+45T/G AND +276G/T SINGLE NUCLEOTIDE POLYMORPHISM OF ADIPONECTIN GENE IN KNEE OSTEOARTHRITIS

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ความหลากหลายทางพันธุกรรมของยีนอะดิโพเนกติน (adiponectin) ที่ตำแหน่ง+45T/G และ +276G/T ในผู้ป่วยโรคข้อเข่าเสื่อม

นายตง จัน

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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ตง จัน : ความหลากหลายทางพันธุกรรมของยีนอะดิโพ เนกติน (adiponectin) ที่ตำแหน่ง+45T/G และ +276G/T ในผู้ป่วยโรคข้อเข่าเสื่อม. (+45T/G AND +276G/T SINGLE NUCLEOTIDE POLYMORPHISM OF ADIPONECTIN GENE IN KNEE OSTEOARTHRITIS) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ศ. สิทธิศักดิ์ หรรษาเวก, 85หน้า.

เป้าหมาย:วัตถุประสงค์ของการศึกษานี้คือเพื่อศึกษาความถี่ของอัลลีลและการกระจายตัวของจีโนไทป์ของความหลากหลายทางพันธุกรรมของยีนอะดิโพเนกตินที่ตำแหน่ง +45T/G(rs2241766) และ +276G/T(rs1501299) ในยีน AdipoQ ปริมาณอะดิโพเนกตินในพลาสมาและความสัมพันธ์ในผู้ป่วยโรคข้อเข่าเสื่อมชาวไทย วิธีทำ: ตัวอย่างพลาสมาได้จากผู้ป่วย 105 คน และคนปกติ 94 คน ตัวอย่างดีเอ็นเอสกัดจากเลือดของผู้ป่วย 202 คน และคนปกติ 196 คน ศึกษาจีโนไทป์ของ +45T/G และ +276G/T โดยใช้เทคนิค polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) และศึกษาปริมาณอะดิโพเนกตินโดยใช้เทคนิค enzyme-linked immunosorbent assay (ELISA) ผลการทดลอง: ไม่พบความแตกต่างอย่างมีนัยสำคัญทางสถิติในแง่ของการกระจายตัวของจีโนไทป์และความถี่ของอัลลีลของ +45T/G และ +276G/T ระหว่างสองกลุ่ม (P>0.05) สำหรับ +276G/T พบความแตกต่างอย่างมีนัยสำคัญทางสถิติระหว่างผู้ป่วยที่มีระดับความรุนแรงในกลุ่ม Kellgren-Lawrence (KL) เกรด 2 และ 3 (P=0.046) และระหว่าง เกรด 2 และ 4 (P=0.037) ในกลุ่มที่มีค่า BMI น้อยกว่า 25 kg/m2 พบความแตกต่างอย่างมีนัยสำคัญใน GG จีโนไทป์ของ +45T/G (P=0.023) ค่าเฉลี่ยของปริมาณอะดิโพเนกตินในพลาสมาของกลุ่มผู้ป่วยโรคข้อเข่าเสื่อมน้อยกว่าในกลุ่มคนปกติ (2.58±0.61,2.78±0.68 μg/ml, P=0.033) ปริมาณอะดิโพเนกตินในพลาสมาของผู้หญิงสูงกว่าผู้ชายทั้งกลุ่มควบคุม กลุ่มผู้ป่วย และกลุ่มที่รวมประชากรทั้งหมดในการศึกษา (P<0.05) นอกจากนี้ปริมาณอะดิโพเนกตินในพลาสมาของผู้หญิงปกติสูงกว่าผู้หญิงที่เป็นโรคข้อเข่าเสื่อมอย่างมีนัยสำคัญทางสถิติ (P<0.001) ปริมาณอะดิโพเนกตินในพลาสมาของ GG จีโนไทป์สูงกว่าของ TT จีโนไทป์ทั้งใน +45T/G และ +276G/T ในกลุ่มคนปกติ (P=0.019 และ P=0.046 ตามลำดับ) สำหรับ GG จีโนไทป์ของทั้งสองตำแหน่งปริมาณอะดิโพเนกตินในพลาสมาของกลุ่มคนปกติสูงกว่ากลุ่มผู้ป่วยอย่างมีนัยสำคัญทางสถิติ (P=0.029 และ P=0.012 ตามลำดับ) ผู้ป่วยโรคข้อเข่าเสื่อมที่มี GG จีโนไทป์ที่ตำแหน่ง +276 G/T มีแนวโน้มที่จะมีความรุนแรงของโรคข้อเข่าเสื่อมมากกว่า GT และ TT จีโนไทป์ สรุป: ความหลากหลายทางพันธุกรรมของยีนอะดิโพเนกตินที่ตำแหน่ง +45T/G และ +276G/T ไม่มีความสัมพันธ์กับความเสี่ยงต่อการเกิดโรคข้อเข่าเสื่อมในชาวไทย แต่อย่างไรก็ตาม GG จีโนไทป์ของ +45T/G และ +276G/T สัมพันธ์กับปริมาณอะดิโพเนกตินในพลาสมาของกลุ่มคนปกติและกลุ่มผู้ป่วย อะดิโพเนกตินในปริมาณสูงอาจมีส่วนช่วยป้องกันการเกิดโรคข้อเข่าเสื่อมโดยเฉพาะอย่างยิ่งในผู้หญิง

สาขาวิชา วิทยาศาสตร์การแพทย์ ลายมือชื่อนิสิต .....

ปีการศึกษา 2556 ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก .....

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DONG ZHAN: +45T/G AND +276G/T SINGLE NUCLEOTIDE POLYMORPHISM OF ADIPONECTIN GENE IN KNEE OSTEOARTHRITIS. ADVISOR: PROF. SITTISAK HONSAWEK, 85pp.

Purpose: The objective of this study was to investigate the allele frequencies and genotype distributions of +45T/G (rs2241766) and +276 G/T (rs1501299) polymorphisms in AdipoQ gene, plasma adiponectin levels, and their associations in Thai knee osteoarthritis (OA) patients. Methods: Plasma samples were collected from 105 patients and 94 controls. Genomic DNA was isolated from peripheral blood of 202 patients and 196 controls. The polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) was used to detect genotypes of +45T/G and +276G/T. The enzyme-linked immunosorbent assay (ELISA) was used to measure plasma adiponectin levels. Results: No statistically significant differences were identified between the two groups with respect to genotype distributions and allele frequencies of +45T/G and +276G/T ($P>0.05$). For +276G/T, significant differences were identified between Kellgren-Lawrence (KL) grade 2 and grade 3 ($P=0.046$), and between KL grade 2 and grade 4 ($P=0.037$) patients in the BMI<25 kg/m2 subgroup, a significant difference was found in the GG genotype of +45T/G ($P=0.023$). The mean plasma adiponectin level of the OA group was lower than that of the control group (2.58±0.61 v.s. 2.78±0.68 μg/ml, $P=0.033$). Female plasma adiponectin levels were higher than male levels in the control, OA and total groups ($P<0.05$). Additionally, plasma adiponectin levels of female controls were statistically higher than females in the OA group ($P<0.001$). Plasma adiponectin levels of the GG genotype were statistically higher than those of TT genotype at both the +45T/G and +276G/T loci in the control group ($P=0.019$, $P=0.046$, respectively). For GG genotypes of the two loci, plasma adiponectin levels of the control group were significantly higher than those of the OA patient group ($P=0.029$, $P=0.012$, respectively). OA patients with the GG genotype at the +276 G/T locus tended to have a higher severity of OA when compared with GT and TT genotypes. Conclusion: The +45T/G and +276G/T polymorphisms were not associated with the susceptibility of knee OA in a Thai population. However, GG genotypes of +45T/G and +276G/T polymorphisms were associated with plasma adiponectin levels in control and OA groups. High plasma adiponectin levels may play a protective role in the pathogenesis of knee OA, especially in Thai women.

Field of Study: Medical Science Student's Signature .....

Academic Year: 2013 Advisor's Signature .....

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LIST OF ABBREVIATIONS

Abbreviation	Full Name
µg	microgram
µl	microliter
ADIPOQ	adiponectin gene
AKT	serin threonine kinase
AMPK	adenosine monophosphate-activated protein kinase
ANOVA	analysis of variance
APN	adiponectin
BMI	body mass index
COL10A1	collagen-type X-alpha 1 chain
CRP	C-reactive protein
ELISA	enzyme-linked immunosorbent assay
EPAS-1	endothelial PAS domain-containing protein 1
ERK	extracellular regulated kinase
HIF-2α	hypoxia inducible factor 2 alpha
IL	interleukin
JNK	Jun N-terminal kinase
kg	kilogram

Abbreviation	Full Name
KL grading system	Kellgren-Lawrence grading system
M	molar
m^2	meter square
MAPK	mitogen activated protein kinase
MEK	mitogen-activated protein kinase kinase
MetS	metabolic syndrome
mg	milligram
ml	milliliter
mM	millimolar
MMP	matrix metalloproteinase
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
nm	nanometer
NSAID	non-steroid anti-inflammatory drug
OA	osteoarthritis
^oC	degree celsius
OD	optical density
OPG	osteoprotegerin
PCR	polymerase chain reaction

Abbreviation	Full Name
pH	potential of hydrogen
PI-3K	phosphoinositide-3 kinase
PKB	protein kinase B
RA	rheumatoid arthritis
RFLP	restriction fragment length polymorphism
ROS	reactive oxygen species
rpm	revolutions per minute
RUNX	Runt-related transcription factor
SNP	single nucleotide polymorphism
T2DM	diabetes mellitus type 2
TIMP	tissue inhibitor of metalloproteinase
TNF	tumor necrosis factor
U	unit
UV	ultraviolet

CHAPTER I
INTRODUCTION

Background and Significance of the Problem

Osteoarthritis (OA) known as a progressive degenerative joint disease has been recognized as a major worldwide health problem. Clinical features of OA include joint pain, stiffness, swelling and crepitus and they are most commonly found in older female populations. Various, complex and multiple factors involve in pathogenesis of OA, such as heredity, development, joint injury and aging, which may initiate degeneration processes of articular cartilage. Lacking or losing protection of the articular cartilage, the subchondral bone eventually is exposed in intra-articular areas. It decreases movement of pair bone in capsular joints and causes symptoms and signs. After drug therapies and rehabilitative exercise, the pain will be reduced and the symptoms will be relieved in OA patients, but the disease cannot be cured completely. Most severe patients eventually undergo joint replacement surgery to improve quality of life.

Beyond the traditional pathological features of OA, it is recognized as an inflammatory disease that can influence many periarticular tissues such as bone, muscles, ligaments and synovium. Inflammatory reaction exactly exerted on the occurrence and development of OA and pro-inflammatory cytokines were considered as potential mediators in this disease [1]. Meanwhile, obesity is considered as a strong risk factor for knee OA [2, 3]. Symptoms and signs of OA and the diagnosis of OA may appear in weight-bearing joints, such as knee joints or non-weight-bearing joints, such as finger joints. Therefore, systemic metabolic factors make a contribution to the high prevalence of OA subjects. In summary, osteoarthritis is not only an inflammatory disease but also a metabolic disease.

In addition to its traditional function of energy storage, adipose tissue is a metabolic and endocrine organ with significance, complication and high activity. Hormones secreted from adipose tissue are named after adipokines that associate with metabolic processes and inflammatory reactions, as well as possessing cytokine-like functions including anti- and pro-inflammatory effects [4]. Adiponectin, one

essential member of the adipokines, is synthesized in differentiated adipocytes and maintains high levels in blood circulation. Besides existing in the bloodstream, adiponectin also presents both in OA synovial fluid and articular chondrocytes [5, 6]. In the joints, the infrapatellar fat pad (IFP) could be a major contributor to adipokine presence [7]. Adiponectin can up-regulate tissue inhibitor of metalloproteinases-2 (TIMP-2) and down-regulates IL-1β-induced MMP-13, protecting cartilage from degeneration. Furthermore, it has been shown that serum levels of adiponectin were elevated in female patients with erosive OA [8]. Several clinical observations observed adiponectin levels were significantly negatively correlated with OA severity in humans in both plasma and synovial fluid [9, 10]. Experimental studies demonstrated that adiponectin expression was negatively regulated by pro-inflammatory cytokines including important pro-inflammatory adipokines IL-6 and TNF-α [11-13]. Adiponectin modulates the function and phenotypes of macrophages that play a central role in inflammation. Adiponectin increases tissue inhibitor of metaroproteinase-1 through expression of IL-10 in human monocyte derived macrophages. In vitro, adiponectin also attenuates LPS-induced up-regulation of TNF-α in cultured macrophages, which is associated with decreased NF-κB activation [14, 15].

As an essential component of the etiology of OA, candidate genes encoding proteins linked to the metabolism of the articular cartilage and inflammation of the synovial membrane have been proved with pathogenesis of OA. A number of gene polymorphisms involved in osteoarthritis have been identified, such as those localized in or adjacent to the encoding sequences for estrogen receptor α [16], interleukin-6 [17] and matrix metalloproteinase-3 [18]. However, adiponectin genetic polymorphisms in OA patients have not been previously demonstrated. There are many genetic variations of the human adiponectin gene reported, including several non-synonymous mutations. The human adiponectin gene is located on chromosome 3q27. It contains three exons within a region of 17kb. The exons 1 and 2 are 76 and 222bp, respectively, sandwiching the large intron 1 of 10.3kb. The exon 3 is approximately 4.28kb. The translation starts at exon 2 and ends at exon 3, leaving exon 1 and part of exon 3 untranslated. Two common single nucleotide

polymorphisms (SNPs) of adiponectin gene: +45T/G (rs2241766) is in exon 2 and +276G/T (rs1501299) is in intron 2. They have been demonstrated to be associated with diabetes, insulin resistance and atherosclerosis [19, 20], and G allele at the +276G/T (rs1501299) locus associated with lower serum adiponectin levels has been documented [21]. Thus the objective of this study was to investigate whether adiponectin gene SNPs, +45T/G (rs2241766) and +276G/T (rs1501299) are associated with osteoarthritis susceptibility.

Objectives

1. To investigate allele frequency and genotype distribution at +45T/G and +276G/T loci in 202 Thai knee osteoarthritis patients and 196 healthy controls as well as among BMI and OA severity subgroups.

2. To investigate adiponectin levels in plasma in Thai knee OA patients and healthy controls as well as gender and OA severity subgroups.

