國 立 交 通 大 學

生 物 科 技 學 院

生物科技學系

碩士論文

小兒心室中膈缺損與第二型和第九型基質金屬蛋白酶基因多型性及酵素活性的相關性

Associations of matrix metalloproteinase-2 and -9 gene polymorphisms and enzyme activity in the children with ventricular septal defects

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指導教授： 林志生 博士

中 華 民 國 101 年 7 月

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小兒心室中膈缺損與第二型和第九型基質金屬蛋白酶基因多型性及酵素活性的相關性

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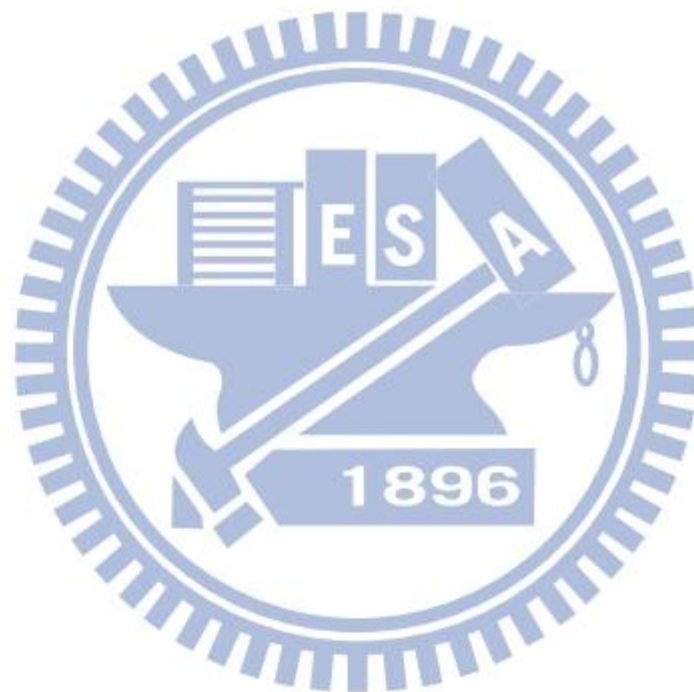
中文摘要

心室中膈缺損(ventricular septal defect ,VSD)為常見的先天性心臟病之一。其VSDs佔所有先天性心臟病的20%，多數病童的中膈缺損會隨著年紀增長而逐漸變小或完全閉合。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 polymorphism, SNP)被指出是造成VSD疾病主要原因之一。基質金屬蛋白酶(matrix metalloproteinases, MMPs)是一類活性依賴於Zn^{2+}等金屬離子且參與降解細胞外基質(extracellular matrix, ECM)的蛋白酶家族，其精密調控著組織中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，且許多心臟疾病也與MMPs的基因多型性有關。據此，我們檢測VSDs的病患的MMPs基因SNPs與其血漿中MMPs活性，來探討MMPs基因SNP和血液中MMPs活性與VSDs之嚴重程度。

自2010年9月起，我們收集了101位7.4 ± 3.8歲VSDs病童的血液樣品，自白血球萃取其基因組DNA，利用聚合酶連鎖反應-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-9 SNPs，包括 -1562C>T、R279Q及R574P，也檢測MMP-2 -735C>T SNP。我們亦分離病童的血漿，以明膠酶電泳(gelatin zymography)測定其MMP-2和MMP-9活性。實驗結果顯示，在所檢測的四個SNPs中，僅有MMP-9 R279Q基因型的分布與VSD的發生有統計上的顯著意義($p < 0.05$)，其VSD組中GA基因型的發生頻率明顯高於控制組(分別為52.2%

及37.2%)。另一方面，我們依病患超音波檢查之VSD/aortic root (Ao)比值判定病童VSD嚴重程度，我們將之分成輕微缺損(VS；VSD/Ao $\leq$ 0.2)、中度缺損(VM；0.2 $<$ VSD/Ao $\leq$ 0.3)及嚴重缺損(VL；VSD/Ao $>$ 0.3) 三組。Gelatin zymography分析結果顯示，在VSD病童血漿中皆可測得較高活性之MMP-2與MMP-9，特別在VSD嚴重缺損病童中，與控制組相較，其MMP-2與MMP-9活性表現分別顯著提升60% ($p < 0.001$) 與50% ($p < 0.01$)。

本研究結果指出MMP-9 SNP R279Q可能與VSD的發生有關，而隨著VSD趨於嚴重時，其血液中MMP-2與MMP-9的活性有增加的趨勢。我們推測MMPs的活性可能影響VSD的嚴重程度或其癒合率，此提供未來繼續探討MMPs影響VSD病理機制十分有價值的線索。



Associations of matrix metalloproteinase-2 and -9 gene polymorphisms and enzyme activity in children with ventricular septal defects

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Abstract

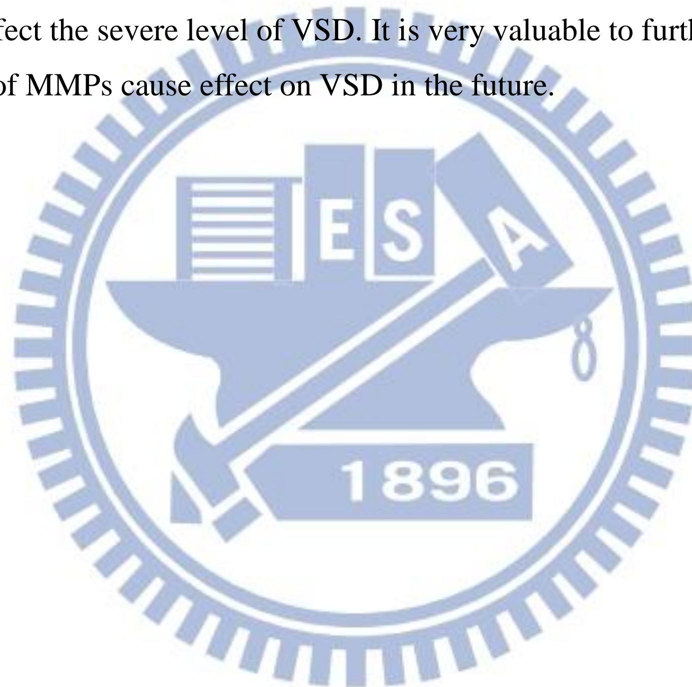
Ventricular septal defect (VSD) is the most common form of congenital heart disease. Approximately 20% of patients in congenital heart disease registries have VSD as a solitary lesion. Rates of spontaneous closure for membranous and muscular VSDs in infant and children were 37% and 50%, respectively, during a mean follow-up of 12 months. Single nucleotide polymorphism (SNP) is considered one of the main factors to VSD formation. Matrix metalloproteinases (MMPs) are a family of zinc-dependent endopeptidases involved in breakdown, and physiological homeostasis of extracellular matrix. MMPs have been reported that play the important roles in embryonic development, cardiomyocyte differentiation and causing cardiac tissue remodeling. Also, many cardiac related diseases are associated to the SNP of MMPs gene. Therefore, we tried to determine the SNPs of MMPs genes and plasma MMPs activity in the patients with VSD and investigate the relation between MMPs and VSD in the aspect of incidence and severe level.

Since September 2010, 101 children with VSD aging from 8 to 13 years old were studied and sampled the blood for genomic DNA preparation. 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 activity were detected by gelatin zymography.

From the four SNPs assay in this study, only the MMP-9 R279Q SNP showed a statistically significant correlation with VSD formation ($p < 0.05$). The frequency of GA genotype in the VSD group was higher than Control group (52.2% and 37.2%, respectively).

On the other hand, according to the definition of VSD/aortic root (Ao) ratio, the patients were classified into three groups: VS (VSD/Ao ratio ≤ 0.2), VM ($0.2 < \text{VSD/Ao} \leq 0.3$), and VL (VSD/Ao > 0.3) which stand for small, medium and large defect, respectively. The MMP-2 activity in the VS, VM and VL groups is about 40%, 50 and 60% higher than that in the Control group, respectively. MMP-9 has the similar trend of MMP-2. MMP-9 activity of VL group is about 50% higher than that in Control group ($p < 0.01$).

In conclusion, MMP-9 R279Q may participate VSD pathogenesis; however, there is need to study further for clarifying the relationship between MMP-9 R279Q polymorphism and VSD. The most valuable found out in this research is that MMP-2 and MMP-9 enzyme activity increases with the defect serious level of VSD increase. Our results hint that MMPs expression may affect the severe level of VSD. It is very valuable to further investigate the exact mechanism of MMPs cause effect on VSD in the future.



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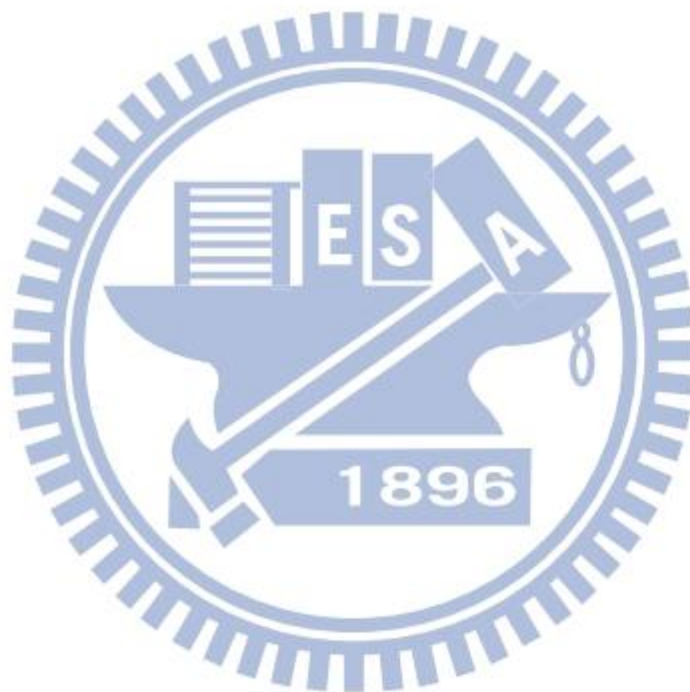

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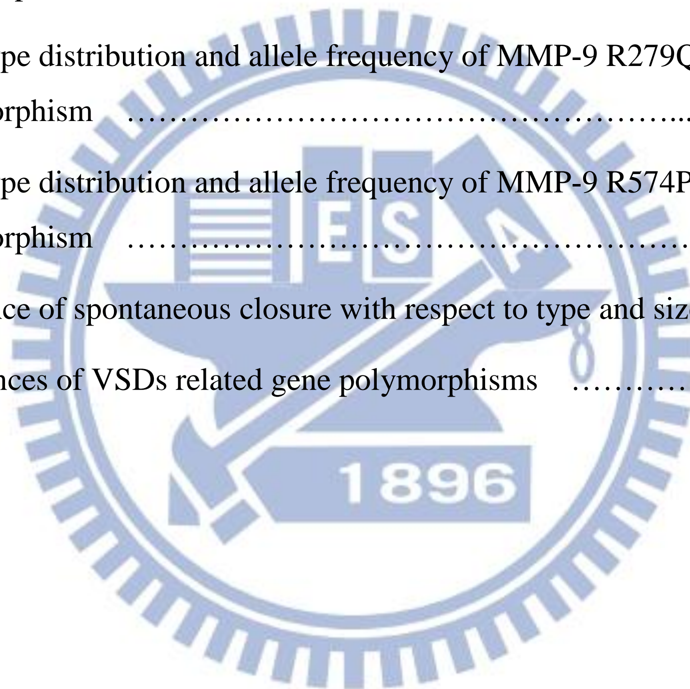

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I. Literature review

1-1. Single-nucleotide polymorphisms

Polymorphism represents natural sequence variants (alleles), which may occur in more than one form. These 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 alternate nucleotide in a given and defined genetic location at a frequency exceeding 1% in a given population. This definition does not include other types of genetic variability like insertions and deletions, and variability in copy number of repeated sequences. They occur once in every 300 nucleotides on average, which means there are roughly 10 million SNPs in the human genome. 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, which underlie individual differences in various biological traits and in susceptibility to disease (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). A non-synonymous polymorphism can differ from missense and nonsense, which the former results in a different amino acid and the latter results in a premature stop codon. Over half of all known disease mutations come from non-synonymous polymorphisms (Stenson, 2009). If gene expression is affected by a SNP this type is referred to as an eSNP (expression SNP) and may 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; denaturating HPLC and gel electrophoresis; hybridization analysis. All of the references information of SNP in this study was obtain from National Center for Biotechnology Information (NCBI)-SNP database.

1-2. Congenital heart defects

1-2-1. Ventricular septal defects

Ventricular septal defects (VSDs) are the most common form of congenital heart disease. The defect can be in any portion of the ventricular septum, and the physiologic consequences can range from trivial to severe. Approximately 20% of patients in congenital heart disease registries have VSD as a solitary lesion (Hoffman and Rudolph, 1965). Although historically the incidence of VSDs 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 echocardiographic studies demonstrated an incidence of VSD in newborns to be 5 to 50 per 1,000 (Tikanoja, 1995). Rates of spontaneous closure for membranous and muscular VSDs 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 course and the VSD persist. The development of echocardiography has provided insight into the mechanisms by which VSDs close spontaneously in a gross point of view (Murphy et al., 1986).

Defects can generally be classified according to their location, either within the muscular septum (muscular defects) or at its margins. Ventricular septal defects at the margins of the muscular septum can be related to hinge-points of the leaflets of the atrioventricular valves (perimembranous), those of the arterial valves (juxta-arterial or subarterial), or both (**Figure 1**) (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 detail molecular insight of spontaneous VSDs closure has been studied, nor studies of association between extracellular matrix (ECM) and spontaneous VSDs closure have been clarified.

