Han B, Wang Y, Yang C, Zhao YL, Shi DQ, Jiang YZ, Shen L, Qiao D, Chen SL, Yu BJ. 2007. VEGF C-634G polymorphism is associated with protection from isolated ventricular septal defect: case-control and TDT studies. Eur J Hum Genet. 15:1246-51.

Yang TT, Hawkes SP. 1992. Role of the 21-kDa protein TIMP-3 in oncogenic transformation of cultured chicken embryo fibroblasts. Proc Natl Acad Sci USA. 89:10676-80.

Yeh CC, Malhotra D, Yang YL, Xu Y, Fan Y, Li H, Mann MJ. 2013. MEK1-induced physiological hypertrophy inhibits chronic post-myocardial infarction remodeling in mice. J Cell Biochem. 114:47-55.

Yi YC, Chou PT, Chen LY, Kuo WH, Ho ES-C, Han CP, Yang SF. 2010. Matrix metalloproteinase-7 (MMP-7) polymorphism is a risk factor for endometrial cancer susceptibility. Clin Chem Lab Med. 48:337-44.

Yong N, Guoping C. 2008. Upregulation of matrix metalloproteinase-9 dependent on hyaluronan synthesis after sciatic nerve injury. Neurosci Lett. 444:259-63.

Yu C, Zhou Y, Miao X, Xiong P, Tan W, Lin D. 2004. Functional haplotypes in the promoter of matrix metalloproteinase-2 predict risk of the occurrence and metastasis of esophageal cancer. Cancer Res. 64:7622-8.

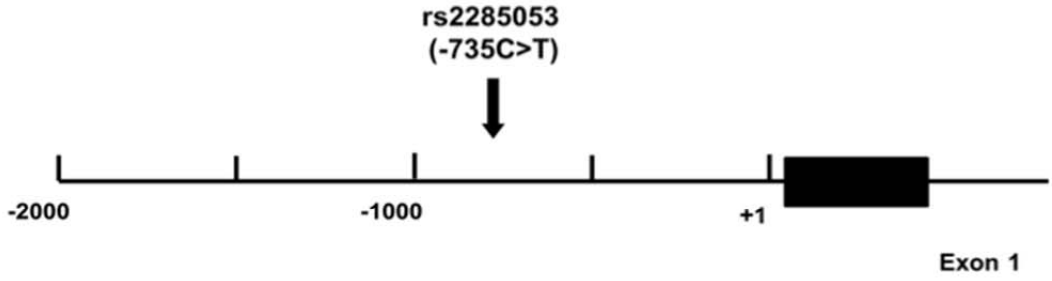
Zhang B, Ye S, Herrmann SM, Eriksson P, de Maat M, Evans A, Arveiler D, Luc G, Cambien F, Hamsten A, Watkins H, Henney AM. 1999. Functional polymorphism in the regulatory region of gelatinase B gene in relation to severity of coronary atherosclerosis. Circulation. 99:1788-94.

Zhang WM, Li XF, Ma ZY, Zhang J, Zhou SH, Li T, Shi L, Li ZZ. 2009. GATA4 and NKX2.5 gene analysis in Chinese Uygur patients with congenital heart disease. Chin Med J (Engl). 122:416-9.

Appendix

Appendix 1. Sketch and diagram of SNPs performed in the study

A.