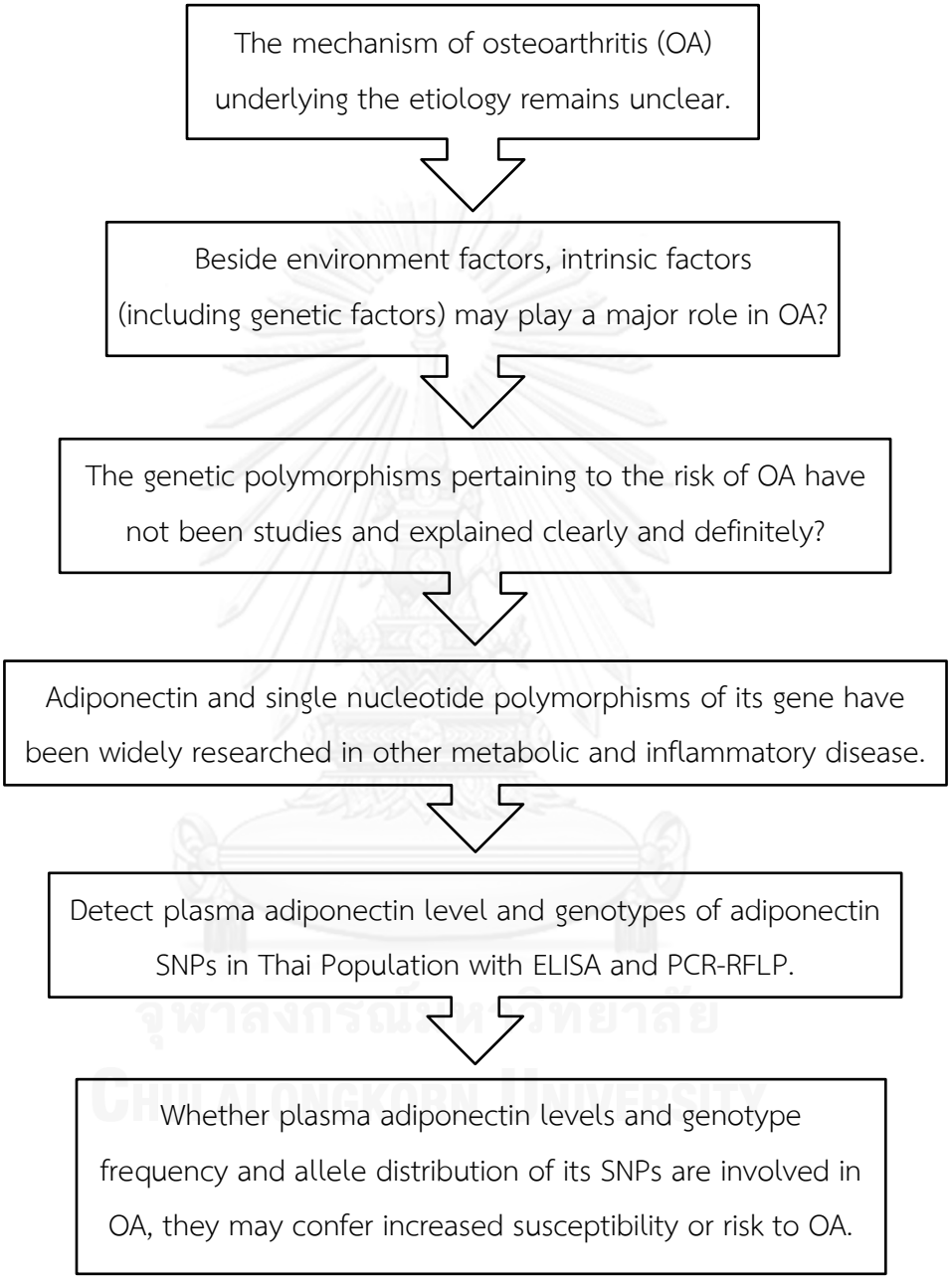
3. To analyze the relationship between plasma adiponectin levels and two SNPs sites in OA group and control group.

Research Hypothesis

1. Adiponectin SNPs, +45T/G (rs2241766) or 276G/T (rs1501299), are associated with knee osteoarthritis.

2. Adiponectin levels in plasma are associated with knee OA and SNPs of adiponectin gene at +45T/G (rs2241766) and/or 276G/T (rs1501299) polymorphisms.

Conceptual Framework



Methodology

1. Case-control Study: A case-control study that is related with the identification of individuals with and without a particular disease is widely used in analysis of genetic and usually retrospective.

2. PCR-RFLP (Polymerase Chain Reaction-Restriction Fragment Length Polymorphism): also named as cleaved amplified polymorphic sequence (CAPS) which is a popular genetic analysis technique. After using PCR to amplify a specific sequence from individual DNA samples, PCR products are broken (digested) into pieces by restriction enzymes. The resulting restriction fragments are separated according to their lengths by gel electrophoresis.

3. ELISA (Enzyme-Linked Immunosorbent Assay): It is a useful test to identify a substance according to antigen-antibody binding and a color change.

CHAPTER II
LITERATURE REVIEW

Concept and Theory

Osteoarthritis (OA) is a disease of joint failure in which pathological changes are observed in all structures of the joint uniformly. Articular cartilage loss in a different and focal manner is the pathological condition of OA with accompanying changes of subchondral bony plate, osteophytes, synovitis, and muscles weakness. Meniscal degeneration can occur in knee OA.

Relevant Research

Osteoarthritis

OA is one of the earliest diseases described diseases by doctors and scientists who have been fighting with this disease for more than 200 years. In 1782, Heberden was the first person to report this disease in his notice of "Digitorum Nodi". After that, Heberden changed the previous name to "Heberden Node" and distinguished it from gout in 1973 [22]. Until 1859, the name rheumatoid arthritis was proposed by Alfred Baring Garrod who made the differences between these two diseases [23]. In 1987, OA was defined as "a heterogeneous group of conditions that lead to joint symptoms and signs, which are associated with defective integrity of articular cartilage, in addition to related changes in the underlying bone at the joint margins" by the American College of Rheumatology (ACR) [24]. Either weight-bearing joints, such as knee joints, hip joints and lumbar spine or non-weight-bearing joints, such as finger joints and toe joints, could be affected by OA through various influences of heavier physical labor, trauma, aging, degeneration and inflammation [25, 26].

Osteoarthritis is the most prevalent form of arthritis and a major cause of pain and disability that affects millions of individuals globally. In 2008, approximately 46 million Americans were affected by OA and this number predicted to increase to 60 million by 2028 [27]. In Asia, where most nations are low-income developing countries, the prevalence of knee OA was 7.9% in a pooled sample of 41,884 patients from 11 community-oriented program for the control of rheumatic diseases (COPCORD) studies, which included younger patients [28]. The prevalence of knee OA

was 16.7% among adults aged >45 years in the Johnston county study and 12.6% among adults in the National Health and Nutrition Examination Survey (NHANES) III study [29]. It is estimated that 20% of the population will be over 60 years and that around 15% will have symptomatic knee OA [29]. OA has been a major public health problem and is a foremost cause of disability causing a heavy socioeconomic burden for developed and developing countries. From the National Health Interview Study of United States, OA and related disorders were the third leading chronic condition resulting in work limitation (1.6 million people or 8.3% of main conditions of impairment), just after heart diseases (10.9%) and back disorders (21.1%) in 1998 [30].

Considering the necessity and importance of experimental and clinical studies for one disease, classification criteria of OA should be established suitably and accepted widely. To date, the most universally accepted classification criteria are those of the American College of Rheumatology. It has categorized OA into two broad types: primary and secondary OA. Primary or idiopathic OA can be localized or generalized resulting from injury or disease, and is mostly a result of natural aging of the joint. This kind of OA occasionally could develop in multiple members of the same family, especially among siblings and identical twins implying a hereditary basis for this condition [24, 31]. Secondary OA may further be caused by post-traumatic, congenital, or due to calcium deposition disease or other bone or systemic diseases and hormone disorders. Generally, secondary OA is a type of OA that is caused by identifiable underlying etiological factor [24].

Although symptoms might remain stable for long periods, pain is the most central and frequent symptom among patients for whose pain it gets worse after exercise and better after resting. After a while, the pain may be present in resting time. Stiffness is another common symptom. When patients wake up in the morning, they may feel hard in their joints. This symptom usually lasts for 30 minutes or less. Activity can improve the function of joints. Some patients can notice a rubbing sound or feeling when they move the joint. Moreover, severe osteoarthritis will cause loss of joint ability. The joint movement is as good compared with before the disease. The diagnosis of OA relies on clinical and radiological features (**Table 1**). Nearly half of patients with radiological features of osteoarthritis have no symptoms and vice

versa. Risk factors for the occurrence and progression of osteoarthritis have been identified, and differ on the basis of the joints involved (**Table 2**).

Table 1 American College of Rheumatology radiological and clinical criteria for osteoarthritis of the knee

Diagnosis of Knee OA	
Knee (clinical) Osteoarthritis if 1, 2, 3, 4 or 1, 2, 5 or 1, 4, 5 are present: 1.Knee pain for most days of previous month 2.Crepitus on active joint motion 3.Morning stiffness lasting 30 min or less 4.Age 38 years or older 5.Bony enlargement of the knee on examination	Knee (clinical and radiographic) Osteoarthritis if 1, 2 or 1, 3, 5, 6 or 1, 4, 5, 6 are present: 1.Knee pain for most days of previous month 2.Osteophytes at joint margins on radiographs 3.Synovial fluid typical of osteoarthritis (laboratory) 4.Age 40 years or older 5.Crepitus on active joint motion 6.Morning stiffness lasting 30 min or less

Table 2 Selected risk factors for the occurrence and progression of osteoarthritis in knees

Knee OA	
Occurrence: Age, sex, physical activity, body-mass index (including obesity), intense sport activities, quadriceps strength, bone density, previous injury, hormone replacement therapy (protective), vitamin D, smoking (protective or deleterious), malalignment (including varus and valgus), genetics	Progression: Age, body-mass index (including obesity), vitamin D, hormone replacement therapy (protective), malalignment (including varus and valgus), chronic joint effusion, synovitis, intense sport activities, subchondral bone oedema on MRI.

OA has long been generally recognized by the failure or excess of the repair process of damaged cartilage due to biochemical changes in the joint. Meanwhile, non-vascularized cartilage restricts the supply of nutrients and oxygen to the chondrocytes that are responsible for the maintenance of a very large amount of extracellular matrix [32]. Numerous studies indicated risk factors for OA. Generally, all factors can be categorized into two broad classes: systemic factors (ageing, gender, race, nutritional factors, bone density, and genetics, which are associated with the development of OA, and local factors (biomechanical factors, muscle strength, exercise, joint injury, obesity and career), which affect biomechanical loading of the joint [33]. Although aging insufficiently develops OA, it is still a strong risk factor for knee OA [34]. Age-related alterations probably exhibit negative roles in articular cartilage. Some studies indicated women were more susceptible than men to OA and to higher disease severity [35]. Numerous studies support the role of ethnicity in the development of OA based on variations among racial and ethnic groups [36]. Some reports suggested that an 18% lower knee extensor strength in women is associated with a higher incidence of OA [37], while others showed that moderate or high quadriceps strength in women significantly reduced, by about 60%, the risk of hip or knee OA [38]. In adults, Felson *et al*. [39] reported that knee injury is the leading modifiable risk factor for OA in men and the second in women (after obesity). Recent evidence shows synovial inflammation corresponds to clinical symptoms such as joint swelling and inflammatory pain, and it is thought to be secondary to cartilage debris and catabolic mediators entering the synovial cavity [40].

In early osteoarthritis, pain and stiffness dominate the other symptoms [41]. Treatment should therefore focus on the reduction of pain and stiffness and on the maintenance and improvement of functional capacities. Furthermore, mitigation joint damage and improvement of quality of life are long-term goals. There are three treatment modalities: non-pharmacological, pharmacological, and surgical [32]. Symptoms can be decreased by recommending lifestyle change for the patient [42, 43], weight reduction, transcutaneous electrical nerve stimulation [44], ultra sound [45], electrotherapy [46], or acupuncture [47]. Considering safety and effectiveness,

paracetamol is the first-choice oral analgesic for osteoarthritis [48-50]. A non-steroidal anti-inflammatory drug (NSAID) is added to patient prescriptions and substituted as a therapeutic option. Meanwhile, the use of opioid analgesics for the treatment of osteoarthritis has risen. Joint replacement is very cost effective in patients with severe symptoms or functional limitations associated with a reduced quality of life, despite conservative treatment [42].

For this oldest disease, with development of medical science and biomedical technology, it is no longer misconceived as a uniform disease. More and more doctors and scientists recognize that osteoarthritis is results from a number of different causes. OA is associated with systemic and local risk factors, epigenetic changes and altered transcriptional regulation to disrupt signaling pathways which lead to the loss of the homeostatic balance between degradation and repair mechanisms in joint tissue [51]. However, the inflammatory and metabolic studies of OA are concentrated by researchers of the world.

Inflammatory Aspect of OA

At one time OA was accepted widely as a non-inflammatory disease in order to distinguish OA from inflammatory arthritis, such as rheumatoid arthritis (RA) [52]. But gradually inflammation has been recognized as a significant contributor to the symptoms and progression of OA [53, 54]. Joint inflammation is a well-accepted feature of OA, notably in the early stages [55]. Inflammation of the synovial membrane [51, 56], which contains metabolically highly active synoviocytes, is physiologically important as it both nourishes chondrocytes via the synovial fluid and joint space and removes metabolites and products of matrix degradation. It is a key factor in OA pathophysiology because of the action of several soluble mediators. In OA patients, imaging, arthroscopy, serological or histological evidence of synovitis is commonly found, even though OA has not been consistently associated with specific immune responses. Hence, treatments that specifically target this previously neglected component of OA could be beneficial for both the symptoms and structural changes that occur in OA. Physicians usually treat patients with non-steroidal anti-inflammatory drugs (NSAIDs) to alleviate symptoms and signs and may

be more effective than simple analgesics, such as paracetamol [57]. Additionally, intra-articular injections of corticosteroids similarly may alleviate both pain and stiffness, not only during acute flares but also as a maintenance therapy. Pain, the predominant symptom in OA, is multidimensional in its nature and mediated through a variety of factors. The pain experience results from interactions between inflammation and other features of the disease, including radiological severity [58], innervation of articular structures [59, 60], central and peripheral sensitization [61] and psychological factors [62]. The precise contribution of inflammation to pain in OA may vary from time to time and from patient to patient. It is currently unclear whether inflammation is a feature of all patients with OA at some stage of their disease, or whether synovitis itself defines one or more disease subgroups. Inflammation may be both a primary event in OA and secondary to other aspects of the disease. Recent studies indicate that histological and serological evidence of synovitis is an early feature in OA and not restricted to patients with end-stage disease undergoing joint replacement surgery [54, 63, 64]. Synovial inflammation may be detected in the presence of mild or severe cartilage changes in OA [63]. Even when inflammation is secondary to other processes within the osteoarthritic joint, synovitis may yet make an important contribution to the symptoms and pathology of the disease. Clinically detectable joint inflammation may predict a worse radiological outcome in OA [65]. Furthermore, in a lapine model of arthritis, joint damage was exacerbated after induction of inflammation in rabbit knees following a meniscal tear [66]. Synovitis, therefore, although not a prerequisite for OA, may lead to a poor clinical outcome [51].

Metabolic Aspect of OA

In obese OA patients, the overload effect on joint cartilage might, in part, explain the greater risk of osteoarthritis in overweight people. Advances in the understanding of the physiology of adipose tissue provide further information about the relation between obesity and osteoarthritis [67]. Indeed, a positive association between obesity and osteoarthritis has been reported for non-weight-bearing joints, such as the hands and not only knee joints [68] suggesting that joint damage might be caused by systemic factors. This phenotype of OA is characterized by its major

causative features- adipokines, hyperglycaemia and hormonal imbalance-and its targeting of mainly middle-aged people (45–65 years old), leading to knee or/and hand OA [69]. And the so-called adipokines, which might provide a metabolic link between obesity and osteoarthritis [70], and which, in addition to weight loss, could become a specific therapeutic target. Some studies have also found that people with Metabolic Syndrome (MetS), distilled into four central features: insulin resistance, visceral obesity, atherogenic dyslipidemia and endothelial dysfunction [71], develop OA at an earlier age and have more generalized pathology, increased inflammation, and augmented intensive pain in the joints, in comparison with patients with OA in the absence of metabolic syndrome [72, 73]. Besides from aging, the metabolism related with OA has grown in recognition to become the second most frequent factor among patients enrolled in clinical studies [69]. As well as associations between OA and the four central components of Metabolic Syndrome, investigators are now assessing how Metabolic Syndrome as a whole is linked to OA [74]. In the Michigan Bone Health and Metabolism Study, in obese women, the presence of two or more cardiometabolic risk factors was associated with more reports of persistent knee pain over the previous 3 years [75]. In the Japanese Research into Osteoarthritis Against Disability (ROAD) study, the odds of OA increased with the presence of each additional component of Metabolic Syndrome [76]. Furthermore, in a Russian study of 1,350 individuals with OA, 62.56% were also diagnosed with Metabolic Syndrome [77]. Therefore, from growing epidemiological evidence, it has been suggested that metabolic syndrome could be part of OA [78] and possibly OA is a metabolic disease.

Adiponectin

Adiponectin is produced mainly by adipocytes, but other cell types, such as endothelial cells and skeletal and cardiac myocytes, can also produce this adipokine. Adiponectin is a 244-amino-acid-long hormone protein and secreted from adipocytes into the bloodstream (**Figure 1**). The first region is a short signal sequence that targets the hormone for secretion outside the cell; the next region is a variable sequence between species; the third region is a 65-amino-acid sequence with similarity to collagenous proteins; the last region is a globular domain of which 3-dimensional structure is similar to TNF-α.