1-2-2. Atrial septal defects

Atrial septal defects (ASDs) represent 6 to 10% of all cardiac anomalies and are more frequent in females than males by about 2:1. ASDs occur in 1 child per 1,500 live births (Samanek, 1992). A prospective echocardiographic study suggested that as many as 24% of newborns have evidence of an opening (3 to 8 mm) in the atrial septum in the first week of life (Fukazawa et al., 1988). However, by a little more than 1 year of age, 92% of the patients were found to have spontaneous closure of the opening, and in most patients, there is evidence of a valve-like opening of the atrial septum that is believed to contribute to closure. Helgason and Jonsdottir (Helgason and Jonsdottir, 1999) reviewed the medical records of all patients in Iceland with a diagnosis of ASD born between 1984 and 1993. ASD was confirmed by 2-D echocardiogram, and data only from patients with secundum ASDs were analyzed. A total of 84 children diagnosed at a mean age of 12 months were followed for 4 years. Spontaneous closure or decreased size was observed in 89% with a 4-mm ASD, 79% with a 5- to 6-mm defect, and only 7% with a defect > 6 mm. Occasionally, spontaneous closure will occur as late as 16 years (Brassard et al., 1999). As the same condition mentioned in the paragraph of VSDs, the underlying mechanism of spontaneous closure of ASDs is till mysterious.

1-3. Matrix metalloproteinase

1-3-1. Structures and functions of MMPs

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 not only play an important role in ECM remodeling in physiologic situations, such as embryonal development, tissue regeneration, and wound repair, also in pathological conditions including rheumatoid arthritis, osteoarthritis, atherosclerotic plaque rupture, tissue ulceration, and in cancer cell invasion and metastasis (Roeb and Matern, 2001; Jones et al., 2003). The MMP family currently consists of 28 enzymes with somewhat different activities. The members are generally divided into six groups according to either structure or preferred substrates and referred to as interstitial collagenases (MMP-1, -8 and -13), stromelysins (MMP-3, -10, -11, and -12), matrilysins (MMP-7 and MMP-26), gelatinases (MMP-2 and MMP-9), membrane-type MMPs (MMP-14, -15, -16, -17, -24, and -25) and others (Visse and Nagase, 2003; Bode and Maskos, 2003). Although MMPs are subclassified based on their ability to degrade 4 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. Zinc-dependent catalytic domain of MMPs 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 activity 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 (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; Overall et al., 1999). The regulation of MMPs occurs 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, tissue inhibitor of metalloproteinases (TIMPs). In addition, there are more and more researchers turn their focus on emphasizing MMPs protein physical functions to the change of nucleotides and the SNPs of MMPs and TIMP correlated with different cardiac diseases and cancers (**Table 1**).

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

In 1978, Sellers et al. were the first to separate a gelatinase activity from collagenase and stromelysin in culture medium from rabbit bone (Sellers et al., 1978). A similar enzyme, acting on basement membrane type IV collagen was reported by Liotta et al. the following year (Liotta et al., 1979). Gelatinase was purified from human skin, mouse tumor cells, rabbit bone, and human gingival. The completed sequence of the human enzyme except for the signal peptide was reported by Collier et al. (Collier et al., 2001). Gelatinase A had a triple

repeat of fibronectin type I domains inserted in the catalytic domain; these participate in binding to the gelatin substrates of the enzyme (Lee et al., 1997; Libson et al., 1995).

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

Harrwas and Krane in 1972 detected a gelatinase activity in rheumatoid synovial fluid. Sopata and Wize described a gelatinase from human polymorphonuclear leukocytes (Sopata and Wize, 1979). Rabbit macrophages produce a very similar enzyme which was able to digest type V collagen (Horwitz et al., 1977). The neutrophil collagenase and gelatinase were resolved in 1980 (Murphy et al., 1980). Purification was achieved in 1983 and sequencing of the cDNA in 1989. An interesting phenomenon, still not fully understood, is the binding of TIMP-1 to proMMP-9 to form a complex (Sakyo et al., 1983; Stetler-Stevenson et al., 1989). Human neutrophil MMP-9 commonly occurs as a complex with lipocalin (Fernández et al., 2005). A series of papers concerned a 95 kDa protein in plasma that binds to gelatin culminated in the identification of this protein as MMP-9 (Makowski and Ramsby, 1998).

1-4. The roles of MMPs in cardiac development

MMP-2 is the earliest MMP known to be expressed during heart development. The process of cardiac looping which converts the single, straight tubular heart into a S-shaped tube and re-positions the primitive heart chambers into their adult anatomical positions before cardiac septation is complete. During the process of making a single heart tube, MMP-2 is expressed in the endocardium, early differentiating cardiomyocytes, and dorsal mesocardium but is soon lost within the myocardium. Blocking MMP-2 activity prevents midline fusion of the primitive heart tubes leading to cardiac bifida (Cai et al., 2000; Linask et al., 2005).

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 (Linask et al., 2005) and increases the incidence of dextrocardia (reversal of the normal anatomical position of the heart, i.e., right-sided heart). In other words, blocking MMP-2 activity prevents midline fusion of the primitive heart tubes leading to cardiac bifida (Linask et al., 2005). Therefore, MMP-2 mediated growth appears to be involved in the direction of cardiac looping.

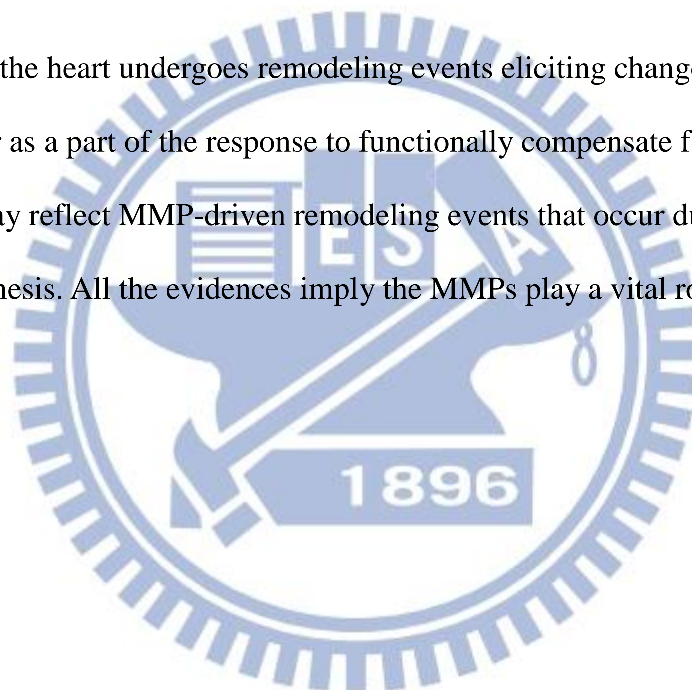
Defects in cardiogenesis during the first three weeks of gestation are usually dangerous. However, anomalous events occurring later in embryonic development often permit the embryo and fetus to make it to term. These anomalies most often manifest themselves as great vessel or cardiac septal defects in 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, endocardial-derived cushion cells and invading 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 in both the atrioventricular and outflow tract regions of the developing heart (Cai et al., 2000, Alexander et al., 1997).

Hyaluronan is abundant in the ECM of the pre-mesenchymal heart (Markwald et al., 1979). Hyaluronan is an esssential mediator of cell migration and invasion for proper heart development (Camenisch et al., 2002). Hyaluronan relate to regulate the expression of multiple MMPs in several cell types and directly induces EMT of the endocardium. Therefore, MMPs play pivotal roles for EMT of the endocardial and cardiac septation. Cells of septum primum adjacent ostium primum express MMP-2, TIMP-2, and TIMP-3 during the formation

and remodeling of the muscular septa. MMPs also involve in remodeling events which are responsible for transfiguring the primitive ventricular myocardial wall into a compact layer and inner trabecular layer. MMPs release bioactive VEGFs from the ECM (Belotti et al., 2003; Sounni et al., 2004) and active VEGF-A increases the expression of several MMPs (Pufe et al., 2004; Wang and Keiser ,1998). Therefore, if MMP processing is required for erbB signaling in the developing heart, the specific temporal and tissue-specific expression of MMPs and TIMPs could dictate where and when particular growth factors modulate cardiac remodeling events.

In a nutshell, the heart undergoes remodeling events eliciting changes in MMP activity and ECM turnover as a part of the response to functionally compensate for the extra load. Such responses may reflect MMP-driven remodeling events that occur during embryonic cardiac morphogenesis. All the evidences imply the MMPs play a vital role in cardiac developing.



II. Materials and Methods

2-1. Patients and sample collection

The blood of 101 children of congenital heart defects will be recruited in this study of Taipei and Hsin-Chu Maykay memorial hospital from the outpatient base in one year period. All the patients of congenital heart defects are enrolled in study when they are already documented by 2-D echocardiography (**Figure 2**). The 152 control subjects contain no genetic diseases patients or the people who did physical examination in hospital, besides, the plasma of 12 health people were classified as control group in MMPs activity assays.

The blood samples will be obtained by nontraumatic needle aspiration from the antecubital vein, with no hemolysis occurring in any of the samples when they receive clinical follow-up at 0, 6, and 12 months. The plasma and white blood cells were separated by centrifuge 2,000 rpm for 10 min, and stored to -80℃.

2-2. Genomic DNA extraction

Genomic DNA for PCR-RFLP genotyping was isolated from the blood of 101 children of congenital heart defects using the genomic DNA Mini kit. Up to 200 μL of whole blood add 30 μL of Proteinase K (10 mg/mL) and mix briefly. After that, incubate the mixture at 60℃ for 15 min. Following then add 200 μL of GB Buffer, mix by shaking vigorously and incubate the mixture in a 60℃ water bath for 15 min. Add 200 μL of absolute ethanol to the sample lysate and immediately mix by shaking vigorously. Transfer the entire mixture (including any precipitate) to the GD Column. Centrifuge at 14,000 x *g* for 5 min. The collections add 400 μL of W1 Buffer to the GD Column and centrifuge at 14,000 x *g* for 1 min. Then add 600 μL of Wash Buffer (ethanol added) to the GD Column and centrifuge at

14,000 x *g* for 1 min. Displace into new collection tube and centrifuge again at 14,000 x *g* for 3 min to dry the column matrix. Finally, add 30 μL of pre-heated Elution Buffer (10 mM Tris-HCl, pH 8.5) to the matrix and last for 5 min; after that, centrifuge at 14,000 x *g* for 1 min to elute the purified DNA. The maximum yield is up to 50 μg. Purified genomic DNA were Stored in the -80°C.

2-3. Genetic analysis

2-3-1. 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 2**. The target sequence was amplified in a 50 μL reaction volume containing 1 μL of genomic DNA, 1 μL each primer (10μM), 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 second, 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 *Sau96* I (New England BioLabs, Ipswich, MA, USA) at 37°C overnight and separated on a 2% agarose gel. *Sau96* I does not digest the T allele but generates 202-bp and 95-bp fragments for the C allele.

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

The primers used to detect this polymorphism were shown in table, which generated a 435-bp fragment containing the -1562C>T site in a 50 μL reaction volume, and the annealing temperature were shown in **Table 2**. 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.

2-3-3. 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 were shown in **Table 2**. 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.

2-3-4. MMP-9 R574P polymorphism

The primers used to detect this polymorphism were shown in **Table 2**, which generated a 246-bp fragment containing the R574P site in a 50 μL reaction volume, and the annealing temperature were shown in 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).

2-4. Gelatin zymography assay

The activity of MMP-2 and MMP-9 in the plasma was measured by gelatin gel zymography as previously has described (Chen et al., 2008). Plasma was mixed with 2× 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℃ in reaction buffer (50 mM Tris-HCl, pH 7.4, 200 mM NaCl, and 5 mM $CaCl_2$). The gels were then stained with Coomossie 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 activities in the gel slabs were 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 controls (Chemicon, Temecula, CA, USA) was contained in each gel as a standard inensity value to normalize smaple intensityand express in arbitrary units.

2-5. Statistical analysis

Data analysis was performed using GraphPad Prism 5 statistical software. Continuous variables were expressed as mean ± standard deviation (SD), and categorical variables were shown as frequencies and percentages. All polymorphisms were tested for confirmation with Hardy–Weinberg expectations in both groups separately. Differences between patients with and without VSDs were examined with the χ^2 test for categorical variables or the Student's t-test and analysis of covariance (ANCOVA) for continuous variables. The association between the MMPs polymorphisms and the risk of VSDs was estimated by odds ratios (ORs) and their 95% confidence intervals (CIs), which were calculated by binary logistic regression models. A 2-tailed $p < 0.05$ was considered statistically significant.

III. Results

3-1. The information of patients

The 101 congenital heart defects patients contained 44 boys and 57 girls were studied at mean age of 8.2 ± 5.0 years old. The blood of CHD patients recruited from hospital containing 90 patients of type 2 VSD, 6 patients of other type VSD, and 5 patients of ASD. All the congenital heart defects patients enrolled in this study were already documented by 2-D echocardiography.

