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ASSOCIATION OF GENETIC VARIATIONS WITH KNEE OSTEOARTHRITIS IN THAIS

Miss Thitiya Poonpet

A Dissertation Submitted in Partial Fulfillment of the Requirements

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โรคข้อเสื่อมเป็นโรคที่พบได้บ่อยในผู้สูงอายุ ลักษณะเด่นของโรคคือกระดูกอ่อนบริเวณรอบข้อเกิดการเสื่อมสลาย โรคนี้เป็นโรคพหุปัจจัยซึ่งเกิดได้ทั้งจากปัจจัยเสี่ยงสำหรับแต่ละบุคคล เช่น อายุ เพศ น้ำหนัก และประวัติการเกิดอุบัติเหตุ รวมถึงจากปัจจัยทางพันธุกรรมด้วย งานวิจัยชิ้นนี้แบ่งออกเป็น 2 ส่วน ส่วนแรกคือการศึกษาความสัมพันธ์ระหว่างการแปรผันในยีน*ADAMTS14* ยีน *ADAM12* และ ยีน *MMP1* กับการเกิดโรคข้อเข่าเสื่อมในชาวไทย โดยทำการศึกษาในกลุ่มผู้ป่วย 108 คน และกลุ่มคนปกติ 119 คน การศึกษาการแปรผันในยีน *ADAMTS14* (rs4747096; A/G) ใช้เทคนิค polymerase chain reaction-restriction fragment length polymorphisms (PCR-RFLP) แต่การศึกษาการแปรผันในยีน *ADAM12* (rs3740199; C/G) และยีน *MMP1* (rs1799750; 2G/1G) ใช้เทคนิคการวิเคราะห์แบบ high resolution melt (HRM) ผลการทดลองพบว่า การแปรผันในยีน *ADAMTS14* สัมพันธ์กับการเกิดโรคข้อเข่าเสื่อมในผู้หญิง และการแปรผันในยีน *ADAM12* สัมพันธ์กับการเกิดโรคข้อเข่าเสื่อมในผู้ชาย แต่ไม่พบความสัมพันธ์ของการแปรผันในยีน *MMP1* กับการเกิดโรคข้อเข่าเสื่อม งานวิจัยส่วนที่สองได้ทำการศึกษาหายีนที่มีรูปแบบการเกิดอัลเทอร์เนทิฟสไปลซิง ที่แตกต่างกันในซิโนวิโอไซต์ระหว่างผู้ป่วยและคนปกติ การประมวลข้อมูลจากไมโครอะเรย์ทำให้พบยีนที่น่าสนใจ 32 ยีน ซึ่ง ยีน *HNRNPU* และยีน *BRD4* ได้รับการคัดเลือกสำหรับการวิจัยในขั้นต่อไป โดยใช้เทคนิค reverse transcriptase–polymerase chain reaction (RT-PCR) และ real-time PCR ผลการทดลองปรากฏว่าไม่พบอัลเทอร์เนทิฟสไปลซิงในยีน *HNRNPU* แต่พบอัลเทอร์เนทิฟสไปลซิงที่บริเวณเอ็กซอน 7 ของยีน *BRD4* สองรูปแบบคือ BRD4[+7] และ BRD4[-7] ซึ่ง BRD4[-7] เป็นไอโซฟอร์มใหม่ที่ยังไม่เคยมีรายงานมาก่อน และมีการแสดงออกลดลงอย่างมีนัยสำคัญทางสถิติในกลุ่มผู้ป่วย แสดงให้เห็นถึงความสัมพันธ์กับการเกิดโรคข้อเข่าเสื่อม โดยสรุปงานวิจัยนี้ค้นพบความสัมพันธ์ระหว่างการแปรผันในยีน *ADAMTS14* กับผู้ป่วยโรคข้อเข่าเสื่อมเพศหญิง และยีน *ADAM12* กับผู้ป่วยโรคข้อเข่าเสื่อมเพศชาย และความสัมพันธ์ของการแสดงออกที่ลดลงของ BRD4[-7] ซึ่งเป็น ไอโซฟอร์มใหม่กับการเกิดโรคข้อเข่าเสื่อม

สาขาวิชา	วิทยาศาสตร์ชีวภาพ	ลายมือชื่อนิสิต...
ปีการศึกษา	2555	ลายมือชื่อ อ. ที่ปรึกษาวิทยานิพนธ์หลัก.........................
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THITIYA POONPET: ASSOCIATION OF GENETIC VARIATIONS WITH KNEE OSTEOARTHRITIS IN THAIS. ADVISOR: ASSIST. PROF. RACHANEEKORN TAMMACHOTE, Ph.D., CO-ADVISOR: PROF. SITTISAK HONSAWEK, M.D., Ph.D.,119 pp.

Osteoarthritis (OA), a common joint disorder in aging people, is characterized by the disintegration and loss of the articular cartilage. In addition to non-genetic factors such as age, sex, body weight and joint injury, OA also has a strong genetic background. This study is divided into two parts. The first part aims to evaluate the association between variations in *ADAMTS14*, *ADAM12* and *MMP1* genes and susceptibility to knee OA in Thai population. The study population consisted of 108 knee OA patients and 119 normal controls. The *ADAMTS14* SNP (rs4747096; A/G) was genotyped by polymerase chain reaction-restriction fragment length polymorphisms (PCR-RFLP), while the *ADAM12* SNP (rs3740199; C/G) and *MMP1* SNP (rs1799750; 2G/1G) were genotyped by high resolution melt (HRM) analysis. The significant associations were achieved from the SNP in *ADAMTS14* with female knee OA patients and *ADAM12* with male knee OA patients. In contrast, no statistically significant association was found from the SNP in *MMP1*. The second part aims to identify genes with alternative splice variants in human synoviocytes. Among 32 candidate gene revealed from microarray data processing, *HNRNPU* and *BRD4* were selected for further analysis by reverse transcriptase–polymerase chain reaction (RT-PCR) and real-time PCR. The results showed no alternative splicing in *HNRNPU* but revealed 2 isoforms of *BRD4* that are BRD4[+7] and BRD4[-7]. The BRD4[-7] is the novel isoform which the expression was significantly decreased in knee OA patients, indicating its possible role in OA pathogenesis. In conclusion, the associations between SNPs in *ADAMTS14* and knee OA in female, *ADAM12* and knee OA in male, and the decreased level of BRD4[-7], a novel isoform of *BRD4*, and knee OA were found in this study.

Field of Study : ⎽⎽⎽⎽Biological Sciences⎽⎽⎽⎽ Student's Signature……………………….

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List of Abbreviations

ACL	Anterior cruciate ligament
ADAM	A disintegrin and metalloproteinase
ADAM12	A disintegrin and metalloproteinases 12
ADAMTS	A disintegrin and metalloproteinases with thrombospondin motifs
ADAMTS14	A disintegrin and metalloproteinases with thrombospondin motifs 14
Brd4	Bromodomain-containing protein 4
DZ	Dizygotic
ECM	Extracellular matrix
EDTA	Ethylenediaminetetra acetic acid
EGF	Epidermal growth factor
GWAS	Genome-wide association studies
hnRNP	Heterogeneous nuclear ribonucleoprotein
HNRNPU	Heterogeneous nuclear ribonucleoprotein U
HRM	High resolution melt
JSN	Joint space narrowing
MMP	Matrix metalloprotease
MMP1	Matrix metalloproteinase1
MZ	Monozygotic
nsSNP	Non-synonymous single nucleotide polymorphism
OA	Osteoarthritis
pNPI	N-propeptides of procollagen type I
P-TEFb	Positive transcription elongation factor b
PSC	Primary sclerosing cholangitis
RFLP	Restriction fragment length polymorphisms
RFU	Relative fluorescence units
RT-PCR	Reverse transcriptase–polymerase chain reaction
SNP	Single nucleotide polymorphisms
SR	Serine/arginine- rich
TβRII-B	Type II TGF-β receptor

THR	Total hip replacement
TJR	Total joint replacement
TKR	Total knee replacement
TSP1	Thrombospondin type 1
VEGF	Vascular endothelial growth factor

CHAPTER I

INTRODUCTION

Osteoarthritis (OA), a late-onset degenerative joint disease, is the main cause of functional incapacity, physical limitation, and reduced quality of life in aging people worldwide. The disease is characterized by the progressive degradation of articular cartilage together with joint space narrowing and subchondral bone changes (sclerosis and osteophyte formation). These features result in joint pain, mild inflammation and restricted motion (Dieppe and Kirwan, 1994; Dieppe and Lohmander, 2005). The most commonly OA affected joints, in descending order, are small joints of the hands, knees, and hips (Hochberg et al., 2012).

In addition to non-genetic factors such as age, sex, body weight and trauma, OA also has a strong genetic background that is likely polygenic in nature. While hand and hip OA have an estimated heritability of 65% and 60%, respectively, knee OA has lower heritability of 40%, and it is more likely to arise from biomechanical factors (Spector and MacGregor, 2004; Chaganti and Lane, 2011). Regarding to public health, knee OA is the most important type of OA because it is a major cause of disability and impairment of day to day activities (Keenan et al., 2006).

Association study is a powerful way towards understanding complex diseases such as OA because it provides an opportunity of quantifying the effects of specific gene variants on disease occurrence. If a variant in a gene is associated with disease

risk, there is a high probability that the gene is involved in disease pathogenesis. The Human Genome Project (The 1000 Genomes Project Consortium, 2010) has shown that approximately 90% of genetic variants are single nucleotide polymorphisms (SNPs). Some SNPs result in the generation or removal of a restriction enzyme recognition site, which can be detected and analyzed by digesting DNA with an appropriate restriction enzyme. Such polymorphisms are termed restriction fragment length polymorphisms (RFLPs). Moreover, SNPs can be precisely distinguished from changes in the melt profile of the PCR product using high resolution melt (HRM) analysis in real-time PCR system.

An increasing number of deleterious SNPs for various diseases have been investigated by HRM analysis. This cost effective technique can be performed in a single-step without the need for allele-specific primers or expensive labeled probes (Reed *et al.*, 2007; Erali *et al.*, 2008). When entire PCR products were melted in the presence of saturated DNA binding dyes, the double-stranded DNA will be differentiated from the single-stranded DNA by the alteration of the fluorescence intensity. Therefore, sequence variants can be precisely distinguished from changes in the melt profile of the PCR product. HRM analysis is an excellent method for SNP genotyping because of the high throughput, sensitivity and accuracy of the system and it is also less toxic and time consuming.

Unbalance between synthesis and degradation of the extracellular matrix (ECM) network in articular cartilage is one of the OA hallmarks. Therefore, the Zn^{2+} dependent matrix metalloproteinases protein family has prompted much interest in OA pathophysiology (Murphy and Nagase, 2008). Genetic associations of SNPs in some members of the matrix metalloproteinase protein families to knee OA have recently been studied. *ADAMTS14* has been recently reported to be a novel OA candidate gene (Rodriguez-Lopez *et al.*, 2009). A non-synonymous single nucleotide polymorphism (nsSNP) of likely deleterious effect in exon 21 of *ADAMTS14* (rs4747096; A/G) was demonstrated to be associated with knee OA. Rodriguez-Lopez *et al*. (2009) found that the G allele in this SNP was associated with women requiring total knee replacement (TKR) because of OA and with symptomatic hand OA in the Caucasian population.

In addition, various association studies had investigated the role of nsSNP (rs3740199; G/C) in the second exon of *ADAM12* in knee OA susceptibility. However, these previous findings have been rather inconsistent because the C allele was found to be significantly associated with female knee OA patients in the United Kingdom (Valdes *et al*., 2004), whereas it showed significant association with male knee OA patients in Estonia (Kerna *et al*., 2009). On the other hand, no significant association was found in either gender in the three other studies (Valdes *et al*., 2006; Rodriguez-Lopez *et al*., 2009; Shin *et al*., 2012).

In 2009, a genetic association study in a Turkish population (157 knee OA patients and 84 healthy controls) had showed significant association between a deleted SNP in the promoter of *MMP1* and knee OA susceptibility (Barlas *et al*., 2009). This SNP (rs1799750) is a deletion of the G nucleotide at the -1,607 position in the promoter region of the gene (GAAAG[-/G]ATATG). Since the adjacent nucleotide of the SNP is also the G nucleotide, this SNP is also known as 2G/1G polymorphism. The results suggested that individuals with 1G/1G and 1G/2G genotypes have a greater risk to develop knee OA than individuals with the 2G/2G genotype (Barlas *et al*., 2009).

However, it is obvious that different population has different genetic characters, and several association studies had indicated the site-specific, sex-specific and ethnic-specific nature of genetic effects on OA. Therefore, association study based on PCR-RFLP and HRM analysis that are simple and reliable techniques can be used to evaluate the role of these SNPs in a Thai population in a case-control study manner.

Apart from gene variation, protein variation plays a significant role in some disease occurrence and progression. Alternative splicing is an important mechanism that allows large protein diversity from the limited number of gene. It enables a single gene to increase its coding capacity, leading to the production of protein isoforms with diverse and even antagonistic functions. Comparing with large complexity of OA, the study about abnormal alternative splicing mechanism in this disease is sparse.

Microarray is a high throughput technique, which normally used to compare the gene expression profiles (mRNA levels) between two interested groups. In general, a probe set spotted on a microarray platform (gene chip) consists of several different polynucleotide probes designed to bind different areas of the same targeted mRNA. If some part of the targeted mRNA is alternatively spliced, the probes bound to that area will show distinct results when compared with the other probes. This basic concept may indicate the existence of different protein isoform or even reveal new candidate genes for OA. Thus, data acquired from microarray analysis may be the new strategy to study the protein variation in OA patients.

Normally, cartilage is the main targeted tissue in OA studies because it is the tissue where disease occurs. Although OA cartilage is easily obtained from patients who underwent TKR surgery, normal cartilage is not easy to obtained. To avoid this problem, synoviocytes from synovial fluid (a yolk-like fluid in the joint cavities) were used as an alternative source for OA study.

From these reasons, this thesis is divided into two main parts. The first part is the association study in three interesting knee OA candidate genes, *ADAMTS14*, *ADAM12* and *MMP1*, which have been shown to be associated with knee OA in non-Thai populations. The second part is the alternative splicing study in synoviocytes for finding genes with distinct protein isoforms in knee OA patients by microarray data analysis.

Objectives

1.	To study the association between variations in *ADAMTS14*, *ADAM12* and *MMP1* genes and the susceptibility to knee OA in Thai population.

2.	To identify genes with alternative splice variants in synoviocytes of knee OA patients.

Significance

Knee OA is an irreversible degenerative joint disease and difficult to detect at the early stage. As one of the most disabling diseases as well as a burden on resources for health care worldwide, OA merits great attention in order to develop helpful preventions and therapeutic methods. Identification of genetic variants associated with knee OA susceptibility will enable detection of individuals at high risk of developing the disease and allow them to carefully utilize their knee for better prevention. In addition, OA study in both genetic and protein variation approaches can contribute more knowledge on the OA pathophysiology on a molecular level, which may open a new area of prevention and therapeutic interventions.

CHAPTER II

LITERATURE REVIEW

2.1 Knee structure

The knee is the largest and the most complicated joint in the human body. It is a hinge type joint that allows flexion and extension. The important role of the knee is related to movement as well as carrying the body weight. The knee is comprised of several parts that work together for correctly functioning (Figure 2.1).
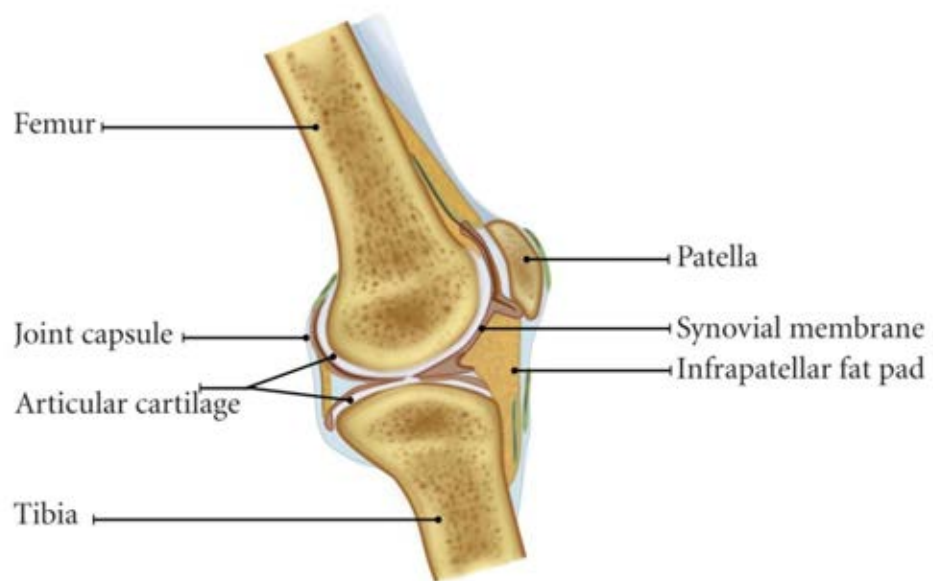
Figure 2.1 Knee structure (drawn by Suparach Seechae)

2.1.1 Bones

The three primary bones (femur, tibia and patella) are part of the knee joint. The femur (thighbone) is the longest, heaviest and strongest bone in our body. The convergence angle of the femur is a main factor in determining of femoral-tibial angle, which is about 175 degrees in the general population. The tibia is the larger bone of the

lower leg (the smaller bone is called fibula). It is the second longest bone (next to the femur) and recognized as the strongest bone for weight bearing. The patella or knee cap is circular-triangular in shape. It protects the anterior surface of the knee joint and increases the angle that the femur was exerted by the tendon (McCracken and Walker, 2001; Platzer, 2009).

2.1.2 Articular cartilage

The end of knee joint bones are covered and protected by the articular cartilage, a thin hyaline cartilage, which is mostly consisted of type II collagen. This smooth and slippery surface helps reduce friction and absorbs shock during movement. Articular cartilage has a limited capacity for self-repairing. In addition, the newly formed cartilage will consist mainly of lesser quality hyaline cartilage. As a result, articular cartilage will crack and tear over time (Drake *et al*., 2010; Maroudas *et al*., 1992; Verzijl *et al.*, 2000).

2.1.3 Joint capsule

The joint capsule is a pocket surrounding a joint. It consists of two membranes that separated by fatty deposits. The white fibrous membrane (outer layer) contains ligaments, and the synovial membrane (inner layer) secretes the synovial fluid for joint-nourishing, lubricating and shock absorbing. The outer layer of joint capsule is avascular and receives oxygen and nutrient from the surrounding blood vessel (Drake *et al*., 2010).

2.1.4 Synovial membrane

Synovial membrane (or synovium) is the soft tissue between the joint cavity and the joint capsule. A dense net of blood vessels in synovium connective tissue provide nutrients for synovium and cartilage. Two intimal cell types are present in synovial membrane. Type A, macrophage-like synoviocytes, are differentiated from blood monocytes. It removes the cell debris and undesirable substances and microbes from the synovial fluid. Type B, fibroblast-like synoviocytes, produces hyaluronic acid (or hyaluronan) for trapping the water to the joint space, as well as lubricin for lubricating the joint surfaces (Drake *et al.*, 2010; Jay *et al.*, 2000).

2.1.5 Synovial fluid

Synovial fluid is a clear, viscous, hyaluronic acid containing fluid found in the cavities of joints. It composes of fluid secreted by synoviam and interstitial fluid from the blood plasma. The roles of synovial fluid are reduction of friction by lubricating the articular cartilage during movement, shock absorption by becoming more viscous the moment pressure is applied, and nutrient and waste transportation by providing oxygen and nutrients and removing metabolic wastes from the synoviocytes and chondrocytes (Hui *et al.*, 2012; Tortora and Derrickson, 2012).

Since the knee supports almost the whole weight of our body and also has limited range of movement, it is vulnerable to both traumatic injury and the development of OA.

2.2 Osteoarthritis (OA)

Osteoarthritis (OA) is a complex degenerative joint disease, which causes pain and restricted joint movements. The elderly suffer the most from this form of arthritis where it reduces functional capacity and quality of life.