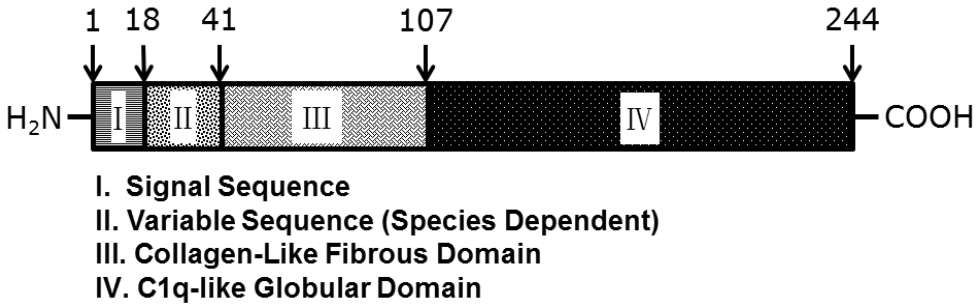


Figure 1 Structure of full length human adiponectin

Adiponectin has four forms: the trimer, the globular former, the hexamer, and the polymer (**Figure 2**). The first is a full-length trimer that is the low-molecular-weight form. A proteolytic enzyme can cleave the trimer to a fragment that is globular adiponectin. The full-length trimer can dimerize to form a hexamer which is the middle-molecular-weight form. The hexamer can then oligomerize to form a polymer. The polymer is the high-molecular-weight form of adiponectin.

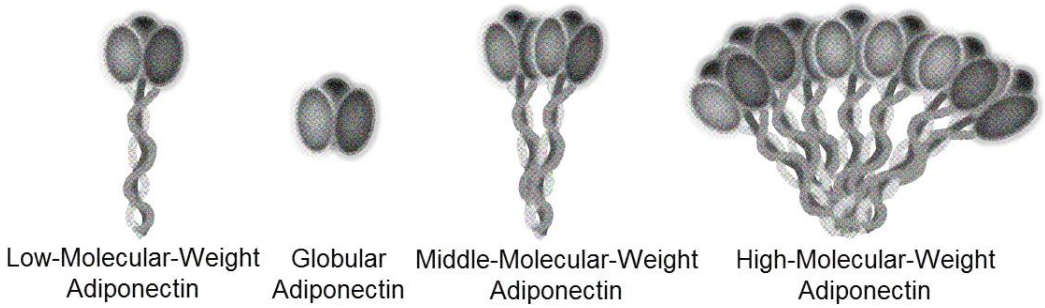


Figure 2 Four different forms of adiponectin

In addition to its traditional function of energy storage, adipose tissue is a metabolic and endocrine organ with significance, complication and high activity. Hormones secreted from adipose tissue are named after adipokines that associate with metabolic processes and inflammatory reactions as well as performing cytokine-like functions including anti- and pro-inflammatory effects [4, 79, 80]. Adiponectin, one essential member of the adipokines, is synthesized in differentiated adipocytes and maintains high levels in blood circulation. The function and effect of adiponectin have been clearly elaborated in anti-diabetic and anti-atherogenic properties. Whether adiponectin conduct resemble actions in OA pathogenesis recently has been discussed more and more. Adiponectin was identified in cartilage, osteophytes,

menisci, synovial membranes, and infrapatellar fat pads taken from the knees of OA patients, with the highest concentrations found in the two latter [81]. Some clinical observations implicate that adiponectin levels were significantly reversely correlated with OA severity in humans in both plasma and synovial fluid [9, 10]. In chondrocytes adiponectin could modulate cartilage destruction through increasing TIMP-2 and decreasing IL-1β induced by MMP-13 [5]. Together previous researches demonstrates that adiponectin may play a protective and active role in the development of OA.

Anti-inflammatory Features of Adiponectin

A number of studies have investigated the association between adiponectin levels and pro-inflammatory factors in various populations. The inflammatory marker C-reactive protein (CRP) levels have been associated positively with BMI [82], suggesting that CRP is a useful biomarker for obesity-linked chronic inflammatory states. CRP mRNA is expressed in human adipose tissue, indicating that adiponectin may participate in the reduction in plasma CRP levels through its ability to negatively regulate CRP expression in adipose tissue. Experimental studies demonstrated that adiponectin expression was negatively regulated by pro-inflammatory cytokines including important pro-inflammatory adipokines IL-6 and TNF-α [11-13]. Meanwhile, it has been indicated by many research results that adiponectin modulates the function and phenotypes of macrophages that play a central role in inflammation. Adiponectin can diminish the expression of class a scavenger receptor (SR-A) in human macrophages and prevents transformation of macrophages to foam cells. Adiponectin increases tissue inhibitor of metaroproteinase-1 through expression of IL-10 in human monocyte derived macrophages. In vitro, adiponectin also attenuates LPS-induced up-regulation of TNF-α in cultured macrophages, which is associated with decreased NF-κB activation [14, 15].

Metabolic Features of Adiponectin

The role of adiponectin, an adipose tissue derived protein known as adipokine, has been studied in OA [81, 83], beside their established role in obesity, metabolic disorders and atherogenesis. Adiponectin was found to be present in OA synovial fluid and articular chondrocytes [84, 85]. Moreover, it was shown that

adiponectin up-regulates tissue inhibitor of metalloproteinases-2 (TIMP-2) and down-regulates IL-1β-induced MMP-13, protecting cartilage from degeneration [84]. Furthermore, it has been shown that serum levels of adiponectin were elevated in female patients with erosive OA [8]. All the above-described data suggest that adiponectin is critically involved in OA pathogenesis [86]. Interestingly, adiponectin, resistin and leptin were found to exhibit different patterns of distribution within the joint and the circulating environment [87] with resistin and adiponectin being elevated in serum and leptin being elevated in the synovial fluid of the joint, especially in women. Thus, it could be argued that the distribution of adipokines may have local effects in the joint and may account for the high prevalence of OA in women. In human studies, the plasma concentration of adiponectin was found to decrease in conditions associated with the metabolic syndrome [88]. The injections of adiponectin recombinant proteins into mice reduced plasma glucose and fatty acid levels and ameliorated insulin resistance [89]. It enhanced fatty acid β-oxidation in skeletal muscle and suppressed hepatic glucose output by decreasing gluconeogenesis [90]. These were in part mediated by the activation of AMP-activated protein kinase [89]. Long-term administration of recombinant adiponectin also induced weight loss.

OA-related SNPs and Adiponectin Gene

Several studies have demonstrated that some individuals are genetically susceptible to OA (Table 3), but some genes and variants have been found to be controversial by other researchers. This is probably due to genetic variations in OA being site ethnic and sex specific. The genes that facilitate this vulnerability are involved in inflammation, extracellular molecules and cartilage development. For example, Hypoxia-inducible factor-2alpha (HIF-2α, encoded by EPAS1) was reported to be the most potent transactivator of COL10A1. A functional single nucleotide polymorphism (SNP) in the human EPAS1 gene was associated with knee OA in a Japanese population [91]. By contrast, according to the research of Honsawek S. *et al.*, the -1612 5A/6A polymorphism genotypes of the MMP-3 gene promoter do not play a role in the development of osteoarthritis in a Thai population [18] .

Table 3 Selected gene associated with knee osteoarthritis

Gene	Protein	Function
Inflammation		
COX-2(PTGS2)	Cyclooxygenase 2 (Prostaglandin G/H SYNTHASE 2)	Osteogenesis and bone repair
IL-1 gene cluster	Interleukin 1α/β, interleukin 1 receptor antagonist (IL1RN)	Stimulate osteoclast activity in vitro, increase production of metalloproteinases and aggrecanases, in turn stimulate cartilage degradation.
IL-10	Interleukin-10	Anti-inflammatory, prevents cartilage destruction by reducing IL-1β and TNF-β expression in articular chondrocytes in mouse model
Extracellular matrix molecules		
ASPN	Asporin, Periodontal ligament-associated protein 1	Associated with cartilage matrix. Suppress TGF-β-mediated effects and reduces proteoglycan accumulation
COMP	Cartilage oligomeric matrix protein	Chondrocytes territorial matrix
CILP	Cartilage intermediate layer protein	Inhibits TGF-β-mediated induction of cartilage matrix genes. Increase in synthesis in early OA cartilage
COL2A1	Collagen Type II	Cartilage collagen
Protease/protein inhibitors		
AACT	A-1-antichymotrypsin	A plasma protease inhibitor involved in cartilage proteoglycan degradation
ADAMTS14	ADAM with thrombospondin motif (ADAMTS) 14	A metalloproteinase
TNA	Teranectin	Extracellular matrix degradation. Induced during the mineralization phase of osteogenesis

Adiponectin is encoded by its gene ADIPOQ located in the chromosomal region 3q27. ADIPOQ spans 16kb and contains three exons. Previous genome-wide linkage scans have identified 3q27 as a susceptibility locus for diabetes [92]. Various single nucleotide polymorphisms (SNPs) in ADIPOQ have been reported to be associated with adiponectin levels and/or metabolism but with inconsistent results. A recent comprehensive review [93] showed that a few ADIPOQ SNPs were associated with adiponectin levels and insulin resistance, but none was consistently associated with diabetes or with adiposity as measured by BMI. As underlined by Menzaghi *et al.*, the lack of consistent findings emphasizes the need for comprehensive characterization of the genetic variation in and around the ADIPOQ gene. They also emphasized the need to address the issue that some ADIPOQ SNPs seem to be associated with adiponectin levels, whereas others seem to be associated with insulin resistance and diabetes-related metabolic traits. However, single nucleotide polymorphisms (SNPs) in ADIPOQ in OA patients have not been researched and reported to be associated with adiponectin levels and the susceptibility of OA. But a study about adiponectin genetic polymorphisms in OA patients has not been presented. There are many genetic variations of the human adiponectin gene reported, including several non-synonymous mutations. The two common researched SNPs, +45T/G (rs2241766) and +276G/T (rs1501299) (Figure 3), have been demonstrated to be associated with diabetes, insulin resistance and atherosclerosis [19, 20]. Additionally, the G allele at the +276G/T (rs1501299) locus is associated with lower serum adiponectin levels [21].

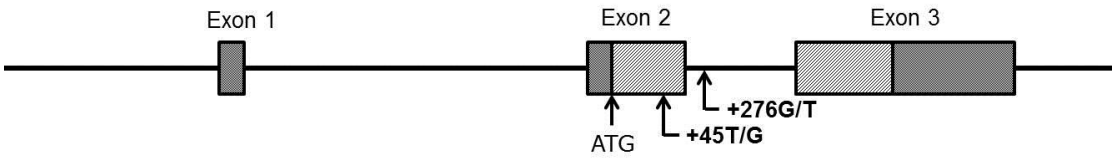


Figure 3 Schematic of genomic structure and polymorphic variants of the adiponectin gene (ADIPOQ). The exon-intron organization of the gene is indicated by closed boxes. Arrows point to the positions of the polymorphic variants identified. There are two common ADIPOQ single nucleotide polymorphisms (SNPs), +45T/G (rs2241766) located in exon 2, and +276G/T (rs1501299) in intron 2.

CHAPTER III

METHODOLOGY

Case-Control Study

A case-control study involves the identification of individuals with and without a particular disease. It is a kind of observational study where two existing groups are identified and compared on the basis of some supposed causal attributes. It is simple to organize and look at one outcome via the calculation of an odd ratio. Few subjects are required in this study to prove or repudiate a hypothesis. Case-control studies are widely used in analysis of genetics and usually retrospective.

Target Population

1. Simple Size

Based on the statistic theory, statisticians provided us following formula to determine the simple size in ours study. The "n" represent how many samples are needed in this research. The "α" represents Type I Error and the " $Z_{\alpha/2}$" represents the desired level of statistical significance. The "e"represents the relative acceptable error, e=0.1.

$$n = \frac{\left(z_{\alpha/2}\right)^2}{4e^2}$$

$$\alpha = 0.05\,, z_{\alpha/2} = 1.96\,, e^2 = 0.1^2$$

$$n = 96.04$$

The number of subjects needed in ours study using a single nucleotide polymorphism assay was greater than 96.04 individuals for every group. Eventually we collected 196 subjects in control group and 202 subjects in OA group.

2. Patients Group

A total of 202 patients aged 50-80 years diagnosed with primary knee osteoarthritis were included in this study. The patients with any systemic inflammatory or autoimmune disorders, or any type of malignant or chronic illness were not included in this study.

3. Diagnosis of OA

The diagnosis of knee OA was based on the criteria of the American College of Rheumatology including primary OA with any symptoms and radiographic signs of OA according to the Kellgren-Lawrence (KL) grading system (**Box**). Clinical characteristics on disease severity were observed by mean of Kellgren-Lawrence grade. Radiographic findings of OA were categorized into Kellgren-Lawrence grade 1, 2, 3, or 4.

Box The Kellgren-Lawrence grading system is the radiological classification of knee OA and is used to determine candidacy for knee replacement

The Kellgren-Lawrence Grading System is based on x- rays and consists of:
Grade I: Unlikely narrowing of the joint space, possible osteophytes.
Grade II: Small osteophytes, possible narrowing of the joint.
Grade III: Multiple, moderately sized osteophytes, definite joint space narrowing, some sclerotic areas, possible deformation of bone ends.
Grade IV: Multiple large osteophytes, severe joint space narrowing, marked sclerosis and definite bony end deformity.
*Joint space narrowing-bone is visible on x-ray but the articular cartilage that covers it is not. A normal joint therefore appears to have a space between the bones. Any decrease in space implies a reduction in cartilage cover.
*Osteophytes - small bony projections that from around joint margins. They are responsible for limiting range of motion and can cause pain.
*Sclerosis-this means 'hardening' and is a sign of osteoarthritis, seen as increased white areas in the bone at the joint margins

4. Control Group

A total of 196 healthy individuals who have no symptoms or signs of OA and other arthritis or any joint diseases were recruited in this study as a control group. Control subjects were consecutively selected among people without personal and family history of OA. Participants were excluded on the basis of having arthropathy due to gout, pseudogout, rheumatoid arthritis (RA), systemic lupus erythematosus, psoriasis, hemochromatosis, previous knee injury, or previous joint infection.

Materials, Equipment and Reagents

1. Materials

- Aluminum Foil (Rainbow Metal Company, USA)
- Barrier Tip: 200 µl (BioScience, USA)
- Clotted Blood and EDTA Tube (Vacuettee, Austria)
- Disposable Gloves
- Glass Pipette: 1 ml, 5 ml, 10 ml (Witeg, Germany)
- Kimwipe Paper
- Microcentrifuge Tube: 0.2 ml, 0.5 ml, 1.5 ml (Bio-Rad, USA)
- Needle, Sterile (Nipro, Thailand)
- Parafilm (American National Can, USA)
- Petri Dish
- Pipette Tip: 10 µl, 200 µl, 1000 µl (AxyGen, USA)
- Plastic Wrap
- Polypropylene Conical Tube, Sterile: 15 ml, 50 ml (Elkay, USA)
- PCR Markers (Bio-Rad, USA)
- Sanitary Tissue Paper (Celox, Thailand)
- Sterile Pasture Pipette (Samco Scientific Corporation, USA)
- Syringe Disposable (Nipro, Japan)
- 96-wells ELISA plate (Nunc, Denmark)

2. Equipment

- Autoclave (Hydroclave Harvey, USA)

- Automatic Adjustable Micropipette (Eppendorf, Germany)
- Balance (Sartorius, Germany)
- Beaker: 50 ml, 100 ml, 200 ml, 500 ml, 1000 ml (Pyrex, USA)
- Centrifuge, Refrigerated Centrifuge (Eppendorf, USA)
- Centrifuge, Microcentrifuge High Speed (Eppendorf, USA)
- Combs (Bio-Rad, Hercules, USA)
- Cuvette 80-100 µl
- Cylinder: 25 ml, 50 ml, 100 ml, 200 ml, 500 ml, 1000 ml (Pyrex, USA)
- Digital Timer
- DNA Thermal Cycler (Thermo Hybaid, USA)
- Electrophoresis Chamber Set (Bio-Rad, USA)
- Freezer -80°C. (Forma Scientific, USA)
- Gel Doc 1000 (Bio-Rad, USA)
- Mitsubishi Video Copy Processor (Bio-Rad, USA)
- Mixing Bolck (Bioer, USA)
- Multi-block Heater (Techne DRI Block, USA)
- Multi-Channel Pipette (Socorex, Switzerland)
- pH Meter (Eutech Cybernataics)
- Pipette Aid (Tecnomara, Switzerland)
- Pipette Rack (Autopack, USA)
- Power Supply Model 250 (Bio-Rad, USA)