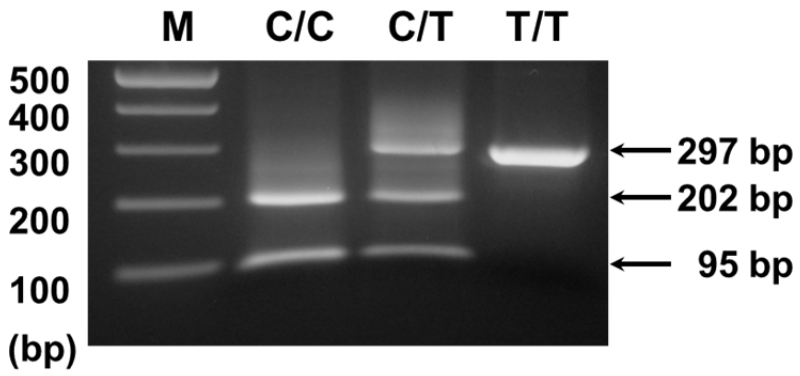


B.

```
1    TGAGTGGGGA  ATTCGTGGAA  CTGAGGGCTC  CTCCCCTTTT  TAGACCATAT
51   AGGGTAAACC  TCCCCACATT  GCCATGGCAT  TTATAAACTG  CCATGGCACT
          ⟶ MMP-2 F
101  GGTGGGTGCT  TCCTTTAACA  TGCTAATGCA  TTATAATTAG  CGTAAAATGA
151  GCAGTGAGGA  TGACCAGAGG  TCGCTTTCTT  TGCCATCTTG  GTTTTGGCTG
201  GCTTCTTCAC  TGCATACTGT  TTTATCAGTG  GGGTCTTTGT  GACCTCTATC
251  TTATTAAACC  AGTCTTGCCC  AATTTCTATC  TCATCCTGTG  ACCGAGAATG
301  CGGACCCTCC  TGGGAGTGCA  GCCCAGCAGG  TCTCAGCCTC  ATTTTACCCA
           T
351  GCCCCCTGTT  CAAGATGGAG  TCGCTCTGGT  TCCAACGTCT  CTAACGCGGG
                                                MMP-2 R ⟵
401  GCCCCTGACT  GCTCTATTTC  CCAAGGTGTA  TCTAGCATCT  CGCACTATAC
451  GAGGCCAAGT  TAAGGCTTAC  ACATTTGCAG  AAGGAAAGAG  GTAAGGA
```

Figure A1. A sketch of human MMP-2 gene and SNP rs2285053. (A) Genomic organization and localization of the SNP rs2285053 of human MMP-2 gene. MMP-2 SNP on promoter region, -735C>T, was marked by the downward arrow in the figure. The black block represents the exon 1 of MMP-2 gene; (B) Sequences of human MMP-2 gene and position of the PCR primers were shown. The arrows represent the PCR primers, and the underlined nucleotide is a genetic variant (MMP-2 -735C>T) in the promoter of MMP-2 gene.

A.



B.

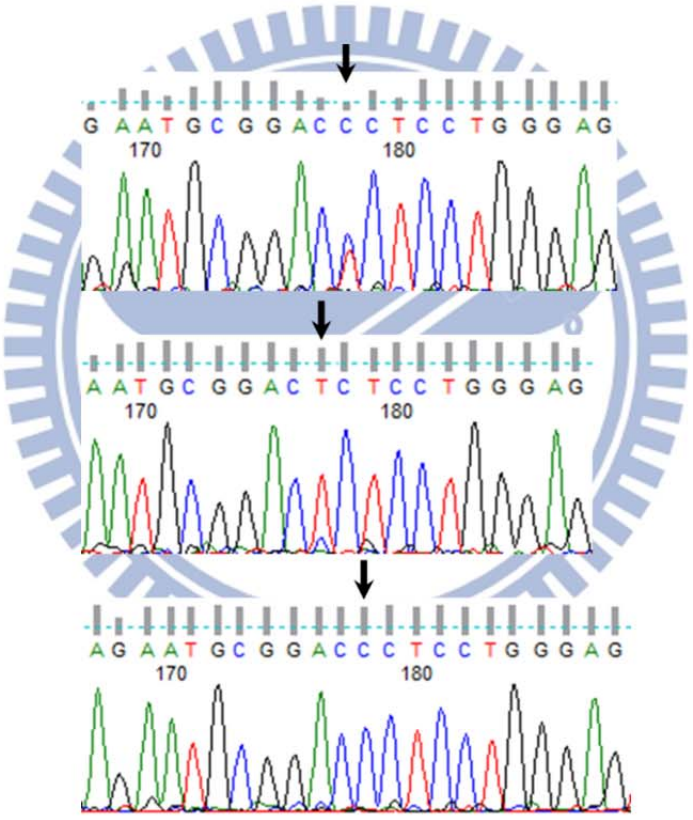


Figure A2. Diagram of MMP-2 SNP rs2285053. (A) Detection of the RFLP for MMP-2 polymorphism rs2285053. After PCR amplifying, the target DNA region (297 bp) was digested by restriction enzyme Sau96 I. Lane 1 represents a 100-bp DNA ladder marker; Lane 2 stands for genotype C/C (202 bp and 95 bp); Lane 3 stands for genotype C/T (297 bp, 202 bp, and 95 bp); Lane 4 stands for genotype T/T (297 bp); (B) Three genotypes of direct sequencing maps for rs2285053 SNP of MMP-2 gene were shown. The black arrows indicate the site of MMP-2 polymorphisms

A.

