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ROLE OF VASCULAR ENDOTHELIAL GROWTH FACTOR (VEGF) IN PATHOGENESIS OF KNEE OSTEOARTHRITIS

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ณัฏฐาภรณ์ แซ่ตัน : บทบาทของวาสคูลาร์เอนโดทีเลียวโกรทแฟคเตอร์ต่อการเกิดโรคข้อเข่าเสื่อม. (ROLE OF VASCULAR ENDOTHELIAL GROWTH FACTOR (VEGF) IN PATHOGENESIS OF KNEE OSTEOARTHRITIS) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ศ. นพ.สิทธิศักดิ์ หรรษาเวก, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: ศ. นพ.ยง ภู่วรวรรณ, 150 หน้า.

โรคข้อเข่าเสื่อมเป็นโรคข้อเสื่อมอักเสบแบบเรื้อรังที่พบได้บ่อยในผู้สูงอายุ สาเหตุของการเกิดโรคข้อเข่าเสื่อมยังไม่เป็นที่ทราบแน่ชัด การศึกษาที่ผ่านมาพบว่าสารวาสคูลาร์เอนโดทีเลียลโกรทแฟคเตอร์ เป็นส่วนหนึ่งในกระบวนการสร้างหลอดเลือดใหม่ และกระบวนการอักเสบซึ่งเกี่ยวข้องกับการดำเนินโรคข้อเข่าเสื่อม วัตถุประสงค์ของการศึกษาวิจัยครั้งนี้ เพื่อศึกษาการแสดงออกของวาสคูลาร์เอนโดทีเลียลโกรทแฟคเตอร์ในระดับ mRNA และระดับโปรตีนในพลาสมา น้ำไขข้อ เยื่อบุข้อเข่า รวมทั้งเซลล์ที่แยกได้จากเยื่อบุข้อเข่าซึ่งเลี้ยงในสภาวะที่มีการจำลองพยาธิสภาพของโรคข้อเข่าเสื่อม ภายใต้สภาวะออกซิเจนต่ำ (hypoxia) ที่ได้รับหรือไม่ได้รับ IL – β รวมถึงศึกษาการแสดงออกของ miR-210 และ miR-223 ซึ่งเป็นไมโครอาร์เอ็นเอที่เกี่ยวข้องกับโรคข้อเข่าเสื่อม นอกจากนี้ได้ทำการศึกษาความหลากหลายทางพันธุกรรมของยีนวาสคูลาร์เอนโดทีเลียลโกรทแฟคเตอร์ ที่ตำแหน่ง -2578C/A, -1154G/A, -634G/C และ +936C/T พบว่า มีระดับของวาสคูลาร์เอนโดทีเลียลโกรทแฟคเตอร์ในน้ำไขข้อของผู้ป่วยโรคข้อเข่าเสื่อมสูงขึ้นอย่างมีนัยสำคัญทางสถิติ ถึงแม้ไม่พบความแตกต่างของวาสคูลาร์เอนโดทีเลียลโกรทแฟคเตอร์ในพลาสมา แต่พบว่ามีความสัมพันธ์เชิงบวกของระดับวาสคูลาร์เอนโดทีเรียลโกรทแฟคเตอร์ทั้งในน้ำไขข้อและในพลาสมากับระดับความรุนแรงของโรคข้อเข่าเสื่อม อย่างไรก็ตามเมื่อทำการตรวจวัดการแสดงออกของวาสคูลาร์เอนโดทีเลียลโกรทแฟคเตอร์ในระดับของสารพันธุกรรมในเยื่อบุข้อเข่าไม่พบความแตกต่างอย่างมีนัยสำคัญทางสถิติเมื่อเปรียบเทียบกับกลุ่มควบคุม ส่วนการแสดงออกของ miR-210 และ miR-223 ในเยื่อบุข้อเข่าของผู้ป่วยโรคข้อเข่าเสื่อมสูงกว่าในกลุ่มควบคุมอย่างมีนัยสำคัญทางสถิติ เมื่อทำการกระตุ้นเซลล์เยื่อบุข้อเข่าให้อยู่ในสภาวะจำลองพยาธิสภาพของโรคพบว่ามีการแสดงออกของวาสคูลาร์เอนโดทีเลียลโกรทแฟคเตอร์รวมถึง miR-210 สูงขึ้นอย่างมีนัยสำคัญทางสถิติในกลุ่มที่ได้รับการกระตุ้นแต่ไม่พบความแตกต่างของการแสดงออกของ miR-223 ผลจากการศึกษาความหลายหลายทางพันธุกรรมของยีนวาสคูลาร์เอนโดทีเลียลโกรทแฟคเตอร์ไม่พบความแตกต่างของการกระจายตัวของจีโนไทป์และความถี่ของอัลลีลระหว่างกลุ่มผู้ป่วยและกลุ่มคนปกติ อย่างไรก็ตามเมื่อทำการทดสอบแบบกลุ่มย่อยโดยแบ่งกลุ่มผู้ป่วยออกตามระดับความรุนแรงของโรค พบการกระจายตัวของ AA จีโนไทป์ ที่ตำแหน่ง -2578C/A มากกว่าในกลุ่มผู้ป่วยที่มีระดับความรุนแรงของโรคน้อยเมื่อเปรียบเทียบกับกลุ่มที่มีระดับความรุนแรงของโรคมาก แสดงว่า AA จีโนไทป์ มีแนวโน้มที่จะเป็นลักษณะทางพันธุกรรมที่ช่วยชะลอการดำเนินโรคข้อเข่าเสื่อม นอกจากนี้ยังพบว่า CC จีโนไทป์ ของ -634G/G ส่งผลต่อการสร้างวาสคูลาร์เอนโดทีเลียลโกรทแฟคเตอร์ที่สูงขึ้นในพลาสมาของผู้ป่วยเมื่อเปรียบเทียบกับคนปกติ การศึกษานี้สรุปได้ว่า วาสคูลาร์เอนโดทีเลียลโกรทแฟคเตอร์มีบทบาทต่อการเกิดโรคข้อเข่าเสื่อมโดยมีการแสดงออกมากขึ้นในน้ำคัดหลั่งที่ตรวจวัดได้จากผู้ป่วยและยังพบมากขึ้นในผู้ป่วยที่มีระดับความรุนแรงสูงขึ้น โกรทแฟคเตอร์ดังกล่าวอาจนำมาใช้เป็นตัวบ่งชี้การดำเนินไปของโรค รวมทั้งวาสคูลาร์เอนโดทีเลียลโกรทแฟคเตอร์อาจเป็นปัจจัยหนึ่งที่ส่งผลต่อการพัฒนาและการดำเนินไปของโรคข้อเข่าเสื่อม