- Reagents Bottle 100 ml, 250 ml, 500 ml, 1000 ml (Duran, USA)
- Refrigerator (Sanyo, Janpan)
- Spectrophotometer (Bio-Rad, USA)
- Stirring-Magnetic Bar
- Test Tube Racks
- Thermometer (IR Thermometer, USA)
- Vortex Mixer (Scientific Industry, USA)
- Water Purification Equipment (Water Pro Ps, Labconco USA)
- Water Bath, Thermostat Shaking (Memment, Germany)
- RolimeterTM (Aircast, USA)
- Microplate Reader (Infinite 200 PRO, Tecan, Switzerland)

3. Reagents

3.1 General Reagents

- Agarose Molecular Grade (Sigma, USA)
- Boric Acid (USB, Hong Kong)
- Bromphenol Blue (Pharmacia, Hong Kong)
- Diethyl Pyrocarbonate (Sigma, USA)
- Ethanol 70%
- Ethidium Bromide (Sigma, USA)
- Hydrochloric Acid (Sigma, USA)
- Sucrose (Sigma, USA)

- Tris (USB, Hong Kong)

3.2 DNA Extraction

- 1× PBS Buffer pH7.2
- RBC Lysis Buffer
- Absolute Ethanol
- Elution Buffer
- Lysis Solution
- Proteinase K
- Wash Buffer

3.3 PCR

- 10 µM Primer Forward (Bio Basic Inc, Thailand)
- 10 µM Primer Reverse (Bio Basic Inc, Thailand)
- 10× PCR Buffer (Fermentus, USA)
- 1× TAE Buffer (Bio Basic Inc, Thailand)
- 2mM Deoxynucleotide Triphosphates (dNTPs) (Fermentus, USA)
- 25mM $MgCl_2$ (Fermentus, USA)
- PCR Marker (Bio-Rad, USA)
- Taq Polymerase Enzyme (Fermentus, USA)

3.4 RFLP

- 10× NE Buffer 3 (New England BioLabs, UK)
- 10× NE Buffer 4 (New England BioLabs, UK)

- Bgl1 (New England BioLabs, UK)
- BspH 1 (New England BioLabs, UK)
- Nuclease-Free Water
- Polyacrylamide Gel
- 0.5×TBE Buffer
- 10% Ammoniumpersulfate
- 30% Polyacrylamide
- 5× TBE Buffer
- TEMED

3.5 ELISA

- DuoSet ELISA kit-human adiponectin(R&D System Inc, USA)
- Reagent Dilution (R&D System Inc, USA)
- Substrate Solution (R&D System Inc, USA)
- Stop Solution 2N H2SO4 (Sigma-Aldrich, USA)
- Tween 20 (Sigma-Aldrich, USA)
- Fetal Bovine Serum

Record of Information and Results of Subjects

For protecting patients' privacy and convenience of data usage, all information of osteoarthritis patients and healthy individuals was conducted clearly and safely. Furthermore, any details related with research results of this study were recorded clearly and truly. Both of them were recorded in Microsoft Excel (**Table 4**).

Table 4 Subjects record form

Name	ID	Gender (F/M)	Age (years)	Weight (kg)	Height (m)	BMI (kg/m^2)	Severity	+45T/G	+276G/T	Adiponectin levels in plasma

Collecting Samples

Peripheral venous blood samples of 8-10 ml were drawn from each individual by standard venipuncture to 2 EDTA tubes.

1. The first tube of 4-5 ml blood was placed in a centrifuge and spun at roughly 4000 rpm for 10 minutes at room temperature. The plasma located at the top of the specimen was then transferred into screw-cap cryovial tubes and stored at -80°C as soon as possible.

2. The second tube of 4-5 ml blood was used to extract Genomic DNA.

Extract Genomic DNA

The products of GE Healthcare "illustra blood genomic Prep Mini Spin Kit" were used to extract the genomic DNA from peripheral venous blood.

1. Samples Thaw: Put frozen samples at room temperature to thaw completely for 20-30 minutes and ensure complete homogenization. Non-frozen sample were used for the next step directly.

2. Blood Cell Lysis: 20 µl of proteinase K was added into the bottom of a 1.5 ml micro-centrifuge tube. Then up to 300 µl of whole blood sample was added. After that, 400 µl of lysis buffer type 10 was added to the tube and mixed well by vortex for 15 seconds. The tubes were incubated at room temperature for 10 minutes with intermittent vortex to aid lysis. At the end of this stage the color of the reaction changed from red to dark brown. The tubes were briefly spun to bring the sample to the bottom of the tube.

3. Genomic DNA Binding: A mini column was assembled in the supplied collection tube. Then, the complete lysate was loaded to the center of the column using a pipette and spun for 1 minute at 11000×g in a micro-centrifuge. After that, the flow-through was discarded and the column placed back inside the collection tube.

4. Wash: 500 µl of lysis buffer type 10 was added to the column and centrifuged for 1 minute at 11000×g. The flow-through of the collection tube was discarded.

5. Wash & Dry: 500 µl of Wash buffer type 6 was added to the column and centrifuged for 3 minutes at 11000 ×g. The collection tube and flow-through was discorded.

6. Elution: The purification column was transferred into a fresh DNase-free micro-centrifuge tube. Add 200 µl of 70°C pre-heated Elution buffer type 5 directly on to the center of the column.

At last, the purified genomic DNA was stored at -20°C.

Polymerase Chain Reaction (PCR)

Polymerase chain reactions (PCR) were conducted to amplify the special sequence of genomic DNA including the +45T/G (rs2241766) and +276G/T (rs1501299) single nucleotide polymorphisms (SNPs).

1. Primers for PCR

The two sets of primers were designed according to a published paper [94].

Primers for the +45T/G (rs2241766) SNP:

Forward 5'-TCCTTTGTAGGTCCCAACT- 3'

Reverse 5'-GCAGCAAAGCCAAAGTCTTG- 3'

Primers for the +276G/T (rs1501299) SNP:

Forward 5'-ACACTGATATAAACGCCATGAA- 3'

Reverse 5'-GCAGCAAAGCCAAAGTCTTC- 3'

2. The components of reaction mixture 25 µl for PCR (**Table 5**)

Table 5 The components of reaction mixture 25 µl for PCR

Components	Volume (µl)	Concentration
10×Tag Buffer	2.5	-
2 mM dNTPs Mix	2.5	0.2 mM
10 µM Forward Primer	1	0.1-1.0 µM
10 µM Reverse Primer	1	0.1-1.0 µM
25 mM MgCl2	2.5	1-4 mM
Template DNA	3	10 pg-1 µg
5 u/µl Taq DNA Polymerase	0.25	1.25 u/reaction
ddH_2O	12.3	to 25 µl
Total Volume	25	

3. The conditions of PCR (**Table 6 & 7**)

Table 6 PCR conditions for +45T/G (rs2241766) polymorphism

Step	Temperature (°C)	Time (min)	No. of cycles
Initial Denaturation	95	15	1
Denaturation	95	0.5	
Annealing	56	0.5	35
Extension	72	1	
Final Extension	72	7	1

Table 7 PCR conditions for +276G/T (rs1501299) polymorphism

Step	Temperature (°C)	Time (min)	No. of cycles
Initial Denaturation	95	10	1
Denaturation	95	0.5	
Annealing	48	1	40
Extension	72	1	
Final Extension	72	7	1

Two kinds of PCR products including +45T/G (rs2241766) and +276G/T (rs1501299) SNPs were electrophoresed in 2.5% agarose gel which contained ethidium bromide and visualized on an ultraviolet transilluminator to identify that the sequences of PCR were the correct molecular weight.

Restriction Fragment Length Polymorphism (RFLP)

The obtained PCR products were digested into pieces by restriction enzymes and resulting restriction fragments were separated according to their lengths by gel electrophoresis. The two restriction enzymes were BspH1 and Bgl1 which could cleave SNPs of +45T/G and +276G/T, respectively (**Table 8**)

Table 8 Restriction enzymes for the two SNPs and their recognized sequences and cut sites

SNP	Enzyme	Recognized Sequence	Cut Site
+45 T/G	BspH1	TCATGA	5′– N|T C A T G A N –3′ 3′– N A G T A C T|N –5′
+276 G/T	Bgl1	GCCNNNNNGGC	5′– N G C C N N N N|N G G C N –3′ 3′– N C G G N|N N N N C C G N –5′

1. The PCR product of +45T/G (rs2241766) was subsequently digested with the enzyme BspH1 in a 37 °C water-bath for 16 hours, which yielded 375bp and 128bp fragments (T allele is major allele of +45T/G). The components of RFLP for +45T/G (rs2241766) are shown in **Table 9**.

Table 9 Components of RFLP for +45T/G locus

Components	Volume (µl)
PCR products	7
1×NEB buffer 3	1
BspH1(10U/µl)	1
Sterile distilled water	1
Total Volume	10

After heat inactivation, the products of digestion were used in 2.5 % agarose gel electrophoresis, which contains ethidium bromide and visualized on an ultraviolet transilluminator.

2. The PCR products of +276G/T (rs1501299) were subsequently digested with the enzyme Bgl1 in a 37 ℃ water-bath for 16 hours, which yielded 147bp and 21bp fragments (G allele is major allele of +276 G/T). The components of RFLP for +276G/T (rs1501299) are shown in **Table 10.**

Table 10 Components of RFLP for +276T/G locus

Components	Volume (µl)
PCR products	7
1×NEB buffer 3	1
Bgl1(10U/µl)	1
Sterile distilled water	1
Total Volume	10

Then digestive products of +45T/G and +276G/T loci were used in 2.5 % agarose gel electrophoresis and 12 % polyacrylamide gel, respectively. The gel contained ethidium bromide and was visualized on an ultraviolet transilluminator. **Table 11** presented the components of the 12 % polyacrylamide gel.

Table 11 Components of 12% polyacrylamide gel

Components	Volume
12 % polyacrylamide	4 ml
5×TEB buffer	2 ml
Deionized water	3.93 ml
10% ammonium persulfate	70 µl
TEMED	3.5 µl

Enzyme-Linked Immunosorbent Assay (ELISA)

A DuoSet ELISA Development kit (human Adiponectin) and reagents from R&D System Inc. were used to measure adiponectin levels in plasma of the control and OA groups.

1. Plasma Preparation: Frozen samples were put at room temperature to thaw completely for 20-30 minutes and complete homogenization was ensured.

2. Capture Antibody Incubation: 96-well microplates were coated with 100ml of capture antibody (2.0 µg/ml) which was diluted in PBS without carrier protein per well overnight at room temperature.

3. Wash Plates: Each well was aspirated and washed with 400 ml of wash buffer. This was repeated three times for a total of three washes. After the last wash, any remaining wash buffer was moved by inverting the plate and blotting it against clean paper towels.

4. Block Plates: 300 ml of reagent diluent with PBS supplemented with 50% fetal calf serum was added to each well and incubated at room temperature for 2 hours. Then, the plates were washed three times with 400 ml of wash buffer per well.

5. 100 ml of samples or standards in reagent diluent with PBS supplemented with 50 % fetal calf serum were added per well and incubated for 2 hours at room temperature. Then, plates were washed three times with 400 ml of wash buffer per well.

6. 100 ml of the detection antibody, diluted in Reagent Diluent was added to each well, covered with a new adhesive strip and incubated for 2 hours at room temperature. Then plates were washed three times with 400 ml wash buffer per well.

7. 100 ml of the working dilution (1:200) of Streptavidin-HRP was added to each well and incubated for 20 minutes at room temperature. Care was taken to avoid placing the plate in direct light. Then, the plates were washed three times with 400 ml wash buffer per well.

8. 100 ml of substrate solution was added to each well and incubated for 20 minutes at room temperature. Care was again taken to avoid placing the plate in direct light.

9. 50 ml of stop solution was added to each well. The plate was gently tapped to ensure thorough mixing.

10. The optical density (OD) of each well was determined immediately using a micro-plate reader. The readings at 450 nm were subtracted at 570 nm to correct for optical imperfections in the plate.

Statistical Analysis

The Hardy-Weinberg equilibrium (HWE) was assessed with Michael H. Court's (2005-2008) online calculator (http://www.tufts.edu/~mcourt01/Documents/Court%20lab%20-%20HW%20calculator.xls). The genotype distributions allele frequencies and *P* values were calculated by the Pearson's Chi-square test in the Statistical Package for Social Sciences software (SPSS, Inc., Chicago, IL, USA, version 17.0). Odds ratios (ORs) and 95% confidence intervals (CIs) of genotypes and alleles were assessed by using the Medcalc® (Medcalc®Software, Mariakerke, Belgium) statistical software program. The linkage disequilibrium (LD), D' and r^2 between these polymorphisms and the haplotypes of them were conducted with the SHEsis online software (http://analysis.bio-x.cn/myAnalysis.php) and Haploview software version 4.1 (Broad Institute Cambridge MA USA). The plasma adiponectin level data were compared between groups by the ANOVA and Student's t-tests. Genotype and allelic frequencies were compared by the Chi-square test in the SPSS (version 17.0). *P* values less than 0.05 were statistically considered significant difference.

CHAPTER IV
RESEARCH RESULTS

Result I: Baseline characteristics of the control group and OA group

The demographic data of the population studied and the number of individuals in each group are shown in **Table 12**. There were significant differences between groups in terms of age, gender and mean body mass index (BMI). In the knee OA patients, the mean age was 68.76±7.76 years. In the healthy controls, the mean age was 62.50±6.17 years (*P*<0.001). The female/male ratio was 136/66 in the knee OA patients and 128/68 in the controls (*P*<0.001). Furthermore, the mean BMI value was significantly different between groups, 26.98±3.71 kg/m2 in the knee OA patients and 24.74±3.96 kg/m2 in the controls, respectively (*P*<0.001). All patients of knee OA were scored according to the Kellgren-Lawrence grading system with 1, 2, 3 or 4.

Table 12 Demographic data of control individuals and OA patients

	Controls	OA	*P*
n	196	202	-
Age (years)	62.502±6.17	68.76±7.76	<0.001
F/M	128/68	136/66	<0.001
BMI (kg/m^2)	24.74±3.96	26.98±3.71	<0.001
KL Grade			
1	-	0	
2	-	55	
3	-	71	
4	-	76	

Result II: The electrophoretic image of +45T/G locus PCR products

The special sequence of genomic DNA including the +45T/G (rs2241766) SNP was amplified by PCR. **Figure 4** shows an example of a 2.5% agarose gel electrophoresis of PCR products which were 503bp in length.

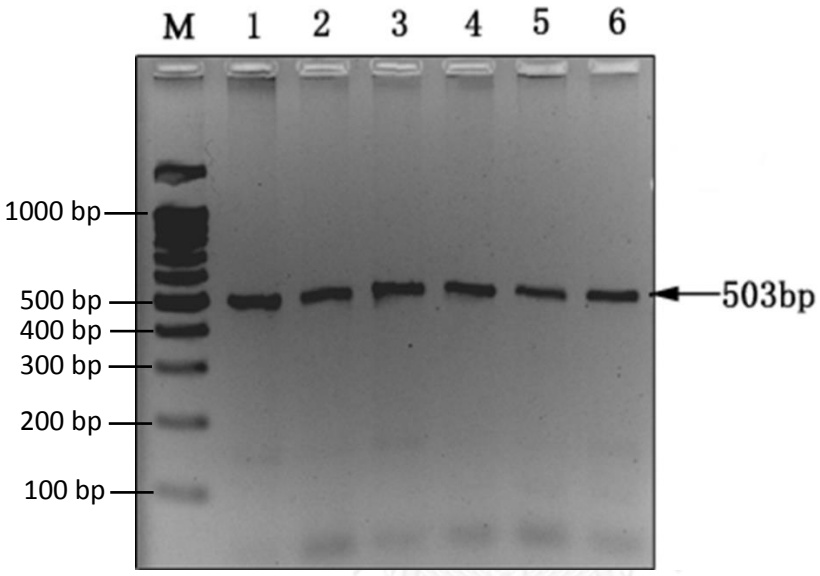


Figure 4: PCR products of +45T/G (rs2241766) of the adiponectin gene on 2.5% agarose gel electrophoresis with ethidium bromide staining and ultraviolet light transillumination. Lane1: molecular weight DNA standard marker, Lane2-7: PCR Products of samples.