2.2.1 Clinical features

The prominent feature of OA is the progressive degradation of the articular cartilage accompanied by changes in subchondral bone and uncertain degrees of intra-articular inflammation. Degradation is first observed at the joint surface in the form of fibrillation, which is associated with the degeneration and loss of collagen II in the cartilage (Hollander *et al.*, 1995). Apart from that, there is a local loss of the other ECM molecules such as aggrecan and proteoglycans, including biglycan and decorin (Lark *et al.*, 1997). These alterations cause the loss of tensile strength and an increase in water content in the cartilage (Poole *et al.*, 2002). Subchondral bone sclerosis and cyst formation, bony outgrowths at the joint margins (osteophytes) and joint space narrowing (JSN) were also found at OA affected joints. These changes result in OA symptoms such as chronic joint pain, limitation of movement, tenderness, crepitus and occasional effusion (Dieppe and Kirwan, 1994; Dieppe and Lohmander, 2005).

2.2.2 Diagnosis and classification of OA

X-rays are commonly used to confirm the clinical diagnosis of OA because it can reveal typical radiographic changes at the joint margin, particularly in the bony outgrowth and JSN. OA severity usually is graded based on radiographic findings

ranging on a 0–4 scale (0=absent, 1=doubtful, 2=minimal, 3=moderate, and 4=severe) developed by Kellgren and Lawrence (1954). However, normal x-rays may have low sensitivity to detect the cartilage loss in primary stages of OA, thus regular physical examination may also be necessary for the detection of pathological symptoms. In addition, people who show radiographic characters because of OA may be completely asymptomatic, thus OA can be classified as radiographic or symptomatic (reviewed in Creamer and Hochberg, 1997; Buckwalter *et al.*, 2004). OA can also be classified as primary OA which is an idiopathic phenomenon of the disease where no obvious initiating factors, and secondary OA which is a consequence of joint injury, destructive artropathies or developmental defects. Moreover, OA can be localized which affects a certain joint, or generalized which affects at least three joints areas (Altman, 1995; Buckwalter *et al.*, 2004; Creamer and Hochberg, 1997).

2.2.3 Prevalence of OA

The prevalence of OA in any kind of joints increases dramatically with advancing age, which is likely a result of several biological abnormalities that arise with aging. It is uncommon in both sexes under the age of 40 years however it is increased almost exponentially after the age of 60 years (Felson *et al.*, 2000; Buckwalter *et al.*, 2004). Generally, OA has a higher prevalence in females than in males. Nevertheless, under the age of 45 years, OA occurs more often in males (Silman, 1993). The existing OA prevalence data is derived mainly from Caucasian populations (Europe and USA) which had lacked information from Asian populations. However, it has been estimated

that approximately 10% of the global population who are over 60 years of age have symptomatic OA (Felson and Zhang, 1998). In developed countries, with extending life expectancy, the number of OA affected people is high, thus future changes in the prevalence of OA is difficult to predict.

2.2.4 Etiopathogenesis of OA

OA is considered as a multifactorial disease, where various individual's risk factors such as age, gender, hormonal status and ethnicity play important roles in the pathogenesis of the disease. Apart from that, the negative correlation between OA and osteoporosis has also been reported (Dequeker, 1996). The result of cross-sectional studies indicates the association between high bone mineral density (or low osteoporosis occurrence) and increased prevalence of hand, hip and knee OA. In addition, local biomechanical factors such as physical activity which requires repeated joint bends, obesity and past history of trauma (particularly in men) make cartilage more vulnerable to injuries and less capable of repair (reviewed in Felson *et al*., 2000; Felson, 2004). Furthermore, OA also has a strong genetic contribution from multiple genes which each gene has relatively modest consequences (Valdes *et al*., 2008a).

2.3 Genetic studies in OA

Several strategies have been used to investigate the genetics contribution in OA, including familial aggregation studies, classical twin studies, linkage analysis and candidate gene association studies.

2.3.1 Familial aggregation studies

This type of genetic study measure the risk ratio for a relative of an OA affected individual compared with the OA prevalence in a population. For affected sibling pairs, the risk is called sib recurrence risk. A study from Nottingham (Lanyon *et al*., 2000) had compared the prevalence of radiographic hip OA in siblings of individuals, who undergone total hip replacement (THR) surgery to the prevalence in controls. A similar study was performed in total knee replacement (TKR) individuals (Neame *et al*., 2004) and a smaller study in Oxford using self-reported total joint replacement (TJR) data (Chitnavis *et al*., 1997). In addition, familial aggregation of generalized OA (Kellgren and Moore, 1952; Riyazi *et al*., 2005), radiographic progression (Botha-Scheepers *et al*., 2007), and specific knee OA phenotypes (anteromedial OA) also have been investigated (McDonnell *et al*., 2007). The results indicate a high familial aggregation, suggesting a strong genetic contribution of OA, which is most striking for hand and hip OA. However, the familial aggregation study results do not come exclusively from genetic factors and may reflect the environmental factors that are shared by family members.

2.3.2 Classic twin studies

An alternative strategy to analyze the actual genetic contribution to OA is called the classic twin studies. This type of genetic study enable quantification of the environmental and genetic contribution of the disease by comparing resemblances of targeted characters in identical (monozygotic, MZ), and non–identical, (dizygotic, DZ)

twins. As MZ twins have identical genetic character, whereas DZ twins share only 50% of their genetic material, the heritability of OA can be calculated from double the difference between the correlations of MZ and DZ twins (Boomsma *et al.*, 2002). Several twin studies have revealed the heritability of the hand, hip, and knee OA, which is between 39% and 65%.

2.3.3 Linkage analysis

To identify chromosomal regions that contain OA susceptibility genes, linkage analysis is performed. The polymorphic loci (genetic markers) were used as tools to track the locus that are jointly inherited with OA. To date, at least five genome-wide linkage analyses have been reported, including studies in Finland, Iceland, United Kingdom, United States and a meta-analysis of studies from Iceland, United Kingdom and United States (reviewed in Valdes and Spector, 2009). The analysis provided evidence that chromosome 2 (2q31.1–2q34), 6 (6p21.1–6q15), 7 (7q34–7q36.3), 11 (11p12–11q13.4) and 15 (15q21.3–15q26.1) were the most likely to harbor susceptibility genes for OA. These chromosome regions include some candidate genes such as *IL1* gene cluster, *MATN3*, *IL4R*, *FRZB*, and *BMP5*.

2.3.4 Association study

The genetic association study is a popular strategy for investigating the relationship between specific genetic variants and disease occurrence. Since 90% of genetic variants in human genome are SNPs (The 1000 Genomes Project Consortium,

2010), most of the association studies were carried out by comparing the allele frequencies of the candidate SNP between patients and control groups. If some alleles were significantly overrepresented in the patient group, it is highly possible to be the OA causing gene. In recent years, genome-wide association studies (GWAS) have unveiled some of the candidate genes for OA susceptibility (Drazen and Phimister, 2007; Valdes *et al.*, 2008b). In addition, SNPs in genes that show significantly different expression level between patients and control are selected for the association study (Valdes *et al.*, 2006). Numerous association studies of OA candidate genes have already been performed on more than 80 genes (Ryder *et al.*, 2008). Notable examples are polymorphisms in *ASPN* (Kizawa *et al.*, 2005), *FRZB* (Loughlin *et al.*, 2004) and *GDF5* (Miyamoto *et al.*, 2007), which were replicated in multiple populations (Teichtahl *et al.*, 2005; Lane *et al.*, 2006; Lories *et al.*, 2006; Rodriguez-Lopez *et al.*, 2006; Valdes *et al.*, 2007). Of these, only a SNP in the 3'untranslated region of the *GDF5* (rs143383; C/T) has demonstrated consistent results, indicating the robust association with OA. Furthermore, this SNP which has been studied on a functional level had revealed that the T allele resulted in a reduced *GDF5* mRNA relative to the C allele (Miyamoto *et al.*, 2007; Southam *et al.*, 2007; Egli *et al.*, 2009). Many other genetic variants have shown association to OA but they have not been sufficiently confirmed.

2.4 Genes involved in OA

A number of genes involved in developmental processes or maintenance of cartilage and bone have been found to be associated with OA occurrence and progression. OA associated genes were categorized into five molecular pathways including inflammation, ECM molecules, Wnt signaling, osteocyte modulation or chondrocyte differentiation, and proteases and their inhibitors (Valdes and Spector, 2009). Interplay between these five molecular pathways has brought about the complexity in OA genetic study. Inflammation caused by joint injuries increases metalloproteinase expression and inhibits chondrocyte proliferation, as well as ECM synthesis, leading to cartilage loss. Inflammation also activates the expression of vascular endothelial growth factor (VEGF), which is an important mediator of angiogenesis and also leads to osteophyte formation. In addition, the loss of homeostasis between the proteolytic enzymes and their inhibitors causes cartilage destruction. ECM molecules and bone differentiation proteins has a direct effect on cartilage volume. Abnormal cell proliferation and differentiation leads to cartilage loss and osteophytes formation. The Wnt signaling pathway also affects osteoblast differentiation and the expression level of β-catenin, VEGF and matrix metalloprotease, which all involve in OA development (reviewed in Tammachote, 2011).

2.5 Proteinase genes involved in OA

Osteoarthritic joints have the imbalance between synthesis and degradation of ECM leading to cartilage destruction. Therefore, metalloproteinases have been considered as the major groups of enzymes responsible for this OA hallmark (Kevorkian et al., 2004; Murphy and Nagase, 2008). Metzincins, a superfamily of Zn^{2+}-metalloproteinases are of particular interest in OA because of their possible genetic factors (Murphy and Nagase, 2008). This large superfamily of enzymes consists of 80 proteins in the human and 93 in the mice (Huxley-Jones et al., 2007), which can be classified into several distinct protein families according to differences in the catalytic site and domain organizations. Recently, the associations between OA and some member of metzincins super family have been reported, including A disintegrin and metalloproteinases with thrombospondin motif (ADAMTS), A disintegrin and metalloproteinases (ADAM), and Matrix metalloproteases (MMP).

2.5.1 ADAMTS

A disintegrin and metalloproteinases with thrombospondin motifs (ADAMTSs) resemble and share a common domain structure with the ADAM proteinases in having a prodomain, an adamalysin/reprolysin like catalytic domain, a disintegrin-like domain and a cysteine-rich domain. However, the ADAMTS proteinases differ from ADAM proteinases in lacking transmembrane domains and are a putative secretory protein. In addition, ADAMTSs possess a thrombospondin type 1 (TSP1) motif, followed by a

spacer region and a variable number of TSP1 repeats which appear to be involved in binding to ECM components, such as aggrecan and procollagen (Kuno *et al.*, 1997; Hurskainen *et al.*, 1999; Apte, 2004). ADAMTSs are implicated in a variety of normal and pathological conditions, including arthritis. There are 19 published vertebrate family members of ADAMTSs, numbered 1–10 and 12–20 to date (Cal *et al.*, 2002; Somerville *et al.*, 2003). *ADAMTS14* is located on chromosome 10q22.1. Although little is still known about its function, ADAMTS14 protein has high similarities in sequence and domain structure to ADAMTS2 (56%) and ADAMTS3 (63%) (Colige *et al.*, 2002), which play a major role in the biosynthesis of collagen precursors. There is evidence that these three enzymes have the ability to cleave the N-propeptides of procollagen type I (pNPI activity) and the homotrimeric procollagen type II before they are incorporated into collagen fibers (Lapiere *et al.*, 1971; Tuderman *et al.*, 1978; Hojima *et al.*, 1989; Hojima *et al.*, 1994). Moreover, ADAMTS14 has been shown to have pNPI activity *in vitro*, and has been suggested as a possible source of residual pNPI activity which is observable in the bone, tendon, cartilage, skin, and other tissues of Ehlers-Danlos syndrome type VIIC patients, dermatosparaxic cattle, and *Adamts2*-null mice (Fernandes *et al.*, 2001; Colige *et al.*, 2002; Le Goff *et al.*, 2006). A nsSNP in *ADAMTS14* (rs4747096) was overrepresented in caucasian women with severe knee OA (Rodriguez-Lopez *et al.*, 2009).

2.5.2 ADAM

A disintegrin and metalloprotease (ADAM) was found to be associated with several complex diseases, such as rheumatoid arthritis, heart disease, cancer and Alzheimer's disease (Duffy *et al*., 2003; Moss and Bartsch, 2004; Mochizuki and Okada, 2007; Kveiborg *et al*., 2008). Among 23 identified human *ADAMs*, *ADAM12* is one of the outstanding candidate genes for OA. It was first identified in mice and initially called meltrin alpha (Yagamihiromasa *et al*., 1995). In 1998, the cloning and characterization of human *ADAM12* gene were completed (Gilpin *et al*., 1998). *ADAM12* is localized on chromosome 10q26.3. The cellular roles of *ADAM12* appear to be normal physiology and pathology critical events. Several studies suggested a regulatory role of human *ADAM12* in bone formation, chondrocyte proliferation and maturation, as well as osteoclast differentiation (Abe *et al*., 1999; Verrier *et al*., 2004; Kveiborg *et al*., 2006; Okada *et al*., 2008). *ADAM12* is up-regulated in OA cartilage and multinucleated giant cells surrounding loosened hip implants (Ma *et al*., 2005; Sato *et al*., 2006). It encodes 2 alternative spliced forms, ADAM12-L and ADAM12-S, which share a common domain structure namely a prodomain, a metalloprotease catalytic domain, a disintegrin-like domain, a cysteine-rich domain and epidermal growth factor (EGF)-like domains. ADAM12-L is a membrane anchored protein containing a transmembrane domain and a cytoplasmic tail, whereas ADAM12-S is a secreted protein containing a unique extended 33 amino acids in the C-terminus (Wewer *et al*., 2006). Both ADAM12-L and ADAM12-S are active enzymes that possess extracellular metalloproteases, also known as

ectodomain shedding, activity, which may regulate diverse physiological functions, such as cell proliferation, differentiation, invasion, and migration (Loechel *et al.*, 1998; Loechel *et al.*, 2000; Shi *et al.*, 2000). In addition to its proteolytic activity, ADAM12 may regulate focal adhesions by promoting cell–cell and cell–extracellular matrix binding through its disintegrin-like and cysteine-rich domains (Eto *et al.*, 2000; Iba *et al.*, 2000; Thodeti *et al.*, 2005). However, ADAM12-L is believed to have intracellular signaling capacities as it interacts with several adaptor and signaling molecules inside the cell through its intracellular domain (reviewed in Kveiborg *et al.*, 2008). Kerna *et al.* (2009) reported that a missense SNP of the *ADAM12* gene (rs3740199) is associated with radiographic knee OA in middle-aged Estonian cohorts.

2.5.3 MMP1

Matrix metalloproteases (MMPs) domain structure begins with the propeptide, the zinc metalloprotease catalytic domain, a linker (hinge) region and C-terminal domains. MMPs are classified according to their substrate specificity into three main groups, including gelatinases, collagenases and stromelysins. Most MMPs are secreted in a latent form but after cleavage by extracellular proteases, the enzymes are activated (Kleiner and Stevenson, 1993), The MMP proteins have important roles in the degradation of extracellular matrix in normal physiological events such as embryonic development, reproduction and tissue remodelling, as well as in disease pathogenesis such as cancer and arthritis (Nagase *et al.*, 2006). Unbalance between synthesis and degradation of the ECM network in articular cartilage is one of the OA hallmarks.

Although the replacement of proteoglycans is rather easily (Fell *et al.*, 1976; Jubb and Fell, 1980), substantial damage to the adult collagen seems to be irreversible due to its very long turnover time (Maroudas *et al.*, 1992; Verzijl *et al.*, 2000). Among the 20 MMPs, MMP-1, -2, -8, -13 and -14 have collagenase activity (Aimes and Quigley, 1995; Nagase and Woessner, 1999; Riley *et al.*, 2002). Interestingly, several MMPs are over expressed in synoviocytes and chondrocytes from OA joints (Wolfe *et al.*, 1993; Keyszer *et al.*, 1995; Borden *et al.*, 1996; Reboul *et al.*, 1996). This phenomenon has been shown to be influenced by SNPs in the promoter region of the genes (Rutter *et al.*, 1998; Ye, 2000).The association of rs1799750, which is the polymorphism of *MMP1*(a gene on chromosome 11q22.3) with knee OA was found in the Turkish population(Barlas *et al.*, 2009)

2.6 Alternative splicing

Apart from gene variation, protein variation plays a significant role in some disease occurrence and progression. Alternative splicing is an important mechanism by which the exons of the RNA produced by a gene transcription (a primary gene transcript or pre-mRNA) are reconnected in multiple ways during RNA splicing. The resulting different mRNAs are translated into different protein isoforms; thus, a single gene may code for several structurally and functionally distinct proteins (Black, 2003). This process occurs as a normal phenomenon in eukaryotes, where it allows large proteomic complexity from a limited number of genes. The control mechanisms of alternative

splicing are various and complex, including the relative abundances and tissue distribution of the serine/arginine- rich (SR) proteins and heterogeneous nuclear ribonucleoprotein (hnRNP).The existence of tissue-specific and/or developmentally regulated splicing factors, the protein-recruiting and elongating properties of the transcription machinery (Caceres and Kornblihtt, 2002) can also occur. In humans, over 80% of genes are alternatively spliced (Matlin *et al*., 2005).

Several important diseases such as cystic fibrosis have been linked to mutations or variations that lead to aberrant splicing and abnormal protein production (Chu *et al*., 1993). Statistics provided by the Human Genome Mutation Database (Stenson *et al*., 2003) revealed that out of 38,177 mutations annotated, 3,659 mutations are on splice sites (Wagner *et al*., 2003). This number, however, underestimates the number of mutations that affect splicing because they do not take into account the intronic and exonic enhancer and silencer elements. In addition, mutations associated with disruptions in *trans*-acting factors may lead to global splicing defects (Garcia-Blanco *et al*., 2004). These essential points determine the need for transcriptomic analysis (rather than genomic analysis) to assess the real impact of alternative splicing on complex diseases such as OA.

2.7 Alternative splicing in OA

Up to now, there are reports about alternative mRNA splicing in some genes in OA patients. These include genes encoding for fibronectin (Rencic *et al.*, 1995; Kriegsmann *et al.*, 2004), vascular endothelial growth factor (VEGF) (Pufe *et al.*, 2001), cathepsin B (Berardi *et al.*, 2001), ADAMTS4 (Wainwright *et al.*, 2006), and type II TGF-β receptor (TβRII-B) (Parker *et al.*, 2007).

2.7.1 Fibronectin

Fibronectin is a large glycoprotein, which connects various molecules in the extracellularmatrix by mediating both cell-matrix and matrix-matrix interactions. Osteoarthritic cartilage contains higher amount of fibronectin than normal cartilage (Burton-Wurster, 1989). Moreover, the alternative splicing of its three exons (IIIA, IIIB, and V) results in large heterogeneity in the structure of fibronectin (Kriegsmannet al., 2004). The relationship between increased fibronectin level in osteoarthritic cartilage and alternative splicing patterns of these three exons was investigated. The results indicated no total differences in splicing of these exons between normal and OA articular cartilage. Therefore, the alternative splicing of cartilage fibronectin do not appear to be associated with the increased fibronectin level in osteoarthritis patients (Rencic et al., 1995).

2.7.2 VEGF

Vascular endothelial growth factor (VEGF) plays an important role in endochondral bone formation, ossification, cartilage remodeling and angiogenesis. It is expressed during development and has almost undetectable level in normal adult cartilage (Neufeld, 1999). Human VEGF presents in several isoforms (121, 165, 189, or 206 amino acids), which all arisen from alternative splicing of a single mRNA. These splice variants have different molecular masses, biologic properties and ability to bind to different VEGF receptors (VEGFRs) (Neufeld, 1999). However, genes expressed during embryogenesis are usually re-expressed in the disease state. Therefore, the expression level of VEGF and its receptors were investigated in osteoarthritic (OA) cartilage. The results showed that increased VEGF productions were recognized in OA cartilage and only VEGF121 and VEGF189 were expressed (Pufe *et al*., 2001).

2.7.3 Cathepsin B

Cathepsin B is expressed at sites of cartilage remodeling in OA. It inhibits cartilage repair, enhances metalloproteinases activity and mediates cartilage neovascularization and mineralization (Baici, 1995). Cathepsin B exists as several splice variants, including the full-length transcript (CB), the variants lacking exon 2 (CB[-2]) or the variants lacking exon 2 and 3 (CB[-2,3]) (Gong, 1993).In recent study, the abundance of all cathepsin B mRNA splice variants in normal and OA cartilage, osteophytes, and cultured chondrocytes were measured. The relative amount of all three splice variants had varied significantly in OA cartilage and osteophytes compared with

normal cartilage. Moreover, the total cathepsin B mRNA and protein levels were significantly higher in OA samples than in normal samples suggesting an important role of the enzyme in OA progression (Berardi *et al.*, 2001).