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

The genomic organization of the human MMP-2 gene and localization of the studied SNPs were shown in **Figure 3A**. In this study, -735C>T polymorphism of MMP-2 was analyzed by PCR-RFLP. The sequence of the human MMP-2 gene and position of the PCR primers which were used to detect MMP-2 -735C>T were shown in **Figure 3B**. Restriction enzyme digestion was performed on MMP-2 -735C>T PCR products using the restriction enzyme *Sau96* I and generate three different length bands between different genotype (**Figure 4A**). We also used direct sequencing to detect MMP-2 polymorphism in patients with VSD, and there were three genotypes of direct sequencing map for MMP-2 -735C>T shown in **Figure 4B**.

The genotype and allele frequencies of MMP-2 -735C>T between groups were reported in **Table 3**. The frequencies of MMP-2 -735C>T genotypes and alleles in the VSD, and control subjects conformed to the Hardy-Weinberg equilibrium. As showed in Table, the allele frequencies of C and T allele were 66.3% and 33.7%; however, the results were similar to control group ($p = 0.92$). The genotype frequency of MMP-2 -735C>T were CC: 47.4%, CT

37.9%, TT 14.7%, but no significant differences was found in the genotype frequencies between the VSDs and the control group (p = 0.1554).

3-3. 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 **Figure 5A, 7A and 9A**. 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.

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

The sequence of the human MMP-9 gene and position of the PCR primers which were used to detect MMP-9 -1562C>T were shown in **Figure 5B**. PCR product digestion by restriction enzyme *Sph* I and generate two different length bands between different genotype (**Figure 6A**). And there were two genotypes of direct sequencing map for MMP-9 -1562C>T were shown in **Figure 6B**.

The distribution of the MMP-9 -1562C>T genotype were described in **Table 4**. However, no MMP-9 T/T genotype was detected in patients with VSD and control group. The distribution of the genotype and allelic status were analyzed by the χ^2 value test.

3-3-2. MMP-9 R279Q polymorphism

The sequence of the human MMP-9 gene and position of the PCR primers which use to detect MMP-9 R279Q were shown in **Figure 7B**. PCR product digestion by restriction enzyme *Sma* I and generate three different length bands (AA genotype: 467-bp, AG genotype:

467-bp, 296-bp, and 171-bp, GG genotype: 296-bp and 171-bp) between different genotype (**Figure 8A**). And there were three genotypes of direct sequencing map for MMP-9 R279Q were shown in **Figure 8B**.

The genotype and allele frequencies of MMP-9 R279Q between VSD and control group were summarized in **Table 5**. As shown in **Table 5**, the genotype frequency of MMP-9 R279Q was GG: 45.6%, GA: 52.2% and AA: 2.2%. The results indicate that the genotype distribution MMP-9 R279Q have statistically significant differences compared to control group which the samples of patients without VSD ($p = 0.0361$); however, there were no significant differences was found in the genotype frequencies between the VSD and the control group ($p = 0.6635$).

3-3-3. MMP-9 R574P polymorphism

The sequence of the human MMP-9 gene and position of the PCR primers which were used to detect MMP-9 R574P were shown in **Figure 9B**. PCR product digestion by restriction enzyme *Nla* IV and generate three different length bands between different genotype (**Figure 10A**). And there were three genotypes of direct sequencing map for MMP-9 R574P were shown in **Figure 10B**. The genotypes and allele frequencies of MMP-9 R574P that found in patients with VSD were reported in **Table 6**. The results shows that the homozygous C/C, heterozygous C/G and homozygous G/G genotypes of MMP-9 R574P were 52.0%, 44.9% and 3.1%, respectively, in the VSD group and 53.2%, 37.6% and 9.2%, respectively, in the control group. There were no significant differences in genotypes ($p = 0.1533$) and allele frequencies ($p = 0.1664$) between groups.

3-4. Plasma MMPs activity in the VSD children with different MMP polymorphisms

In order to investigate the differences of each MMPs activity in VSD patients that have different genotype of each MMPs polymorphism, we used the gelatin zymography to detect MMP-2 and MMP-9 activity in plasma from congenital heart defects children.

3-4-1. MMP-2 activity

MMP-2 activity in sample plasma was detected by gelatin zymography and samples were classified according to genotype of patients on MMP-2-735, which has CC, CT, TT genotype and each group contained 45, 39, and 13 samples, respectively. Used standard MMP-2 as positive controls (Chemicon, Temecula, CA, USA) to calculate MMP-2 activity of each group and collected the statistic as shown in **Figure 11**. MMP-2 activities have no significant difference in MMP-2 subgroups, but TT allele has the highest MMP-2 activity in MMP-2 -735C>T group ($p = 0.052$).

3-4-2. MMP-9 activity

For investigating the relation of MMP-9 activity in the VSDs children and MMP-9 SNP, three SNP were chosen, MMP-9 -1562C>T, MMP-9 R279Q and MMP-9 R574P. According to different genotype of patients underwent classification. **Figure 12A** shows the part of MMP-9 R279Q and is departed in GG, GA, AA and each group have 42, 50, 2 samples, sequentially. **Figure 12B** shows the part of MMP-9 R574P and is departed in CC, CG, GG and recruit 51, 44, 3 samples, respectively. **Figure 12C** shows the part of MMP-9 -1562C>T and is classified in CC, CT and includes 69 and 21 patients, respectively. We used standard MMP-9 as positive controls (Chemicon, Temecula, CA, USA) to calculate MMP-9 activity of each group. As the data showed, MMP-9 activities have no significant difference in the subgroups of MMP-9 genotypes.

3-5. The relationship between plasma MMPs activity and VSD severity

Anatomically, VSDs are classified in to three defect levels: large, medium and small. The large defect is defined as the size is larger than two-thirds of the aortic root diameter; the medium size is in between two-thirds and one-third of the aortic root diameter; and small if the size is less than one-third of the aortic root diameter (Hornberger et al., 1989; Kabra and Srivastava, 2010). **Figure 13** shows the Echocardiograms of normal, small defect and medium defect. The sample of patients with VSD conducted in this study are relative not severe population, which the severe population means surgery needed. According to the definition of VSD/aortic root (Ao) ratio, the patients were classified into three groups: VSD/Ao ratio ≤ 0.2 is VS group; $0.2 < \text{VSD/Ao} \leq 0.3$ is VM group; and VSD/Ao > 0.3 is considered to VL group. The VS, VM and VL stand for small, medium and large defect, respectively; each group includes 12, 45, 25 and 15 patients, respectively. The MMPs activity was detected by zymography, and the results were shown in **Figure 14**.

3-5-1. MMP-2 activity

According to figure **Figure 14A**, the MMP-2 activity of VS group is 1.4 fold higher than control group ($p < 0.01$), besides, VM and VL group MMP-2 activity are 1.6 fold higher than control group ($p < 0.01$, $p < 0.001$, respectively).

3-5-2. MMP-9 activity

In the part of MMP-9 activity, the patients also were separately assigned into VS, VM and VL group, and three of them were compared to control group (no VSD). **Figure 14B** shows that there is no significantly difference between MMP-9 activity of VS defect level and control group; but when defect level rises to VM defect level, the MMP-9 activity

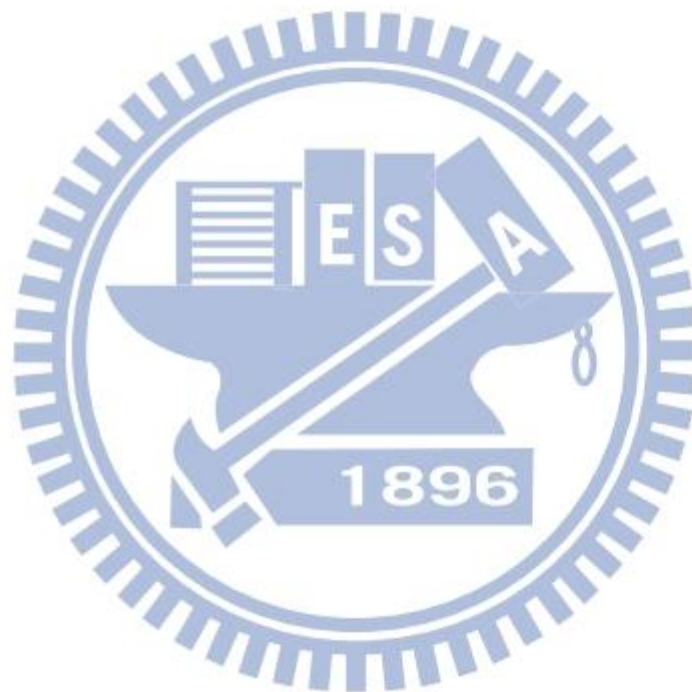
significantly increases ($p < 0.05$); as defect level considered large also performs same trend which MMP-9 activity of VL group is about 1.5 fold higher than control group.

3-6. Investigating the relationship between MMPs activity and spontaneous closure rate of VSD

The patients with VSD were total diagnosed with echocardiographic examination twice with the interval of six months. Patients were initially assigned by first echocardiographic examination result based on t VSD/Ao ratio and then all samples were classified into VS, VM and VL groups. For the purpose of investigating the relation between MMPs activity and spontaneous closure rate of VSD, the ratio of VSD to aortic root from second echocardiographic examination result were compared to first ratio. If the compared ratio value were above 15 % , then the patient with VSD were viewed as happened spontaneous closure of VSD; if the value below 15%, then the patient with VSD were considered as happened no spontaneous closure or personal equation. There total were 73 patients conducted in this experiment, the statistic table of spontaneous closure classification were shown at **Table 7**. If the patient with VSD were operated or had only one time echocardiographic examination, then will not include in this study. The relative MMPs activity was defined as the spontaneous closure MMPs activity to the basic of non-spontaneous closure MMPs activity.

Figure 15A denotes that the MMP-2 activity in spontaneous closure subgroup of VS group is significantly lower than non-spontaneous closure subgroup ($p < 0.05$), indicating that the mechanism of spontaneous closure might suppress the activity of MMP-2. However, the unusual point is that the phenomenon presented in VM and VL subgroups are opposite to VS, which non-spontaneous closure subgroup owns not significantly but higher MMP-2 activity

than spontaneous closure subgroup. The study of MMP-9 also demonstrates same trend in MMP-2 as showed in **Figure 15B**.



IV. Discussion

The first description of MMP-like activity was related to the metamorphosis of the tadpole (Brinckerhoff and Matrisian, 2002). This and many other subsequent discoveries strongly suggest that this well-characterized family of metalloproteinases is also intimately involved in normal tissue growth and ECM remodeling (Stamenkovic, 2003). Morphogenesis and tissue growth, remodeling, and repair are sentinel features of childhood and adolescence. Heart morphogenesis involves the characteristic mechanisms of cell proliferation, cell rearrangements, cell to cell, cell to matrix interactions, and tissue remodeling. Linask et al. 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 (Linask et al., 2005), although little is known about the production, secretion, and clearance of these important proteinases throughout normal growth and development in human heart

At present, information suggests that the septum has both mesenchymal and muscular components (Contreras-Ramos et al., 2008). The mesenchymal element originates mainly from fusion of the conotruncal and atrioventricular endocardial cushions. Mechanisms that initiate development of the muscular septum are less well defined, but at least two processes have been proposed. 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 inwards (Goor et al., 1970). The second process is an alternative hypo thesis 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 (De La Cruz and Moreno-Rodriguez, 1998).

Gene polymorphism also is considered an essential cause of VSD formation; especially the TBX5 and GATA4 SNP are frequently discussed. These gene both express in the heart and their interaction is vital for normal cardiac septation (Maitra et al.,2009). TBX5 is a vital gene during embryonic differentiation which affects cardiac and upper limbs development. When TBX5 occurs mutation may cause the loss of this gene function of and therefore cause cardiac and upper limbs malformations (Basson et al.,1997). A TBX5 polymorphismis also associated with ventricular septal defect in the Chinese Han population (Liu et al., 2009). There are many researches point out that GATA4 gene mutationis related to the happen of ASD and VSD (Zhang et al., 2009; Chen et al., 2010[b]; Rajagopal et al.,2007). The other VSD related genes were list in **Table 8**. According to above, this research is based on studying the association of MMPs SNP and MMPs activity to the formation of VSDs and may participate the mechanism of spontaneous closure of VSDs.

To realize MMPs polymorphism from National Center for Biotechnology Information (NCBI)-SNP database, which had registered over 700 SNPs of MMP-2 and 300 SNPs of MMP-9. The relatively important and correlative literatures were organized and shown in **Appendix 1** for MMP-2 and **Appendix 2** for MMP-9. As MMPs play important role in many physiological functions, MMP polymorphism also has proven to be relative to many diseases. Gene transcription is the primary point of regulation of MMPs, which implicates that sequence changes in the promoter may be important for the transcription and cause effect in protein levels and cell physiology.

Two of MMPs polymorphisms in promoter region were detected in this study: MMP-2 -735C>T and MMP-9 -1562 C>T. The reason of chosen these two promoter will described as

following. The C>T 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 -1562 C>T polymorphism in the promoter region of MMP-9 exerts an T allelic effect on MMP-9 promoter strength and results in losing a nuclear repressor protein binding site which decreasing MMP- 9 expression as the T allele is present, and thus increasing the enzyme expression compared to the C allele (Morgan et al., 2003; Zhang et al., 1999).

Besides, if SNPs occurred at gene exon sequence, the structure of enzyme may change which further affects itself activity. So, this study also examined SNP in *MMP-9* rs17576 (Q279R) and rs2250889 (R574P). SNP in MMP-9 rs17576 (Q279R) is a non-conservative amino acid substitution that modifies an amino acid residue within the highly conserved gelatinase-specific fibronectin type II domain (FN2) (Allan et al., 1995). Although the specific functional consequences of the substitution of proline for arginine coded by rs2250889 in MMP-9 (R574P) have not been studied yet, the location of SNP rs2250889 in the C-terminal hemopexin-like domain could 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 (Rodríguez-Pla et al.,2008).