B.

```
  1   GATGTTCATT   GGTTAGTGAA   CTTTAGAACT   TCAACTTTTC   TGTAAAGGAA
 51   GTTAATTATC   TCCATCTCAC   AGTCTCATTT   ATTAGATAAG   CATATAAAAT
            MMP-9-1 F
101   GCCTGGCACA   TAGTAGGCCC   TTTAAATACA   GCTTATTGGG   CCGGGCGCCA
151   TGGCTCATGC   CCGTAATCCT   AGCACTTTGG   GAGGCCAGGT   GGGCAGATCA
201   CTTGAGTCAG   AAGTTCGAAA   CCAGCCTGGT   CAACGTAGTG   AAACCCCATC
251   TCTACTAAAA   ATACAAAAAA   TTTAGCCAGG   CGTGGTGGCG   CACGCCTATA
                                                        G
301   ATACCAGCTA   CTCGGGAGGC   TGAGGCAGGA   GAATTGCTTG   AACCCGGGAG
351   GCAGATGTTG   CAGTGAGCCG   AGATCACGCC   ACTGCACTCC   AGCCTGGGTG
401   ACAGAGTGAT   ACTACACCCC   CCAAAAATAA   AATAAAATAA   ATAAATACAA
451   CTTTTTGAGT   TGTTAGCAGG   TTTTTCCCAA   ATAGGGCTTT   GAAGAAGGTG
501   AATATAGACC   CTGCCCGATG   CCGGCTGGCT   AGGAAGAAAG   GAGTGAGGGA
                                MMP-9-1 R
551   GGCTGCTGGT   GTGGGAGGCT   TGGGAGGGAG   GCTTGGCATA   AGTGTGATAA
601   TTGGGGCTGG   AGATTTGGCT   GCATGGAGCA   GGGCTGGAGA
```

Figure A3. A sketch of human MMP-9 gene and SNP rs3918242. (A) Genomic organization and localization of the SNP rs3918242 of human MMP-9 gene. Each exon of MMP-9 gene is represented with a box; exon length and intron length are not on the scale. The arrow marks the position of SNPs that analysis in the study; (B) Sequence of human MMP-9 gene and position of the PCR primers were shown. The arrows represent the PCR primers, and the underlined nucleotide is a genetic variant (MMP-9 -1562) in the promoter region of MMP-9 gene.

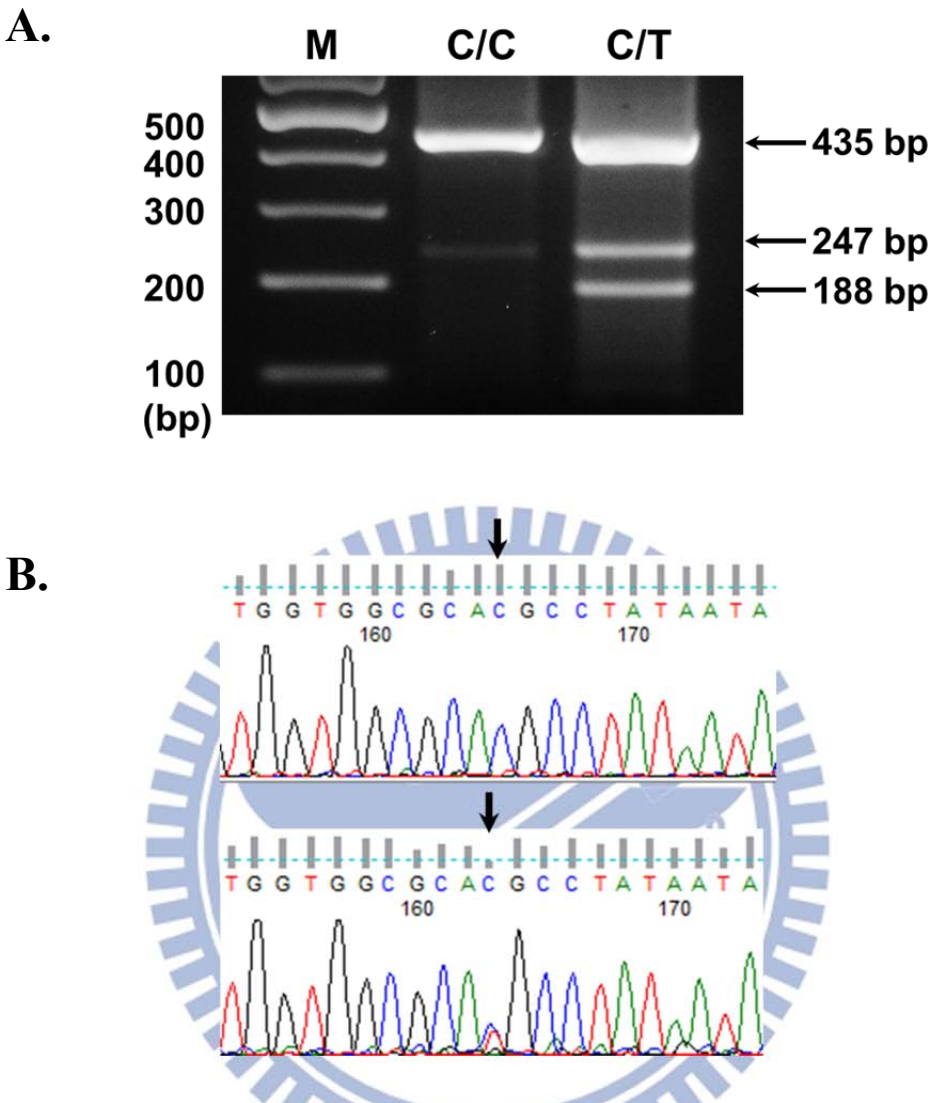


Figure A4. Diagram of MMP-9 SNP rs391824. (A) Detection of the RFLP for MMP-9 polymorphism rs3918242. After PCR amplifying, the target DNA region (435 bp) were digested by restriction enzyme *Sph* I. Lane 1 represents a 100-bp DNA ladder marker; Lane 2 stands for genotype C/C (435 bp); Lane 3 stands for genotype C/T (435 bp, 247 bp, and 188 bp).; (B) Two genotypes of direct sequencing maps for rs3918242 SNP of MMP-9 gene were shown. The black arrows indicate the site of MMP-9 polymorphisms.