2.7.4 ADAMTS4

The substrate for ADAMTS4 is proteoglycan (aggrecan) of articular cartilage (Gao, 2004). RT-PCR analysis using primers that were designed to bind across the exon 8/9 junction of ADAMTS4 resulted in two products, an expected full length product (541 bp) and a smaller product (380 bp) that is missing 161 bp from the 5′ end of exon 9 leading to a new C-terminal domain in which exon 8 joins to a cryptic 3′ splice site within exon 9. This alteration in the C-terminal domain of ADAMTS4 affects its substrate specificity. Full length ADAMTS4 is partly active and can cleave aggrecan within the chondroitin sulphate-rich region. In contrast, the smaller ADAMTS4 is fully active and can cleave the Glu373–Ala374 bond of aggrecan. It was suggested that alternative splicing of ADAMTS4 produced low levels but fully active form of an alternative transcript, which was secreted into the synovial fluid. This protein isoform may be a factor in progressive aggrecan loss of superficial zone in patients with arthritic diseases (Wainwright *et al.*, 2006).

2.7.5 TβRII-B

Transforming growth factor-β (TGF-β) has critical roles in chondrocyte proliferation and matrix synthesis by unclear mechanisms. However, evidences of TGF-β–dysregulation in osteoarthritis are increasing. Types I and II TGF-β receptors are major receptors for TGF-β signal transduction (Van den Berg, 1999). To determine its role in TGF-β signaling, the expression of TβRII-B, a splice form of the type II TGF-β receptor, was investigated in human cartilage, human chondrocyte cell lines and human primary chondrocytes. The results showed that the TβRII-B expression can be detected in normal and osteoarthritic cartilage. Furthermore, it forms heteromeric complexes with the types I and II TGF-β receptors and other molecules (betaglycan and endoglin). In conclusion, enhanced TGF-β signaling and responses in chondrocytes caused by over expression of TβRII-B, indicating its key role in the regulation of TGF-β activity (Parker *et al*., 2007).

Comparing with its large complexity, alternative splicing studies in OA is still little. Additional studies need to be performed to identify high-impact OA causing genes.

CHAPTER III

METHODOLOGY

3.1 Association study

    3.1.1 Study population

        The subjects of this study were 227 Thais, 108 OA patients and 119 healthy controls. The 108 cases (88 women and 20 men; mean age 70 years, range 51-91 years) were primary knee OA patients with severe clinical and radiographic symptoms diagnosed by expert clinicians. They all had undergone total knee replacement (TKR) surgery at Thammasat Commemoration Hospital. The indications of TKR surgery were the absence of femoral joint space, no responses to conservative treatment longer than six months, and grades 3-4 of radiographic signs of OA according to the Kellgren–Lawrence grading system (Kellgren and Lawrence, 1957). The control population consisted of 119 healthy individuals (93 women and 26 men; mean age 55 years, range 50-60 years). They were healthy individuals who have annual check-up and routine screening in Chulalongkorn Memorial Hospital and were enrolled in a study about the role of cytokines and gene polymorphism in knee osteoarthritis. All were diagnosed by physicians to have no symptoms or signs of OA, other arthritis or any joint disorders (chronic pain, inflammation, tenderness, swelling or limited range of motion), and to have no family history of those diseases. The patients with any systemic inflammatory or

autoimmune disorders, or any type of malignant or chronic illness were not included in this study.

3.1.2 Ethic approval

The protocol for taking blood sample from knee OA patients has been approved by the Clinical Research Ethics Committee of the Faculty of Medicine, Thammasat University (protocol No. MTU-OT-4-CR010-010/53), whereas the protocol for taking blood sample from healthy controls has been approved by the Clinical Research Ethics Committee of Faculty of Medicine, Chulalongkorn University (protocol No. 533/54). All research participants provided written informed consents after study procedures were explained.

3.1.3 Sample collection and DNA extraction

Peripheral blood samples were taken from each participant by standard venipuncture and kept at 4 °C in tubes containing ethylenediaminetetra acetic acid (EDTA) until use. Genomic DNA was extracted from whole blood using a commercially available innuPREP Blood DNA Master Kit (Analytikjena, Germany) according to the manufacturer's procedure. The DNA concentration and purity were estimated by measuring the absorbance at the wavelength of 260 and 280 nm. After that, DNA was diluted to the working concentration of 50 ng/µl and used as a template in SNP genotyping.

3.1.4 SNP genotyping

a) *ADAMTS14* (rs4747096; A/G)

The rs4747096 polymorphism was genotyped by the PCR-RFLP technique. The mutated 5'-GGAACTTGGGACGCCAGAGGGGCAGTGGGTGCCACAATCCG-3' forward primer (41 bases) and the 5'-AGGGAGGTGAAGGTCACACA-3' reverse primer (20 bases) were used for amplification of the interested area. The PCR was conducted in a 20 µl volume containing 2 µl of 10X *Taq* buffer, 1.5 mmol MgCl$_2$, 0.5 mmol dNTPs, 0.2 µmol of each primer, 100 ng of genomic DNA template and 0.4 U *Taq* DNA polymerase (Fermentas Inc., USA). The PCR program consisted of the following steps. The initial denaturation 5 min at 94 °C, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 54 °C for 30 s, and extension at 72 °C for 1 min. The last step is final extension of 72 °C for 5 min. The PCR product (196 bp) was incubated overnight at 37 °C with the *BspE*I restriction enzyme (New England BioLabs, USA) which can cut PCR products with the G allele. After incubation, the mixtures were electrophoresed in 3% agarose and visualized by ethidium bromide staining and ultraviolet light transillumination. The expected fragment was 196 bp in AA, 158 and 38 bp in GG and 196, 158 and 38 bp in AG genotypes, respectively. For accuracy testing, several samples with different genotypes were subjected to standard sequencing.

b) *ADAM12* (rs3740199; C/G)

Genotyping of the rs3740199 polymorphism was performed by HRM analysis with a pair of unlabeled standard primers. Amplification was performed on the CFX96[TM] real-time PCR detection system (Bio-Rad Laboratories, Inc.). The short segments close to the SNP were amplified by the forward primer 5'-TCACTGGGATCCAGAGGT-3' (18 bases) and the reverse primer 5'-TGAAGTTGTCAGTGCCTCT-3' (19 bases) to limit the resulting amplicon to 50 bp. Each 20 µl reaction mixture containing 100 ng of genomic DNA, 0.15µM of each primer, 10 µl of EvaGreen Super mix (Bio-Rad) and 6.8 µl of sterile dH$_2$O. The amplification protocol was initial denaturation at 98 °C for 30 sec; follow by 40 cycles consisting of 98 °C for 2 sec, 60 °C for 10 sec and a plate read. After that, samples were heated to 98 °C for 30 sec and cooled to 70 °C for 30 sec. A melt curve was generated by slowly heating from 70 °C to 90°C with 0.2 °C increments, 10 sec dwell times. The melt profile was tracked by fluorescence intensity from a plate read at each temperature. The melt profiles of each sample were analyzed using Precission Melt Analysis[TM] software (Bio-Rad). The fluorescence data were normalized, and displayed as difference plotting. The software discriminates sequence variations by automatic grouping the samples into groups of different genotypes based on similar melt profiles. In order to confirm HRM accuracy, a sample was genotyped twice and several samples from each genotype were subjected to standard sequencing.

c) *MMP1* (rs1799750; 2G/1G)

Genotyping of the rs1799750 polymorphism was also performed by HRM analysis on the CFX96^{TM} real-time PCR detection system (Bio-Rad Laboratories, Inc.). As this polymorphism is the nucleotide deletion instead of the nucleotide substitution, it is easier to see the differences in melt profile between genotypes, and the amplicon size can be slightly increased. The forward primer 5'-GCCACTTAGATGAGGAAATTGTAG-3' (24 bases) and the reverse primer 5'-CGTCAAGACTGATATCTTACTCAT-3' (24 bases) were used to amplify the interested region. The resulting amplicon of the 1G genotype is 110 bp, whereas the resulting amplicon of the 2G genotype is 111 bp. The real-time PCR was conducted in a 10 µl reaction mixture containing 50 ng of genomic DNA, 0.2 µM of each primer, 5 µl of EvaGreen Super mix (Bio-Rad) and 3.2 µl of sterile dH_2O. The amplification protocol was initial denaturation at 98 ºC for 30 sec; follow by 40 cycles consisting of 98 ºC for 2 sec, 54 ºC for 10 sec and a plate read. After that, samples were heated to 98 ºC for 30 sec and cooled to 55 ºC for 30 sec. A melt curve was generated by slowly heating from 55 ºC to 80 ºC with 0.2 ºC increments, 10 sec dwell times. The melt profile was tracked by fluorescence intensity from a plate read at each temperature. The melt profiles of each sample were analyzed using Precission Melt Analysis^{TM} software (Bio-Rad). The fluorescence data were normalized, and displayed as difference plotting. The software discriminates sequence variations by automatic grouping the

samples into groups of different genotypes based on similar melt profiles. In order to confirm HRM accuracy, a sample was genotyped twice and several samples from each genotype were subjected to standard sequencing.

3.1.5 Statistical analysis

The Excel 2007 (Microsoft® Excel®) was used for comparison of participants' demographic data. Chi-square test was used for the binary traits (gender), whereas unpaired Student's t-test was used for the quantitative traits (age, weight, height and BMI). The allele frequencies and genotype proportions in patient and control groups were investigated for Hardy–Weinberg equilibrium (HWE) as mentioned in the following website; http://www.oege.org/software/hardy-weinberg.html (Rodriguez et al., 2009). The association between each SNP and knee OA susceptibility was evaluated by comparing the allele frequencies and genotype distributions among OA patients and normal controls using the program StatCalc (AcaStat Software, USA) in term of the odds ratio (OR) with 95% confidence intervals (CI). $P \leq 0.05$ and the 95%CI that not includes 1 were accepted to be statistically significant. The results from male and female were also compared separately to determine the effect of SNPs in each gender.

3.1.6 Haplotype analysis

Although SNP genotyping of these three genes were carried out in the same study population, haplotype analysis is possible only for *ADAMTS14* and *ADAM12* because it located closely on the same chromosome. The rs4747096 (A/G) and the rs3740199 (C/G) give rise to four haplotypes (AC, AG, GC and GG). To investigate the combinatorial effect of alleles from these two SNPs, frequencies of each haplotype were compared between patient and control groups using StatCalc Program.

3.2 Alternative splicing study

3.2.1 Bioinformatic analysis

This part of the study used the gene expression (microarray) data from a research article, which identified mRNA expression profiles in the rheumatoid arthritis (RA), osteoarthritis and healthy synovial membrane (Huber *et al.*, 2008). The study used 2 gene chips (GPL96 and GPL97); therefore it generated 2 sets of corresponding expression data (GSE12021-GPL96 and GSE12021-GPL97).

To begin the bioinformatics analysis, all needed information including gene chip files (platform) and expression files were downloaded from the Gene Expression Omnibus (GEO) dataset in NCBI (accession number: GSE12021). The platform file provides probes' ID and gene symbol (Figure 3.1), while the expression file provides expression data of each probe in each participant (Figure 3.2). All files were processed using Excel 2007 (Microsoft® Excel®).

Figure 3.1 A gene chip platform (GPL96) opened with Excel 2007

Figure 3.2 An expression file (GSE12021-GPL96) opened with Excel 2007

The expression files consist of the information from 10 OA patients (GSM302876, GSM302880, GSM302930, GSM303326, GSM303341, GSM303356, GSM303358, GSM303360, GSM303362 and GSM303370), 9 normal controls (GSM302859, GSM302864, GSM302866, GSM302870, GSM303522, GSM303523, GSM303525, GSM303531 and GSM303533) and 12 RA patients (GSM302872, GSM302882, GSM302896, GSM302900, GSM302924, GSM302927, GSM302933, GSM302943, GSM303364, GSM303366, GSM303368 and GSM303520). However, the information from 12 RA patients was excluded from our bioinformatic analysis. For the first set of data analysis, all necessary data from a platform (GPL96) and its corresponding expression file (GSE12021-GPL96) were combined into a starting file (Figure 3.3).

Figure 3.3 A combined starting file (GSE12021-GPL96). SV NC = Normal synovial membrane, SV OA = Osteoarthritic synovial membrane

The average expression level of each probe was calculated in both control and OA groups. After that, the values were compared to examine the trend of gene expression from each probe in OA patients (Figure 3.4).

Figure 3.4 The trend of gene expression from each probe in OA patients after compared with controls. Up = increased expression, Down = decreased expression

FTEST function was used to determine if variance between control and patient groups within the same probe ID are equal (i.e. homoscedastic) or not equal (i.e. heteroscedastic) (Figure 3.5).

Figure 3.5 The F-test values of each probe ID

If p-value from F-test is more than 0.05, the variances are homoscedastic (represented by numeric 2), but if p-value from F-test is equal or less than 0.05, the variance are heteroscedastic (represented by numeric 3) (Figure 3.6).

Figure 3.6 The variance types of each probe ID

TTEST function for two independent samples was used to compare the average expression level from each probe ID between controls and patients, with appropriate adjusted for equality of the variance of each probe ID (Figure 3.7).

	AB8		f_x =TTEST(D8:L8,N8:W8,2,AA8)						
	A	B	C	M	X	Y	Z	AA	AB
6		Reference		Average Control	Average OA				
7	ID	ID_REF	Gene Symbol	(9 samples)	(10 samples)	Expression	f-test	Variance	t-test
8	1007_s_at	1007_s_at	DDR1	493.15	537.35	Up	0.0817	2	0.583954
9	1053_at	1053_at	RFC2	168.82	229.19	Up	0.0172	3	0.049087
10	117_at	117_at	HSPA6	414.31	278.74	Down	0.4964	2	0.032805
11	121_at	121_at	PAX8	999.87	1078.93	Up	0.2672	2	0.444857
12	1255_g_at	1255_g_at	GUCA1A	47.73	172.04	Up	0.0000	3	0.02704
13	1294_at	1294_at	UBA7	613.91	574.61	Down	0.0316	3	0.628856
14	1316_at	1316_at	THRA	98.59	93.42	Down	0.6757	2	0.670534
15	1320_at	1320_at	PTPN21	48.56	47.20	Down	0.2286	2	0.918463
16	1405_i_at	1405_i_at	CCL5	207.39	215.35	Up	0.4658	2	0.925225
17	1431_at	1431_at	CYP2E1	60.90	42.95	Down	0.2294	2	0.070222
18	1438_at	1438_at	EPHB3	82.25	127.00	Up	0.0046	3	0.341858
19	1487_at	1487_at	ESRRA	563.92	503.04	Down	0.9555	2	0.230106
20	1494_f_at	1494_f_at	CYP2A6	224.73	175.59	Down	0.8877	2	0.115104

Figure 3.7 The Student's *t*-Test values of each probe ID

The gene symbol of probe IDs that showed significantly different expression between OA and control groups by TTEST function at 95% (p≤0.05) and 99% (p≤0.01) confidence were revealed and sorted alphabetically (Figure 3.8).

	AA4		f_x										
	A	B	C	M	X	Y	Z	AA	AB	AC	AD	AI	AJ
6		Reference		Average	Average						Significant level		
7	ID	ID_REF	Gene Symbol	Control	OA	Expression	f-test	Variance	t-test	0.05	0.01	4815	2220
8	1007_s_at	1007_s_at	DDR1	493.15	537.349	Up	0.0817	2	0.583954			A4GNT	AAK1
9	1053_at	1053_at	RFC2	168.824	229.194	Up	0.0172	3	0.049087 RFC2			AAK1	AAMP
10	117_at	117_at	HSPA6	414.307	278.741	Down	0.4964	2	0.032805 HSPA6			AAMP	ABHD10
11	121_at	121_at	PAX8	999.866	1078.93	Up	0.2672	2	0.444857			AARSD1	ABHD14A
12	1255_g_at	1255_g_at	GUCA1A	47.734	172.042	Up	0.0000	3	0.02704 GUCA1A			AASS	ABHD2
13	1294_at	1294_at	UBA7	613.907	574.614	Down	0.0316	3	0.628856			AATK	ABI2
14	1316_at	1316_at	THRA	98.5867	93.4166	Down	0.6757	2	0.670534			ABAT	ABI2
15	1320_at	1320_at	PTPN21	48.5601	47.2032	Down	0.2286	2	0.918463			ABCA1	ACBD3
16	1405_i_at	1405_i_at	CCL5	207.388	215.349	Up	0.4658	2	0.925225			ABCA2	ACO2
17	1431_at	1431_at	CYP2E1	60.8969	42.9511	Down	0.2294	2	0.070222			ABCA5	ACP1
18	1438_at	1438_at	EPHB3	82.2489	127.001	Up	0.0046	3	0.341858			ABCB11	ACTB
4813	205279_s_	205279_s_	GLRB	150.013	201.122	Up	0.2003	2	0.114237			ZNF79	
4814	205280_at	205280_at	GLRB	39.0043	103.676	Up	0.1176	2	0.008171 GLRB	GLRB		ZNF821	
4815	205281_s_	205281_s_	PIGA	152.252	116.585	Down	0.0291	3	0.262304			ZNF821	
4816	205282_s_	205282_at	LRP8	113.031	128.856	Up	0.8341	2	0.353255			ZNHIT1	
4817	205283_at	205283_at	FKTN	250.948	198.307	Down	0.3148	2	0.113266			ZNRF4	
4818	205284_at	205284_at	URB2	323.73	174.152	Down	0.0008	3	0.103845			ZSCAN18	
4819	205285_s_	205285_s_	FYB	190.529	111.913	Down	0.6364	2	0.027351 FYB			ZXDA	
4820	205286_at	205286_at	TFAP2C	117.826	101.453	Down	0.1060	2	0.439851			ZXDC	
4821	205287_s_	205287_s_	TFAP2C	11.0374	12.7495	Up	0.7661	2	0.501595			ZZEF1	
4822	205288_at	205288_at	CDC14A	24.0663	28.0024	Up	0.2978	2	0.486536			ZZEF1	

Figure 3.8 The gene symbol of probe IDs that showed significantly different expression at p≤0.05 (4815 probes) and p≤0.01 (2220 probes)

After that, all gene symbols in the combined starting file were screened for genes that were detected by more than 1 probe ID using a complex function as shown in Figure 3.9

Figure 3.9 Screening for genes that were detected by more than one probe ID

Then, the candidate genes that showed both significantly different expressions at 99% confidence and were detected by more than 1 probe IDs were revealed by the VLOOKUP function (Figure 3.10).

Figure 3.10 Some candidate genes from the first set of microarray data processing

All processing steps were also done in another set of data (GPL97 and GSE12021-GPL97), so two set of analyzed results from two platforms were obtained. Then the VLOOKUP function was used again to find the concordance results (candidate genes) from two platforms (Figure 3.11).

Figure 3.11 Some of the concordant candidate genes from 2 platforms

After all, candidate genes that have at least one probe ID, which showed significantly different expression (p<0.01) in OA samples when compared with controls and showed different expression pattern (up or down) when compared with other probes that detect the same gene were selected for further processes.

3.2.2 Primer designing

All important data from candidate genes were retrieved again to analyze the interesting targeted areas for further analysis. Microarray probes' information of both GPL96 and GPL97 were downloaded from the Affymetrix website and opened with Excel 2007 (Figure 3.12).

Figure 3.12 Probes' detail from the Affymetrix website opened with Excel 2007

The binding area of microarray probes that showed distinct result comparing with other probes that bind to the same genes were indicated (Figure 3.13). Candidate genes with probes that bind to the area suitable for primer designing, have high fold change of expression between patient and control groups and showed high expression signal were chosen for further analysis.

Figure 3.13 The binding area of microarray probes that showed different result when compared with other probe within the same probe set were underline

Among all candidate genes, *HNRNPU* and *BRD4* were selected to further investigations. For *HNRNPU*, the interesting probe ID (200593_s_at) showed down expression level in OA patients (Normal/OA=1441/829, *p*-value=0.002), meaning that some part of this area may be spliced out. Although the another probe ID (236244_at) was also showed down expression level in OA patients, the expression signal from this probe ID is too low (Table 3.1)

Table 3.1 Details of probe IDs targeted to *HNRNPU* (Down = significantly decreased expression in OA patients, Same = not significantly different expression between patients and controls).