All SNPs above have more potential in ruling and causing MMP-2 and MMP-9 to significant change than other SNPs, which making them to be the target SNP in this research. In this study, the SNPs of the MMPs genes, such as MMP-2 (-735C>T) and MMP-9 (-1562C>T, R279Q, and R574P) were determined by the polymerase chain reaction, restriction fragment length polymorphism method (**Table 2**). 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 (**Table 3**). This result suggest that C>T

polymorphism located at nucleotide -735 may not influence the incidence of VSD or not the core factor in Taiwan individuals.

MMP-2 activity in VSD patient plasma 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 that C>T polymorphism located at nucleotide -735 should significantly diminish promoter activity (Yu et al., 2004); 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 which is different from the results of Yu C et al, suggesting C allele enhanced MMP-2 protein transcription which corresponds with higher MMP-2 activity (Yu et al., 2004).

Polymorphisms in the promoter of MMP-9 have been implicated in the regulation of gene expression and susceptibility to various diseases. The results of genotype distribution and allele frequencies of MMP-9 -1562 C>T between the VSD and the control group were shown in **Table 4.** The results show that polymorphism in the promoter of MMP-9 -1562 C>T has no correlation to VSD. However, there is no VSD patient have TT genotype of MMP-9 -1562 C>T in our study. We speculate that lack of T allelic genotype of MMP-9 polymorphism is relative to population and region, which is correspond to other disease studies conducted in Asian population also had few or even no T allelic genotype of MMP-9 polymorphism in their samples (Chen et al., 2010[a]; Lacchini et al., 2010; Buss et al., 2009).

The polymorphism at position -1562 were expected to change the promoter activity of MMP-9 (Zhang et al., 1999; Van den Steen et al., 2002); however, our results showed no relationship between genotypes of MMP-9 -1562 polymorphism and their activity. Our results

communicates with Demacq et al. whose plasma samples were recruited from healthy subjects (Demacq et al., 2006).

In detection of MMP-9 codon polymorphisms: MMP-9 Q279R and R574P, our data demonstrated that there is an association of the MMP-9 Q279R polymorphism and risk of VSD (**Table 5** and **Table 6**). The importance of MMP-9 Q279R is based on the ability of changing MMP-9 protein structure and their substrate binding affinity which related to many diseases, such as melanoma, lumbar-disc herniation, type 2 diabetes, and pelvic organ prolapse (Ahluwalia et al., 2009; Cotignola et al., 2007; Hirose et al., 2008; Chen et al., 2010[a]). The GA genotype distribution in VSD group is much higher than control group. Although there were few subjects of AA genotype in VSD group, there is an obvious difference in genotype distribution to control group.

Blankenberg et al. found that the G allele of MMP-9 R574P polymorphism was overrepresented in patients with histologically confirmed giant cell arteritis (GCA) (Blankenberg et al., 2003); however, we do not find any significant differences in both the genotype and allele distribution between the VSD and control groups in Taiwanese population. According to VSD patients different genotypes of MMP-9 Q279R and R574P polymorphism, there is no significant difference showed in MMP-9 activity detection, which indicates these SNPs may cause effect in protein structure and substrate binding efficiency but no notable effect in enzyme activity.

The VSD patients according to their serious level were further categorized to four groups. All groups were conducted of the study that whether the serious level is correlative to MMPs activity. The most valuable find out in this research is that MMP-2 and MMP-9 enzyme activity increases with the defect serious level of VSDs increase (**Figure 14**). Indicating that

MMPs expression may affect the severe level of VSDs, and interfere the closure rate of VSDs.

The rate of spontaneous closure of VSDs has been reported to be between 11% and 70.8% in various researches (Kidd et al., 1993; Mehta and Chidambaram, 1992; Ahunbay et al., 1999; Eroğlu et al., 2003). In our study, spontaneous closure was detected in 23% of perimembranous defect (**Table 7**). Commonly, patient with small defect is considered more easy to happen spontaneous closure for not violent blood stream; but in our study, our samples showed that patients with severe defect are more possible to happen spontaneous closure, which the possibility of spontaneous closure are 38% and 14% for severe and slight VSD, respectively.

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). This study proposes that in vivo proteases are very possible relative to spontaneous closure of VSD. **Figure 15** demonstrates that spontaneous closure subgroup of VS group owns lower MMPs activity than the other subgroup, and the results are opposite to VM and VL group, which their spontaneous closure subgroup own higher MMPs activity. Combined the results from **Figure 14** and **Figure 15**, the reasons of VM and VL group have higher MMPs activity expressed in spontaneous closure subgroup are suspected to their severe defect level and the role of MMPs participating in spontaneous closure of VSD. However, the role of MMPs participating in spontaneous closure of VSD is advantage to closure or not still unknown. The further research of mechanism about MMPs in VSD closure is needed in the future.

V. Conclusions

Ventricular septal defect is one of the most common form of congenital heart diseases, and there are about 50% children patients will spontaneous closure in 12 months; however, it still remains vague about the mechanisms of VSD occurrence and its' spontaneous closure. VSD children patients' blood were collected and processed with extracting their genome for MMPs genotyping and separating plasma for MMPs activity evaluation. The genotypes of the MMP2 -735C>T polymorphisms and MMP9 -1562C>T, R279Q and R574P polymorphisms were determined using PCR-RFLP. Plasma MMPs activity was determined by zymography in VSDs children with different MMP polymorphisms.

The results in this study indicate that the R279Q polymorphism of MMP-9 gene is significantly associated with VSD risk in Taiwan children. However, both enzyme activity and genotype distribution of another MMPs SNP in this study were not associated with VSD. Furthermore, VSD patients according to echocardiography were classified into different severe group for investigating the relationship between plasma MMPs activity and VSD severity and the relationship between MMPs activity and spontaneous closure rate of VSD. We find that when the severity of VSD rises then MMP-2 and MMP-9 activities increase. And the experiments results show that MMPs participate in spontaneous closure mechanism of different severity VSD.

This study finds out the potential role of MMP SNPs in regulation of ECM effector and the recovery of the congenital defects in children. Also help physician to elucidate the different activities among different MMPs members in these common clinical situation. The work may offer the predictable factor about which group of cardiac defects will recovery spontaneously. Besides, the results may point-out the novel targets which are significantly in

the process of heart recovery, and the researches might modulate the natural remodeling process by regulating the target through pharmacological therapy.

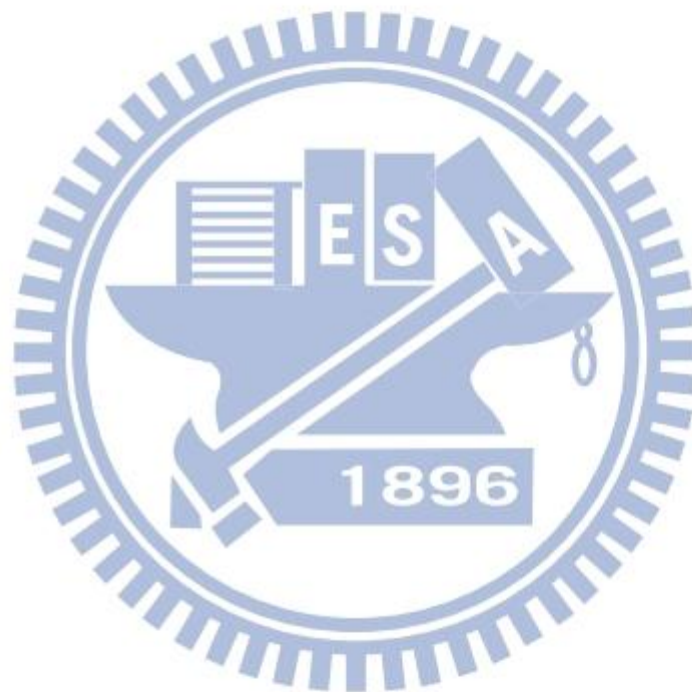


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Table 1. Outlines of the information of MMPs and TIMPs SNPs defined and relationship with particular diseases

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., 117:5172-5181, 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., 404:119-123, 2009[b]
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., 63:182-185, 2008
TIMP-2 (-418, rs8179090)	GG GC CC	Both genotype distribution and allele frequency of the TIMP-2 -418G>C polymorphism were significantly different between the atrial fibrillation and control group (*p* = 0.005 and *p* = 0.001, respectively).	Gai et al., 411:719-724, 2010

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

Targeting gene (position, dbSNP rs# cluster ID)	Genotypes	Relationship with particular diseases	References
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., 39:555-562, 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.	Rodríguez-Pla et al., 58:1849-1853, 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., 408:105-109, 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., 149:222-224, 2010

Table 2. Sequences of the primers used for MMP-2 and MMP-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-GCCCCTTCCTTATCGCCGAC R-ACTCAGCCGCCCCTACGTTTG	246	65	*Nla* IV	CC (65 and 182 bp) CG (65, 182 and 246 bp) GG (247 bp)

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

Genotypes and alleles	VSD (%) (n = 95)	Control (%) (n = 136)	OR (95% CI)	*P* value
CC	45 (47.4)	55 (40.4)	1 (ref.)	
CT	36 (37.9)	68 (50.0)	0.65 (0.37-1.14)	0.130
TT	14 (14.7)	13 (9.6)	1.32 (0.56-3.09)	0.526
C allele	126 (66.3)	178 (65.4)	1 (ref.)	
T allele	64 (33.7)	94 (34.6)	0.96 (0.65-1.42)	0.845

CI, confidence interval; OR, odds ratio.

Genotype distribution of SNPs between groups: $\chi^2 = 3.723$, df = 2, $P = 0.155$.

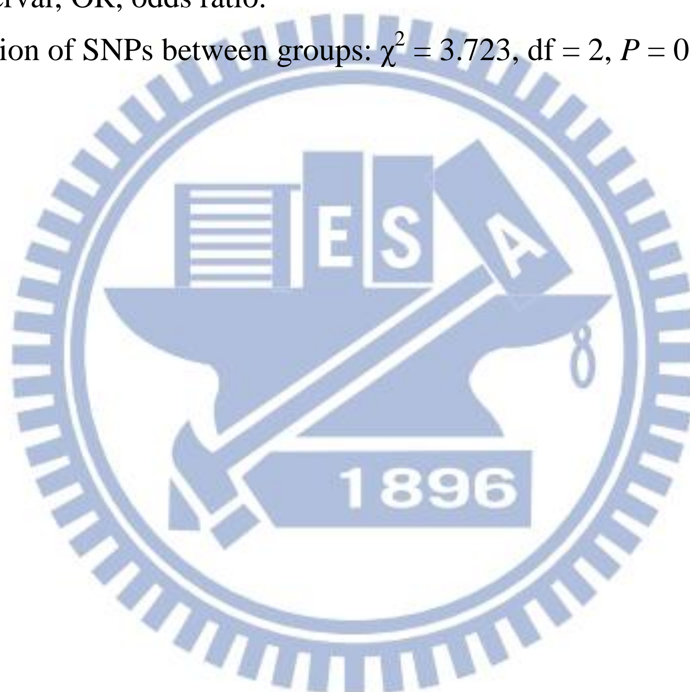


Table 4. Genotype distribution and allele frequency of MMP-9 -1562C>T polymorphism

Genotypes and alleles	VSD (%) (n = 90)	Control (%) (n = 98)	OR (95% CI)	*P* value
CC	69 (76.7)	77 (78.6)	1 (ref.)	
CT	21 (23.3)	21 (21.4)	1.17 (0.56-2.22)	0.754
TT	0 (0)	0 (0)	-	-
C allele	159 (88.3)	175 (89.3)	1 (ref.)	
T allele	21 (11.7)	21 (10.7)	1.10 (0.58-2.09)	0.770

CI, confidence interval; OR, odds ratio.
Genotype distribution of SNPs between groups: $\chi^2 = 0.098$, df = 1, $P = 0.754$.

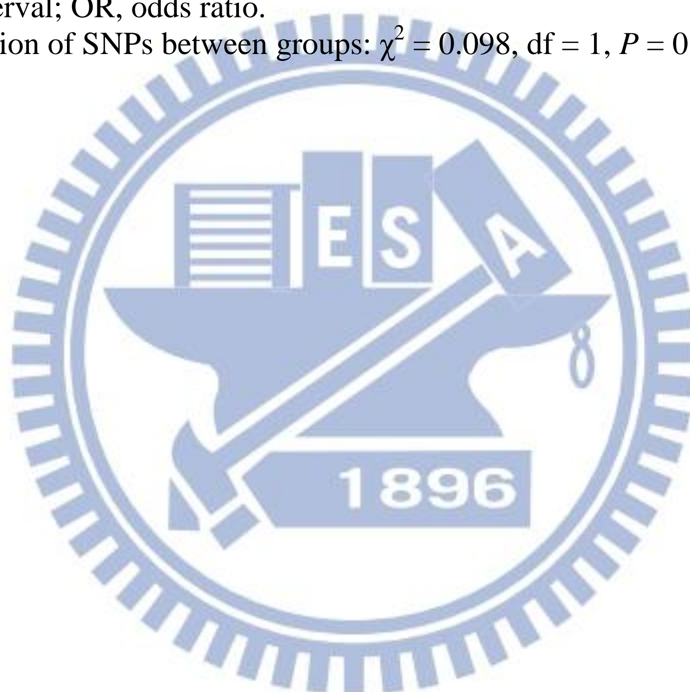


Table 5. Genotype distribution and allele frequency of MMP-9 R279Q polymorphism

Genotypes and alleles	VSD (%) (n = 90)	Control (%) (n = 129)	OR (95% CI)	*P* value
GG	41 (45.6)	71 (55.0)	1 (ref.)	
GA	47 (52.2)	48 (37.2)	1.70 (0.97-2.96)	0.062
AA	2 (2.2)	10 (7.8)	0.35 (0.07-1.66)	0.168
G allele	129 (71.7)	190 (73.6)	1 (ref.)	
A allele	51 (28.3)	68 (26.4)	1.11 (0.72-1.69)	0.647

CI, confidence interval; OR, odds ratio.