A.

B.

```
 1    GAGTGAGTGA   GGGGGCTCGC   CGAGGGCTGG   GGGCGCCCAC   CACCCTTGAT
51    GGTCCTGGGT   TCTAATTCCA   GCTCTGCCAC   TAGTGCTGTG   TGGCCTGCAA
      ⟶ MMP-9-2 F
101   TTCACCCTCC    CGCACTCTGG   GCCCAATTTT   CTCATCTGAG   AAATGATGAG

151   AGATGGGATG   AACTGCAGAC   CATCCATGGG   TCAAAGAACA   GGACACACTT
201   GGGGGTTATA   ATGTGCTGTC   TCCGCCTTCT   CCCCCTTTCC   CACATCCTCC
251   TCGCCCCAGG   ACTCTACACC   CAGGACGGCA   ATGCTGATGG   GAAACCCTGC
                               T
301   CAGTTTCCAT   TCATCTTCCA   AGGCCAATCC   TACTCCGCCT   GCACCACGGA
351   CGGTCGCTCC   GACGGCTACC   GCTGGTGCGC   CACCACCGCC   AACTACGACC
401   GGGACAAGCT   CTTCGGCTTC   TGCCCGACCC   GAGGTACCTC   CACCCTGTCT
451   ACCAGGTTCA   GCCCCGCCCT   CTCATCATGT   ATTGGCCCCC   AAAACGCGGC
501   TCTTCCCTCC   CATCAGTTTG   TCTTTCCACT   CTCATTGGTC   CTCAGGACGA
551   CCGTGACTCC    GCCCACCTAC   ACCACATTTC   CACCACTATC   CCTGACTTCC
           MMP-9-2 R ⟵
601   AATGGCCCCG    CCCCAGCCAC   TAAGGTTCGG   CCTTTTCTGC   CCAGCTGGCC
651   GCCTCTTCCT    TGGTCTGGTG
```

Figure A5. A sketch of human MMP-9 gene and SNP rs17576. (A) Genomic organization and localization of the SNP rs17576 of human MMP-9 gene. Each exon of MMP-9 gene is represented with a box; exon length and intron length are not on the scale. The arrow marks the position of SNPs that analysis in the study; (B) Sequence of human MMP-9 gene and position of the PCR primers were shown. The arrows represent the PCR primers, and the underlined nucleotide is a genetic variant (MMP-9 R279Q) in MMP-9 gene.