Result III: The electrophoretic image of +45T/G locus digested products

After digestion with the enzyme BspH1, the 503bp-length PCR products of +45T/G (rs2241766) were yielded into 375bp and/or 128bp fragments (major T allele of +45T/G). Using 2.5% agarose gel electrophoresis for digested PCR products, the genotypes of this locus were visible according to the different positions and quality of bands, which are shown in **Figure 5**. Homozygous TT and GG corresponded to the presence of 375bp and 503bp fragments, respectively, whereas the heterozygous GT corresponded to the presence of both 375bp and 503bp fragments.

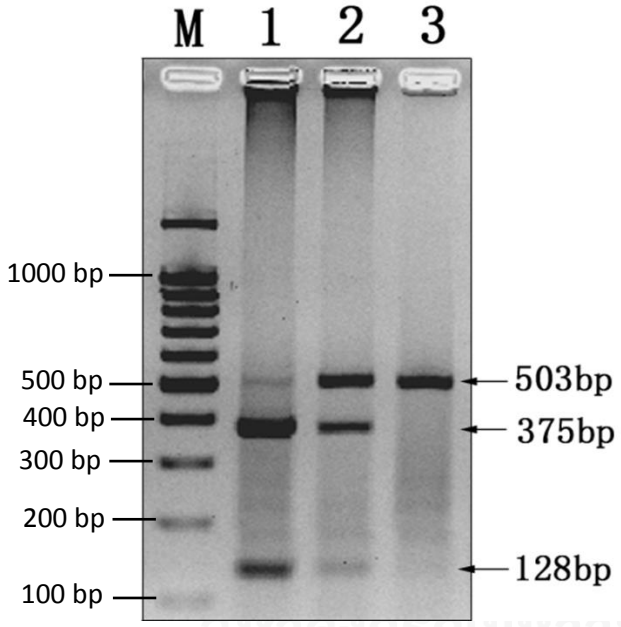


Figure 5: Digested PCR products with BspH1 of +45T/G (rs2241766) in 2.5% agarose gel electrophoresis with ethidium bromide staining and ultraviolet light transillumination. Lane 1: molecular weight DNA standard marker, Lane 2: homozygote for T/T genotype of sample 1, Lane 3: heterozygote T/G genotype of sample 2, Lane 4: homozygote for G/G genotype of sample 3.

Result IV: The electrophoretic image of +276G/T locus PCR products

The special sequence of genomic DNA including the +276G/T (rs1501299) SNP was amplified by PCR. **Figure 6** shows an example of a 2.5% agarose gel electrophoretic of PCR products which were 168bp in length.

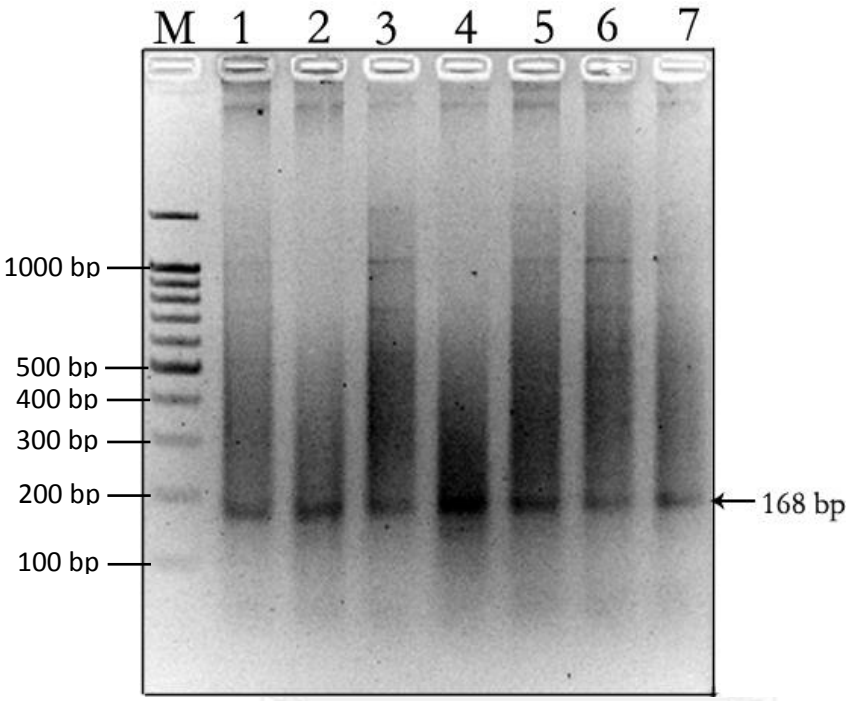


Figure 6 PCR products of +276G/T (rs1501299) of the adiponectin gene on 2.5 % agarose gel electrophoresis with ethidium bromide staining and ultraviolet light transillumination. Lane 1: molecular weight DNA standard marker, Lane 2-9: PCR Products of samples.

Result V: The electrophoretic image of +276G/T locus digested products

After digestion with the enzyme Bgl1, the 168bp-length PCR products of +276G/T (rs1501299) yielded into 147bp and/or 21bp fragments (major G allele of +276T/G). Through 12 % polyacrylamide gel electrophoresis for digested PCR products, the genotypes of this locus were visible according to different positions and quantity of bands as shown in **Figure 7**. Homozygous TT and GG corresponded to the presence of 168bp and 147bp fragments, respectively, whereas the heterozygous GT corresponded to the presence of both 168bp and 147bp fragments.

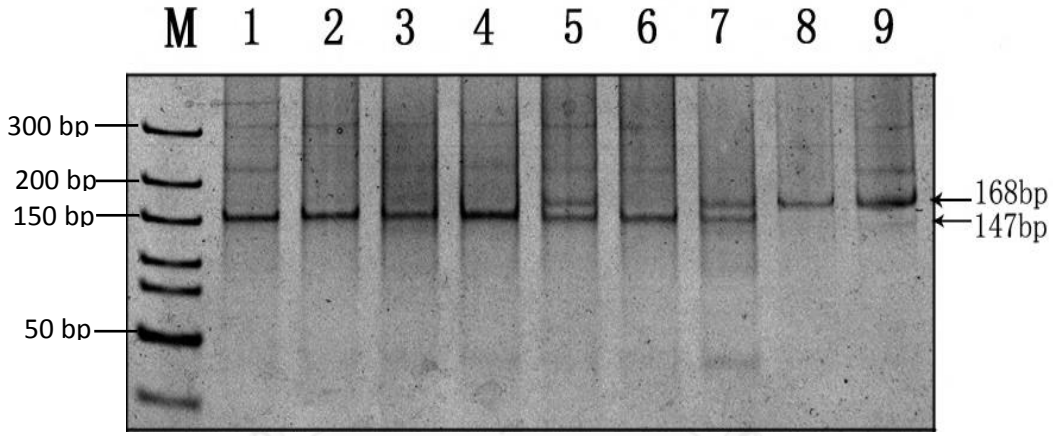


Figure 7 Digested PCR products with Bgl1 of +276G/T (rs1501299) in 12% polyacrylamide gel electrophoresis with ethidium bromide staining and ultraviolet light transillumination. Lane 1: molecular weight DNA standard marker, Lanes 2-5 and 7: homozygote for G/G genotype of samples 1-4 and 6, Lanes 6 and 8: heterozygote T/G genotype of sample 5 and 7, Lanes 9 and 10: homozygote for G/G genotype of samples 8 and 9.

Result VI: Genotype and allelic frequencies of +45T/G locus

The distributions of the genotypes conformed to the Hardy-Weinberg equilibrium in healthy controls and knee OA patients (*P*>0.05). The genotypes and allele frequencies of adiponectin gene SNPs in the whole population study are presented in **Table 13**. A statistically significant difference between healthy controls and knee OA patients groups could not be found in +45T/G (rs2241766) genotypes (*P*=0.267). The T allele frequency was 68.11 % in control group and 64.60% in OA group, and the G allele frequency was 31.89% in control group and 35.40% in OA group (*P*=0.295). According to statistical analysis, the genotype distributions and allele frequencies of +45T/G (rs2241766) polymorphism were not associated with the risk of knee OA in our studied population.

Table 13 Genotype distributions, allele frequencies of adiponectin gene +45T/G (rs2241766) SNP in control and OA groups

+45T/G (rs2241766)	SNP	Control		OA		OR (95%CI)	*P*
		n	%	n	%		
genotype	TT	96	48.98	84	41.60	1	-
	TG	75	38.27	93	46.00	1.417 (0.929-2.162)	0.106
	GG	25	12.76	25	12.40	1.143 (0.611-2.139)	0.676
allele	T	267	68.11	261	64.60	1	-
	G	125	31.89	143	35.40	1.170 (0.872-1.571)	0.295

Result VII: Genotype and allelic frequencies of +276G/T locus

The distributions of the genotypes conformed to the Hardy-Weinberg equilibrium in healthy controls and knee OA patients (*P*>0.05). The genotypes and allele frequencies of adiponectin gene SNP in the whole population study were presented in **Table 14**. A statistically significant difference between healthy controls and knee OA patients could not be found in +276G/T (rs1501299) genotypes (*P*=0.889). The equivalent statistical result was indicated in the allele frequencies of +276G/T (rs1501299) SNP. The G allele frequency was 71.68% in control group and 71.29% in OA group, and the T allele frequency was 28.32% in control group and 28.71% in OA group (*P*=0.9014). There was no association between the genotype distributions or allele frequencies of the +276G/T polymorphism and the risk of knee OA in our studied population.

Table 14 Genotype distributions and allele frequencies of the adiponectin gene +276G/T (rs1501299) SNP in control and OA groups

+276T/G rs1501299	SNP	Control		OA		OR(95%CI)	*P*
		n	%	n	%		
genotype	GG	102	52.00	106	52.50	1	-
	GT	77	39.30	76	37.60	0.950 (0.626-1.442)	0.809
	TT	17	8.70	20	9.90	1.132 (0.561-2.283)	0.729
allele	G	281	71.68	288	71.29	1	
	T	111	28.32	116	28.71	1.020 (0.750-1.387)	0.901

Result VIII: Haplotype distributions of +45T/G and +276G/T loci

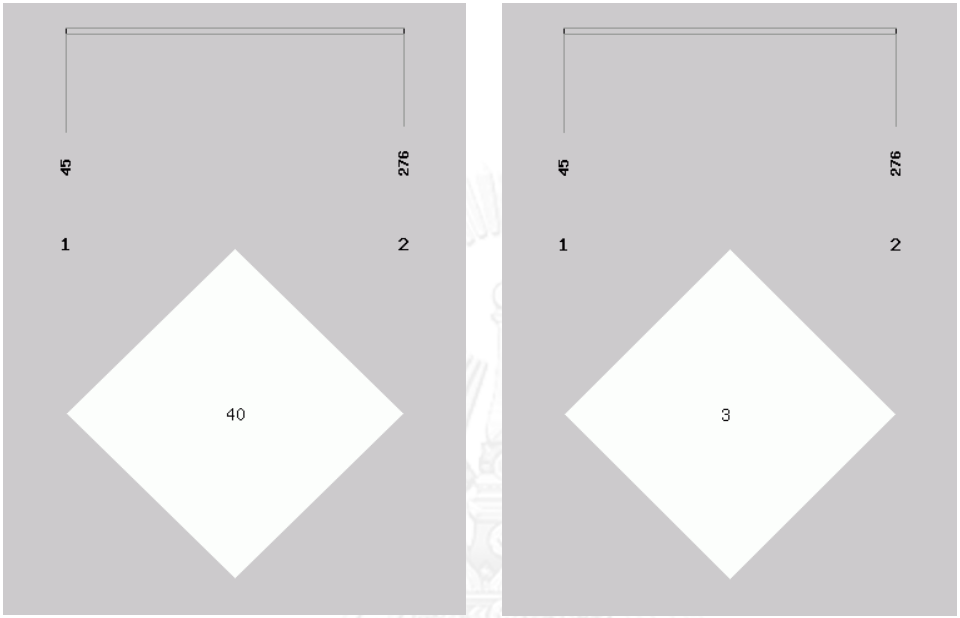
All frequencies of haplotypes TG, TT, GG and GT were less than 50% in knee OA patients and healthy controls. The frequencies of the haplotype TG were highest percentages in both control group (45.10%) and in OA group (42.00%). However, *P*<0.05 could not be found in haplotype frequency analysis (**Table 15**).

Table 15 Haplotype distributions of adiponectin polymorphisms (+45T/G and +276G/T) in the control and OA groups

Haplotype		Control		OA		OR (95% CI)	*P*
+45T/G locus	+276G/T locus	n	freq.	n	freq.		
T	G	176.90	0.451	169.66	0.420	0.880 (0.665-1.165)	0.373
T	T	90.10	0.230	91.34	0.226	0.979 (0.703-1.363)	0.900
G	G	104.10	0.266	118.34	0.293	1.146 (0.840-1.562)	0.390
G	T	20.90	0.053	24.66	0.061	1.154 (0.633-2.103)	0.640

Result IX: Linkage disequilibrium test of +45T/G and +276G/T loci

For haplotype analysis with +45 T/G and +276 G/T loci polymorphisms of the adiponectin gene, the linkage disequilibrium of them is shown in **Figure 8**.



D': 0.403 r^2: 0.033

Figure 8 Haplotype analysis of SNPs +45T/G (rs2241766) and +276G/T (rs1501299) in Thai population. Linkage disequilibrium (LD) in subjects is represented as a white square (SHEsis Software, ver. Online & Haploview software version 4.1). The correlation coefficient of the frequencies r^2 is 0.033.

Result X: The OA severity and +45T/G locus genotypes

As demonstrated in **Table 16**, the genotypes of the adiponectin gene +45T/G (rs2241766) among different radiographic severity of OA patients were investigated. There were no significant differences between KL grade 2 and KL grade 3 at +45T/G (rs2241766) genotypes (*P*=0.414), as well as between KL grade 2 and KL grade 4 (*P*=0.360), and between grade 3 and 4 (**P*=0.995). The allele frequency of the +45T/G (rs2241766) SNP was no significant differences (*P*=0.338 was not listed in this table).

Table 16 Based on radiographic severity of OA, genotype distribution of adiponectin gene +45T/G SNP in OA patients

OA severity	Genotype			*P*	**P*
	TT	TG	GG		
grade 2	27	21	6	-	-
grade 3	28	36	7	0.414	-
grade 4	29	36	12	0.360	0.995

P value for difference in distribution of genotype between grade 2 and grade 3 or grade 4. **P* value for genotype distributions between grade 3 and grade 4.

Result XI: The OA severity and +276 G/T locus genotypes

In **Table 17**, the association between genotypes of the adiponectin gene +276G/T (rs1501299) SNP and radiographic severity of OA patients was analyzed. There were significant differences between KL grade 2 and KL grade 3 at +276G/T (rs1501299) genotypes (*P*=0.037), as well as between KL grade 2 and KL grade 4 (*P*=0.046). Moreover, there was a statistically significant difference between KL grade 2 and the genotype summation of grade 3 and 4 (*P*=0.016). However, there was no significant difference between KL grade 3 and 4 (*P*=0.906). The allele frequency of +276G/T (rs1501299) SNP was not significant different.

Table 17 Based on radiographic severity of OA, genotype distribution of adiponectin gene +276 G/T SNP in OA patients

OA severity	Genotype			*P*	**P*
	GG	GT	TT		
grade 2	20	29	5	-	-
grade 3	40	22	9	0.037	-
grade 4	44	25	8	0.046	0.906

P value for difference in distribution of genotype between grade 2 and grade 3 or grade 4. **P* value for genotype distribution between grade 3 and grade 4.

Result XII: The BMI classification and +45T/G or +276G/T locus genotypes

The population of this study was divided into two subgroups based on their body mass index: BMI<25kg/m^2 and BMI⩾25 kg/m^2. The genotypes and allele frequency of the adiponectin gene polymorphisms according to BMI are presented in **Table 18**. In the BMI<25 kg/m^2 subgroup, there was significant difference in the genotype of +45T/G polymorphism (*P*=0.023). But in the BMI⩾25 kg/m^2 subgroup of same locus, the *P* value was greater than 0.05 (*P*=0.551). There were no statistically significant differences of genotypes between two groups in BMI<25 and BMI⩾25 kg/m^2 subgroups (*P*=0.204, *P*=0.279, respectively) of +276G/T (rs1501299) polymorphism. In addition, there were no significant differences of all frequencies between the control group and OA group in BMI<25 and BMI⩾25 kg/m^2 subgroups.