Genotype distribution of SNPs between groups: $\chi^2 = 6.645$, df = 2, $P = 0.036$*.

* indicates $p < 0.05$ *vs.* the Control group.

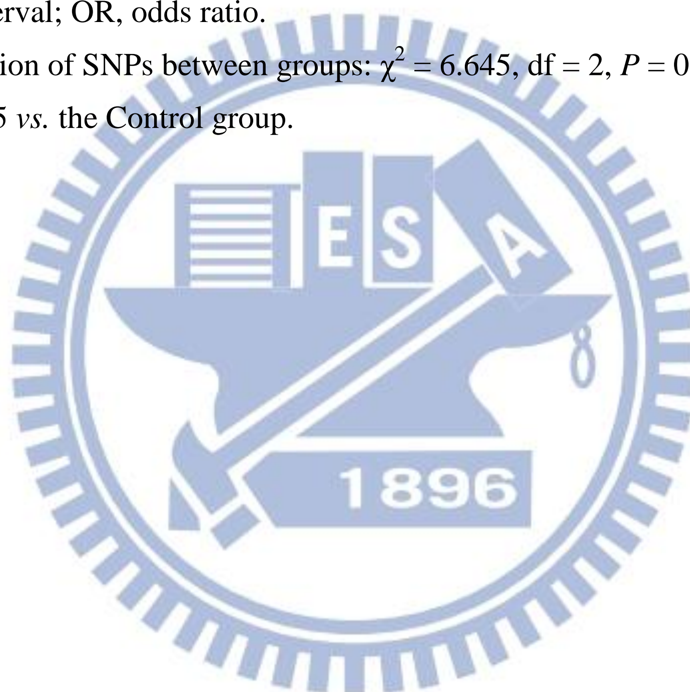


Table 6. Genotype distribution and allele frequency of MMP-9 R574P polymorphism

Genotypes and alleles	**VSD (%) (n = 98)**	**Control (%) (n = 109)**	**OR (95% CI)**	***P* value**
CC	51 (52.0)	58 (53.2)	1 (ref.)	
CG	44 (44.9)	41 (37.6)	1.22 (0.69-2.15)	0.492
GG	3 (3.1)	10 (9.2)	0.34 (0.09-1.31)	0.104
C allele	146 (74.5)	157 (72.0)	1 (ref.)	
G allele	50 (25.5)	61 (28.0)	0.88 (0.57-1.36)	0.571

CI, confidence interval; OR, odds ratio.
Genotype distribution of SNPs between groups: $\chi^2 = 3.751$, df = 2, $P = 0.153$.

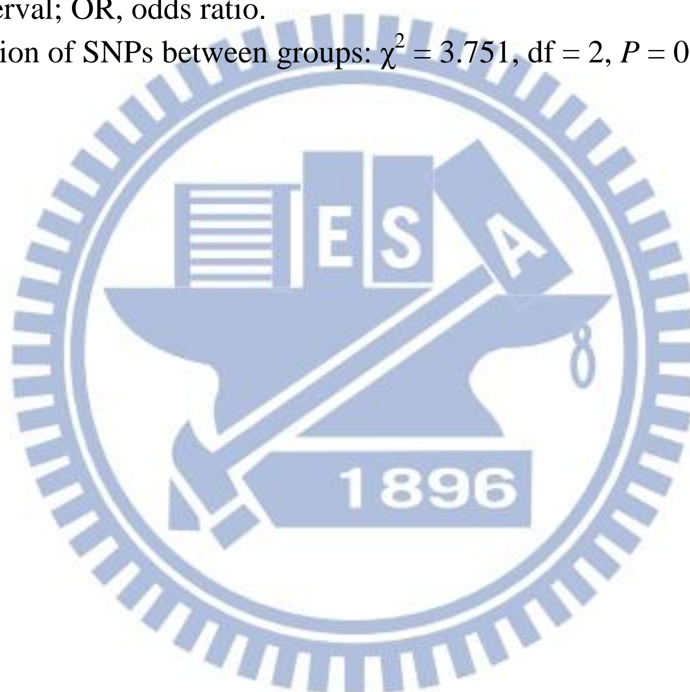


Table 7. Incidence of spontaneous closure with respect to type and size of defect

Size of VSD[a]	No. of patients	Defect reduced (%)	Without change (%)
Small	36	5 (13.9)	31 (86.1)
Medium	24	7 (29.2)	17 (70.8)
Large	13	5 (38.5)	8 (61.5)
Total	73	17 (23.3)	56 (76.7)

[a] According to the definition of VSD/aortic root (Ao) ratio, the patients were classified into three groups: VSD/Ao ratio ≤ 0.2 is small group; $0.2 < \text{VSD/Ao} \le 0.3$ is medium group; and VSD/Ao > 0.3 is considered to large group.

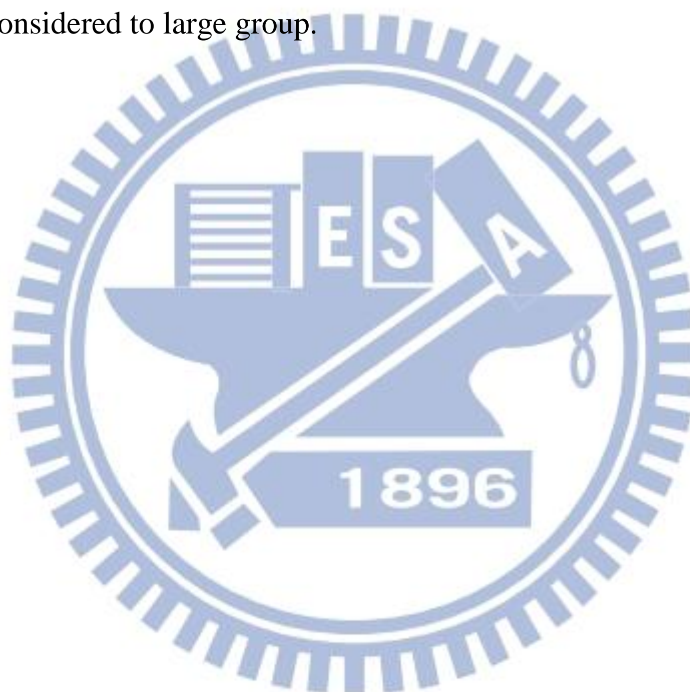


Table 8. References of VSDs related gene polymorphisms

Gene	Important findings	References
VEGF	The -634C allele was in a significant protective association against VSD, suggesting that VEGF dysregulation was involved in the pathological processes of VSD	Xie et al., 15:1246-1251, 2007
TBX20	TBX20-I121M adopts a more fluid tertiary structure leading to enhanced interactions with cofactors and more stable transcriptional complexes on target DNA sequences.	Posch et al., 47:230-235, 2010
GATA4	A novel M310V mutation in GATA4 gene that is located in the NLS region and leads to hereditary ASD in a Chinese family.	Chen et al., 140:684-687, 2010
NFATc1	Based on stratification analyses by congenital heart disease types, individuals that the three times repeat in intron 7 of NFATc1, 56 nucleotides (nt) downstream of the 3' boundary of exon 7 were postulated to have a higher risk of perimembranous ventricular septal defect.	Gu et al., 49:592-600, 2011
22q11	22q11.2 deletion can be detected in isolated TOF, VSD and PDA of three Chinese ethnic groups, without detectable 22q11.2, deletion in those isolated ASD patients examined thus far.	Jiang et al., 105:216-223, 2005
TBX5	SNP rs11067075 within the TBX5 gene had significant correlation with ventricular septal defect (P=0.0037) by single marker association analysis. TBX5 is associated with the occurrence of ventricular septal defect and may be a predisposing gene to congenital heart disease in Han Chinese	Liu et al., 122:30-34, 2009

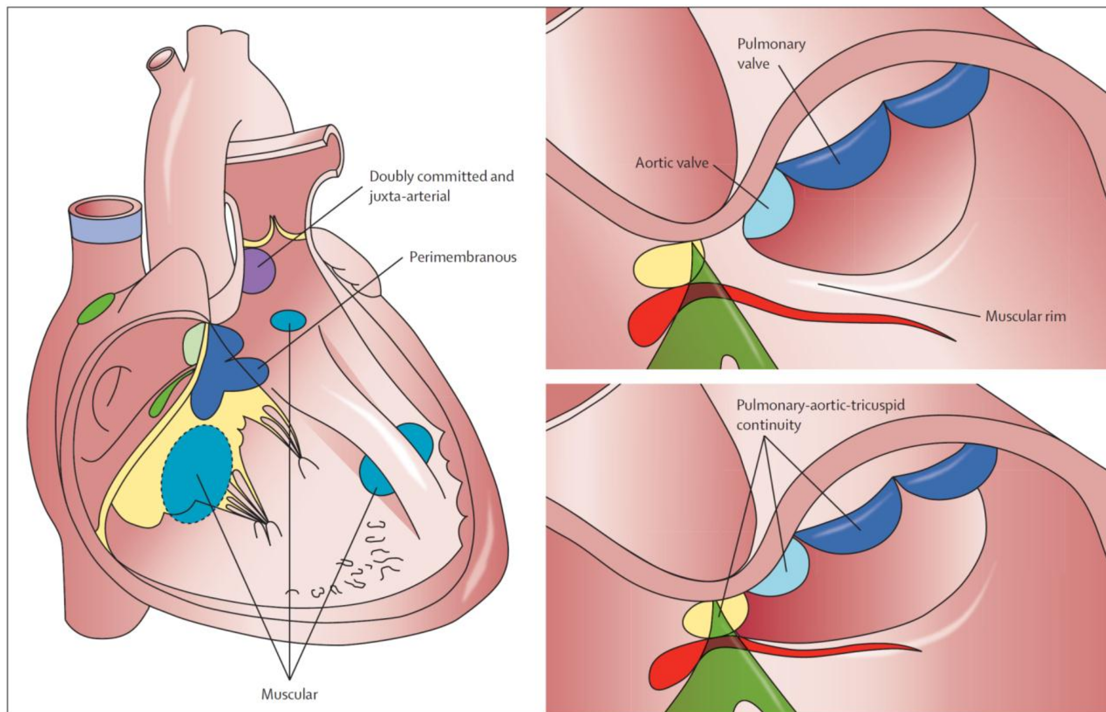
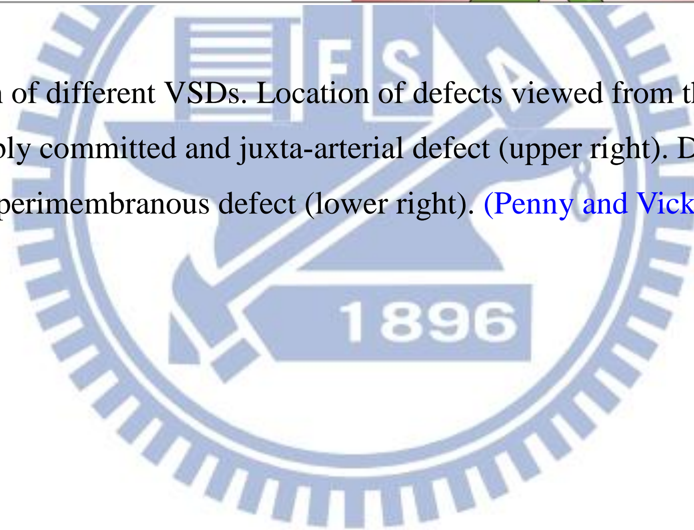
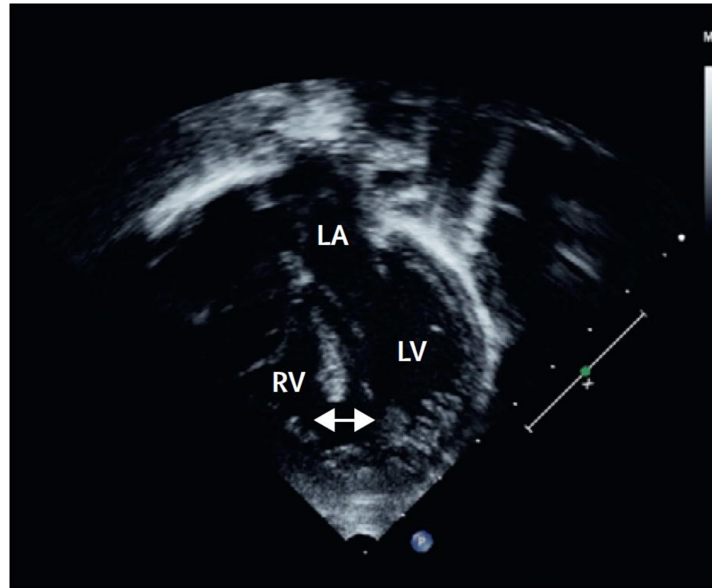


Figure 1. Location of different VSDs. Location of defects viewed from the right ventricle (left). Typical doubly committed and juxta-arterial defect (upper right). Doubly committed, juxta-arterial, and perimembranous defect (lower right). (Penny and Vick, 2011)



A.