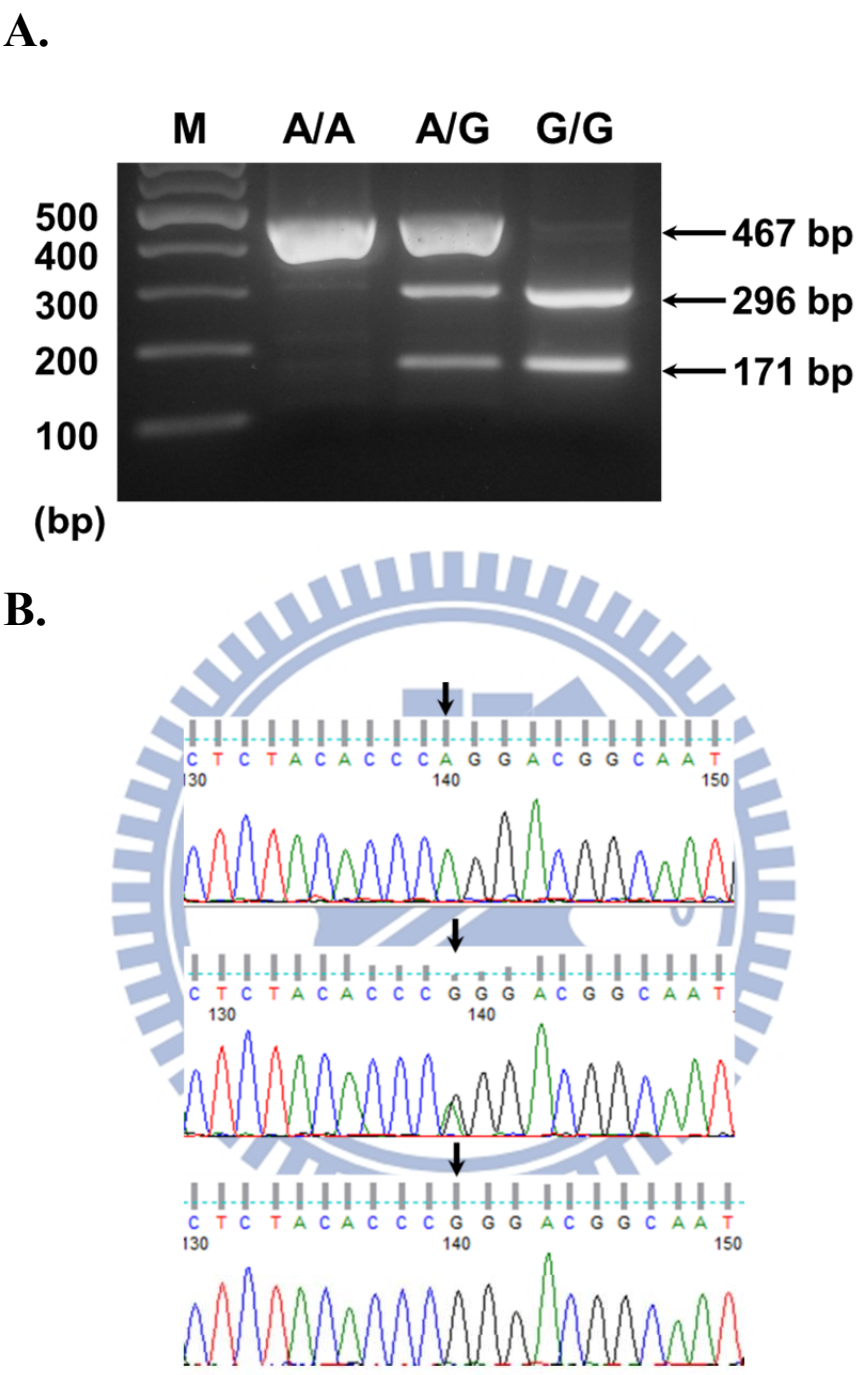
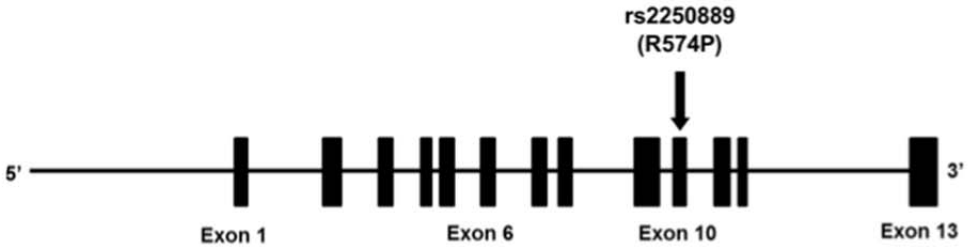


Figure A6. Diagram of MMP-9 SNP rs17576. (A) Detection of the RFLP for MMP-9 polymorphism rs17576. After PCR amplifying, the target DNA region (467 bp) were digested by restriction enzyme *Sma* I. Lane 1 represents a 100-bp DNA ladder marker; Lane 2 stands for genotype A/A (467 bp); Lane 3 stands for genotype A/G (467 bp, 296 bp, and 171 bp); Lane 4 stands for genotype G/G (296 bp and 171 bp); (B) Three genotypes of direct sequencing maps for rs17576 SNP of MMP-9 gene were shown. The black arrows indicate the site of MMP-9 polymorphisms.

A.



B.

1	CCACTGGCCC	TGTGTCCAAG	GCTTAGAGCC	CGTCCTTTCC	CTCCTCGCTT
51	TCTCAGGAAG	TACTGGCGAT	TCTCTGAGGG	CAGGGGGAGC	CGGCCGCAGG
		MMP-9-3 F			
101	GCCCCTTCCT	TATCGCCGAC	AAGTGGCCCG	CGCTGCCCCG	CAAGCTGGAC
151	TCGGTCTTTG	AGGAGCGGCT	CTCCAAGAAG	CTTTTCTTCT	TCTCTGGTTA
		C			
201	GTTACCTACT	TTCCCTCCCC	CGCCCGGTCA	ATCCCCATCA	GTCAAGGAGG
251	CTCAAGAGAC	CATCGATAAC	CCACGAAACG	TCTTGTGCGT	TTTAGAAAAA
301	TACGCCCCCT	GGCGGACGCA	GTTTAGCAAA	CGTAGGGGCG	CTGAGTTTCT
					MMP-9-3 R
351	GCCCCCTCCT	CTCCACGCCC	TCGCGTCGCT	CTACCCAGCG	CCTCTGCCCC
401	TGGGTTGCAG	GGACTGCGGG	CACGCGGGCT	AGGAAAGGCC	TCGCCGGAAT

Figure A7. A sketch of human MMP-9 gene and SNP rs2250889. (A) Genomic organization and localization of the SNP rs2250889 of human MMP-9 gene. Each exon of MMP-9 gene is represented with a box; exon length and intron length are not on the scale. The arrow marks the position of SNP that analysis in the study; (B) Sequence of human MMP-9 gene and position of the PCR primers were shown. The arrows represent the PCR primers, and the underlined nucleotide is a genetic variant (MMP-9 R574P) in MMP-9 gene.