Table 18 Based on BMI<25 and BMI⩾25, association between the genotype and allele frequencies and the risk of osteoarthritis in control and OA groups.

		BMI<25		*P*	BMI⩾25		*P*
		controls	OA		controls	OA	
+45T/G genotype	TT	34	28		13	55	
	TG	24	31	0.023	20	55	0.551
	GG	9	1		6	22	
+45T/G allele	T allele (%)	92 (68.66)	87 (72.50)	0.571	46 (58.97)	165 (62.50)	0.542
	G allele (%)	42 (31.34)	33 (27.50)		32 (42.03)	99 (37.50)	
+276G/T genotype	GG	41	28		22	72	
	GT	22	29	0.204	16	46	0.279
	TT	4	3		1	14	
+276G/T allele	G allele (%)	104 (77.61)	85 (70.83)	0.216	60 (76.92)	190 (71.97)	0.433
	T allele (%)	30 (22.39)	35 (29.17)		18 (23.08)	74 (28.03)	

P value for difference in distribution of genotypes or alleles between control group and OA group.

Result XIII: Adiponectin levels in healthy controls and knee OA patients

Plasma adiponectin concentrations of healthy controls and knee OA patients were shown in **Figure 9**. The mean value of plasma adiponectin in OA group was lower than that of the control group, and the difference was statistically significant (2.58±0.60 vs. 2.78±0.68 μg/ml, *P*=0.033).

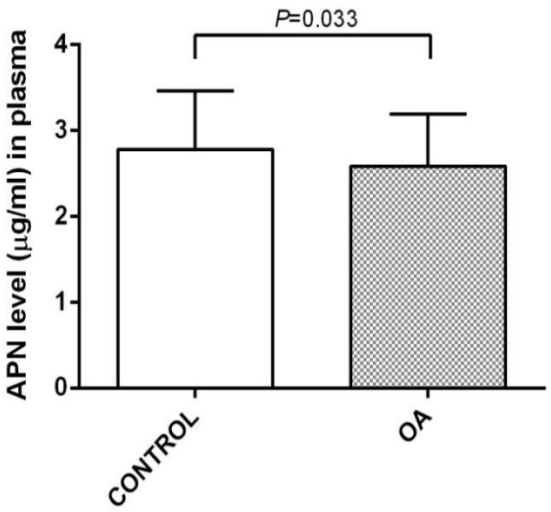


Figure 9 Adiponectin levels in plasma between control and OA groups.

Result XIV: Adiponectin levels in females and males

In view of gender, that females had higher prevalence percentage of knee OA than males, the association of plasma adiponectin levels and gender were analyzed as shown in **Figure 10**. Compared with adiponectin levels in plasma of males, those of females were higher in the control group, OA group and total group (P<0.001, P=0.04, P=0.001). As well, adiponectin levels in plasma of females were significantly different between the control group and OA group (P<0.001). However, plasma adiponectin levels of males were not significantly different between control group and OA group (P=0.286).

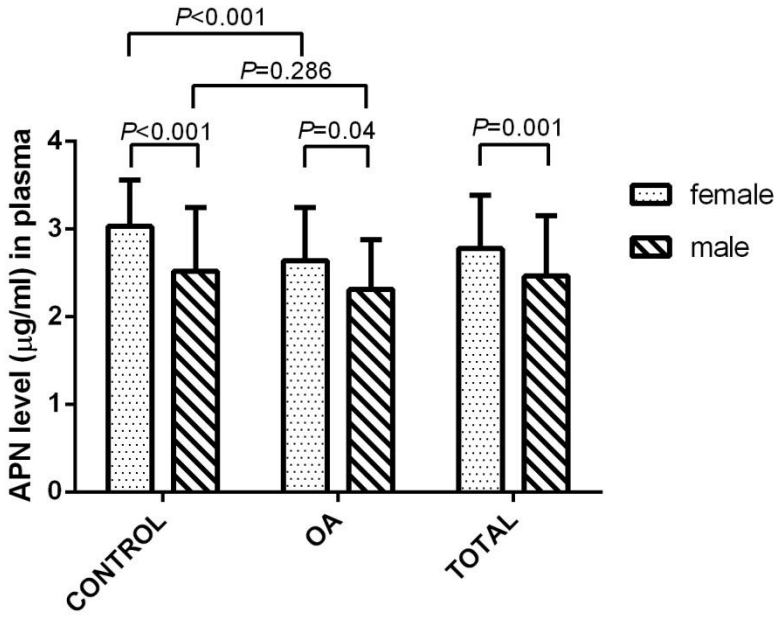


Figure 10 Comparison of plasma adiponectin levels between female and male in control group, OA group and total subjects.

Result XV: The OA severity and adiponectin levels

As demonstrated in **Figure 11**, the values of plasma adiponectin levels in the control group and KL grade 2, 3, 4 groups were 2.78±0.68, 2.57±0.56, 2.65±0.51, 2.53±0.76 μg/mL, respectively. The differences were not statistically significant (*P*>0.05).

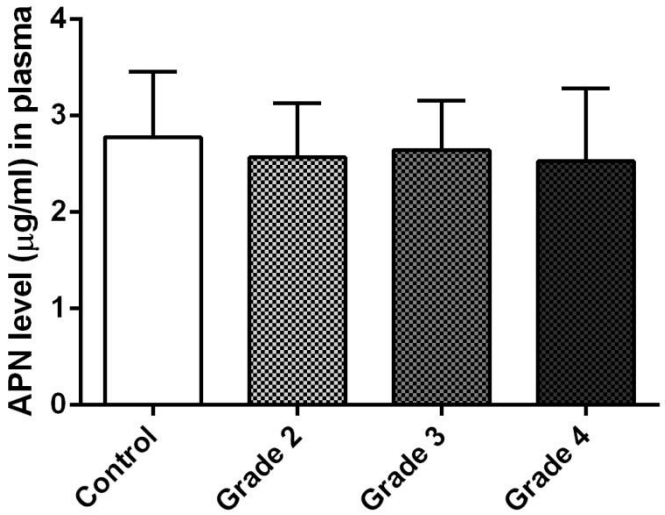


Figure 11 Plasma adiponectin levels of OA patients severity classified by the Kellgren-Lawrence grading scale and healthy controls.

Result XVI: +45T/G genotypes and adiponectin levels

The plasma adiponectin concentrations of three different +45 T/G locus genotypes (T/T, T/G and G/G) in healthy controls and knee OA patients are shown in **Figure 12**. In OA group, the mean values of plasma adiponectin in the TT, TG and GG were 2.63±0.66, 2.57±0.53, 2.50±0.77 μg/ml, of which the differences were not statistically significant ($P>0.05$). In control group, the mean value of plasma adiponectin in the TT was lowest among the three genotypes (2.66±0.66, 2.78±.69, 3.16±.63 μg/ml, respectively). A significant difference was found between the GG and TT genotypes ($P=0.019$), suggesting that plasma adiponectin levels of GG genotype were significantly higher than those of the TT genotype at the +45T/G polymorphism of control group.

In the GG genotype of the +45T/G locus, the mean value of plasma adiponectin of control group was higher than that of OA group, and the difference was statistically significant ($P=0.029$).

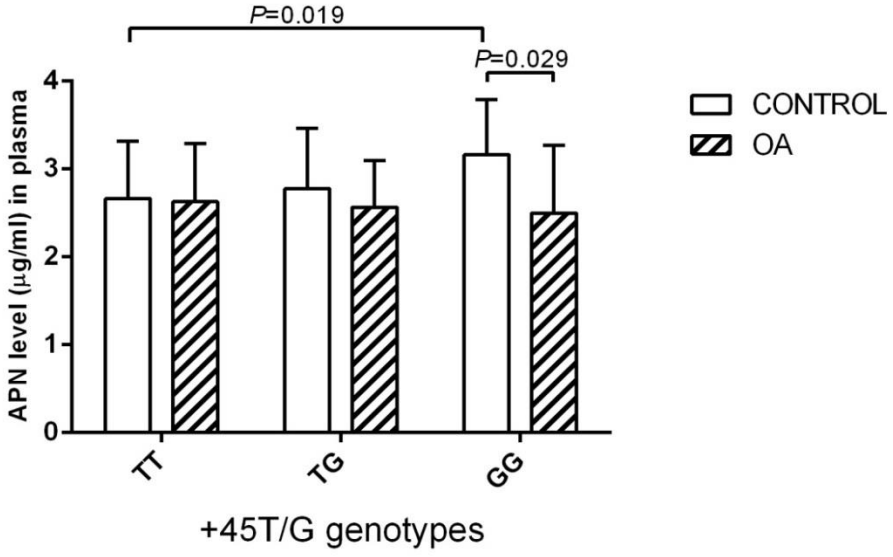


Figure 12 Genotypes of +45T/G locus and their plasma adiponectin levels in control group and OA group.

Result XVII: +276G/T genotypes and adiponectin levels

The plasma adiponectin concentrations of three different +276 G/T locus genotypes (T/T, T/G and G/G) in healthy controls and knee OA patients are shown in **Figure 13**. In OA group, the mean values of plasma adiponectin in the TT, GT and GG were 2.92±0.57, 2.58±0.63 and 2.53±0.59 μg/ml, of which the differences were not statistically significant (P>0.05). In control group, the mean value of plasma adiponectin in TT was lowest among three genotypes (2.34±0.88, 2.81±0.66, 2.84±0.63 μg/mL, respectively). The significant difference was found between GG and TT genotype (P=0.046) indicate that plasma adiponectin level of GG genotype was significantly higher than that of TT genotype at +276G/T polymorphism of healthy controls.

In GG genotype of +276 G/T locus, the mean value of plasma adiponectin of control group were significantly than those of OA patients group, and the difference was statistically significant (P=0.012).

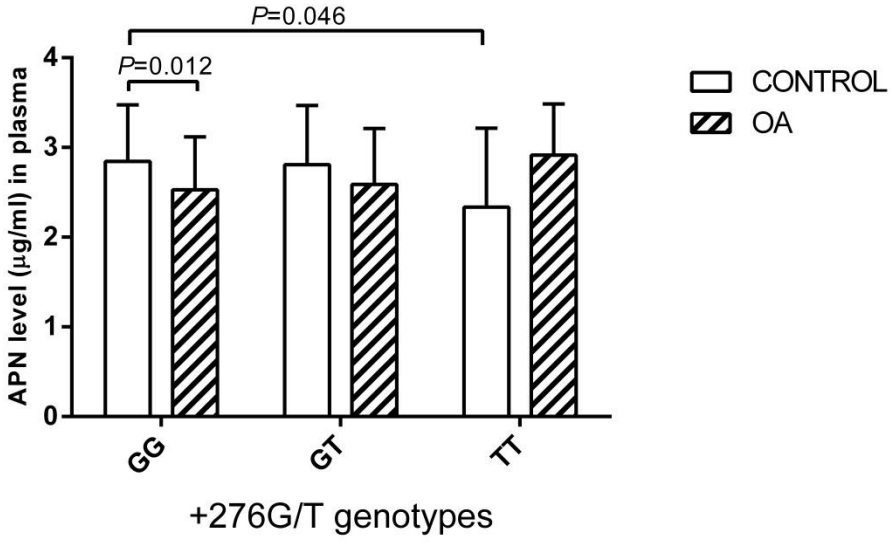


Figure 13 Genotypes of +276 G/T locus and their plasma adiponectin levels in control group and OA group.

CHAPTER V
DISCUSSIONS & CONCLUSIONS

Discussions

This study is the first to examine possible interactions between BMI and adiponectin gene polymorphisms for knee osteoarthritis as well as between radiographic severities of OA. It is additionally the first to investigate the adiponectin levels in plasma of healthy controls and knee OA patients. The population of this study was ethnically homogeneous according to the Hardy-Weinberg equilibrium, which makes the possibility of confounding ethnic heterogeneity less possible. Compared with other diseases, OA is a polygenic disease on the basis of the epidemiologic and genetics study.

Depending on the study of knee OA, overall prevalence of radiographic knee OA in American subjects 45 years of age and older varies between 19.2% and 27.8%, with women more predisposed than men. Similarly, in the largest European survey, the Zoetermeer Community Survey in the Netherlands, the prevalence of radiographic knee OA was higher in women than in men of 45 years and older with 22,800 participates compared to 14,100 out of 100,000 citizens [141]. A more global perspective of knee and hip OA prevalence has also been reported by the World Health Organization [95]. In NHANES III, the overall prevalence of knee OA worldwide increased to 37.4% in subjects 60 years of age and older. It is likely that the protocol employed for NHANES III knee examination biased the prevalence estimates to be lower than the true population values, since only a single anterior/posterior non-weight-bearing radiograph for each knee was examined [96]. As did the first national Health and Nutrition Examination Survey (HANES I) [97], NHANES III also reported that the prevalence of radiographic knee OA was significantly higher in non-Hispanic African Americans than in non-Hispanic Caucasians or Mexican Americans (52.4%, 36.2%, and 37.6%, respectively) [96]. More recent studies have demonstrated that there was significantly greater severity of knee OA in African Americans than in Caucasians [98]. This study evaluated not only the tibiofemoral but also the patellofemoral compartment and demonstrated an increase of tricompartmental disease in African Americans. In fact, care must be taken when evaluating

radiographic prevalence of knee OA. Indeed, most studies evaluated the tibiofemoral compartment, thus underestimating the tibiopatellar compartment, which can be a major source of radiographic and clinical OA, particularly in a younger population [99].

With the help of twin studies, the importance of genetic determination of cartilage volume was demonstrated [100], as well as the heritability of OA progression [101]. Linkage analysis, a method based on the tendency of several loci to be inherited together, revealed a large number of genomic regions on many different chromosomes including the X chromosome, which might lead to OA susceptibility [102]. Moreover, genetic studies exploring either the entire human genome or regions have already shown an association with OA, aim to identify OA-specific loci. A summary of the most recent data revealed that the gene variants identified so far have only a minor effect size and are not suited to be clinically useful biomarkers [102]. However, the combination of several genes might be of help in future studies to identify individuals at high risk for progression and adjust the treatment strategies according to their genetic risk factors.

Knee osteoarthritis is very common in the elderly. Consistently, our study showed that there was a significant difference of population age between knee OA patients and healthy controls. The mean age of knee OA patients was higher than that of healthy controls. However, it cannot be concluded that OA is a consequence of ageing. The relationship between ageing and osteoarthritis results from age-related changes in multiple components of the musculoskeletal system rather than ageing itself [103]. Aging of chondrocytes and their matrix affect stability of homeostasis to release pro-inflammatory mediators and matrix degrading enzymes under oxidative stress. A previous study showed that an increase of reactive oxygen species (ROS) levels could be related with the aging of chondrocytes [5, 104]. Increased ROS levels through signal pathways could decrease matrix synthesis, inhibit growth factors expressions and increase the productions of MMPs (Matrix Metalloproteinases) and cytokines that result in matrix loss and OA occurrence [103]. These related cell signal pathways including Mitogen-Activated Protein (MAP) kinase pathways, which include Extracellular signal-Regulated kinases (ERK), Jun N-terminal kinases (JNK), p38 [105] and the phosphoinositide 3 kinase-serine threonine kinase/protein kinase B (PI-

3K-Akt/PKB) pathway, which is necessary for chondrocyte proteoglycan synthesis, and the mitogen activated protein kinase/extracellular regulated kinase (MEK-ERK) MAP kinase pathway, which inhibits proteoglycan synthesis [103]. Aging changes including excessive levels of ROS could play an important role in the imbalance of anabolic and catabolic signaling. An alteration in the level of anabolic and catabolic activity represents a loss in homeostasis. A number of the chronic degenerative conditions associated with aging appear to result from an age-related loss in the ability of cells and tissues in the body to maintain homeostasis, particularly when put under stress. OA is rare in young adults and even serious joint injuries rarely manifest as OA until years later, suggesting that young joint tissues can compensate, to some degree, to abnormal mechanical stress. But with aging, the ability to compensate and maintain homeostasis declines. The elderly who experience a joint injury develop OA much more rapidly than younger adults with a similar injury [106]. In the cartilage matrix and chondrocyte, variations related with age result in the tissues around the knee joint are unable to the keep balance of anabolic and catabolic maintenance as mechanical stress leads to matrix loss and destruction. Age-related oxidative stress and damage may play a central role in cartilage aging through modulation of cell signaling pathways that regulate anabolic and catabolic activity. Although the use of general anti-oxidants as therapies for aging-related diseases has not met with much success to date, it is possible that modulating the activity of a specific set of redox-regulated pathways may be more effective.