B.

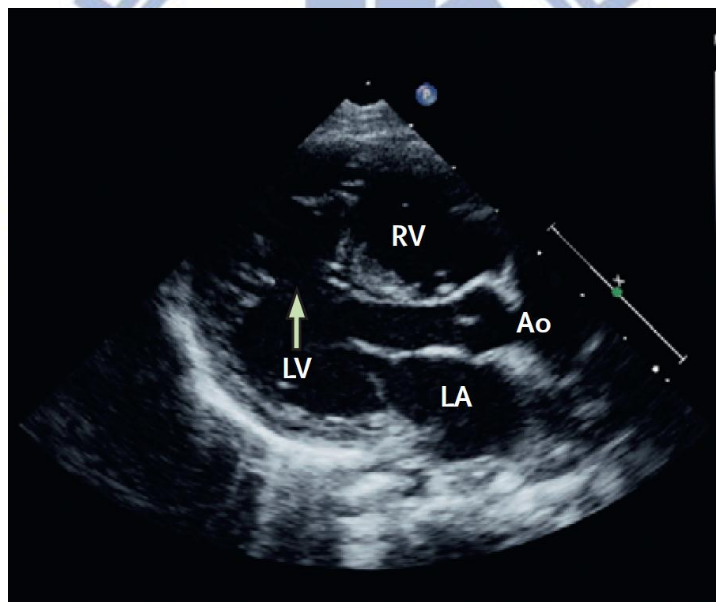
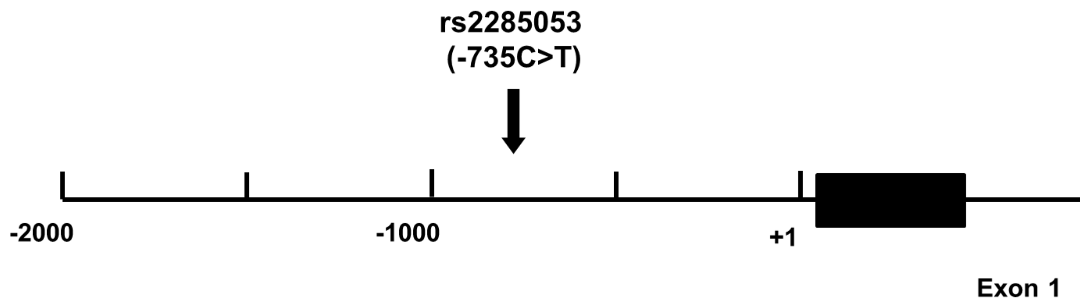


Figure 2. Echocardiograms of VSDs. Echocardiograms show apical four-chamber (**A**) and parasternal long-axis view (**B**) of a large apical muscular defect (arrows). LA, left atrium; LV, left ventricle; RV, right ventricle; Ao, aortic valve. (Penny and Vick, 2011)

A.

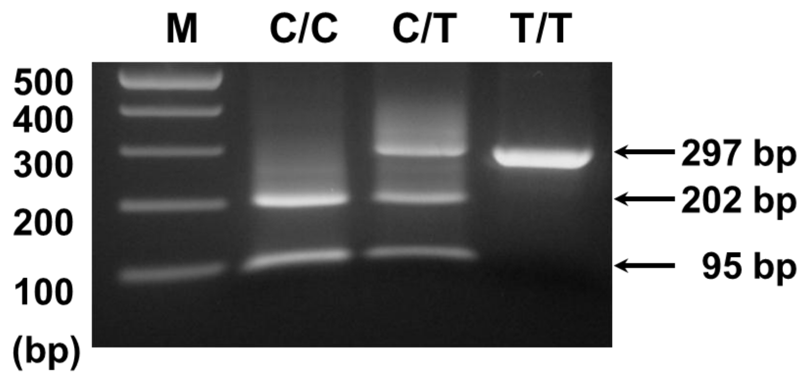


B.

```
1     TGAGTGGGGA  ATTCGTGGAA  CTGAGGGCTC  CTCCCCTTTT  TAGACCATAT
51    AGGGTAAACC  TCCCCACATT  GCCATGGCAT  TTATAAACTG  CCATGGCACT
101   GGTGGGTGCT  TCCTTTAACA  TGCTAATGCA  TTATAATTAG  CGTAAAATGA
      MMP-2 F   ──→
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 3. Genomic organization and localization of the SNP rs2285053 of human MMP-2 gene. MMP-2 SNP on promoter region, -735C>T, were marked by the downward arrow in the figure. The black block represents the exon 1 of MMP-2 gene (**A**). Sequences of human MMP-2 gene and position of the PCR primers were shown. The shadowed parts represent the PCR primers, and the underlined nucleotide is a genetic variant (MMP-2 -735C>T) in the promoter of MMP-2 gene (**B**).

A.



B.

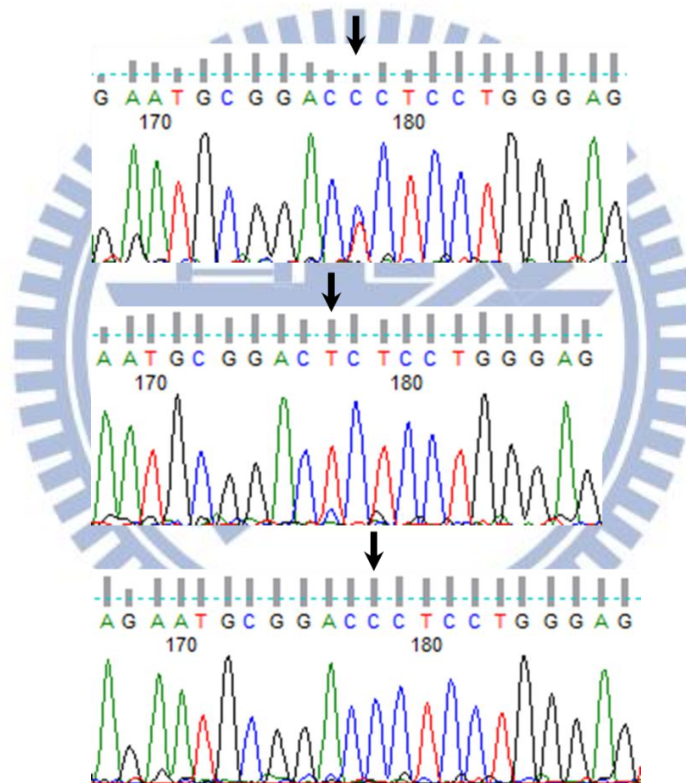
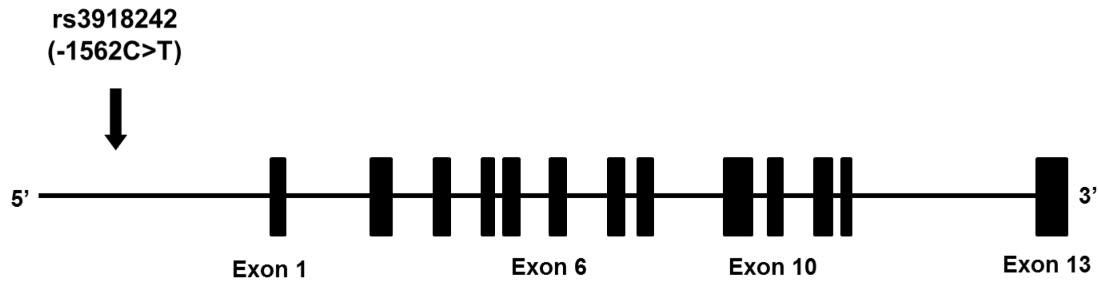


Figure 4. Detection of the RFLP for MMP-2 polymorphism rs2285053. After PCR amplifying, the target DNA region (297 bp) was digested by restriction enzyme *Sau96* I. Lane 1 represents a 100-bp DNA ladder marker; Lane 2 stands for genotype C/C (202 bp and 95 bp); Lane 3 stands for genotype C/T (297 bp,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

A.

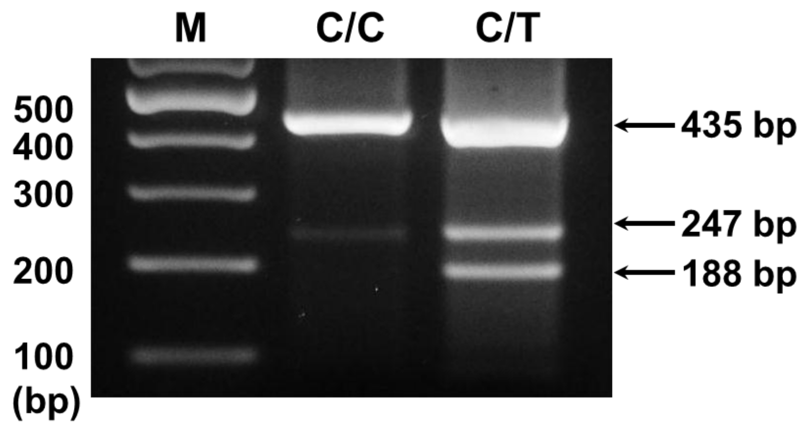


B.

```
1     GATGTTCATT   GGTTAGTGAA   CTTTAGAACT   TCAACTTTTC   TGTAAAGGAA
51    GTTAATTATC   TCCATCTCAC   AGTCTCATTT   ATTAGATAAG   CATATAAAAT
101   GCCTGGCACA   TAGTAGGCCC   TTTAAATACA   GCTTATTGGG   CCGGGCGCCA
       MMP-9-1 F   ——→
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 5. 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. (**A**). Sequence of human MMP-9 gene and position of the PCR primers were shown. The shadowed parts represent the PCR primers, and the underlined nucleotide is a genetic variant (MMP-9 -1562) in the promoter of MMP-9 gene (**B**).

A.



B.

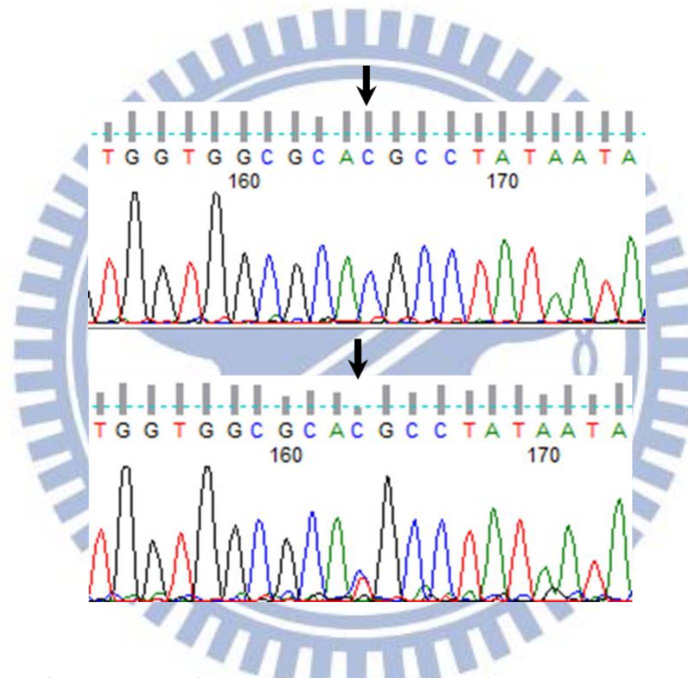
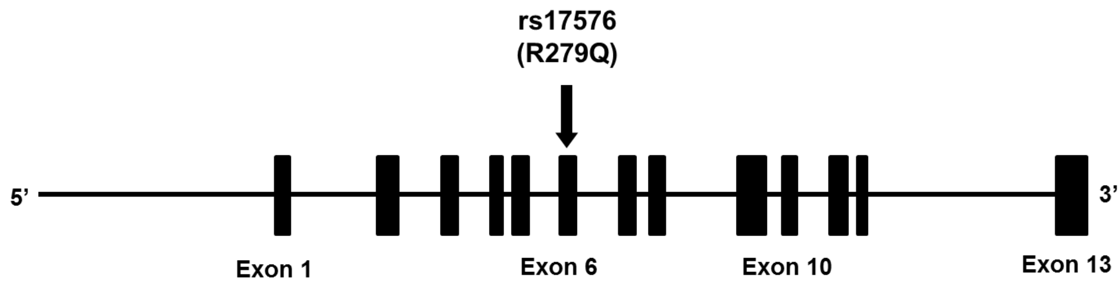


Figure 6. Detection of the RFLP for MMP-9 polymorphism rs3918242. After PCR amplifying, the target DNA region (435 bp) were digested by restriction enzyme *Sph* I. Lane 1 represents a100-bp DNA ladder marker; Lane 2 stands for genotype C/C (435 bp); Lane 3 stands for genotype C/T (435 bp, 247 bp, and 188 bp) (**A**). Two genotypes of direct sequencing map for rs3918242 SNP of MMP-9 gene were shown. The black arrows indicate the site of MMP-9 polymorphisms (**B**).

A.