Gene	Probe ID	OA	Control	t-test	Expression	Binding area
HNRNPU	200593_s_at	829	1440	0.002	Down	Exon 10-12
HNRNPU	200594_x_at	4723	5217	0.217	Same	
HNRNPU	216855_s_at	40	85	0.037	Same	
HNRNPU	225805_at	1072	688	0.045	Same	
HNRNPU	235603_at	504	448	0.484	Same	
HNRNPU	236244_at	70	182	0.001	Down	Exon 13

The interesting probe ID of *HNRNPU* binds across the exon 10 to the exon 12 of the gene, so the primers were designed to cover this area (Figure 3.14).
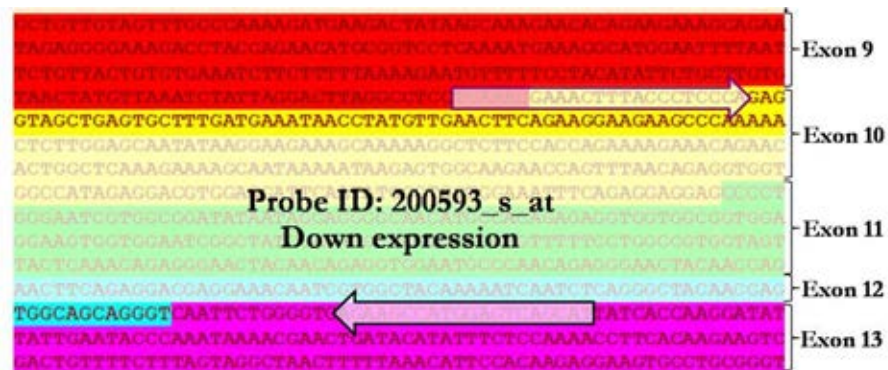
Figure 3.14 The area for *HNRNPU* primer designing

For *BRD4*, the interesting probe ID (202102_s_at) also showed down expression level in OA patients (Normal/OA=1487/730, p-value=0.006). Although the other probe IDs (226052_at and 226054_at) were also showed down expression level in OA patients, the binding area of these two probes is not suitable for primer designing (Table 3.2).

Table 3.2 Details of probe IDs targeted to *BRD4* (Down = significantly decreased expression in OA patients, Same = not significantly different expression between patients and controls).

Gene	Probe ID	OA	Control	t-test	Expression	Binding area
BRD4	202102_s_at	730	1487	0.006	Down	Exon 6-8
BRD4	202103_at	197	246	0.11	same	
BRD4	226052_at	2715	3520	0.002	Down	Intron
BRD4	226054_at	1034	1473	0.006	Down	Last exon
BRD4	239000_at	54	60	0.756	same	
BRD4	240360_at	45	42	0.844	same	

Since the interesting probe ID of *BRD4* binds across the exon 6 to the exon 8 of

the gene, so the primers were designed to cover this area (Figure 3.15).

Figure 3.15 The area for *BRD4* primer designing

3.2.3 Study population

In total, 12 participants were included in this part of the study. The patient

group consisted of 7 individuals, 3 men and 4 women, with a median age of 59 years

(range 45–71 years). They all had grades 3-4 of radiographic signs of knee OA

according to the Kellgren–Lawrence grading system (Kellgren and Lawrence, 1957) and

undergone TKR surgery at Thammasat Commemoration Hospital. The control group

consisted of 5 men, with a median age of 29 years (range 25–33 years). They had knee

surgery due to the anterior cruciate ligament (ACL) injury at Thammasat

Commemoration Hospital. All controls were diagnosed by physicians to have no

symptoms or signs of OA.

3.2.4 Ethic approval

The protocol for taking the synovial fluid samples from all participants has been approved by the Clinical Research Ethics Committee of the Faculty of Medicine, Thammasat University (protocol No. MTU-EC-OT-4-049/55). All research participants provided written informed consents after study procedures were explained.

3.2.5 Synovial fluid collection and RNA extraction

Synovial fluid samples were obtained from 7 individuals with severe knee OA symptoms and 5 individuals with torn anterior cruciate ligament (ACL), during their knee surgery. All samples were transferred on ice to the laboratory and subjected to RNA extraction immediately. Synoviocytes were obtained from synovial fluids as described by Scanu *et al*. (2007). Briefly, synovial fluids were centrifuged at 1,500 rpm for 10 minutes. After carefully discarded the fluid, the cell pellet was suspended in sterile distilled water and used for total RNA extraction by innuPREP RNA minikit (Analytikjena, Germany), according to the manufacturer's instructions.

3.2.6 RT-PCR

To confirm the result from bioinformatics analysis, RT-PCR was applied to two interesting genes, *HNRNPU* and *BRD4*. RNA was reverse transcribed to cDNA by SuperScript III reverse transcriptase (Invitrogen, USA) priming by random hexamer, according to the manufacturer's instructions. The PCR was conducted in the total

volume of 20 µl containing 2 µl of 10X *Taq* buffer, 1.5 mmol MgCl$_2$, 0.5 mmol dNTPs, 0.2

µmol of each primer, 2µl of cDNA and 0.4 U *Taq* DNA polymerase (Fermentas Inc.,

USA). *GAPDH* was used as internal control to evaluate cDNA quality and quantity in

each sample. Primer details of all three studied genes were shown in Table 3.3.

Table 3.3 Primer details of genes studied by RT-PCR

Gene	Primer	Sequence (5'→3')	bases	Product size (bp)
GAPDH	forward	CCACATCGCTCAGACAC	17	146
	reverse	ATGTAGTTGAGGTCAATGAAGG	22	
HNRNPU	forward	TGAAAGGAATTACCCTCCCA	20	551
	reverse	ATGCTGACTCCATGGCTTCT	20	
BRD4	forward	CCCAGCACCAGAGAAGAGC	19	537
	reverse	GCTTTGAGCTGCTCCTGGA	19	

The PCR program of all three genes consisted of the following steps. The initial

denaturation 5 min at 94 °C, followed by 35 cycles of denaturation at 94 °C for 30 s,

annealing at 56 °C for 30 s, and extension at 72 °C for 1 min. The last step is final

extension of 72°C for 5 min. PCR products were electrophoresed in 3% agarose and

visualized by ethidium bromide staining and ultraviolet light transillumination. PCR

products that showed more than one band sizes were positive results for alternative

splicing study. All interesting bands were cut under the UV light and extracted from the

gel by MiniElute Gel Extraction kit (QIAGEN, USA). The sequence of suspicious bands was revealed by standard DNA sequencing.

3.2.7 Real-time PCR

BRD4 was further investigated by real-time PCR. Primers were designed according to DNA sequencing results to confirm the existing and the expression level of the discovered alternative splice variant. *GAPDH* was selected as the reference gene, and the *GAPDH* primers from RT-PCR analysis were used. The first step of real-time PCR is to find the common protocol, which can be used with all three primer sets to eliminate the inter-run errors. Standard curves were generated using serial dilutions of a sample to evaluate the reaction efficiency of each primer set. The real-time PCR was performed in 7 knee OA and 5 control samples by CFX96^{TM}Real-time PCR Detection System (Bio-Rad Laboratories, Inc.) in three replicates. The 10 µl reaction mixture containing 1 µl of cDNA, 0.25 µM of each primer, 5 µl of EvaGreen Super mix (Bio-Rad) and 3 µl of sterile dH$_2$O. The amplification protocol was initial denaturation at 95 °C for 30 sec; followed by 40 cycles consisting of 95 °C for 5 sec, 54 °C for 30 sec and a plate read. A melt curve was generated to check specificity of the reaction by slowly heating the PCR product from 70 °C to 90 °C with 0.5 °C increments. Some real-time PCR products were also subjected to 2% agarose gel electrophoresis to test whether the product sizes were correct.

3.2.8 Tissue specificity of the novel splice variant

To investigate whether the newly discovered splice variant is expressed in a tissue specific manner, real-time PCR was carried out using cDNA obtained from different sources including OA synovial fluid, cultured OA synoviocyte, OA cartilage, cultured OA chondrocyte and cultured dermal fibroblast (the passage of all cultured cell is not over 3). The cDNA of cultured dermal fibroblast was obtained from Molecular Genetic Center, Faculty of Medicine, Chulalongkorn University. Only one sample from each source was studied, and all cDNA samples came from different individuals. The primer sets and the real-time PCR protocol were the same as described above.

3.2.9 Real-time PCR result calculations

The real-time PCR results, in the term of threshold cycle (C_T) values, were exported to Excel 2007. The relative expression level (normalized to *GAPDH*) of common and the newly discovered isoforms were compared between OA patients and controls, or synovial fluids and different tissue/cell types, using $2^{-\Delta\Delta C_T}$ formula as described by Livak (Livak and Schmittgen, 2001). The statistical analysis was performed by student's *t*-Test in the Excel 2007 at 95% confidence.

CHAPTER IV

RESULTS

4.1 Association study

4.1.1 Study population

Demographic characteristics and the number of individuals in each group of the population studied are illustrated in Table 4.1. In this study, there were no significant differences between OA patients and healthy controls in terms of gender, weight, height, and body mass index (BMI). However, the average age of the knee OA group was significantly greater than that of the control group.

Table 4.1 Characteristics of knee OA patients and normal controls

Variables	Knee OA patients n=108	Controls n=119	P-value
Female (%)	88 (81.5)	93 (78.2)	0.53
Age (years), mean (SD)	69.94 (8.41)	54.50 (2.72)	< 0.001*
Weight (kg), mean (SD)	63.57 (10.92)	63.48 (12.11)	0.95
Height (cm), mean (SD)	155.59 (7.05)	157.18 (6.25)	0.06
BMI (kg/m^2), mean (SD)	26.29 (4.45)	25.65 (4.25)	0.27

Note: *statistically significant

4.1.2 SNP genotyping

a) *ADAMTS14* (rs4747096; A/G)

The rs4747096 polymorphism was genotyped by PCR-RFLP. PCR product size was 196 bp. *BspE*I cleaved the PCR product with G allele into 158- and 38-bp fragments. An example of an electrophoretic gel displaying PCR products after

digestion with *BspE*I is shown in Figure 4.1. As the smallest fragment (38 bp) was difficult to be visualized, the presence of a 196-bp fragment corresponded to the AA genotype, the 158-bp fragment corresponded to the GG genotype, and the 196- and 158-bp fragments corresponded to the AG genotype.
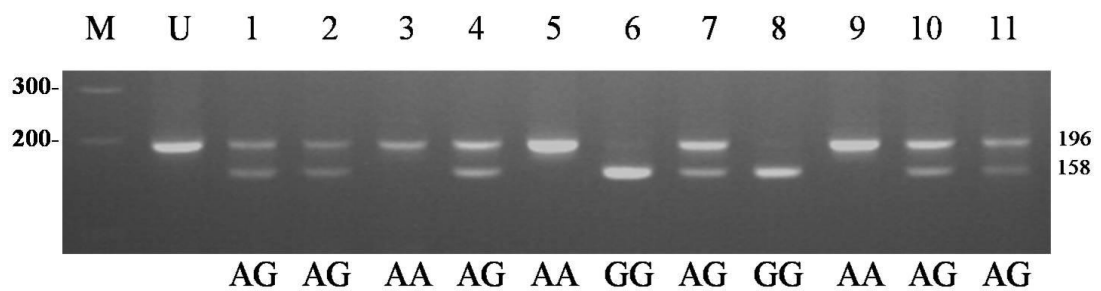
Figure 4.1 *ADAMTS14* rs4747096 genotyping by PCR-RFLP. The PCR product was digested with *BspE*I, which cleaved the PCR products with G alleles, and subjected to electrophoresis on a 3% agarose gel. M = 100 bp DNA standard marker; U = uncut PCR product; 1-11 = sample 1-11. Genotypes of each sample are shown in the bottom.

The genotype and allelic frequencies of rs4747096 are summarized in Table 4.2. Interestingly, the genotype distribution among the controls was not in Hardy–Weinberg equilibrium (HWE) due to the overrepresentation of the rare homozygous genotype (GG), whereas the genotypes of patients were found to be in HWE (P> 0.05). AA was the most common genotype in OA patients and normal controls (46.3% and 40.3%, respectively).

Table 4.2 Genotype distribution and allelic frequency of *ADAMTS14* polymorphism between knee OA patients and normal controls

rs4747096	OA	Control	OR (95% CI)	*P*-value
Genotype (%)				
AA				
Whole group	50 (46.3%)	48 (40.3%)	2.17 (0.92-5.19)	0.054
Female	40 (45.5%)	35 (37.6%)	**2.79 (1.05-7.59)**	**0.023**
Male	10 (50.0%)	13 (50.0%)	0.77 (0.09-6.35)	0.56
AG				
Whole group	46 (42.6%)	46 (38.7%)	2.08 (0.87-5.02)	0.07
Female	39 (44.3%)	36 (38.7%)	2.65 (0.99-7.19)	0.031
Male	7 (35.0%)	10 (38.5%)	0.70 (0.07-6.39)	0.54
GG				
Whole group	12 (11.1%)	25 (21.0%)	1*	
Female	9 (10.2%)	22 (23.7%)	1*	
Male	3 (15.0%)	3 (11.5%)	1*	
Allele (%)				
A				
Whole group	146 (67.6%)	142 (59.7%)	1.41 (0.94-2.11)	0.08
Female	119 (67.6%)	106 (57.0%)	**1.58 (1.00-2.45)**	**0.038**
Male	27 (67.5%)	36 (69.2%)	0.92 (0.35-2.46)	0.86
G				
Whole group	70 (32.4%)	96 (40.3%)	1*	
Female	57 (32.4%)	80 (43.0%)	1*	
Male	13 (32.5%)	16 (30.8%)	1*	

*Reference.

We found a significant association between the AA genotype and knee OA in female patients (P= 0.023). This genotype was linked to a higher risk of knee OA in females (OR = 2.79, 95%CI = 1.05-7.59). In addition, the A allele was also significantly associated with female knee OA patients (OR = 1.58; 95% CI = 1.00–2.45; P= 0.038). Moreover, a marginal significant association was found between the AG genotype and knee OA in female patients (OR = 2.65; 95% CI = 0.99–7.19; P= 0.031). In contrast, the rs4747096 polymorphism was not associated with OA susceptibility in males in this study.

b) *ADAM12* (rs3740199; C/G)

HRM analysis performed by CFX96 real-time PCR system enabled us to identify the rs3740199 polymorphism (C/G) in each participant. The small amplicon size (50 bp) enhanced the melting temperature difference between the GG, GC and CC genotypes. The unique melt profiles regarding their melt curve shape and/or melting temperature (Tm) from different genotypes were scored by Precision Melt Analysis[TM] software. As illustrated in Figure 4.2, the melt profiles of all samples were normalized (Figure 4.2A), and the most common genotype (GC) was used as the baseline (red lines) for difference curve plotting (Figure 4.2B). Although both homozygous genotypes showed similar melt curve shapes, the GG genotypes (green lines) displayed higher difference relative fluorescence units (RFU) than the CC genotypes (blue lines) when compared with the baseline. The auto-grouping function of the program differentiated melting behavior of each sample and clustered similar melt profiles into three groups as shown in Figure

4.2C. The average genotype calling efficiency was 99.1% in patients and 98.7% in controls.
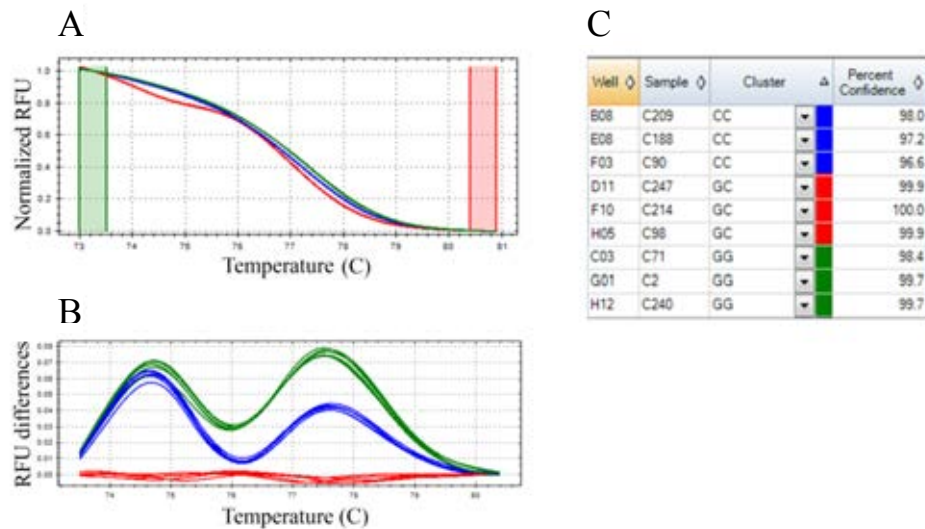
Figure 4.2. The rs3740199 SNP genotypes were investigated by HRM analysis using Precision Melt Analysis software. A, melt profile of the GG (green lines), CC (blue lines) and GC genotypes (red lines) after normalization; B, difference curve converted from data in Fig. 1A using the GC genotype as the baseline. C, melt profiles were computed by the Precision Melt Analysis program to discriminate sequence variation by clustering similar melt profiles into the same group. RFU, relative fluorescence units.

The allelic and genotype frequencies of rs3740199 in this study population are presented in Table 4.3. All genotypes among both patients and controls were in Hardy–Weinberg equilibrium ($P>0.05$). The overall allelic frequencies for the C allele and the G allele were 51.1% and 48.9%, respectively. GC was the most frequent genotype in both OA patients (52.8%) and control group (49.6%). Interestingly, we found remarkable association between the C allele and knee OA in male patients (OR=4.05, 95%CI=1.54-10.82, P=0.001).

Table 4.3 Genotype distribution and allelic frequency of *ADAM12* polymorphism between knee OA patients and normal controls

rs3740199	OA	Control	OR (95% CI)	*P*-value
Genotype (%)				
CC				
Whole group	26 (24.1%)	32 (26.9%)	0.91 (0.40-2.06)	0.81
Female	16 (18.2%)	28 (30.1%)	0.42 (0.16-1.11)	0.053
Male	10 (50.0%)	4 (15.4%)	**13.75 (1.59-155.82)**	**0.004**
GC				
Whole group	57 (52.8%)	59 (49.6%)	1.08 (0.54-2.18)	0.81
Female	49 (55.7%)	48 (51.6%)	0.75 (0.34-1.69)	0.46
Male	8 (40.0%)	11 (42.3%)	4.00 (0.56-35.21)	0. 11
GG				
Whole group	25 (23.1%)	28 (23.5%)	1*	
Female	23 (26.1%)	17 (18.3%)	1*	
Male	2 (10.0%)	11 (42.3%)	1*	
Allele (%)				
C				
Whole group	109 (50.5%)	123 (51.7%)	0.95 (0.65-1.40)	0.80
Female	81 (46.0%)	104 (55.9%)	0.67 (0.43-1.04)	0.06
Male	28 (70.0%)	19 (36.54%)	**4.05 (1.54-10.82)**	**0.001**
G				
Whole group	107 (49.5%)	115 (48.3%)	1*	
Female	95 (54.0%)	82 (44.1%)	1*	
Male	12 (30.0%)	33 (63.46%)	1*	

*Reference.