Adiponectin is derived from adipocytes, has anti-inflammatory and anti-atherogenic effects, as well as multiple beneficial effects on metabolism [107]. Previous studies demonstrated that adiponectin modulated the function and phenotypes of macrophages in chronic inflammation [17] and suppressed the production of TNF-alpha [16]. Moreover, it was shown that adiponectin up-regulated tissue inhibitor of metalloproteinases-2 (TIMP-2) [108] and down-regulated IL-1β-induced MMP-13 [109], protecting cartilage from degeneration. Furthermore, Honsawek S *et al.* have reported that as a protective factor, adiponectin in plasma and synovial fluid decreased significantly with increasing of OA severity.

Some studies on the pathogenesis of OA support that adiponectin plays a role in inflammation and cartilage destruction in osteoarthritis. Adiponectin levels in plasma were positively correlated with biomarkers of OA, such as MMP-3, either adiponectin-induced the expression of nitric oxide NO, IL-6 and MMP-1, 3 in OA cartilage and in primary chondrocytes in vitro by the mitogen-activated protein kinase (MAPK) pathway. However the pathogenesis of OA is controversial. From this study, plasma adiponectin concentration in the control group was greater than that of the OA patients group. The positive function of adiponectin in bone biology was supported by other studies showing that this hormone and its receptors are expressed in osteoblasts [110, 111]. Adiponectin has also been found to stimulate the proliferation and mineralization of osteoblasts via the AdipoR1- and AMP-activated kinase (AMPK) signaling pathway in autocrine and/or paracrine fashions [112]. Adiponectin seems to stimulate the receptor activator of nuclear factor k B ligand (RANKL) pathway, to inhibit the production of osteoprotegerin (OPG) in human osteoblasts, and to indirectly promote osteoclastogenesis [113]. Moreover, adiponectin has been shown to bind to some growth factors [114]. Adiponectin was also found to enhance bone morphogenetic protein-2 expression in osteoblastic cells, through the involvement of the AdipoR1, AMPK, p38, and NF-κB signaling pathways [115]. In another study using primary human and rat osteoblasts, adiponectin stimulated osteoblast growth but inhibited osteoclastogenesis, probably via an effect on stromal cells [116]. Interestingly, the adiponectin knockout mouse displayed increased bone mass, suggesting that adiponectin may also have indirect effects on bone, possibly through modulating growth factor action or insulin sensitivity [116]. In the mouse ATDC5 cell line, an *in vitro* model of chondrogenesis, adiponectin increased chondrocyte proliferation, proteoglycan synthesis, and matrix mineralization, through upregulation of the expression of type II collagen, aggrecan, Runx2, type X collagen, alkaline phosphatase and MMP-9 activity, suggesting the possibility of a direct role in chondrocyte proliferation and differentiation [117]. Experimental studies also showed contrasting effects of adiponectin on joint inflammation. Recent reports showed that adenovirus-mediated gene transfer of

adiponectin reduced the severity of collagen-induced arthritis in mice, thus preventing inflammation and joint destruction [118].

The significant increase in OA prevalence in post-menopausal women has led to many investigations of the hormonal implication in the pathophysiology of OA. Although conflicting data have been shown [119], there is now increasing evidence that estrogen influences the activity of joint tissues through complex molecular pathways that act at multiple levels [120]. On the other hand, it was also reported that women on estrogen replacement therapy were less likely to require total knee replacement [121, 122]. Indeed, those who had taken estrogen for 10 years or longer had a greater decrease in the risk for hip OA; however, there was no significant reduction in disease symptoms [122]. Our study examined plasma adiponectin levels which of female from control group was greater than those from OA group. Given that low-circulating adiponectin levels are associated with increased body fat [123], it was hypothesized that this adipokine may contribute to the metabolic changes occurring during the transition to menopause. Contradictory findings have been reported with respect to adiponectin levels across the menopausal transition. Previous studies showed a decrease in adiponectin levels in postmenopausal women [124]. Adiposity levels may also account for these differences, as lower serum adiponectin levels were earlier reported in obese than in normal weight postmenopausal women [125]. Moreover, Soni *et al.* [126] found higher circulating adiponectin levels with increasing weight loss in overweight postmenopausal women undergoing an 18-month lifestyle intervention trial. Interestingly, adiponectin was found to be correlated negatively with changes in intra-abdominal fat but not with subcutaneous adipose tissue or total percent body fat in healthy pre- and post-menopausal women [127]. As central obesity is often linked with insulin resistance, type 2 diabetes and the risk of OA.

Until now, two-loci polymorphisms in the adiponectin gene have been investigated widely, +45T/G (rs2241766) in exon 2 and +276G/T (rs1501299) in intron 2. The two loci polymorphisms have been identified to associate with a number of diseases related with metabolism and inflammation. Whether these polymorphisms can affect the susceptibility of knee osteoarthritis is still to be defined. In the current

study, we analyzed the effect of +45T/G (rs2241766) and +276G/T (rs1501299) polymorphisms on the risk of knee OA in a Thai population. Our findings indicated that the frequency of alleles and the distribution of genotypes were not significantly different between the control group and OA group. An association has been found between adiponectin concentration and OA severity that worked in concert with the previous finding that +276G/T (rs1501299) were significantly associated with fasting serum adiponectin levels in the Chingford study [128] by Kyriakou T *et al.* However, the samples of their study were collected from Caucasians and adiponectin concentration were assayed with overnight fasting serum, because variations of SNPs can occur in different populations and because different requirements in blood sampling may influence the adipocyte metabolism inducing different adiponectin concentration. Polymorphisms of adiponectin promoters also interact with SNP loci to affect adiponectin levels.

Obesity has been assumed as an additional impact on mechanical or non-mechanical destruction for the joints, as well as those findings that demonstrated the importance of the metabolic effect and inflammatory process of obesity in OA pathophysiology. In French population [129], a study implicated that the +45T/G polymorphism was associated with body mass index (BMI) in non-diabetic subjects and G allele was associated with higher BMI. In contrast, a study of Guzman-Ornelas MO. *et al.* suggested that that +45T/G polymorphism is not associated with BMI in a Mexican-Mestizo population [107]. Besides the +45T/G polymorphism, a nearby +276G/T polymorphism was not associated with body weight in Italian non-diabetic subjects, but the T allele of +276G/T was associated with higher BMI [108]. However, the G allele of the +276G/T was presented to have an association with higher BMI in Greek women with polycystic ovary syndrome (PCOS) [109]. In a Swedish obesity study that suggested the +45T/G and +276G/T polymorphisms were associated with obesity [128]. Consistently, our study indicated that the GG genotype distribution of +45T/G was significantly different between the control group and OA group in BMI<25 kg/m^2. According to this interesting result, we demonstrated that the GG genotype of +45T/G polymorphism exhibited a positive association for decreasing the risk of OA in a non-obese population. Alleles of +45T/G polymorphism did not play

similar functions. Nevertheless, our study also indicated that the genotype distribution and the allele frequency of +276G/T polymorphisms were not significantly different between the OA patients and controls in BMI<25 and BMI≥25 kg/m^2 classes. This statistical result is supported by a previous report which indicated a dose-response relationship between BMI and progression of knee OA was deficient [129]. Body mass index (BMI) could not be changed with the variance of body composition, probably because fat mass increases at the same time skeletal muscle mass decreases. This development between fat mass and skeletal muscle mass give rise to sarcopenic obesity, in which the weight or BMI variation of OA patients could not be observed. Lee S. *et al.* demonstrated the association between sarcopenic obesity and knee OA was closer than non-sarcopenic obesity [130]. Their study confirmed our experimental data and statistical results that BMI did not associate with the genotype distribution and allele frequency of +276G/T polymorphisms between controls and OA patients.

Two common variants of the +45T/G and +276 G/T polymorphisms have been widely studied. The results were conflicting, with the association dependent on both the location and type of sample. The T allele of +45T/G has been associated with lower serum adiponectin in samples from French [131], and Canadian healthy volunteers [132]. Low allele-specific mRNA expression of the T allele has also been demonstrated in adipose tissue taken from healthy volunteers from Taiwan [133]. In these cohorts the +45T was associated with T2DM, and adverse lipid profiles in obesity, respectively. However, the other allele, the G allele of +45G/T SNP has been associated with lower serum adiponectin in the healthy Amish [134], Swedish women [135], and healthy Spanish [136]. The G allele of +45G/T locus was also associated with obesity in Swedish individuals [128]. According to our findings at +45T/G polymorphism, T allele and G allele frequencies (68.11% and 31.89%) of control group were less than those of 1000 genomes allele frequencies from the world (86.00% and 14.00% were shown in **Figure 14**). The allele percentages were changed by about 4.00% and closer to the Asian allele frequencies (72.00% and 28.00%) from 1000 genomes (**Figure 14**). However, the allele frequencies of the Ensemble project were composed of the Chinese and Japanese population. The allele frequencies of

Thai population were not investigated before our study at +45T/G polymorphism. Furthermore, the GG genotype of the +45T/G polymorphism was associated with higher adiponectin levels in plasma than the TT genotype, but only in control group. Additionally, our study revealed that plasma adiponectin level in OA group was significantly lower than control group with same GG genotype at +45T/G locus. Consistently, GG genotype carriers had higher serum adiponectin levels and TT genotype carriers had a higher total body fat mass and biceps and triceps skinfold thickness [107]. The distribution of body fat storage can play different roles in adiponectin production and/or function during the maintenance of the inflammatory process in obesity. As a result of this association of the polymorphic allele with distribution of body fat storage, they suggest that genetic variation in the ADIPOQ gene may modulate the levels of serum adiponectin.

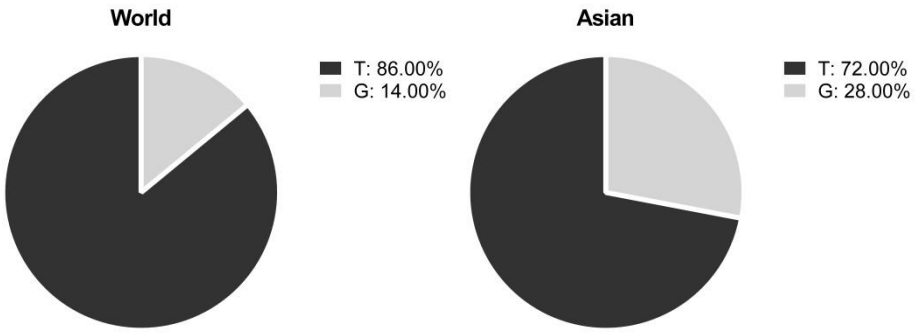


Figure 14 The 1000 genomes allele frequencies at +45T/G (rs2241766) polymorphism from the Ensemble project (www.ensemble.org).

For the +276G/T polymorphism, different associations showed in different samples. The type G allele was associated with lower serum adiponectin (France, Greece, and Spain) and also low serum adiponectin in the health professionals follow up study in the United States [137]. However, the type T allele had been associated with lower serum adiponectin from healthy volunteers in Italy-Lazio [108]. Our study investigated G allele and T allele frequencies (71.68% and 28.32%) of control group, which were varied by about 3.00%, compared with those of 1000 genomes allele frequencies (68.00% and 32.00%) from the world (**Figure 15**). The allele percentages were consistent with the Asian allele frequencies (71.00% and 29.00%) from 1000 genomes (**Figure 15**). But genome frequencies of the Asia were

composed of the Chinese and Japanese population. Our study first investigated allele frequencies at +276G/T locus of adiponectin gene in Thai population. Furthermore, our study showed that the GG genotype of +276G/T polymorphism was associated with higher adiponectin levels in plasma than the TT genotype, but only in control group. Moreover, our study presented that plasma adiponectin level of OA group was lower than control group with the same GG genotype at +276G/T locus. A previous study demonstrated a decline in adiponectin in all genotype groups with the greatest decline among those carrying the rare T allele of the +276 G/T SNP. Interestingly, for severity of knee OA patients, the adiponectin genotype at +276 was significantly different between KL grade 2 and grade 3 or 4, which demonstrated that OA patients of the GG genotype were more likely to develop OA severity in KL grade than those of the GT and TT genotypes. An association has been found between the adiponectin concentration or genotype at the +276 G/T (rs1501299) and OA severity which worked in concert with the previous finding that the T allele of +276G/T (rs1501299) was significantly associated with elevated serum adiponectin levels in diabetic men by Lu Qi *et al*. [21] and the Chingford study [138] by Kyriakou T *et al*. In general, lacking the T allele, the GG genotype in OA patients could not play a role as a regulator in the expression of adiponectin leading to a progression in OA severity and increasing of KL grade.

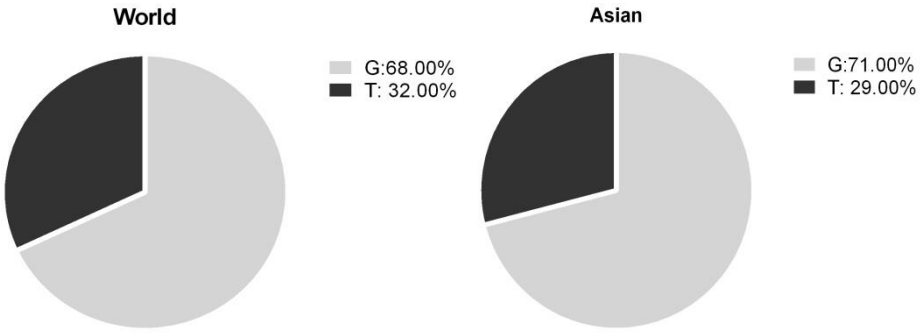


Figure 15 The 1000 genomes allele frequencies at +276G/T (rs1501299) polymorphism from the Ensemble project (www.ensemble.org).

How the +276G/T SNP affects the adiponectin gene function is still questionable. The possible effect on gene expression of SNPs with no apparently biological significance cannot be ruled out and, in fact, it has been recently reported

for the adiponectin gene [133]. It appears more likely that this SNP is in linkage disequilibrium with another mutation either within, or in other genes close to, the adiponectin gene that determines its negative effects. One study had previously shown that the +276G/T SNP is in almost complete linkage disequilibrium with an 'A' insertion in the 3' UTR of the adiponectin gene (SNP +2019) [139]. 3' UTR regions are generally recognized to play a central role in the regulation of gene expression and the +2019 insertion may disrupt one of the regulatory elements of the adiponectin 3'UTR region, affecting mRNA processing, translation or degradation. Interestingly, polymorphisms in the 3'UTR of other genes affected mRNA stability [142].

When these SNPs have been combined to generate a risk haplotype, the two SNPs were found to be in strong linkage disequilibrium in Italy [139]. A risk haplotype was generated and much stronger associations were observed when carriers were compared with non-carriers. The haplotype was associated with higher body weight, waist circumference, blood pressure, and HDL/total cholesterol ratio. The haplotype was also associated with lower serum adiponectin and higher risk of T2DM in American participants [139]. The reason for this variation is unclear. Although transcription enhancers have been described in adiponectin gene introns [140], the variability of the associations with the +45 and +276 SNPs and their position and effect on transcription (exon 2 and intron 2, where no regulatory sites have been described) suggests that the SNPs are either in linkage disequilibrium with another functional SNP for which they act as markers or they are being influenced by the environment. While the linkage disequilibrium is different between racial groups, it is unlikely to be so in subjects from Europe. Interaction between genotype and environmental factors, such as obesity should, therefore, be considered in interpreting these results.

The discrepancy persists in several studies regarding to the association of the SNP with OA and many possibilities are conceivable for further research. The susceptibility of candidate genes for OA has previously been demonstrated by some studies, but variants will be controversial by other researchers. This study included a relatively small number of participants in this single-center trial study. It is necessary to conduct additional observations under administration of multiple centers with a

larger increased sample size. Multiple risk factors contribute to osteoarthritis including mechanical stress, inflammation, obesity, aging, and genetic alteration. The susceptibility of OA could vary in different populations. Environmental factors may influence the genetic contributions to the susceptibility of OA. Haplotype analysis is beneficial to explain the functional variation responsible for adiponectin expression. In the future, more research in *in vitro* cell culture is needed to elucidate the function of adiponectin in OA. Additional investigation *in vivo* is required to observe the association between other SNPs and OA.