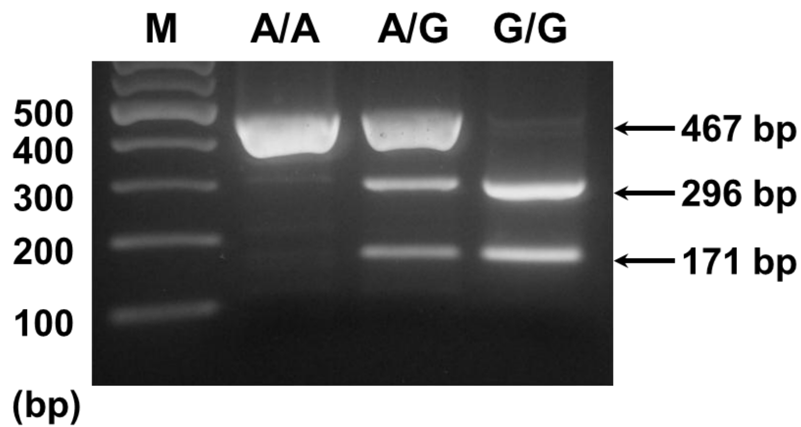


B.

```
  1    GAGTGAGTGA    GGGGGCTCGC    CGAGGGCTGG    GGGCGCCCAC    CACCCTTGAT
 51    GGTCCTGGGT    TCTAATTCCA    GCTCTGCCAC    TAGTGCTGTG    TGGCCTGCAA
101    TTCACCCTCC    CGCACTCTGG    GCCCAATTTT    CTCATCTGAG    AAATGATGAG
        MMP-9-2 F  ------>
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 7. 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. (**A**). Sequence of human MMP-9 gene and position of the PCR primers were shown. The shadowed parts represent the PCR primers, and the underlined nucleotide is a genetic variant (MMP-9 R279Q) in MMP-9 gene (**B**).

A.



B.

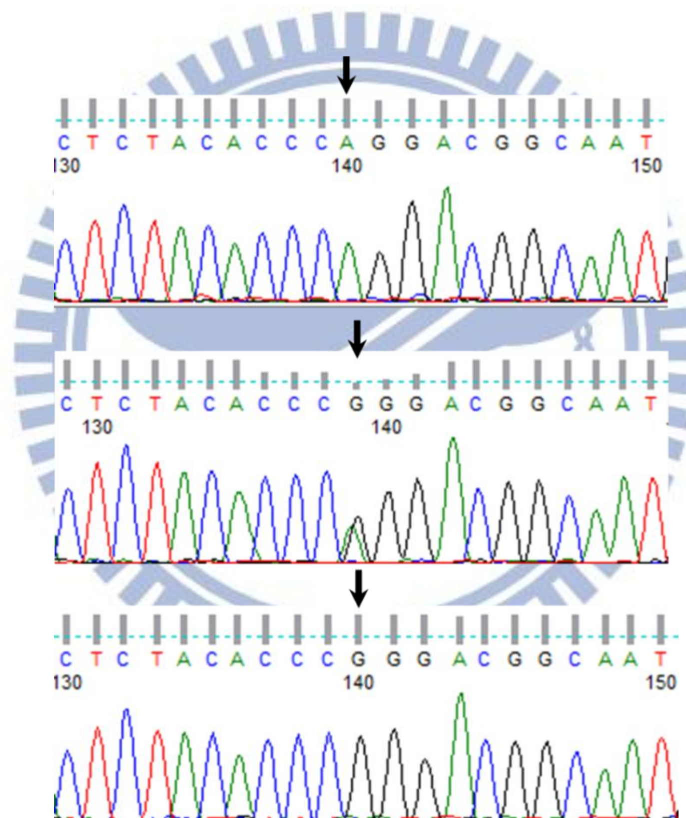
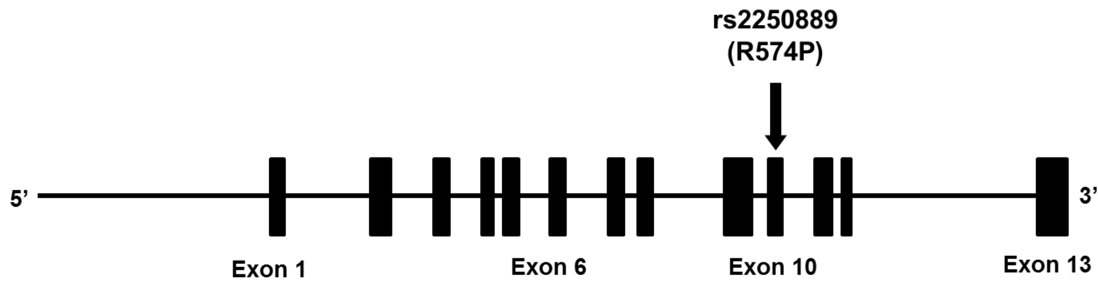


Figure 8. Detection of the RFLP for MMP-9 polymorphism rs17576. After PCR amplifying, the target DNA region (467 bp) were digested by restriction enzyme *Sma* I. Lane 1 represents a 100-bp DNA ladder marker; Lane 2 stands for genotype A/A (467 bp); Lane 3 stands for genotype A/G (467 bp, 296 bp, and 171 bp); Lane 4 stands for genotype G/G (296 bp and 171 bp) (**A**). Three genotypes of direct sequencing map for rs17576 SNP of MMP-9 gene were shown. The black arrows indicate the site of MMP-9 polymorphisms (**B**).

A.

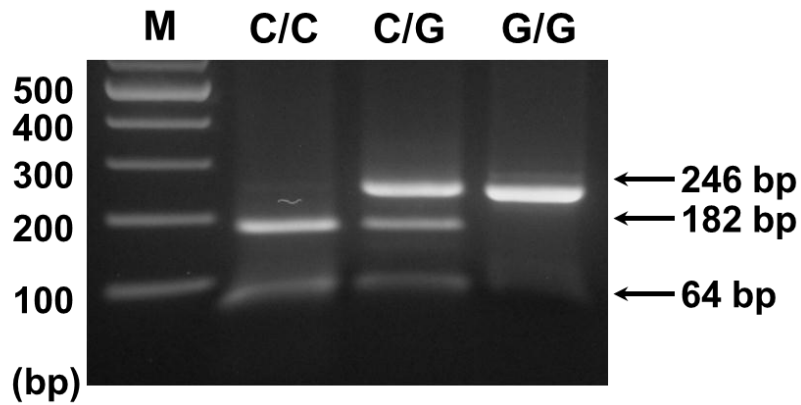


B.

```
1      CCACTGGCCC   TGTGTCCAAG   GCTTAGAGCC   CGTCCTTTCC   CTCCTCGCTT
51     TCTCAGGAAG   TACTGGCGAT   TCTCTGAGGG   CAGGGGGAGC   CGGCCGCAGG
101    GCCCCTTCCT   TATCGCCGAC   AAGTGGCCCG   CGCTGCCCCG   CAAGCTGGAC
       MMP-9-3 F  →
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 9. 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 (**A**). Sequence of human MMP-9 gene and position of the PCR primers were shown. The shadowed parts represent the PCR primers, and the underlined nucleotide is a genetic variant (MMP-9 R574P) in MMP-9 gene (**B**).

A.



B.

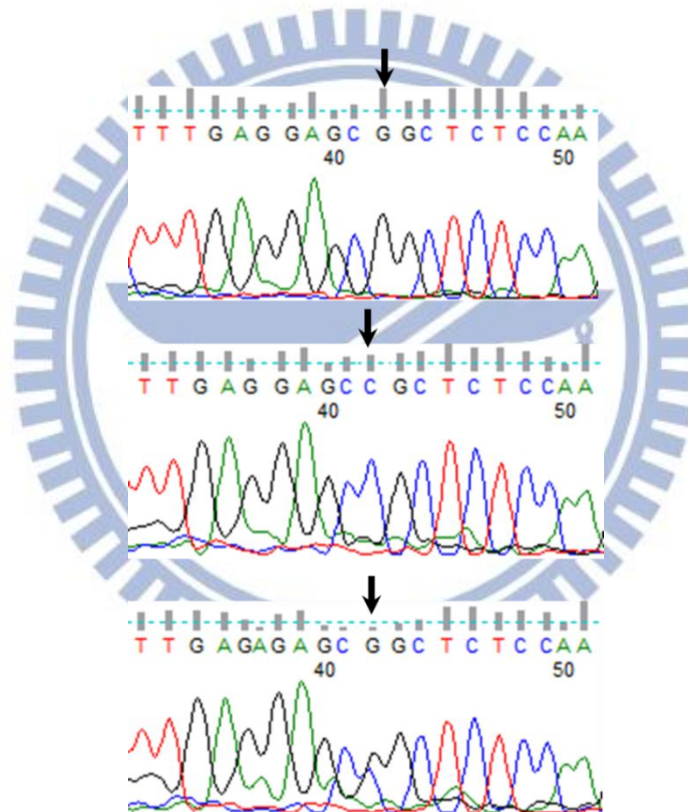


Figure 10. Detection of the RFLP for MMP-9 polymorphism rs2250889. After PCR amplifying, the target DNA region (246 bp) were digested by restriction enzyme *Nla* IV. Lane 1 represents a 100-bp DNA ladder marker; Lane 2 stands for genotype C/C (182 bp and 64 bp); Lane 3 stands for genotype C/G (246 bp, 182 bp, and 64 bp); Lane 4 stands for genotype G/G (246 bp) (**A**). Three genotypes of direct sequencing map for rs2250889 SNP of MMP-9 SNP were shown. The black arrows indicate the site of MMP-9 polymorphisms (**B**).

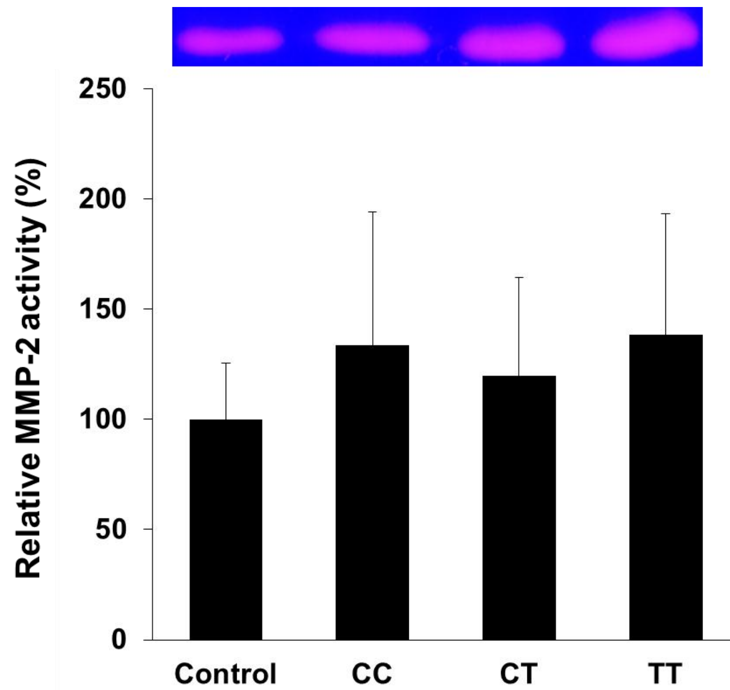
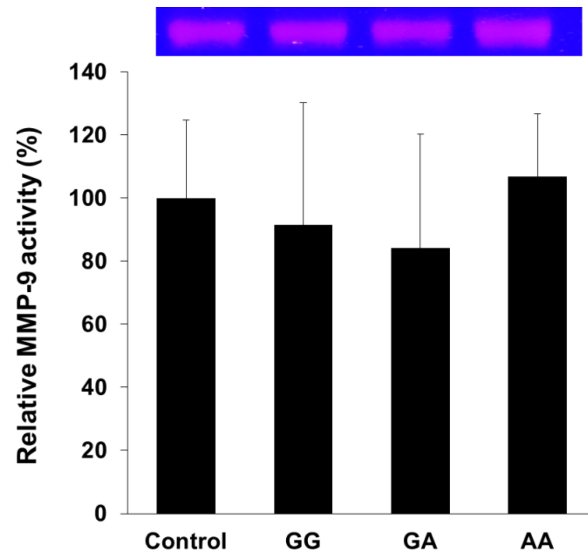
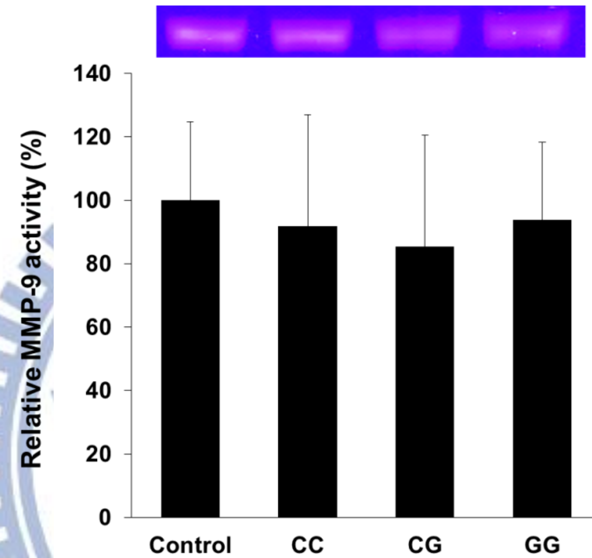


Figure 11. Plasma MMP-2 activity in the children with VSDs. The relative MMP-2 activities of three different genotype of MMP-2 -735C>T, were detected by gelatin zymography. As the data shown, there is no significant difference among MMP-2 genotype, although TT allele has the highest MMP-2 activity in MMP-2 -735C>T group (p = 0.052).