Furthermore, the results indicated that males with the CC genotype seem to have almost 14 times higher risk for having knee OA than males with the GG genotype (OR=13.75, 95%CI=1.59-155.82, P=0.004). No significant relations between rs3740199 SNP and the knee OA susceptibility were found in pooled or female groups.

c) *MMP1* (rs1799750; 2G/1G)

The unique melt profiles regarding mainly on their melting temperature (Tm) and melt curve shape from different genotypes were analyzed by Precision Melt Analysis™ software. As demonstrated in Figure 4.3, the fluorescent signal from all samples were normalized (Figure 4.3A), and the most common melt pattern in a run (mostly came from the 2G/1G genotype) was used as the baseline (red lines) for plotting difference curves (Figure4.3B). The 1G/1G genotypes (blue lines) displayed lower difference relative fluorescence units (RFU) than the 2G/2G genotypes (green lines) when compare with the baseline. The melting pattern of all samples were differentiated and clustered by the auto-grouping function of the program into three groups according to similar melt profiles as illustrated in Figure 4.3C. The average genotype calling efficiency was 98.58% in patients and 98.70% in controls.
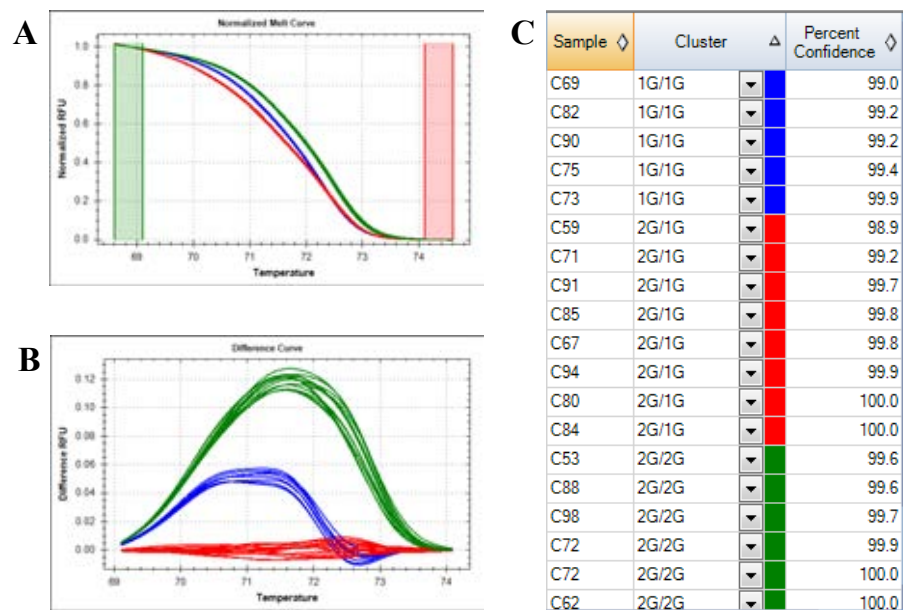
Figure 4.3 The rs1799750 SNP genotypes of the study population were investigated by HRM analysis using Precision Melt Analysis software. A, melt profile of the 2G/2G (green lines), 2G/1G (red lines) and 1G/1G genotypes (blue lines) after normalization; B, difference curve plotting from data in Fig. 1A using the 2G/1G genotype as the baseline. C, Melt profiles were discriminated by the auto-grouping function of the program into 3 genotypes. RFU, relative fluorescence units.

The comparison of allelic and genotype frequencies of the SNP between case and control are demonstrated in Table 4.4. The prevalence of all genotypes in patient and control groups were in Hardy–Weinberg equilibrium ($P>0.05$), showing normal distribution of the 2G and 1G allele. The overall allelic frequencies for the 2G allele and the 1G allele were 57.8% and 42.2%, respectively. The 2G/1G genotype was the most frequent genotype in both OA patients (44.4%) and control group (45.4%). There was no statistically significant difference between patient and control groups regarding genotype distributions or allele frequencies in pooled or gender stratified groups.

Table 4.4 Genotype distribution and allelic frequency of *MMP1* polymorphism between knee OA patients and normal controls

rs1799750	OA	Control	OR (95% CI)	*P*-value
Genotype (%)				
1G/1G				
Whole group	20 (18.5%)	25 (21.0%)	0.80 (0.36-1.78)	0.55
Female	15 (17.0%)	19 (20.4%)	0.81 (0.33-2.02)	0.63
Male	5 (25. 0%)	6 (23.1%)	0.71 (0.10-4.78)	0.69
2G/1G				
Whole group	48 (44.4%)	54 (45.4%)	0.89 (0.47-1.67)	0.69
Female	40 (45.5%)	40 (43.0%)	1.03 (0.51-2.08)	0.93
Male	8 (40. 0%)	14 (53.8%)	0.49 (0.10-2.43)	0.32
2G/2G				
Whole group	40 (37.1%)	40 (33.6%)	1*	
Female	33 (37.5%)	34 (36.6%)	1*	
Male	7 (35.0%)	6 (23.1%)	1*	
Allele (%)				
1G				
Whole group	88 (40.7%)	104 (43.7%)	0.89 (0.60-1.31)	0.52
Female	70 (39.8%)	78 (41.9%)	0.91 (0.59-1.42)	0.68
Male	18 (45. 0%)	26 (50.0%)	0.82 (0.33-2.03)	0.63
2G				
Whole group	128 (59.3%)	134 (56.3%)	1*	
Female	106 (60.2%)	108 (58.1%)	1*	
Male	22 (55. 0%)	26 (50.0%)	1*	

*Reference.

4.1.3 Haplotype analysis

ADAMTS14 and ADAM12 SNP allele combinations (haplotypes) were compare between patient and control groups to investigate the combinatorial effects of these two SNPs on OA susceptibility. As the G allele from both SNPs seem to have no effect on OA susceptibility, The GG haplotype was used as the reference haplotype. No association was observed between any haplotype and knee OA (Table 4.5).

Table 4.5 The rs4747096/rs3740199 haplotype analysis

Haplotype of rs4747096/ rs3740199	OA	Control	OR (95% CI)	P-value
AC				
Whole group	73 (30.5%)	69 (27.8%)	1.18 (0.68-2.05)	0.54
Female	58 (29.0%)	56 (28.3%)	1.06 (0.57-1.96)	0.84
Male	15 (38.5%)	13 (26.0%)	1.92 (0.46-8.18)	0.31
AG				
Whole group	73 (30.5%)	69 (27.8%)	1.18 (0.68-2.05)	0.54
Female	65 (32.5%)	50 (25.2%)	1.33 (0.72-2.47)	0.33
Male	8 (20.5%)	19 (38.0%)	0.7 (0.16-3.13)	0.60
GC				
Whole group	48 (20.1%)	60 (24.2%)	0.89 (0.49-1.61)	0.68
Female	38 (19.0%)	52 (26.3%)	0.75 (0.39-1.44)	0.35
Male	10 (25.6%)	8 (16.0%)	2.08 (0.43-10.45)	0.30
GG				
Whole group	45 (18.9%)	50 (20.2%)	1*	
Female	39 (19.5%)	40 (20.2%)	1*	
Male	6 (15.4%)	10 (20.0%)	1*	

*Reference.

4.2 Alternative splicing study

4.2.1 Bioinformatic analysis

Microarray data processing had revealed 32 candidate genes with high possibility to have alternative splicing variants in knee OA patients compared with controls (see in Appendix). Due to the availability of primer designing, as explained in Methodology, *HNRNPU* and *BRD4* were selected for further studies.

4.2.2 RT-PCR of *HNRUPU* and *BRD4* genes

RT-PCR analysis of *HNRNPU* using a primer set designed to amplify across the exon 10 to the exon 12 resulted in the amplification of the expected 551-bp product without any evidence of the alternative splice variant (Figure 4.4)
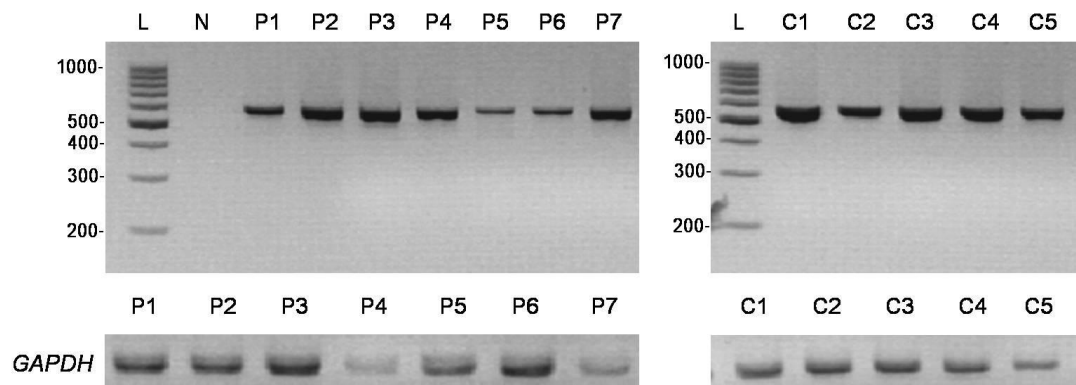
Figure 4.4 RT-PCR results of *HNRNPU* show the expected band size of 551 bp without any evidence of the alternative splice variant. The bottom panels showed comparative band intensity of *GAPDH*. L=100 bp DNA ladder, N=Negative control, P=Patient, C=Control.

In contrast, the primers for *BRD4*, which were designed to amplify across the exon 6 to the exon 8 generated a 537-bp expected product together with a smaller product (Figure 4.5). However, in some samples with small amount of PCR products, the smaller band was difficult to be visualized.
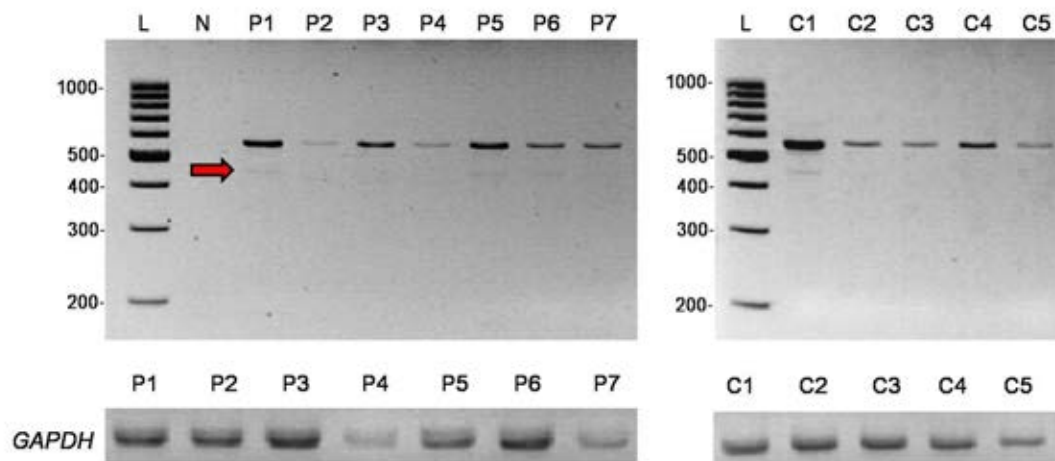
Figure 4.5 RT-PCR results of *BRD4* show the expected band size of 537 bp and a smaller band in some samples (emphasized by the arrow), indicating an alternative splice variant. The bottom panels showed comparative band intensity of *GAPDH*. L=100 bp DNA ladder, N=Negative control, P=Patient, C=Control.

4.2.3 Sequencing results of *BRD4*

Samples with obvious positive result of *BRD4* gene were selected to enrich PCR product, especially for the small band. After 3% agarose gel electrophoresis, the best quality bands of large and small products were cut out, extracted from the gel and subjected to standard sequencing. Interestingly, the results showed that the large band corresponds to the exon 6 to the exon 8 of *BRD4*, whereas the small band corresponds to the exon 6 to the exon 8 *of BRD4*, with the entire exon 7 (129 bp) missing, leading to the exon 6/8 junction as shown in Figure 4.6 (the complete sequencing data is in the

Appendix). Importantly, BRD4 with the entire exon 7 missing has never been reported in any database, suggesting that it is the new isoform.
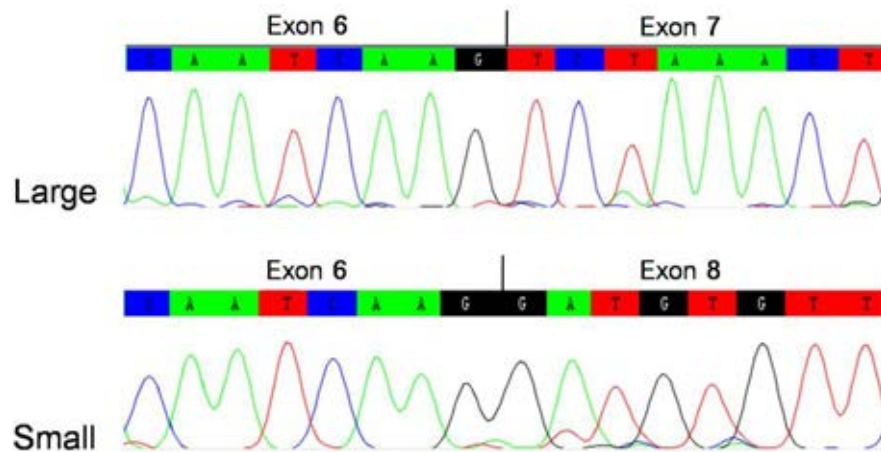
Figure 4.6 Sequencing results of *BRD4* RT-PCR products. The upper panel shows the exon 6/7 junction in the large band, whereas the lower panel shows the exon 6/8 junction in the small band.

4.2.4 Primer designing results for real-time PCR of *BRD4*

The DNA sequencing results of the large and small bands from *BRD4* RT-PCR products revealed two BRD4 isoforms, BRD4[+7] and BRD4[-7]. More specific primers were designed to amplify these two splice variants by real-time PCR. For BRD4[+7], the forward primer was designed to bind to the exon 6; while the reverse primer was designed to bind the exon 7 (Figure 4.7)
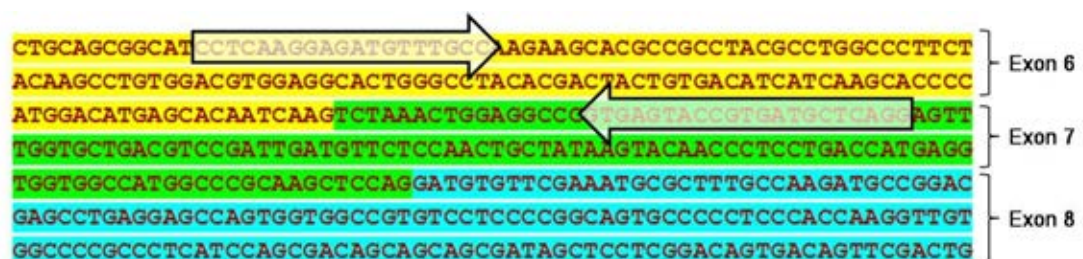
Figure 4.7 The binding area of real-time PCR primers for BRD4[+7] isoform

For BRD4[-7] isoform, the sequence that corresponds to the exon 7 of *BRD4* was missing, leading to the exon 6/8 junction, so the forward primer was the same as for BRD4[+7] isoform, but the reverse primer was designed to bind the exon 6/8 junction (Figure 4.8)
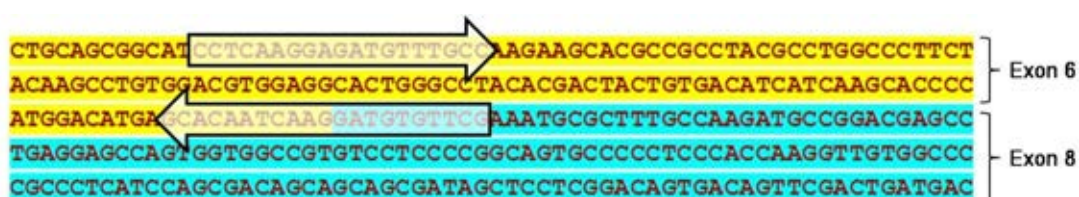
Figure 4.8 The binding area of real-time PCR primers for BRD4[-7] isoform

Primer details of all 3 genes for Real-time PCR were summarized in Table 4.6.

Table 4.6 Real-time PCR primers information

Gene	Primer	Sequence (5'→3')	bases	Product size (bp)
GAPDH	Forward	CCACATCGCTCAGACAC	17	146
	Reverse	ATGTAGTTGAGGTCAATGAAGG	22	
BRD4[+7]	Forward	CCTCAAGGAGATGTTTGCC	19	170
	Reverse	CCTGAGCATCACGGTACTCAC	21	
BRD4[-7]	Forward	CCTCAAGGAGATGTTTGCC	19	143
	Reverse	CGAACACATCCTTGATTGTGC	21	

4.2.5 Expression of BRD4 isoforms analyzed by real-time PCR

a) Serial dilution

After standard curves were performed using serial dilutions, the efficiency of each primer set was calculated. At the primer concentration of 250 nm and 54 ℃ annealing temperature, all three primer sets showed acceptable reaction efficiencies

(95-105%), which were 103.5% for *GAPDH*, 95.8% for BRD4[+7] isoform and 104.8% for

BRD4[-7] isoform (Figure 4.9-4.11).
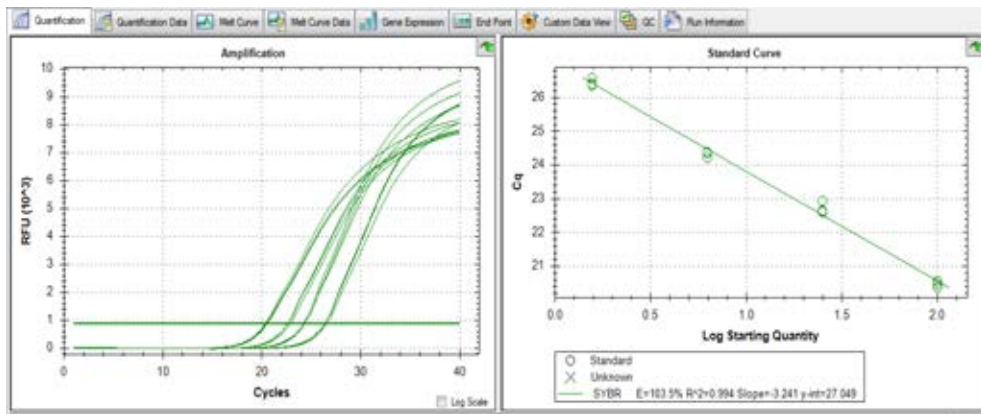
Figure 4.9 The standard curve for calculating the efficiency of *GAPDH* primers
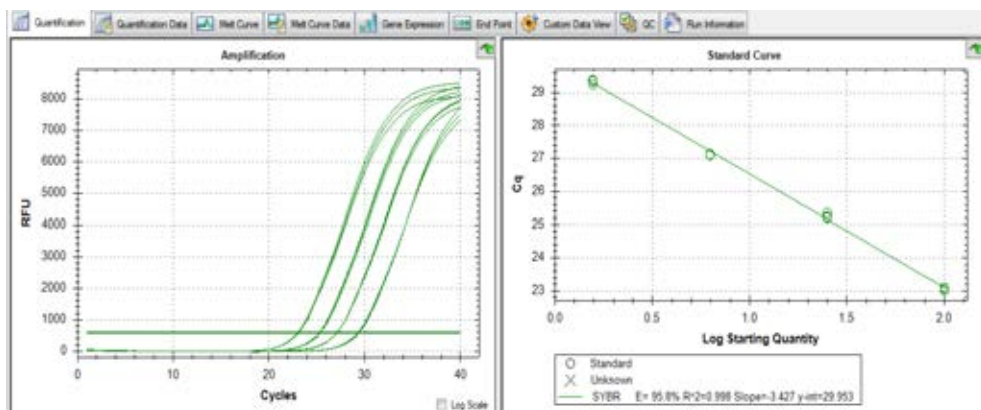
Figure 4.10 The standard curve for calculating the efficiency of BRD4[+7] primers
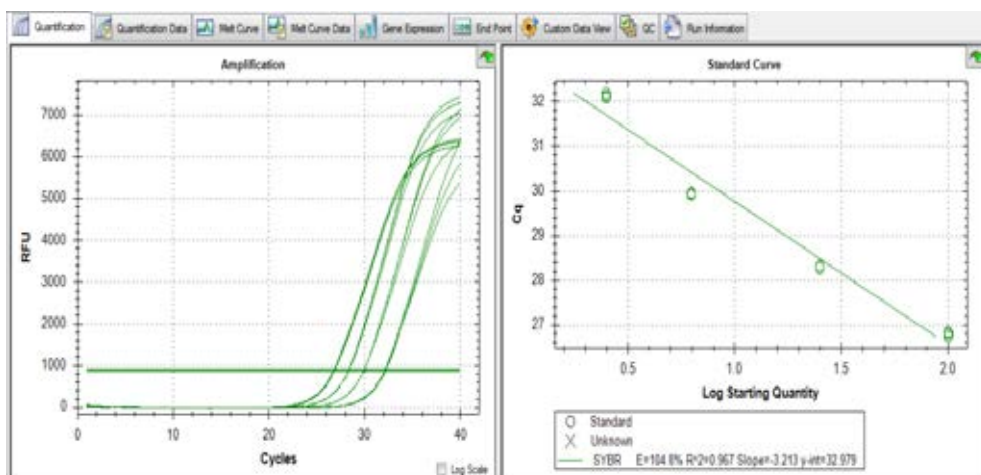
Figure 4.11 The standard curve for calculating the efficiency of BRD4[-7] primers

b) Real time PCR result

The real-time PCR results, in the terms of threshold cycle (C_T) values, of each sample were shown in the Appendix. The gene expression level has negative correlation with the C_T values, meaning that samples with higher C_T values have lower gene expression level. To determine the specificity of the real-time PCR primers, melting temperature of each real-time PCR product was indicated (see in the Appendix), and some PCR products were subjected to 2% agarose gel electrophoresis. The results showed that all three primer sets generated correct PCR products, without nonspecific band (Figure 4.12)
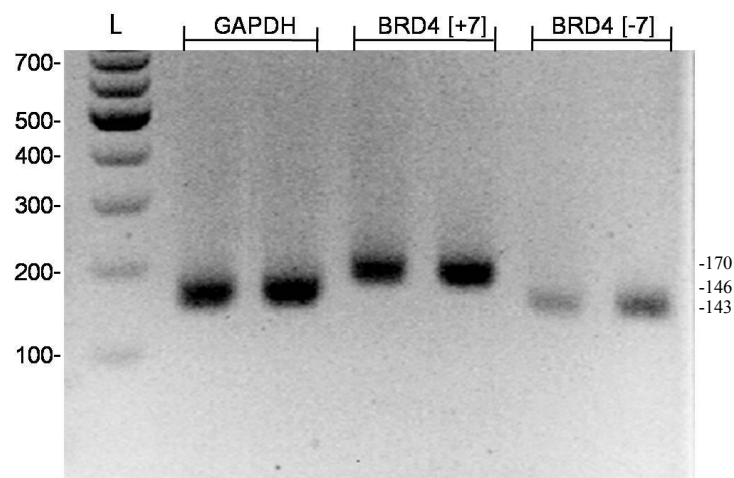
Figure 4.12 The sizes of GAPDH, BRD4[+7] and BRD4[-7] real-time PCR products

c) Normalized fold expression ($2^{-\Delta\Delta_{CT}}$)