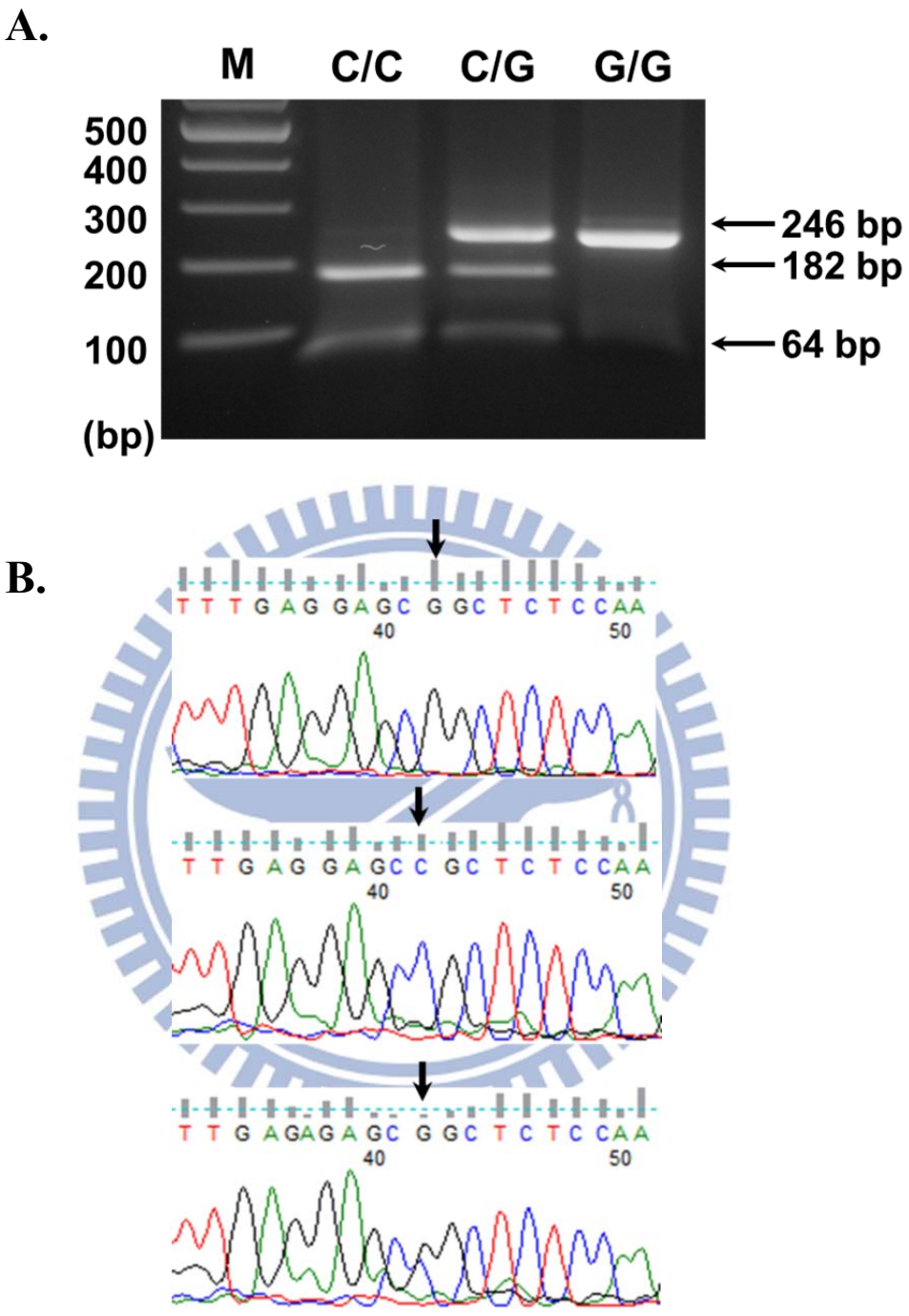


Figure A8. Diagram of MMP-9 SNP rs2250889. (A) Detection of the RFLP for MMP-9 polymorphism rs2250889. After PCR amplifying, the target DNA region (246 bp) were digested by restriction enzyme *Nla* IV. Lane 1 represents a 100-bp DNA ladder marker; Lane 2 stands for genotype C/C (182 bp and 64 bp); Lane 3 stands for genotype C/G (246 bp, 182 bp, and 64 bp); Lane 4 stands for genotype G/G (246 bp); (B) Three genotypes of direct sequencing maps for rs2250889 SNP of MMP-9 were shown. The black arrows indicate the site of MMP-9 polymorphisms.