A. MMP-9 R279Q



B. MMP-9 R574P



C. MMP-9 -1562C>T

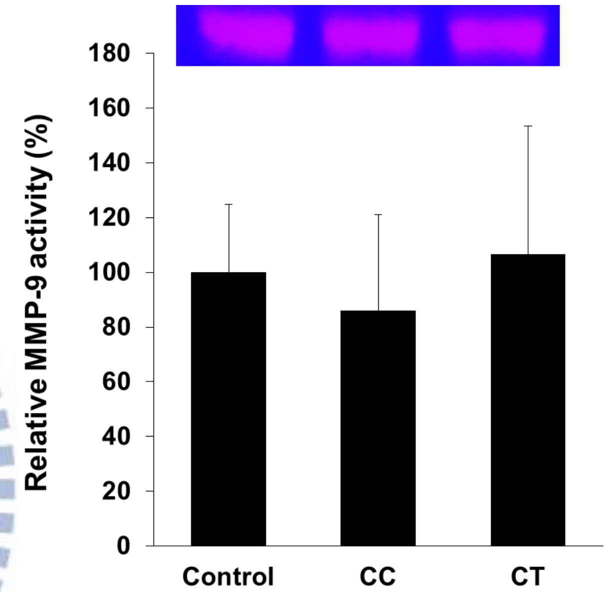
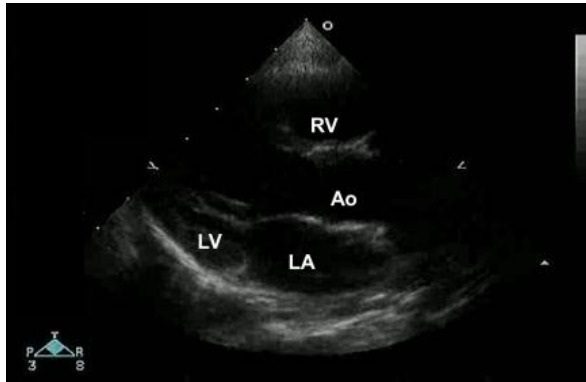
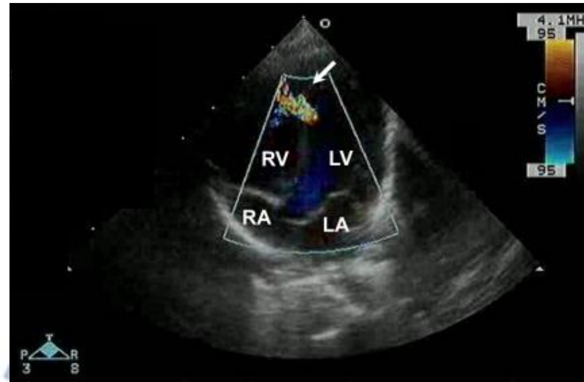


Figure 12. Plasma MMP-9 activity in the children with VSDs. The relative MMP-9 activities of three different genotype of MMP-9 R279Q (A), MMP-9 R574P (**B**) and MMP-9 -1562C>T (**C**) were detected by gelatin zymography. As the data shown, MMP-9 activity has no significant difference among MMP-9 genotypes.

A.



B.



C.

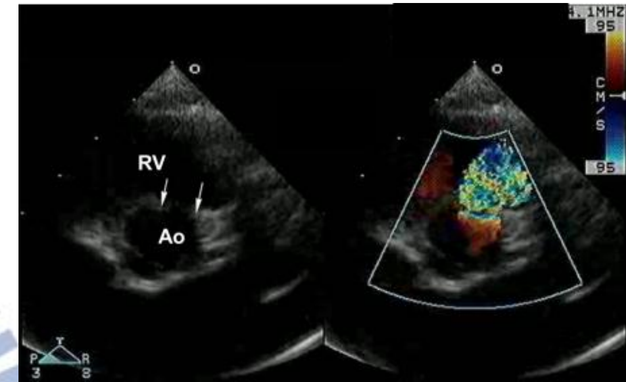
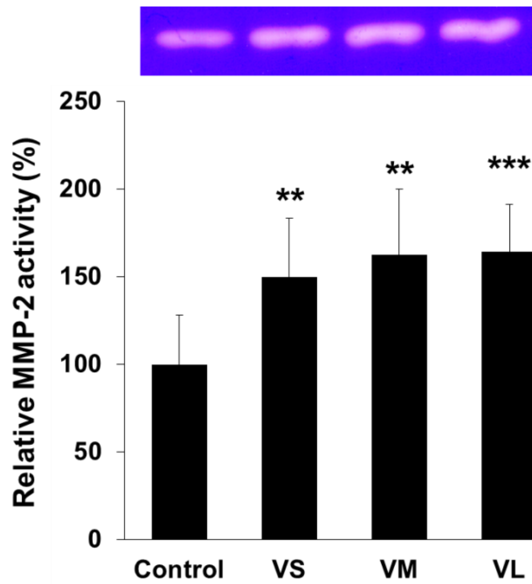


Figure 13. Echocardiographic examination for the valuation of VSDs severity. Echocardiograms show normal (**A**), small size of perimembraneous VSD (**B**) and medium size of subarterial VSD (**C**). The white arrows indicate the site of VSD. LA, left atrium; LV, left ventricle; RV, right ventricle; Ao, aortic valve.

A. MMP-2



B. MMP-9

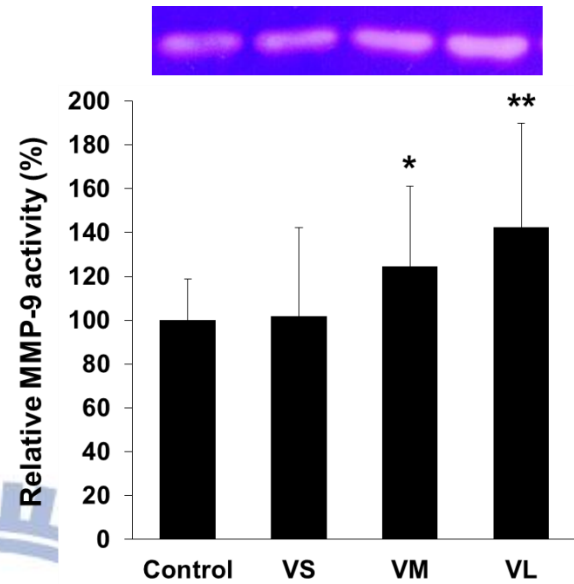
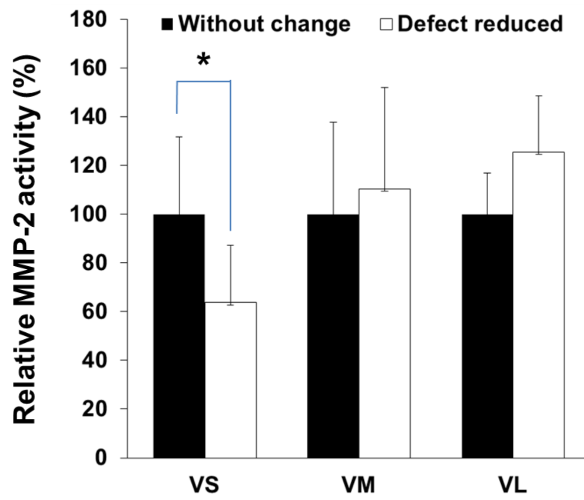


Figure 14. Plasma MMP-2 and MMP-9 activity of the children with different VSD severity. The gelatin zymography show the activity of plasma MMP-2 (**A**) and MMP-9 (**B**) of the four groups, including Control, VS (small VSD defects), VM (medium VSD defects) and VL (large VSD defects); each group includes 12, 45, 25 and 15 patients, respectively. *, ** and *** indicate $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively, compared with Control group.

A. MMP-2



B. MMP-9

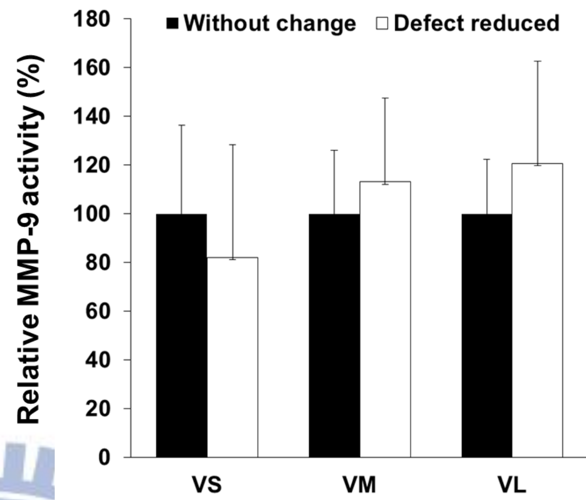


Figure 15. Relation of MMPs activity and spontaneous closure in different severe levels of VSD patients. The gelatin zymography shows the activity of plasma MMP-2 (**A**) and MMP-9 (**B**). The three groups stand for different severe levels of VSD patients, and each level differ to two subgroups which were clarified by the state of VSD closure. * indicate $p < 0.05$ compared with No change group.

Appendix 1. Database information of MMP-2 SNPs

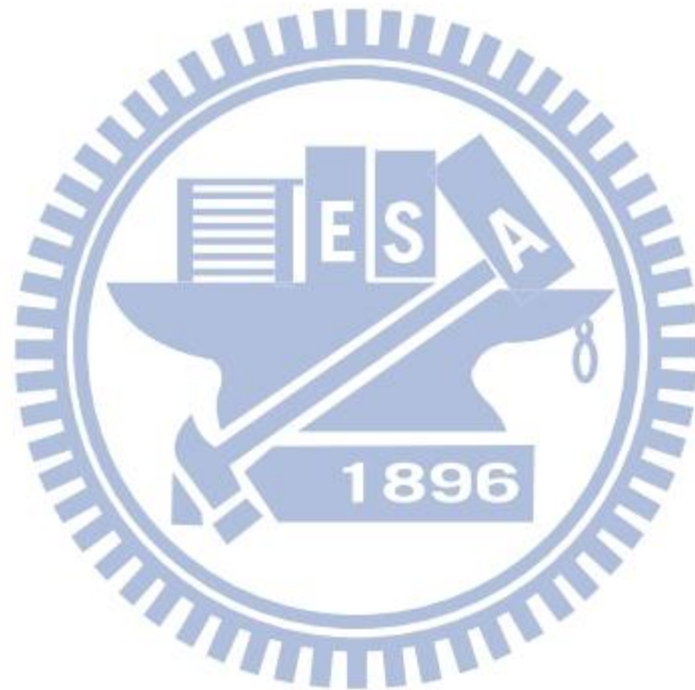
dbSNP rs# cluster ID	mRNA position	Hetero-zygosity	MAF	Function	dbSNP allele	Protein residue	Amino acid position	Reference
rs243866		0.181	0.138	5' near gene	A/G			Manso et al., 2010; Mossböck et al., 2010
rs243865		0.199	0.142	5' near gene	(> 6 bp)			Katz et al., 2011; Low et al., 2011; Lacchini et al., 2012
rs17859821		0.173	0.154	5' near gene	A/G			Hua et al., 2009[a]; Hua et al., 2009[b]
rs243864		0.206	0.138	5' near gene	G/T			Beeghly-Fadiel et al., 2009; Hua et al., 2009[a]; Hua et al., 2009[b]
rs2285053		0.500	0.175	5' near gene	C/T			Han et al., 2008; Beeghly-Fadiel et al., 2009; 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., 2009; Manso et al., 2010
rs865094		0.422	0.296	intron	A/G			Beeghly-Fadiel et al., 2009
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., 2009
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., 2009; Manso et al., 2010
				contig reference	C	Thr [T]	250	Han et al., 2008; Beeghly-Fadiel et al., 2009; 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., 2009
rs2241145		0.500	0.483	intron	C/G			Fatar et al., 2008; Beeghly-Fadiel et al., 2009; Manso et al., 2010
rs2241146		0.219	0.103	intron	A/G			Beeghly-Fadiel et al., 2009
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., 2009; Manso et al., 2010
				contig reference	T	Asp [D]	383	Han et al., 2008; Beeghly-Fadiel et al., 2009; Manso et al., 2010
rs12599775		0.173	0.047	intron	C/G			Beeghly-Fadiel et al., 2009
rs243847		0.434	0.347	intron	C/T			Beeghly-Fadiel et al., 2009; Low et al., 2011
rs2192852		0.483		intron	-/A/G			Beeghly-Fadiel et al., 2009
rs12923011		0.291	0.223	intron	C/T			Beeghly-Fadiel et al., 2009
rs243845		0.448	0.338	intron	C/T			Beeghly-Fadiel et al., 2009
rs243844		0.450	0.310	intron	A/G			Beeghly-Fadiel et al., 2009

rs2287074	1691	0.446	0.339	synonymous	A	Thr [T]	460	
				synonymous	C	Thr [T]	460	
				contig reference	G	Thr [T]	460	
rs243842		0.427	0.310	intron	C/T			Beeghly-Fadiel et al., 2009; Manso et al., 2010
rs183112		0.378	0.242	intron	A/G			Beeghly-Fadiel et al., 2009; 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., 2009
rs9923304		0.405	0.320	intron	C/T			Beeghly-Fadiel et al., 2009
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., 2009
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., 2009
				contig reference	C	Pro [P]	614	Beeghly-Fadiel et al., 2009
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., 2009

MAF, minor allele frequency



Appendix 2. 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 etal., 2011
rs3918242		0.290	0.153	5' near gene	C/T			Planello et al., 2011; 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	Rodríguez-Pla et al., 2008
				contig reference	C	Asn [N]	127	Rodríguez-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., 2010
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., 2010; Wu et al., 2010
				missense	C	Pro [P]	668	
				missense	T	Leu [L]	668	
				contig reference	G	Arg [R]	668	
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