The expression levels of BRD4[+7] and BRD4[-7] isoforms in synovial fluid were measured in patient and control groups (normalized by the expression of *GAPDH*), as well as in different tissue/cell types to determine whether these isoforms expressed differently in each target group. The relative expression levels (normalized by *GAPDH*) between BRD4[+7] and BRD4[-7] isoforms were compared within the OA patient group or the control group (displayed as normalized fold expression when the expression of BRD4[+7] is one) as shown in Figure 4.13. The results showed that the expression levels of BRD4[-7] isoform were 21% of BRD4[+7] isoform in the control group (*p*=0.004) and 12% in the OA patient group (*p*=0.005).
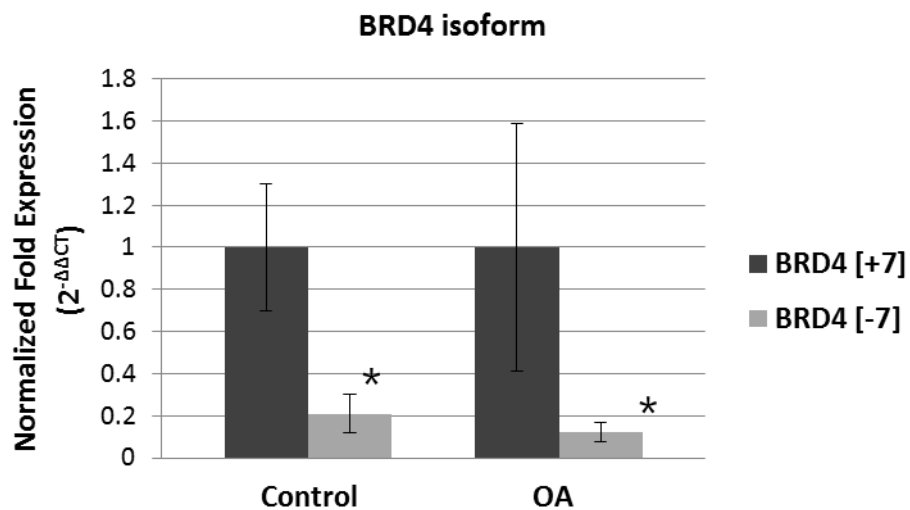
Figure 4.13 Normalized fold expression of BRD4[-7] compared with BRD4[+7] within control or OA patient group (* indicate statistically significant difference at 99% confidence)

Moreover, the relative expression levels (normalized by *GAPDH*) of each BRD4 isoform were compared between the OA patient and control groups (displayed as normalized fold expression when the expression of control group is one). The results suggested that the expression level of BRD4[+7] isoform in the patient group was 64% of the control group (p=0.07), whereas the expression level of BRD4[-7] isoform in the patient group was 40% of the control group (p=0.025) as shown in Figure 4.14.
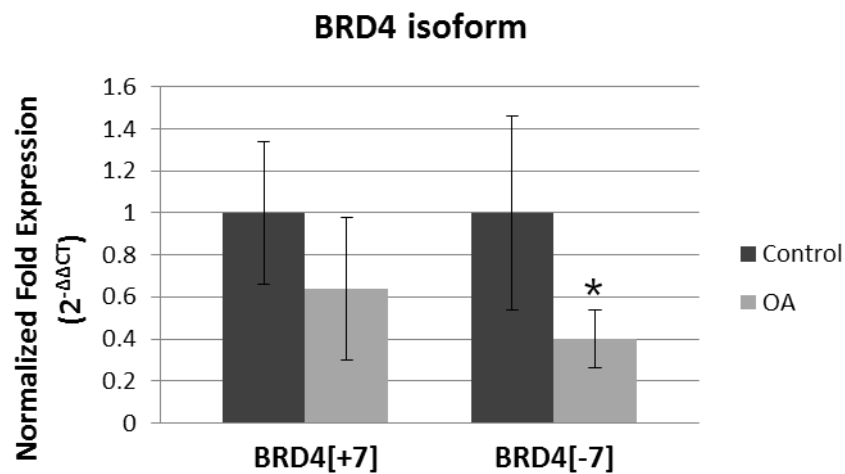
Figure 4.14 Normalized fold expression of each BRD4 isoform in the OA patient group compared with the control group (* indicates statistical difference at 95% confidence)

Moreover, to determine whether BRD4 isoforms are expressed in a tissue specific manner, the real-time PCR was carried out using cDNA from OA synovial fluid, cultured OA synoviocyte, OA cartilage, cultured OA chondrocyte and dermal fibroblast. The result showed that BRD4[+7] isoform was expressed in all tested sources as shown in Figure 4.15
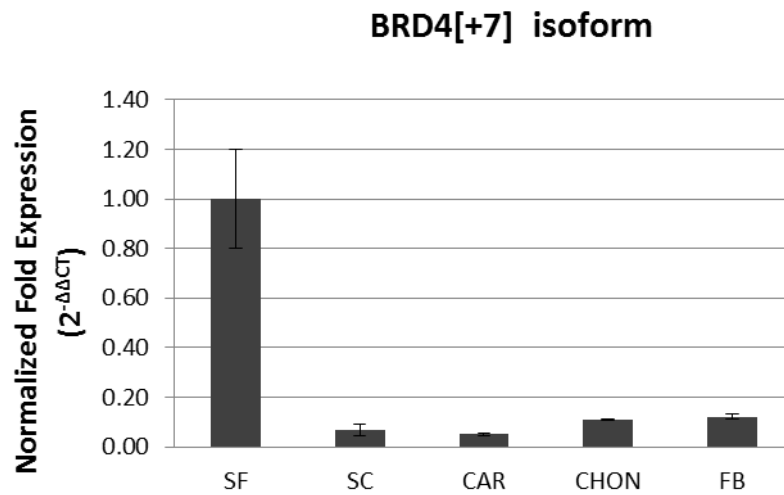
BRD4[+7] isoform

Figure 4.15 Normalized fold expression of BRD4[+7] isoform in different sources compared with the OA synovial fluid. SF=OA synovial fluid, SC=cultured OA synoviocyte, CAR=OA cartilage, CHON=cultured OA chondrocyte and FB=dermal fibroblast.

In addition, the expression of BRD4[-7] isoform was also detected in all tested sources as shown in Figure 4.16.
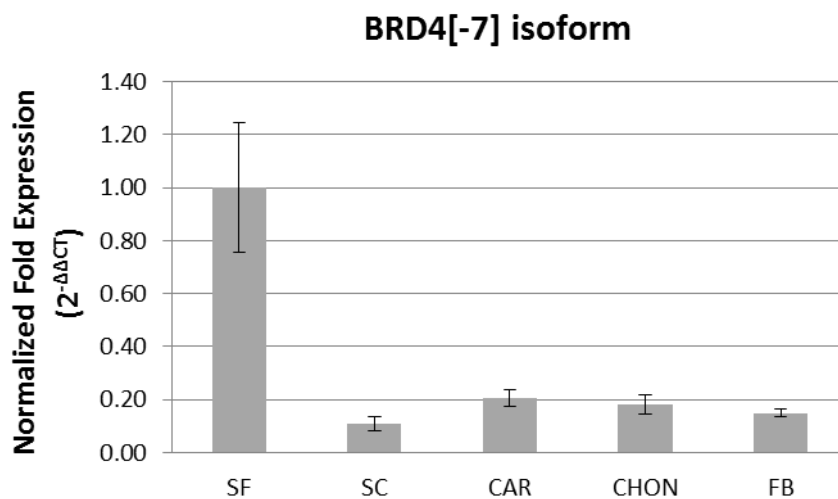
BRD4[-7] isoform

Figure 4.16 Normalized fold expression of BRD4[-7] isoform in different sources compared with the OA synovial fluid. SF=OA synovial fluid, SC=cultured OA synoviocyte, CAR=OA cartilage, CHON=cultured OA chondrocyte and FB=dermal fibroblast.

CHAPTER V

DISCUSSION

5.1 Association study

5.1.1 SNP genotyping techniques

PCR-RFLP is a common technique for SNP genotyping because it is simple, cheap and does not require specific instruments. However, it is a time and labor consuming technique. In addition, some SNPs are not related to any restriction enzyme recognition site, so mutated primer had to be used to generate the site like in this study. In contrast, HRM analysis is a high throughput SNP genotyping technique. This one-step method allows rapid SNP genotyping by real-time PCR system without the need for expensive labeled probes or allele-specific primers. However, it needs more optimization steps and more expensive equipments and reagents than PCR-RFLP.

5.1.2 *ADAMTS14* SNP (rs4747096; A/G)

In the present study, the association between rs4747096 nsSNP in *ADAMTS14*, a recently discovered OA candidate gene, and presence of knee OA in Thai population was evaluated. Originally, this nsSNP designates a change of glutamic acid (G<u>A</u>A) to glycine (G<u>G</u>A) in the COOH-terminal domain of ADAMTS14 protein. The allele G is the ancestral allele as it exists in *ADAMTS14* orthologues in nonhuman primate such as chimpanzee, whereas the A allele is more common in humans than the G allele (Sherry *et al*., 2001). Recently, there was evidence of the T allele, which determines the amino

acid valine (G<u>T</u>A) in the rs4747096 database. The existence of the new allele seems to complicate the analysis of rs4747096 role in OA. However, this new variation was discovered in genomic DNA from cancer patients (Futreal *et al*., 2004), and was not appeared in any population in The 1000 Genome Project (The 1000 Genomes Project Consortium, 2010), including Asian population even though the whole genome sequencing method was used as genotyping technique. Thus, the T allele was neglected in this study.

Genotype distributions and allele frequencies in Thai population showed an opposite trend to the previous study (Rodriguez-Lopez *et al*., 2009). The result from this study demonstrated that rs4747096 nsSNP was associated with susceptibility to knee OA in female patients undergoing total knee replacement surgery. The A allele is associated with increased risk of knee OA, and individuals with the AA genotype carried the highest risk in comparison with the other genotypes. In contrast, Rodriguez-Lopez study (2009) reported that the G allele was overrepresented and associated with knee OA in Caucasian women. The reason for these contradictory results may be due to differences in ethnicity or shared environmental factors. However, the non-associated results from male population are consistent.

It was believed that the substitution of glutamic acid (acidic) to glycine (nonpolar) in ADAMTS14 (E1049G) may result in abnormal ability of the protein in processing collagen precursor and finally leading to OA. The positive results of both A

and G alleles obtained from the present and previous studies may indicate that the amino acid substitution determined by rs4747096 in *ADAMTS14* is not the direct damaging factor contributing to OA. However, this polymorphic locus may be linked to the OA causative gene on the same chromosome and the SNP is inherited jointly with the disease.

The number of the ancestral G-allele in Thais was higher than Caucasians especially in women (38% vs 20%). These figures are consistent with HapMap reports showing that the G allele frequency of this SNP in East Asian population and European population was approximately 37% and 19%, respectively (The International HapMap Consortium, 2003). The genotype distribution of the control group in this study did not fit the HWE, which indicated that specific disturbing influences were introduced. One possible influence is a selective pressure for the G allele as the GG genotype seemed to be the protective genotype.

5.1.3 *ADAM12* SNP (rs3740199; C/G)

Although the G allele in rs3740199 is the ancestral allele and considered as major allele in African (59%), American (57%) and European (56%) populations, it frequency is lower than the C allele in Asian population (49%) (The International HapMap Consortium, 2003) as well as in this study (49%). Very recently, the third allele (A) discovered from The ClinSeq Project was added to the database of this SNP (Biesecker *et al.*, 2009). However, SNP genotyping technique used in this study was

HRM analysis, which is a high sensitivity and accuracy technique. The HRM results did not show any evidence of the A allele in our study population as the melt profile of all samples were categorized into just three groups (GG, GC or CC) in every run.

This SNP (c.142G>C) in the second exon determines a change of glycine (nonpolar) to arginine (positively charge) in the prodomain that involves in ADAM12 maturation. The metalloprotease domain of ADAM12, which is kept inactive inside the cell through a cysteine-switch mechanism, will be activated by the prodomain removal (Loechel *et al*., 1999; Cao *et al*., 2002; Wewer *et al*., 2006). Interestingly, the prodomain may also play the regulatory roles in the extracellular matrix as it remains associated with the catalytic domain by noncovalent bond after enzyme maturation (Kawaguchi *et al*., 2002; Wewer *et al*., 2006) and also mediated transportation and folding of the protein (Cao *et al*., 2002). The proteolytical activity of ADAM12 seem to be essential for cleavage of IGF-1-IGFBP-5 complex, a process that increase bioavailability of insulin-like growth factor -1 (IGF-1), resulting in chondrocyte proliferation (Okada *et al*., 2008). It has also been reported that inadequate anabolic growth factors such as IGF-1 may leads to cartilage degradation (Trippel *et al*., 2004). Thus, the amino acid substitution in the prodomain resulting from rs3740199 may affect proteolytical activity of ADAM12 and give rise to insufficiency of IGF-I, which in turn will cause the loss of articular cartilage and finally initiate OA.

5.1.4 *MMP1* SNP (rs1799750; 2G/1G)

In this study, the relationship between a SNP (rs1799750; -1,607 1G/2G) in *MMP1,* a gene on chromosome 11q22.3, and knee OA susceptibility in a Thai population was investigated using HRM analysis. The 2G allele is the ancestral allele and considered as the major allele in Asian (67%), American (56%) and European (51%) populations, whereas its frequency is lower than the 1G allele in African population (45% vs 55%) (The International HapMap Consortium, 2003). This promoter polymorphism has been shown to influence the transcriptional regulation of the MMP-1 protein. The presence of the 2G allele creates the consensus sequence for the Ets family of transcription factors (AAGGAT). Thus, the 2G allele possesses greater transcriptional activity than the 1G allele (Rutter *et al*., 1998; Ye, 2000; Price *et al*., 2001).

The results from several previous studies investigated the role of this SNP were diverse. There was a study indicating that the 2G allele frequency was significantly higher in ovarian cancer patient group than in the control group. Moreover, the *MMP1* expression levels in ovarian cancer patients carrying 2G alleles were increased significantly when compared with 1G homozygotes (Kanamori *et al*., 1999), and clinical researches in colorectal tumors and lung cancer also showed the similar results (Ghilardi *et al*., 2001; Zhu *et al*., 2001). On the other hand, studies in sarcoidosis (Ninomiya *et al*., 2004), endobronchial tuberculosis (Kuo *et al*., 2008), primary sclerosing cholangitis (PSC) (Wiencke *et al*., 2004), as well as the previous study in

knee OA (Barlas *et al.*, 2009) have shown a significant increase in 1G/1G or 1G/2G genotypes in patients. Nevertheless, there was no significant association neither from the 2G allele nor the 1G allele in the present study. The possible explanation for our finding may base on the fact that after joint injury and inflammation, the mean levels of proMMP-1 and MMP-1 activities were increased (Tchetverikov *et al.*, 2005), so the increased levels of activated MMP-1 in OA joint may be a consequence, not a cause of the disease.

5.1.5 The combinatorial effect of studied SNPs

Although the SNPs in *ADAMTS14*, *ADAM12* and *MMP1* have been shown to be associated with knee OA in previous studies, only SNPs in *ADAMTS14* and *ADAM12* showed significantly association with knee OA in Thai population. However, the SNPs from these two genes seem to have no additive effects on each other because it showed significantly association in different genders (*ADAMTS14* in female and *ADAM12* in male). Moreover, haplotype analysis of allele combinations from SNPs in these two genes showed no evidence of its combinatorial effect on knee OA occurrence.

5.1.6 Factors affecting association study results

Although explanations for the inconsistent results between the previous and the present studies are still unclear, several possibilities could be mentioned. Firstly, different characteristics of the study populations including population size, age and gender ratio may have contributed to the contradictory results. Secondly, it is obvious

that given populations have their own gene pool, therefore it was not surprise to see differences in allele frequencies and genotype distributions from population with different ethnicity. As Thais are mixed racial population, gene flow or migration can also be influences that alter the existing gene frequency. Lastly, unique environmental factors or gene-environment interactions in each population such as diets, lifestyles, and selected physical activity may affect the genetic roles of investigated gene to knee OA susceptibility. The results from this study draw attention to the importance of each population's own genetic association study and SNP database. However, the potential limitation in this study to be stated is the small study population size, especially in the male group. Additional investigations conducted in a larger sample size or different ethnicities are still required to validate the present findings.

5.2 Alternative splicing study

5.2.1 Bioinformatic analysis

Microarray is a useful technique to compare gene expression (mRNA) levels between target groups in the large scale. The running of microarray experiment is not difficult as long as equipments and expenses are available. In contrast, processing of the data generated from microarray experiment is much more complicated. In this study, the new strategy for searching new candidate genes with alternative splice variants from microarray data has introduced. The basic concept is to find genes with at least one probe shows significantly different expression when compared between OA patients

and controls, and different expression pattern when compared with the other probes within the same probe set. This concept could also be applied to identify gene with alternative splicing in other diseases.

5.2.2 RT-PCR and real-time PCR

The high rate of false positive result from microarray due to nonspecific binding of probes is well known. Hence, bioinformatic results were confirmed with other molecular methods, which are RT-PCR and real-time PCR. RT-PCR is very useful technique for the first trial of alternative splicing study because it enables primers designing to bind across multiple exons or the large exon area with unclear alternative splice sites. A critical step of RT-PCR to be mentioned is reverse transcription. Both oligodT and random hexamer can be used in the first strand cDNA synthesis step, with the higher specificity from oligodT, however, random hexamer should be used if the target area is distant from the poly-A tail of mRNA. In contrast, real-time PCR is a useful technique for determining the expression level of the targeted transcript variants. Nevertheless, the limitation of real-time PCR is the product size, which should not larger than 250 bp, so it should be carry out when the targeted areas is definite. In this study, microarray data processing has revealed 32 candidate genes with alternative splicing in OA patients. However, only *HNRNPU* and *BRD4* were selected for further analyses because of the explicit probe results and availability for primer designing.

5.2.3 *HNRNPU*

HNRNPU is located on chromosome 1q44. This gene encodes heterogeneous nuclear ribonucleoprotein U, which is also known as scaffold attachment factor A. It belongs to the ubiquitously expressed heterogeneous nuclear ribonucleoproteins (hnRNPs) subfamily (Kiledjian andDreyfuss,1992). The hnRNPs are RNA binding proteins associated with pre-mRNA processing in the nucleus and influence mRNA metabolism and transport. The hnRNP proteins have nucleic acid binding properties because it contains the RNA binding domain and the scaffold-associated region (SAR)-specific bipartite DNA-binding domain. This protein is also believed to be involved in large ribonucleoprotein packaging (Fackelmayer and Richter, 1994a). All of the hnRNPs are localized in the nucleus but some are also present in the cytoplasm. To date, at least two alternatively spliced variants of this gene have been identified (Fackelmayer and Richter, 1994b). However, neither alternative splicing at targeted area nor association of the HNRNPU and knee OA pathogenesis were observed in this study.