Appendix 2. Database information of MMP-2 SNPs

dbSNP rs# cluster ID	mRNA position	Hetero-zygosity	MAF	Function	dbSNP allele	Protein residue	Amino acid position	Reference
rs243866		0.181	0.138	5' near gene	A/G			(Manso et al., 2010); (Mossböck et al., 2010)
rs243865		0.199	0.142	5' near gene	(> 6 bp)			(Katz et al., 2011); (Low et al., 2011); (Lacchini et al., 2011)
rs17859821		0.173	0.154	5' near gene	A/G			(Hua et al., 2009a); (Hua et al., 2009b)
rs243864		0.206	0.138	5' near gene	G/T			(Beeghly-Fadiel et al., 2011); (Hua et al., 2009a); (Hua et al., 2009b)
rs2285053		0.500	0.175	5' near gene	C/T			(Han et al., 2008); (Beeghly-Fadiel et al., 2011); (Yi et al., 2010)
rs857403		0.380	0.283	intron	A/T			(Wang et al., 2008); (Manso et al., 2010)
rs1030868		0.466	0.344	intron	C/T			(Fatar et al., 2008)
rs1477017		0.457	0.346	intron	A/G			(Jacobs et al., 2008); (Beeghly-Fadiel et al., 2011); (Manso et al., 2010)

rs865094		0.422	0.296	intron	A/G			(Beeghly-Fadiel et al., 2011)
rs17301608		0.482	0.399	intron	C/T			(Jacobs et al., 2008); (Manso et al., 2010)
rs11646643		0.367	0.252	intron	A/G			(Beeghly-Fadiel et al., 2011)
rs1132896	989	0.373	0.214	synonymous	C	Gly [G]	226	(Han et al., 2008); (Manso et al., 2010)
				contig reference	G	Gly [G]	226	(Han et al., 2008); (Manso et al., 2010)
rs1053605	1061	0.134	0.083	synonymous	T	Thr [T]	250	(Han et al., 2008); (Beeghly-Fadiel et al., 2011); (Manso et al., 2010)
				contig reference	C	Thr [T]	250	(Han et al., 2008); (Beeghly-Fadiel et al., 2011); (Manso et al., 2010)
rs17859889		0.083	0.058	intron	C/T			(Ban et al., 2010)
rs9302671		0.356	0.252	intron	G/T			(Beeghly-Fadiel et al., 2011)
rs2241145		0.500	0.483	intron	C/G			(Fatar et al., 2008); (Beeghly-Fadiel et al., 2011); (Manso et al., 2010)
rs2241146		0.219	0.103	intron	A/G			(Beeghly-Fadiel et al., 2011)

rs9928731		0.500	0.483	intron	C/T			(Wojciechowski et al., 2010)
rs243849	1460	0.339	0.227	synonymous	C	Asp [D]	383	(Han et al., 2008); (Beeghly-Fadiel et al., 2011); (Manso et al., 2010)
				contig reference	T	Asp [D]	383	(Han et al., 2008); (Beeghly-Fadiel et al., 2011); (Manso et al., 2010)
rs12599775		0.173	0.047	intron	C/G			(Beeghly-Fadiel et al., 2011)
rs243847		0.434	0.347	intron	C/T			(Beeghly-Fadiel et al., 2011); (Low et al., 2011)
rs2192852		0.483		intron	-/A/G			(Beeghly-Fadiel et al., 2011)
rs12923011		0.291	0.223	intron	C/T			(Beeghly-Fadiel et al., 2011)
rs243845		0.448	0.338	intron	C/T			(Beeghly-Fadiel et al., 2011)
rs243844		0.450	0.310	intron	A/G			(Beeghly-Fadiel et al., 2011)
rs243842		0.427	0.310	intron	C/T			(Beeghly-Fadiel et al., 2011); (Manso et al., 2010)
rs183112		0.378	0.242	intron	A/G			(Beeghly-Fadiel et al., 2011); (Manso et al., 2010)
rs1992116		0.407	0.308	intron	C/T			(Palacios et al., 2009); (Manso et al., 2010)

rs243839		0.484	0.370	intron	A/G			(Beeghly-Fadiel et al., 2011)
rs9923304		0.405	0.320	intron	C/T			(Beeghly-Fadiel et al., 2011)
rs2287076		0.409	0.322	intron	C/T			(Fatar et al., 2008)
rs11639960		0.368	0.227	intron	A/G			(Jacobs et al., 2008); (Beeghly-Fadiel et al., 2011)
rs14070	2117	0.450	0.336	synonymous	T	Phe [F]	602	(Han et al., 2008)
				contig reference	C	Phe [F]	602	(Han et al., 2008)
rs11541998	2153	0.125	0.053	synonymous	G	Pro [P]	614	(Beeghly-Fadiel et al., 2011)
				contig reference	C	Pro [P]	614	(Beeghly-Fadiel et al., 2011)
rs7201	2554	0.409	0.332	3' UTR	A/C			(Fatar et al., 2008)
rs17860021	2694	0.046	0.013	3' UTR	A/G			(Ban et al., 2010)
rs243831		0.266	0.151	3' near gene	G/T			(Beeghly-Fadiel et al., 2011)