General Conclusions

In summary, our study suggested that the +45T/G (rs2241766) and +276G/T (rs1501299) polymorphisms were not associated with knee OA susceptibility in our Thai population. However, the GG genotype of the +45T/G (rs2241766) polymorphism is demonstrated to decrease the risk of OA in a Thai population with BMI<25 kg/m2. The knee OA patients with the adiponectin GG genotype at the +276G/T locus seemed to have a higher potential risk in the severity of OA than those having the GT and TT genotypes. High adiponectin in Thai plasma probably play a protective role in the pathogenesis of knee OA, especially in Thai women. The GG genotypes of the +45T/G (rs2241766) and +276G/T (rs1501299) polymorphisms were associated with adiponectin levels in plasma between healthy controls and knee OA patients. All these studies encourage us to further discover the importance of adiponectin and its single nucleotide polymorphisms in osteoarthritis.

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APPENDIX

VITA

Personal Information:

Full Name: Dong Zhan Nationality: P.R .China, the Han

Date of Birth: December, 1984 Birth Place: Yunnan Province

Sex: Male Marital Status: Married

Height: 174cm Weight: 67kg

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Work Experience:

July, 2008-Present: Anatomy Department of Kunming Medical University, as a teacher, making fifties of qualified and wonderful dissections for our exhibition hall and teaching progression by myself with the warmhearted and patient help and guidance of our leaders, professors and teachers.

October,2009-April,2010: Department of Anatomy, National University of Singapore, as a dissector, help them make 20 samples and one of my article had been published.

Education background:

June, 2012-Present: Faculty of Medicine, Chulalongkorn University in Thailand, study medical sciences for Master Degree and research about osteoarthritis.

September, 2003-July, 2008: Bachelor of Clinical Medicine, School of the Clinical Medicine, Kunming Medical University, Yunnan Province

Social Practice:

October 20-22th, 2013**:** made a short presentation about "association between adiponectin gene polymorphism and knee osteoarthritis" in 35th Annual

Meeting of RCOST (the royal college of orthopaedic surgeons of Thailand) on Pattaya, Thailand.

December, 2006-December, 2007: Kunming General Hospital of People Liberty Army, as an internship doctor studying diagnoses and treatments against all kinds of diseases and improving clinical skills.

March 8th, 2007: WORLD KIDNEY DAY, disseminated harms of primitive and chronic kidney, had people knowing how to prevent chronic kidney disease in our daily lives and let policy-makers and doctors pay more attention to "this silent killer", with doctors from Kunming General Hospital of P.L.A.

October,2006-January, 2007**:** made a dissection, with other two classmates, exposed whole human body arteries, which was placed in the human anatomical exhibition hall of KMMU.

2005-2006: took part in the special team, organized by teachers of Anatomy Department of KMMU, in which all of students were interested in human anatomical knowledge and dissection operating technique. We completed a number of samples with teachers' assistance, such as coronary arteries, spinal cord, moving out encephalon, etc.

2004-2005**:** as a tutor, helped four Junior and Senior Middle School students with their mathematics, physics and biology, twice a week, respectively.

Personal Honors:

2004 The third grade scholarship of Kunming Medical University

2005 The outstanding servers' scholarship of Kunming Medical University

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2007 The third grade scholarship of Kunming Medical University

2008 The Superior Graduate of Kunming Medical University in

Published Papers:

1. Zhan D, Yuktanandana P, Anomasiri W, Tanavalee A, Honsawek S. Association of adiponectin+ 276G/T polymorphism with knee osteoarthritis. Biomedical Reports 2014;2:229-32.

2. Zhan D, Zhao Y, Sun J, Ling E-A, Yip GW. High origin of radial arteries: a report of two rare cases. The Scientific World Journal 2010;10:1999-2002.

BIOMEDICAL REPORTS

Association of adiponectin +276G/T polymorphism with knee osteoarthritis

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Abstract. Osteoarthritis (OA) is a prevalent, degenerative joint disorder leading to the destruction of articular cartilage, osteophyte formation and subchondral bone sclerosis. Genetic and environmental factors are involved in the development of OA. The role of adiponectin gene polymorphisms in OA has not yet been established. The aim of this study was to investigate the association of adiponectin +276G/T (rs1501299) gene polymorphism with knee OA. Genotype distributions and allelic frequencies of adiponectin gene, +276G/T polymorphism were determined in a total of 200 subjects (100 knee OA patients and 100 healthy controls). Single-nucleotide polymorphism (SNP) of the adiponectin +276G/T gene was genotyped by polymerase chain reaction (PCR)-restriction fragment length polymorphism analysis. The genotype distribution of +276G/T SNP was observed in the Hardy-Weinberg equilibrium for OA patients and controls. No statistically significant difference was identified between the two groups with respect to genotype distributions and allelic frequencies (P>0.05). The T- and G-allele frequencies were indicated as 24.5 and 75.5%, respectively, in OA patients, whereas the frequency was 23-70% in the control group. Findings of this study therefore suggest that the +276G/T SNP was not associated with susceptibility to knee OA.

Introduction

Osteoarthritis (OA) is a degenerative joint disorder leading to stiffness, reduced motion, swelling, crepitus, substantial morbidity and disability (1). It is characterized by synovitis, osteophyte formation, subchondral sclerosis and progressive destruction of articular cartilage, which results in pain and loss of joint mobilization. In recent years, numerous genetic factors have been identified and implicated in causing OA. Previously, OA was recognized as a non-inflammatory arthropathy. Nevertheless, results of previous studies have demonstrated that an inflammatory process plays a vital role in the pathogenesis of OA (2,3). Pro-inflammatory cytokines are considered to play a role as key mediators in the disease (4). A number of gene polymorphisms associated with the development of knee OA have been recently studied, such as those localized in or adjacent to the encoding sequences for growth differentiation factor 5 (5), estrogen receptor α (6), calcitonin (7), interleukin (IL)-6 (8), SMAD3 (9) and matrix metalloproteinase-3 (10).

Adiponectin is a 30-kDa protein encoded by the ADIPOQ gene located on chromosome 3q27 consisting of three exons and two introns. It assembles into complexes of different size, known as trimers (low molecular weight), hexamers (middle molecular weight) and higher order oligomeric complexes (high molecular weight) prior to being secreted (11). Adiponectin has been found to be associated with type of lifestyle and plays a substantial role in the development of metabolic diseases, such as diabetes mellitus and coronary heart disease (12). Although adiponectin may act as an anti-inflammatory mediator in many conditions, its role in joint diseases remains controversial.

To the best of our knowledge, there are currently no published studies regarding the role of adiponectin +276G/T polymorphism in OA patients. In this case-control study, we hypothesized that the +276G/T single-nucleotide polymorphism (SNP) of the adiponectin in intron 2 would contribute to the susceptibility of knee OA. The aim of this study was to examine the association between adiponectin +276G/T polymorphism and primary knee OA in the Thai population.

Materials and methods

Study population. A total of 100 patients diagnosed with primary knee OA (75 females and 25 males; mean age, 68.2±0.9 years) and 100 control individuals who had no symptoms or signs of OA, other types of arthritis, or any joint diseases (80 females and 20 males; mean age, 67.0±1.1 years)

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Key words: adiponectin, case-control study, single-nucleotide polymorphism, knee osteoarthritis, Thai population

were recruited in this study. The diagnosis of knee OA was based on the criteria of the American College of Rheumatology, which included primary OA with any symptoms and radiographic signs of OA according to the Kellgren-Lawrence (KL) grading system (13). Radiographic findings of OA were classified as KL grade 1, 2, 3, or 4. The control subjects were consecutively selected among individuals without a personal and family history of OA. Subjects were excluded on the basis of having arthropathy due to gout, pseudogout, rheumatoid arthritis (RA), systemic lupus erythematosus, psoriasis, hemochromatosis, previous knee injury, or previous joint infection. Patients with any systemic inflammatory or autoimmune disorders, or any type of malignant or chronic illness were not included in this study.

This case-control study was approved by the Institutional Review Board on Human Research of the Faculty of Medicine, Chulalongkorn University, Thailand. The present study was conducted in accordance with the guidelines of the Declaration of Helsinki. Written informed consent was obtained from all the subjects prior to their participation in the study.

Genotyping of adiponectin gene. Peripheral venous blood samples were obtained from each subject by standard venipuncture. Genomic DNA was isolated from buffy coats by using illustra blood genomicPrep Midi Flow kit (GE Healthcare, Little Chalfont, UK) and the samples were stored at -20˚C for subsequent analysis. The adiponectin +276G/T (rs1501299) genotypes were determined by polymerase chain reaction (PCR)-restriction fragment length polymorphism analysis. The +276G/T SNP in the adiponectin gene was genotyped by the amplification of genomic DNA using the primers (14): forward, 5'-ACACTGATATAAACGCCATGAA-3' and reverse, 5'-GCAGCAAAGCCAAAGTCTTG-3'. The amplification conditions were as follows: 95˚C for 10 min, followed by 40 amplification cycles at 95˚C for 30 sec, 50˚C for 30 sec, 72˚C for 60 sec and a final extension at 72˚C for 7 min. The amplified PCR product was 168 bp in length. The polymorphism was typed using the enzyme *Bgl*1 (New England Biolabs, Beverly, MA, USA), which yielded 147- and 21-bp fragments (G allele of +276G/T).

In the genotyping experiments, the digestion fragments were subjected to electrophoresis on 12% polyacrylamide gel containing ethidium bromide and visualized on an ultraviolet transilluminator. An example of an electrophoretic gel showing PCR product digestion with *Bgl*1 is shown in Fig. 1. Homozygous GG and TT corresponded to the presence of 147- and 168-bp fragments, respectively, whereas the heterozygous GT corresponded to the presence of both 147- and 168-bp fragments.

Statistical analysis. The Statistical Package for Social Sciences software (SPSS, Inc., Chicago, IL, USA), version 16.0 for Windows was used for statistical analysis. The demographic and clinical data were compared between groups by the Chi-square and Student's t-tests. Genotype and allelic frequencies were compared by the Chi-square test. Allele and genotype proportions were evaluated for Hardy-Weinberg equilibrium. P<0.05 was considered to indicate a statistically significant difference.

Table I. Demographic data of OA patients and control individuals.

Clinical characteristics	Controls	OA patients
No.	100	100
Age (years)	67.0±1.1	68.2±0.9
Female/male	80/20	75/25
BMI (kg/m^2)	24.5±3.7	27.3±3.9
KL grade		
1	-	0
2	-	31
3	-	39
4	-	30

OA, osteoarthritis; BMI, body mass index; KL, Kellgren-Lawrence.

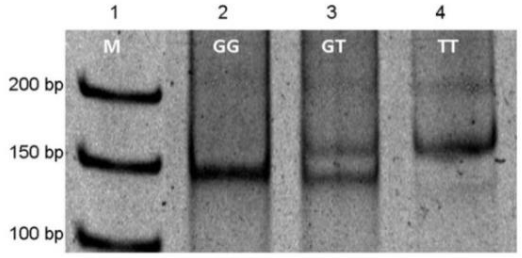

Figure 1. Genotypes of adiponectin polymorphism on 12% polyacrylamide gel electrophoresis with ethidium bromide staining and ultraviolet light transillumination. Lane 1, molecular weight DNA standard marker; lane 2, 147 bp represents homozygous GG; lane 3, 147 and 168 bp represent heterozygous GT; lane 4, 168 bp represents homozygous TT.

Results

Patient characteristics. Demographic data of the population studied and the number of individuals in each group are shown in Table I. There were no significant differences between groups in terms of age, gender and mean body mass index (BMI). In the knee OA patients, the mean age was 68.2±0.9 years. In the healthy controls, the mean age was 67.0±1.1 years (P=0.2). The female/male ratio was 75/25 in the knee OA patients and 80/20 in the controls (P=0.4). Furthermore, the mean BMI value was not significantly different between groups, 27.3±3.9 in the knee OA patients and 24.5±3.7 kg/m^2 in the controls, respectively (P=0.5).

Genotype and allelic frequencies of adiponectin +276G/T polymorphism. GG was the most frequent genotype in the OA patients and control groups and the genotype frequency was within the Hardy-Weinberg equilibrium. There was no statistically significant difference between the groups with respect to genotype distribution (P=0.84) (Table II). The T- and G- frequencies were indicated as 24.5 and 75.5%, respectively, in OA patients, whereas the frequency was 23-70% in the control group. According to the adiponectin

Table II. Genotype distribution and allelic frequency of adiponectin +276G/T single-nucleotide polymorphism between OA patients and controls.

	Genotype distribution				Allelic frequencies		
+276G/T SNP	GG	GT	TT	P-value	G allele (%)	T allele (%)	P-value
Controls	59	36	5	0.84	154 (77.0)	46 (23.0)	0.72
OA patients	58	35	7		151 (75.5)	49 (24.5)	

P-value for difference in distribution of genotypes and allelic frequencies between controls and OA patients. OA, osteoarthritis; SNP, single-nucleotide polymorphism.

+276G/T polymorphism genotypes, there was no association between the genotypes of adiponectin +276G/T polymorphism and the clinical characteristics of the OA patients and controls.

Discussion

OA is a common cause of degenerative joint disease and functional limitation and disability in the elderly. The knee is the most clinically significant site of primary OA involvement. Although great efforts have been made to elucidate the pathophysiology of OA, the genetic factors underlying the development of OA remain unclear. Results of recent studies have shown that there are several candidate genes associated with knee OA (5-10).

Adiponectin, a 244-amino acid polypeptide, represents the highest proportion of all adipokines in the circulation. Adiponectin is structurally homologue to complement factor C1q and tumor necrosis factor-α (TNF-α) (15). It has been shown that adiponectin exerts an anti-inflammatory effect by reducing the release of pro-inflammatory cytokines, e.g., TNF-α and IL-6, and inducing the expression of anti-inflammatory cytokines (16-18). Moreover, numerous studies have shown that adiponectin is capable of counteracting insulin resistance, artherosclerosis and inflammatory processes (15,16,19-21). However, whether adiponectin plays pro- or anti-inflammatory roles in joint disease pathogenesis remains the subject of debate. Recent data have revealed that adiponectin may be secreted by synovial fibroblasts, chondrocytes and infrapatellar fat pad in patients with OA and RA, which led to the increased production of IL-6, IL-8, matrix metalloproteinase and nitric oxide (22,23). These mediators promoted inflammation and joint destruction (16,19). By contrast, Chen *et al* (24) suggested that adiponectin might play a protective role in OA by inducing tissue inhibitor of metalloproteinase-2 expression and suppressing IL-1β-induced matrix metalloproteinase-13 production.

In the present study, we aimed to screen for a susceptibility gene that could facilitate the early diagnosis of OA. Such a genetic screen would enable the identification of individuals who are at a high risk for developing OA. Whether the adiponectin genetic polymorphism at +276G/T influences the susceptibility or severity in patients with knee OA is not fully examined. To address this issue, we investigated the effect of adiponectin +276G/T polymorphism on the risk of knee OA in the Thai population. To the best of our knowledge, this is the first report to evaluate the association between adiponectin +276G/T polymorphism and OA. Our findings demonstrate that the percentage of the adiponectin +276G/T polymorphism allele and the distribution of genotypes were not significantly different between the OA patients and controls.

Limitations of this study involved the relatively small number of enrolled subjects. Further studies conducted on a random sample of multiple centers with larger sample sizes are required to determine whether these findings can be extrapolated to other populations. In addition, this study investigated only one polymorphism in most of the genes, potentially missing any association to a specific polymorphism. Haplotype analysis is necessary for understanding the functional variation responsible for the adiponectin expression and may provide further knowledge on the pathways responsible for the relationship of adiponectin gene with OA. Another limitation is the lack of information regarding the level and source of adiponectin. More studies are in progress to gain insight into adiponectin production and expression.

In conclusion, the present study has suggested that the +276G/T polymorphism genotypes of adiponectin gene do not confer increased susceptibility to knee OA in the Thai population. Additional studies in different and large populations of OA patients are required to elucidate the precise role of adiponectin +276G/T polymorphism and OA.

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