5.2.4 *BRD4*

Bromodomain-containing protein 4 (Brd4) is encoded from *BRD4* gene, which is located on chromosome 19p13.12. It is a nuclear protein which belongs to the BET (bromodomain and extraterminal domain) family. The member of this protein family usually carry two tandem bromodomains, BDI and BDII, which bind preferentially to acetylated chromatin, and an extra terminal (ET) domain, which confers transcription

activation in pTEFb-independent manner. These domains are conserved from yeasts to humans (ShailaRahman, 2011). *BRD4* is expressed in many tissues and plays an essential role in cell growth, cell cycle regulation, DNA replication and cancer progression. In addition, it has been implicated in virus-host pathogenesis as some human papilloma viruses (HPV) use Brd4 as a cellular adaptor for anchoring viral genomes to host chromosomes (Wu and Chiang, 2007).

Recent studies have revealed that Brd4 has significant roles in several diseases, including acutemyeloid leukemia (Zuber*et al*., 2011), Burkitt's lymphoma (Mertz *et al*., 2011), multiple myeloma (Delmore*et al*., 2011), colon cancer (Rodriguez *et al*., 2011), NUTmidline carcinoma (French *et al*., 2003), and inflammatory disease (Nicodeme*et al*., 2010). Two spliced variants of *BRD4* have been reported in NCBI. The long isoform, (NM_058243.2) encodes the full length Brd4 (1362 amino acid), whereas the short isoform (NM_014299.2) encodes shorter Brd4 (722 amino acid) with a distinct C-terminus, lacking the P-TEFb-interacting domain (PID). Both Brd4 protein isoforms share complete BDI, BDII and ET domains.

Both BDI and BDII domains of Brd4 play critical role in chromatin binding. It can bind acetylated K14 on histone H3 and K5/12 on H4 to regulate transcription of many genes, so it can be found in various transcription complexes (Dey, 2003; Wu, 2007). Although BDI and BDII show less homology (44%) to each other, amino acid residues essential for acetyl-lysine binding in both domains are highly conserved

(Nakamura *et al.*, 2007). It has been shown that only one bromodomain of Brd4 is enough for acetylated chromatin targeting and transcriptional regulation (Wu *et al.*, 2006; Nakamura *et al.*, 2007). However, Brd4 mutants with only one bromodomain associate with chromatin more weakly than BRD4 with two bromodomains (Dey, 2003). Apart from binding acetylated chromatin, BDI and BDII also interact with other nonhistone proteins to regulate other cellular activities (Jang *et al.*, 2005; Yang *et al.*, 2005). The BDII domain of Brd4 has been shown to interact with triacetylatedcyclin T1, leading to the binding of human positive transcription elongation factor b (P-TEFb) to Brd4 (Schroder *et al.*, 2012).

In this study, a primer set designed to amplify across exon 6 to exon 8 of BRD4*,* which is the binding area of a microarray probe that showed down expression level, generates 2 band sizes. The DNA sequencing results suggest that the large band is the PCR product of Brd4 known isoform (BRD4[+7]) and the smaller band is the PCR product of Brd4 novel isoform, which the entire exon 7 (129 bp) is spliced out (BRD4[-7]). After combining the data from many sources, results indicate that exon 7 of *BRD4* corresponds to amino acid 405-447 of BRD4 protein, and it belongs to αA, αB, and αC, as well as BC loop of BDII domain. Interestingly, the missing amino acid residues in BRD4[-7] isoform includes Tyr-432, Asn-433, and Val-439 which are conserved and likely important for acetyl-lysine binding, and Gly-419, Ala-420, Tyr-430, Lys-431, Gln-447 which involved in homodimeric interactions. Taken together, these data suggesting

that BRD4[-7] isoform may have less acetyl-lysine binding ability, and it plays important role in transcriptional regulation rather than chromatin targeting. Moreover, since the BDII domain is essential for the recruitment of P-TEFb, the novel isoform of BRD4 which several parts of BDII domain are missing may target to different transcription factors.

However, BRD4[-7] isoform is expressed in both patient and control groups, indicating its important role in normal physiology. As demonstrated earlier, some alternative splicing studies emphasized the effect of protein isoforms proportion on OA pathogenesis. Thus the expression level of BRD4[+7] isoform and BRD4[-7] isoform should be quantitated in patient and control groups. Obviously, RT-PCR is not sensitive enough for this purpose, so real time PCR was performed.

Real time PCR show that BRD4[-7] isoform is expressed in small amount compared with BRD4[+7] isoform in both patient and control groups. In addition, significantly decreased expression of BRD4[-7] isoform in OA patient group was observed, concordant with the microarray result used in bioinformatic analysis. Moreover, the real-time PCR results from different tissue/cell types showed that BRD4[-7] isoform is expressed not only in OA synovial fluid but also in OA cultured synoviocyte, OA cartilage, OA cultured chondrocyte and dermal fibroblast. Since only one sample from each source was used for tissue specificity test, statistical test is not possible. Therefore, additional studies in a larger sample size or other tissue/cell types should be

carried out to determine the exact expression level of BRD4[-7] isoform in each tissue/cell type.

However, the results from this study seem to indicate that although BRD4[-7] isoform is produced in small amount when compared with BRD4[+7] isoform, it's function is very essential for normal physiology. Therefore, decreased expression of BRD4[-7] isoform may lead to abnormalities including OA.

The limitation of alternative splicing part is the small number of study population. Although normal synovial fluid is not difficult to obtained as normal cartilage, all normal synovial fluid used in this study were obtain from patients with knee injury. All are young men, with the average age of 29 years, while the patient group consisted of 3 men and 4 women, with the average age of 59 years. This leads to big differences about sex ratio and age between patient and control groups. Therefore, the effect of these differences could not be neglected.

CHAPTER VI

CONCLUSION

This genetic study on knee OA in a Thai population is divided into two parts. The first part is association study and the second part is alternative splicing study. In the first part, associations between the SNP in *ADAMTS14*, *ADAM12* and *MMP1* and knee OA were determined. *ADAMTS14* (rs4747096; A/G) SNP genotyping was carried out by PCR-RFLP technique, whereas *ADAM12* (rs3740199; C/G) and *MMP1* (rs1799750; 2G/1G) SNPs genotyping were carried out by HRM analysis. The results showed that the A allele of rs4747096 in *ADAMTS14* was associated with knee OA in female patients and individuals with the AA genotype are at higher risk of having knee OA when compared with the other genotypes. In addition, both C allele and CC genotype from rs3740199 in *ADAM12* were strongly associated with knee OA susceptibility in male. In contrast, the 2G/1G polymorphism in the *MMP1* promoter (rs1799750) was not associated with knee OA occurrence in both genders. Haplotype analysis was carried out to investigate the combinatorial effect of alleles from *ADAMTS14* and *ADAM12* SNPs. However, the results showed lack of associations between all tested haplotypes and knee OA. This may be the result from distinct effect of each SNP in each gender. Therefore, the role of *ADAMTS14* and *ADAM12* in knee OA seems to be remarkably gender-dependent.

For alternative splicing study, microarray data processing has reviewed 32 candidate genes with high possibility to have alternative splicing pattern in knee OA

patients. Two candidate genes, *HNRNPU* and *BRD4*, were subjected to RT-PCR, but only *BRD4* showed positive results. The sequencing results of two band sizes generated from RT-PCR of *BRD4* indicate the existing of BRD4 known isoform (BRD4[+7]) and BRD4 novel isoform (BRD4[-7]) in synovial fluid. The expression levels of both isoforms were quantitated by real-time PCR. The results showed that BRD4[-7] isoform is exist in small amount relative to BRD4[+7] isoform in synovial fluid. In addition, its expression is decreased in knee OA patient, indicating it possible role in knee OA pathogenesis. Moreover, tissue specificity test has revealed that this BRD4 novel isoform is also expressed in the other tissue/cell types including cultured OA synoviocyte, OA cartilage, cultured OA chondrocyte and dermal fibroblast.

Since this study has shown that the SNP in *ADAMTS14* and *ADAM12* are associated with knee OA in female and male, respectively, genotypes from these two genes could be used as a genetic marker for identifying the individuals, whose knee should be carefully used and particularly taken care because of higher risk for developing knee OA. Moreover, the challenge will be to connect the biology of these genes to the development and progression of osteoarthritis and to evaluate its role in different genders, as well as to investigate the therapeutic potential of these genes for disease prevention and treatment. A possible way to investigate the authentic role of these SNPs in knee OA susceptibility is the functional study. Gene with the variety of targeted SNP alleles should be constructed and expressed to evaluate the effect of

each allele on protein folding, enzyme activity, cellular localization and protein-protein interaction across both genders.

In addition, the function of BRD4[-7] isoform discovered from this study and the effect of its low expression level on knee OA pathogenesis is need to be further studied. Sequence of Brd4 BDII domain lacking the exon 7 should be constructed and expressed to investigate its specific properties, such as protein folding, cellular localization and expression level in different tissues. In addition, the functions of this new isoform, especially on chromatin binding and transcriptional regulation, as well as its targeted molecules need to be clarified.

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APPENDIX

APPENDIX

The 32 candidate genes from bioinformatic analysis (* Expression: Down = decreased expression in patients, Up = increased expression in patients, same = same expression between patients and controls)

Number	Gene	Probe ID	t-test	Expression*
1	*AKAP13*	208325_s_at	0.019	same
	AKAP13	209534_x_at	0.002	Down
	AKAP13	221718_s_at	0.004	Down
	AKAP13	222023_at	0.003	Down
	AKAP13	222024_s_at	0.001	Down
	AKAP13	224884_at	0.004	Down
	AKAP13	227039_at	0.107	same
	AKAP13	232188_at	0.001	Down
2	*BCLAF1*	201083_s_at	0.485	same
	BCLAF1	201084_s_at	0.374	same
	BCLAF1	201101_s_at	0.021	same
	BCLAF1	214499_s_at	0.000	Down
	BCLAF1	229454_at	0.001	Down
	BCLAF1	239897_at	0.207	same
3	*BRD4*	202102_s_at	0.006	Down
	BRD4	202103_at	0.110	same
	BRD4	226052_at	0.002	Down
	BRD4	226054_at	0.006	Down
	BRD4	239000_at	0.756	same
	BRD4	240360_at	0.844	same
4	*CALD1*	201615_x_at	0.003	Down
	CALD1	201616_s_at	0.073	same
	CALD1	201617_x_at	0.737	same

Number	Gene	Probe ID	t-test	Expression*
	CALD1	205525_at	0.011	same
	CALD1	212077_at	0.609	same
	CALD1	214880_x_at	0.726	same
	CALD1	215198_s_at	0.011	same
	CALD1	215199_at	0.012	same
	CALD1	231881_at	0.005	Down
	CALD1	235834_at	0.101	same
	CALD1	243084_at	0.012	same
5	*CD44*	204489_s_at	0.711	same
	CD44	204490_s_at	0.836	same
	CD44	209835_x_at	0.911	same
	CD44	210916_s_at	0.129	same
	CD44	212014_x_at	0.857	same
	CD44	212063_at	0.042	same
	CD44	216056_at	0.077	same
	CD44	217523_at	0.005	Down
	CD44	229221_at	0.902	same
	CD44	234411_x_at	0.001	Down
	CD44	234418_x_at	0.413	same
6	*CHD7*	218829_s_at	0.006	Down
	CHD7	220619_at	0.190	same
	CHD7	222755_s_at	0.173	same
	CHD7	226123_at	0.001	Down
7	*CHD9*	212615_at	0.247	same
	CHD9	212616_at	0.564	same
	CHD9	220586_at	0.008	Down
	CHD9	229586_at	0.000	Down
	CHD9	235388_at	0.000	Down
	CHD9	239654_at	0.003	Down

Number	Gene	Probe ID	t-test	Expression*
	CHD9	242157_at	0.144	same
	CHD9	243660_at	0.575	same
8	*CLMN*	213839_at	0.006	Down
	CLMN	221042_s_at	0.501	same
	CLMN	225757_s_at	0.001	Down
	CLMN	225759_x_at	0.821	same
9	*DLG1*	202514_at	0.348	same
	DLG1	202515_at	0.885	same
	DLG1	202516_s_at	0.350	same
	DLG1	215988_s_at	0.926	same
	DLG1	217208_s_at	0.000	Down
	DLG1	229703_at	0.668	same
	DLG1	230229_at	0.009	Down
10	*FAM162A*	220942_x_at	0.000	Up
	FAM162A	221533_at	0.864	same
	FAM162A	223193_x_at	0.007	Up
	FAM162A	224345_x_at	0.051	same
11	*FKSG49*	208120_x_at	0.000	Down
	FKSG49	211454_x_at	0.000	Down
	FKSG49	224284_x_at	0.013	same
	FKSG49	224288_x_at	0.004	Down
12	*GAPDH*	212581_x_at	0.039	same
	GAPDH	213453_x_at	0.745	same
	GAPDH	217398_x_at	0.257	same
	GAPDH	AFFX-hum_alu_at	0.000	Down
	GAPDH	AFFX-HUMGAPDH/M33197_3_at	0.297	same
	GAPDH	AFFX-HUMGAPDH/M33197_5_at	0.396	same
	GAPDH	AFFX-hum_alu_at	0.007	Down

Number	Gene	Probe ID	t-test	Expression*
	GAPDH	AFFX-HUMGAPDH/M33197_3_at	0.482	same
	GAPDH	AFFX-HUMGAPDH/M33197_5_at	0.137	same
13	*HIPK2*	213763_at	0.015	same
	HIPK2	219028_at	0.000	Down
	HIPK2	224016_at	0.001	Down
	HIPK2	224065_at	0.056	same
	HIPK2	224066_s_at	0.108	same
	HIPK2	225097_at	0.477	same
	HIPK2	225115_at	0.130	same
	HIPK2	225116_at	0.671	same
	HIPK2	225368_at	0.363	same
	HIPK2	240294_at	0.732	same
14	*HNRNPU*	200593_s_at	0.002	Down
	HNRNPU	200594_x_at	0.217	same
	HNRNPU	216855_s_at	0.037	same
	HNRNPU	225805_at	0.045	same
	HNRNPU	235603_at	0.484	same
	HNRNPU	236244_at	0.001	Down
15	*IGF1R*	203627_at	0.040	same
	IGF1R	203628_at	0.007	Down
	IGF1R	208441_at	0.019	same
	IGF1R	225330_at	0.950	same
	IGF1R	243358_at	0.001	Down
16	*MAPK8IP3*	213177_at	0.140	same
	MAPK8IP3	213178_s_at	0.006	Down
	MAPK8IP3	216137_s_at	0.229	same
	MAPK8IP3	216139_s_at	0.022	same
	MAPK8IP3	230162_s_at	0.005	Down
	MAPK8IP3	232085_at	0.612	same

Number	Gene	Probe ID	t-test	Expression*
17	*MGEA5*	200898_s_at	0.107	same
	MGEA5	200899_s_at	0.734	same
	MGEA5	214972_at	0.010	Down
	MGEA5	223494_at	0.074	same
	MGEA5	235868_at	0.001	Down
18	*MLL*	212076_at	0.003	Down
	MLL	212078_s_at	0.004	Down
	MLL	212079_s_at	0.100	same
	MLL	212080_at	0.362	same
	MLL	216624_s_at	0.596	same
	MLL	220546_at	0.046	same
	MLL	226981_at	0.003	Down
	MLL	229935_s_at	0.203	same
	MLL	244110_at	0.814	same
19	*NFIB*	209289_at	0.667	same
	NFIB	209290_s_at	0.684	same
	NFIB	211466_at	0.009	Down
	NFIB	211467_s_at	0.514	same
	NFIB	213029_at	0.640	same
	NFIB	213032_at	0.076	same
	NFIB	213033_s_at	0.551	same
	NFIB	230291_s_at	0.893	same
	NFIB	233304_at	0.000	Down
	NFIB	233394_at	0.871	same
20	*NRP2*	210841_s_at	0.000	Down
	NRP2	210842_at	0.641	same
	NRP2	211844_s_at	0.474	same
	NRP2	214632_at	0.260	same
	NRP2	223510_at	0.008	Down

Number	Gene	Probe ID	t-test	Expression*
	NRP2	225566_at	0.855	same
	NRP2	228102_at	0.071	same
	NRP2	228103_s_at	0.418	same
	NRP2	228699_at	0.024	same
	NRP2	229225_at	0.096	same
	NRP2	230410_at	0.602	same
21	*OSBPL10*	216755_at	0.001	Down
	OSBPL10	217017_at	0.706	same
	OSBPL10	219073_s_at	0.930	same
	OSBPL10	222818_at	0.524	same
	OSBPL10	231656_x_at	0.004	Down
22	*PHF20L1*	219606_at	0.193	same
	PHF20L1	222133_s_at	0.000	Down
	PHF20L1	226942_at	0.237	same
	PHF20L1	227523_s_at	0.008	Down
	PHF20L1	230098_at	0.499	same
	PHF20L1	231967_at	0.647	same
23	*QKI*	212262_at	0.707	same
	QKI	212263_at	0.255	same
	QKI	212265_at	0.004	Down
	QKI	212636_at	0.109	same
	QKI	214541_s_at	0.970	same
	QKI	214543_x_at	0.750	same
	QKI	228540_at	0.539	same
	QKI	236154_at	0.815	same
	QKI	241938_at	0.010	Down
24	*SFRS15*	222310_at	0.012	same
	SFRS15	222311_s_at	0.000	Down

Number	Gene	Probe ID	t-test	Expression*
	SFRS15	226082_s_at	0.464	same
	SFRS15	233753_at	0.486	same
	SFRS15	243759_at	0.002	Down
25	SFRS18	212176_at	0.004	Down
	SFRS18	212177_at	0.000	Down
	SFRS18	212179_at	0.539	same
	SFRS18	225507_at	0.000	Down
	SFRS18	226412_at	0.251	same
	SFRS18	230375_at	0.012	same
26	SGCD	210329_s_at	0.004	Up
	SGCD	210330_at	0.135	same
	SGCD	213543_at	0.157	same
	SGCD	214492_at	0.072	same
	SGCD	228602_at	0.006	Down
	SGCD	230730_at	0.161	same
27	SPG7	202104_s_at	0.002	Down
	SPG7	214494_s_at	0.010	same
	SPG7	230884_s_at	0.000	Down
	SPG7	230885_at	0.113	same
	SPG7	235325_at	0.543	same
	SPG7	238286_at	0.075	same
28	SUB1	212857_x_at	0.420	same
	SUB1	214512_s_at	0.007	Up
	SUB1	221727_at	0.133	same
	SUB1	224586_x_at	0.056	same
	SUB1	224587_at	0.001	Up
	SUB1	237784_at	0.207	same