MAF, minor allele frequency

Appendix 3. Database information of MMP-9 SNPs

dbSNP rs# cluster ID	mRNA position	Hetero-zygosity	MAF	Function	dbSNP allele	Protein residue	Amino acid position	Reference
rs3918240		0.490	0.453	5' near gene	C/T			(Haq et al., 2010)
rs3918278		0.035	0.035	5' near gene	(> 6 bp)			(Haq et al., 2010)
rs3918241		0.249	0.154	5' near gene	A/T			(Haq et al., 2010); (Pinto et al., 2010); (Beeghly-Fadiel et al., 2011)
rs3918242		0.290	0.153	5' near gene	C/T			(Pinto et al., 2010); (Skarmoutsou et al., 2011); (Belo et al., 2012)
rs2234681		0.722		5' near gene	(> 6 bp)			(Belo et al., 2012)
rs1805088	78	0.046	0.029	missense	A	Asp [D]	20	(Nan et al., 2008)
				missense	G	Gly [G]	20	(Nan et al., 2008)
				missense	T	Val [V]	20	(Nan et al., 2008)
				contig reference	C	Ala [A]	20	(Nan et al., 2008)
rs3918249		0.487	0.483	intron	C/T			(Haq et al., 2010); (Pinto et al., 2010)
rs3918251		0.500	0.491	intron	(> 6 bp)			(Haq et al., 2010)

rs3918252	400	0.003		missense	G	Lys [K]	127	(Rodriguez-Pla et al., 2008)
				contig reference	C	Asn [N]	127	(Rodriguez-Pla et al., 2008)
rs25650	499	0.180		nonsense	G	xxx [X]	160	(Haq et al., 2010)
				contig reference	C	Tyr [Y]	160	(Haq et al., 2010)
rs8125581	512	0.002		missense	A	Asn [N]	165	(Cotignola et al., 2007)
				contig reference	G	Asp [D]	165	(Cotignola et al., 2007)
rs3918253		0.400	0.281	intron	C/T			(Haq et al., 2010); (Manso et al., 2010); (Pinto et al., 2010)
rs2274755		0.282	0.162	intron	(> 6 bp)			(Haq et al., 2010); (Pinto et al., 2010)
rs2236416		0.229	0.156	intron	A/G			(Haq et al., 2010); (Manso et al., 2010); (Pinto et al., 2010)
rs3918256		0.399	0.281	intron	A/G			(Haq et al., 2010); (Pinto et al., 2010)
rs3787268		0.400	0.213	intron	(> 6 bp)			(Haq et al., 2010); (Pinto et al., 2010); (Wang et al., 2008)
rs35691798	1730	0.003		missense	G	Val [V]	571	(Cotignola et al., 2007)

				contig reference	T	Phe [F]	571	(Cotignola et al., 2007)
rs2250889	1740	0.213	0.153	missense	C	Pro [P]	574	(Chen et al., 2010); (Haq et al., 2010); (Kim et al., 2011)
				contig reference	G	Arg [R]	574	(Chen et al., 2010); (Haq et al., 2010); (Kim et al., 2011)
rs13969	1840	0.468	0.408	synonymous	C	Gly [G]	607	(Cheong et al., 2008); (Haq et al., 2010)
				contig reference	A	Gly [G]	607	(Cheong et al., 2008); (Haq et al., 2010)
rs17577	2022	0.280	0.170	missense	A	Gln [Q]	668	(Wang et al., 2008); (Wu et al., 2010)
				missense	C	Pro [P]	668	(Wang et al., 2008); (Wu et al., 2010)
				missense	T	Leu [L]	668	(Wang et al., 2008); (Wu et al., 2010)
				contig reference	G	Arg [R]	668	(Wang et al., 2008); (Wu et al., 2010)
rs3918261		0.315	0.181	intron	A/G			(Haq et al., 2010); (Pinto et al., 2010)
rs3918262		0.321	0.215	intron	(> 6 bp)			(Haq et al., 2010)

rs13925	2101	0.242	0.162	synonymous	A	Val [V]	694	(Haq et al., 2010)
				contig reference	G	Val [V]	694	(Haq et al., 2010)
rs20544	2146	0.381	0.277	3' UTR	(> 6 bp)			(Haq et al., 2010)
rs3918270		0.268	0.170	3' near gene	A/G			(Pinto et al., 2010)

MAF, minor allele frequency