Number	Gene	Probe ID	t-test	Expression*
29	*TMCO3*	220240_s_at	0.000	Up
	TMCO3	220241_at	0.012	same
	TMCO3	226050_at	0.010	Up
	TMCO3	230317_x_at	0.479	same
30	*UBTF*	202692_s_at	0.015	same
	UBTF	214881_s_at	0.006	Down
	UBTF	225982_at	0.037	same
	UBTF	239920_at	0.005	Down
31	*USP34*	207365_x_at	0.000	Down
	USP34	212065_s_at	0.994	same
	USP34	212066_s_at	0.018	same
	USP34	212980_at	0.020	same
	USP34	215013_s_at	0.012	same
	USP34	233595_at	0.002	Down
	USP34	242647_at	0.001	Down
32	*USP48*	220078_at	0.003	Down
	USP48	220079_s_at	0.496	same
	USP48	225925_s_at	0.587	same
	USP48	229812_at	0.654	same
	USP48	232621_at	0.002	Down
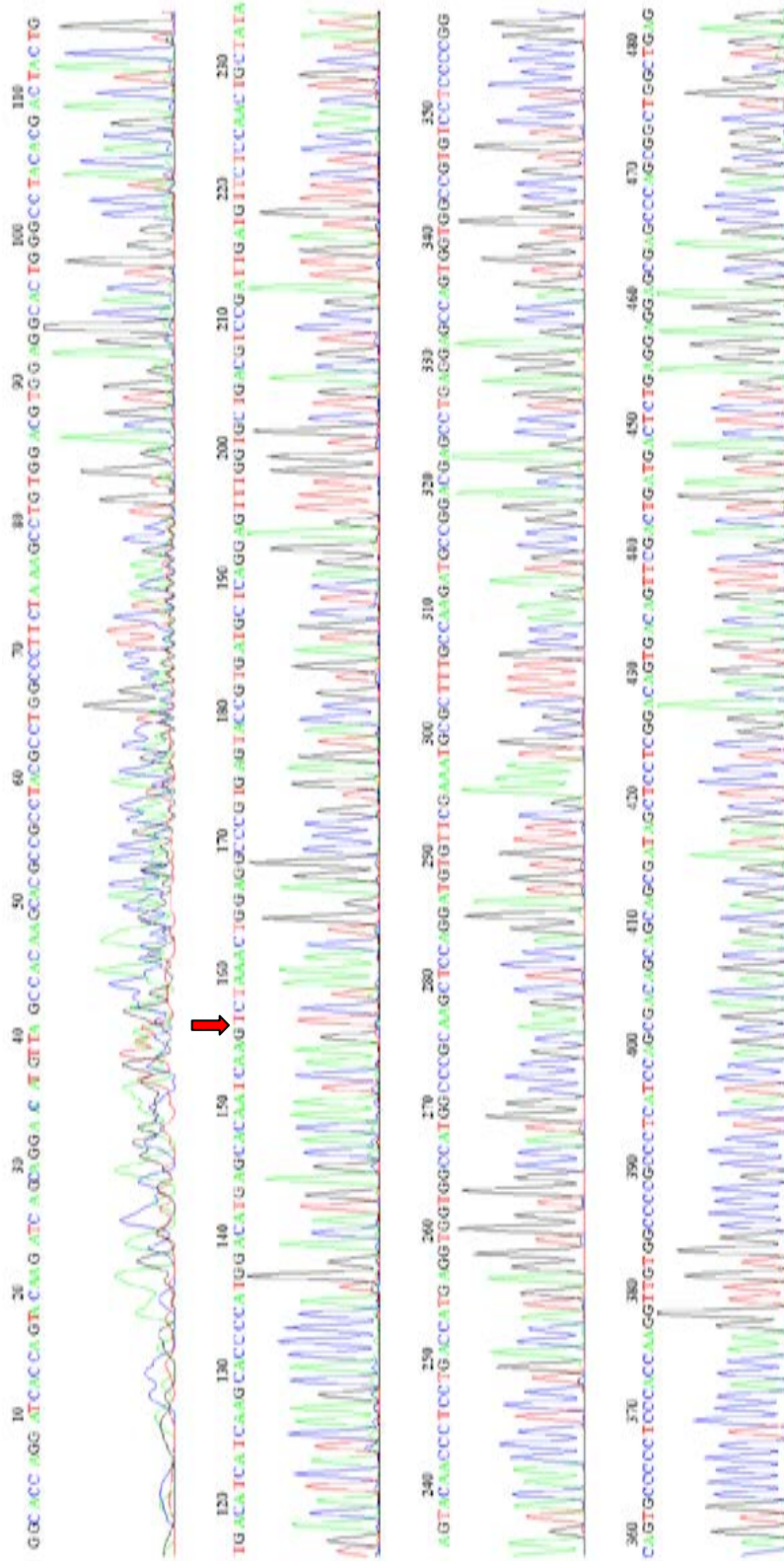
Sequencing result of BRD4[+7] isoform (arrow indicates alternative splice site)
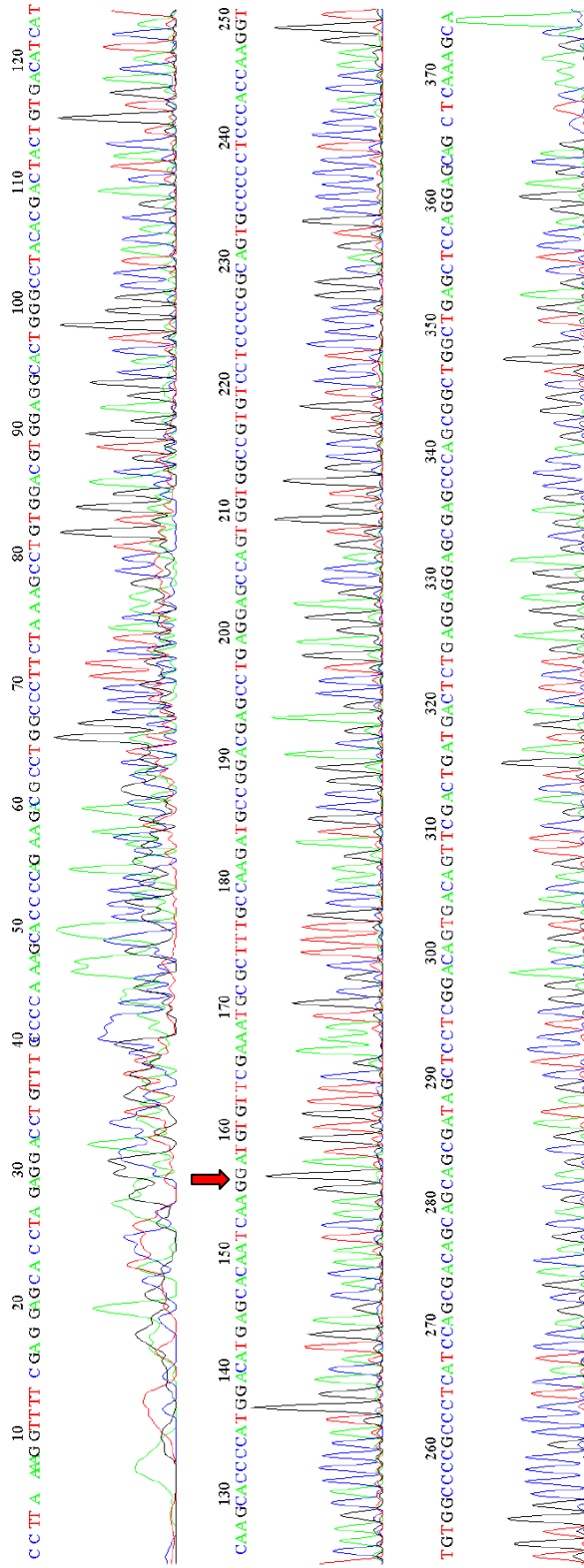
Sequencing result of BRD4[-7] isoform (arrow indicates alternative splice site)

Real-time PCR results of the control group

Well	Fluor	Content	Target	Sample	Threshold Cycle (C_T)	Melt Temperature
A02	SYBR	Neg Ctrl	GAPDH		N/A	N/A
A03	SYBR	Neg Ctrl	GAPDH		N/A	N/A
A04	SYBR	Neg Ctrl	GAPDH		N/A	N/A
A05	SYBR	Neg Ctrl	large		N/A	N/A
A06	SYBR	Neg Ctrl	Large		N/A	N/A
A07	SYBR	Neg Ctrl	Large		N/A	N/A
A08	SYBR	Neg Ctrl	Small		N/A	N/A
A09	SYBR	Neg Ctrl	Small		N/A	N/A
A10	SYBR	Neg Ctrl	Small		N/A	N/A
D02	SYBR	Unkn	GAPDH	C1	20.65	82.50
D03	SYBR	Unkn	GAPDH	C1	20.51	82.50
D04	SYBR	Unkn	GAPDH	C1	20.44	82.50
D05	SYBR	Unkn	Large	C1	25.08	84.50
D06	SYBR	Unkn	Large	C1	25.22	84.50
D07	SYBR	Unkn	Large	C1	25.11	84.50
D08	SYBR	Unkn	Small	C1	27.80	84.00
D09	SYBR	Unkn	Small	C1	27.94	84.00
D10	SYBR	Unkn	Small	C1	27.74	84.00
E02	SYBR	Unkn	GAPDH	C2	22.01	82.50
E03	SYBR	Unkn	GAPDH	C2	21.97	82.50
E04	SYBR	Unkn	GAPDH	C2	22.07	82.50
E05	SYBR	Unkn	Large	C2	27.26	84.50
E06	SYBR	Unkn	Large	C2	27.36	84.50
E07	SYBR	Unkn	Large	C2	27.30	84.50
E08	SYBR	Unkn	Small	C2	28.82	84.00
E09	SYBR	Unkn	Small	C2	28.70	83.50
E10	SYBR	Unkn	Small	C2	28.74	84.00

Well	Fluor	Content	Target	Sample	Threshold Cycle (C_T)	Melt Temperature
G02	SYBR	Unkn	GAPDH	C3	24.43	82.50
G03	SYBR	Unkn	GAPDH	C3	24.43	82.50
G04	SYBR	Unkn	GAPDH	C3	24.43	82.50
G05	SYBR	Unkn	Large	C3	29.70	84.00
G06	SYBR	Unkn	Large	C3	30.03	84.00
G07	SYBR	Unkn	Large	C3	29.50	84.50
G08	SYBR	Unkn	Small	C3	30.79	83.00
G09	SYBR	Unkn	Small	C3	30.94	83.50
G10	SYBR	Unkn	Small	C3	31.02	83.50
E02	SYBR	Unkn	GAPDH	C4	22.75	82.50
E03	SYBR	Unkn	GAPDH	C4	22.65	82.50
E04	SYBR	Unkn	GAPDH	C4	22.67	82.50
E05	SYBR	Unkn	Large	C4	27.43	84.00
E06	SYBR	Unkn	Large	C4	27.52	84.00
E07	SYBR	Unkn	Large	C4	27.18	84.00
E08	SYBR	Unkn	Small	C4	31.01	83.00
E09	SYBR	Unkn	Small	C4	30.74	83.00
E10	SYBR	Unkn	Small	C4	31.37	83.50
H02	SYBR	Unkn	GAPDH	C5	24.11	82.50
H03	SYBR	Unkn	GAPDH	C5	23.66	82.50
H04	SYBR	Unkn	GAPDH	C5	23.75	82.50
H05	SYBR	Unkn	Large	C5	28.06	84.00
H06	SYBR	Unkn	Large	C5	28.44	83.50
H07	SYBR	Unkn	Large	C5	27.68	83.50
H08	SYBR	Unkn	Small	C5	30.72	83.00
H09	SYBR	Unkn	Small	C5	30.77	83.50
H10	SYBR	Unkn	Small	C5	31.63	83.50

Real-time PCR results of the patient group

Well	Fluor	Content	Target	Sample	Threshold Cycle (C$_T$)	Melt Temperature
A02	SYBR	Neg Ctrl	GAPDH		N/A	N/A
A03	SYBR	Neg Ctrl	GAPDH		N/A	N/A
A04	SYBR	Neg Ctrl	GAPDH		N/A	N/A
A05	SYBR	Neg Ctrl	Large		N/A	N/A
A06	SYBR	Neg Ctrl	Large		N/A	N/A
A07	SYBR	Neg Ctrl	Large		N/A	N/A
A08	SYBR	Neg Ctrl	Small		N/A	N/A
A09	SYBR	Neg Ctrl	Small		N/A	N/A
A10	SYBR	Neg Ctrl	Small		N/A	N/A
C02	SYBR	Unkn	GAPDH	P1	21.90	82.50
C03	SYBR	Unkn	GAPDH	P1	22.11	82.00
C04	SYBR	Unkn	GAPDH	P1	21.60	82.50
C05	SYBR	Unkn	Large	P1	26.77	84.00
C06	SYBR	Unkn	Large	P1	26.67	84.00
C07	SYBR	Unkn	Large	P1	26.89	84.00
C08	SYBR	Unkn	Small	P1	33.71	83.00
C09	SYBR	Unkn	Small	P1	30.65	83.00
C10	SYBR	Unkn	Small	P1	31.03	83.00
G02	SYBR	Unkn	GAPDH	P2	24.22	82.00
G03	SYBR	Unkn	GAPDH	P2	24.08	82.00
G04	SYBR	Unkn	GAPDH	P2	24.52	82.00
G05	SYBR	Unkn	Large	P2	29.80	84.00
G06	SYBR	Unkn	Large	P2	31.17	84.00
G07	SYBR	Unkn	Large	P2	29.70	84.00
G08	SYBR	Unkn	Small	P2	32.49	84.00
G09	SYBR	Unkn	Small	P2	32.30	84.00

Well	Fluor	Content	Target	Sample	Threshold Cycle (C_T)	Melt Temperature
G10	SYBR	Unkn	Small	P2	32.08	84.00
D02	SYBR	Unkn	GAPDH	P3	22.62	82.50
D03	SYBR	Unkn	GAPDH	P3	22.61	82.50
D04	SYBR	Unkn	GAPDH	P3	22.64	82.50
D05	SYBR	Unkn	Large	P3	27.08	84.00
D06	SYBR	Unkn	Large	P3	27.01	84.00
D07	SYBR	Unkn	Large	P3	27.24	84.00
D08	SYBR	Unkn	Small	P3	31.66	83.00
D09	SYBR	Unkn	Small	P3	31.36	83.00
D10	SYBR	Unkn	Small	P3	31.97	83.00
F02	SYBR	Unkn	GAPDH	P4	24.47	82.00
F03	SYBR	Unkn	GAPDH	P4	24.32	82.00
F04	SYBR	Unkn	GAPDH	P4	24.24	82.00
F05	SYBR	Unkn	Large	P4	30.02	84.00
F06	SYBR	Unkn	Large	P4	30.09	84.00
F07	SYBR	Unkn	Large	P4	29.94	84.00
F08	SYBR	Unkn	Small	P4	32.72	84.00
F09	SYBR	Unkn	Small	P4	32.41	84.00
F10	SYBR	Unkn	Small	P4	32.94	84.00
D02	SYBR	Unkn	GAPDH	P5	21.58	82.00
D03	SYBR	Unkn	GAPDH	P5	21.34	82.00
D04	SYBR	Unkn	GAPDH	P5	21.46	82.00
D05	SYBR	Unkn	Large	P5	27.27	84.00
D06	SYBR	Unkn	Large	P5	27.24	84.00
D07	SYBR	Unkn	Large	P5	27.28	84.00
D08	SYBR	Unkn	Small	P5	29.77	84.00
D09	SYBR	Unkn	Small	P5	30.08	84.00

Well	Fluor	Content	Target	Sample	Threshold Cycle (C$_T$)	Melt Temperature
D10	SYBR	Unkn	Small	P5	29.96	84.00
C02	SYBR	Unkn	GAPDH	P6	21.81	82.00
C03	SYBR	Unkn	GAPDH	P6	21.92	82.00
C04	SYBR	Unkn	GAPDH	P6	21.80	82.00
C05	SYBR	Unkn	Large	P6	28.05	84.00
C06	SYBR	Unkn	Large	P6	27.78	84.00
C07	SYBR	Unkn	Large	P6	28.26	84.00
C08	SYBR	Unkn	Small	P6	30.51	84.00
C09	SYBR	Unkn	Small	P6	30.16	84.00
C10	SYBR	Unkn	Small	P6	30.03	84.00
B02	SYBR	Unkn	GAPDH	P7	23.31	82.00
B03	SYBR	Unkn	GAPDH	P7	23.17	82.00
B04	SYBR	Unkn	GAPDH	P7	23.05	82.00
B05	SYBR	Unkn	Large	P7	29.78	84.00
B06	SYBR	Unkn	Large	P7	29.31	84.00
B07	SYBR	Unkn	Large	P7	29.70	84.00
B08	SYBR	Unkn	Small	P7	31.86	84.00
B09	SYBR	Unkn	Small	P7	31.19	84.00
B10	SYBR	Unkn	Small	P7	31.49	84.00

Real-time PCR results of the different cDNA resources (SF=OA synovial fluid, SC= cultured OA synoviocyte, CAR=OA cartilage, CHON=cultured OA chondrocyte, SF= skin fibroblast)

Well	Fluor	Content	Target	Sample	Threshold Cycle (C$_T$)	Melt Temperature
A02	SYBR	Neg Ctrl	GAPDH		N/A	N/A
A03	SYBR	Neg Ctrl	GAPDH		N/A	N/A

Well	Fluor	Content	Target	Sample	Threshold Cycle (C_T)	Melt Temperature
A04	SYBR	Neg Ctrl	GAPDH		N/A	N/A
A05	SYBR	Neg Ctrl	Large		N/A	N/A
A06	SYBR	Neg Ctrl	Large		N/A	N/A
A07	SYBR	Neg Ctrl	Large		N/A	N/A
A08	SYBR	Neg Ctrl	Small		N/A	N/A
A09	SYBR	Neg Ctrl	Small		N/A	N/A
A10	SYBR	Neg Ctrl	Small		N/A	N/A
H02	SYBR	Unkn	GAPDH	SF	26.52	82.50
H03	SYBR	Unkn	GAPDH	SF	26.53	82.50
H04	SYBR	Unkn	GAPDH	SF	26.48	82.50
H05	SYBR	Unkn	Large	SF	28.49	84.00
H06	SYBR	Unkn	Large	SF	28.28	84.00
H07	SYBR	Unkn	Large	SF	28.81	84.00
H08	SYBR	Unkn	Small	SF	31.35	83.50
H09	SYBR	Unkn	Small	SF	31.96	84.00
H10	SYBR	Unkn	Small	SF	31.22	84.00
G02	SYBR	Unkn	GAPDH	SC	21.53	82.50
G03	SYBR	Unkn	GAPDH	SC	21.36	82.50
G04	SYBR	Unkn	GAPDH	SC	21.18	82.50
G05	SYBR	Unkn	Large	SC	26.95	84.50
G06	SYBR	Unkn	Large	SC	27.31	84.50
G07	SYBR	Unkn	Large	SC	27.56	84.50
G08	SYBR	Unkn	Small	SC	29.90	84.00
G09	SYBR	Unkn	Small	SC	29.70	84.00
G10	SYBR	Unkn	Small	SC	28.98	84.00
C02	SYBR	Unkn	GAPDH	CAR	24.45	82.50
C03	SYBR	Unkn	GAPDH	CAR	24.38	82.50

Well	Fluor	Content	Target	Sample	Threshold Cycle (C_T)	Melt Temperature
C04	SYBR	Unkn	GAPDH	CAR	24.72	82.50
C05	SYBR	Unkn	Large	CAR	30.77	84.50
C06	SYBR	Unkn	Large	CAR	30.83	84.50
C07	SYBR	Unkn	Large	CAR	30.92	84.00
C08	SYBR	Unkn	Small	CAR	31.73	83.50
C09	SYBR	Unkn	Small	CAR	31.86	83.50
C10	SYBR	Unkn	Small	CAR	31.79	84.00
E02	SYBR	Unkn	GAPDH	CHON	23.29	82.50
E03	SYBR	Unkn	GAPDH	CHON	23.14	82.50
E04	SYBR	Unkn	GAPDH	CHON	23.12	82.50
E05	SYBR	Unkn	Large	CHON	28.50	84.50
E06	SYBR	Unkn	Large	CHON	28.37	84.50
E07	SYBR	Unkn	Large	CHON	28.34	84.50
E08	SYBR	Unkn	Small	CHON	30.43	84.50
E09	SYBR	Unkn	Small	CHON	30.83	84.50
E10	SYBR	Unkn	Small	CHON	30.67	84.50
D02	SYBR	Unkn	GAPDH	FB	19.14	82.50
D03	SYBR	Unkn	GAPDH	FB	19.06	82.50
D04	SYBR	Unkn	GAPDH	FB	19.02	82.50
D05	SYBR	Unkn	Large	FB	24.23	84.50
D06	SYBR	Unkn	Large	FB	24.00	84.50
D07	SYBR	Unkn	Large	FB	24.17	84.50
D08	SYBR	Unkn	Small	FB	26.75	84.50
D09	SYBR	Unkn	Small	FB	26.92	84.50
D10	SYBR	Unkn	Small	FB	26.89	84.50

BIOGRAPHY

Miss Thitiya Poonpet was born on the 24th of August 1983 in Phetchaburi, Thailand. She is a daughter of Mr. Aumpon and Mrs. Akaraya Poonpet with two brothers and one sister. She graduated her Bachelor's Degree of Science in Genetics with the 2nd class honor from Chulalongkorn University in 2006. After worked as a biology teacher at Benjamatheputit Phetchaburi School for two years, she returned back to the student life. She was awarded Chulalongkorn University Graduate Scholarship to Commemorate the 72nd Anniversary of His Majesty King Bhumibol Adulyadej to study in Biological Science Program since 2008.

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