สาขาวิชา ชีวเวชศาสตร์ ลายมือชื่อนิสิต .....

ปีการศึกษา 2556 ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก .....

ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม .....

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KEYWORDS: OSTEOARTHRITIS / VASCULAR ENDOTHELIAL GROWTH FACTOR / SYNOVIAL INFLAMMATION / DISEASE SEVERITY / MIRNA / SYNOVIOCYTES / SINGLE NUCLEOTIDE POLYMORPHISM

NATTHAPHON SAETAN: ROLE OF VASCULAR ENDOTHELIAL GROWTH FACTOR (VEGF) IN PATHOGENESIS OF KNEE OSTEOARTHRITIS. ADVISOR: PROF. SITTISAK HONSAWEK, M.D., Ph.D., CO-ADVISOR: PROF. YONG POOVORAWAN, M.D., 150 pp.

Recent evidences suggest that angiogenesis and inflammation contribute to the development and progression of knee osteoarthritis (OA). Vascular endothelial growth factor (VEGF) is a potential angiogenic factor associated with the pathogenesis of OA. The etiology of OA is poorly understood and many risk factors can influence OA. The purposes of this study was to investigate VEGF mRNA and protein levels in plasma, synovial fluid (SF), and synovial tissue from knee OA patients and *in vitro* under osteoarthritic-like conditions (hypoxia with or without interleukin-1β treatment) using fibroblast like synoviocytes (FLS) isolated from knee OA patients. Additionally, this study was designed to determine the associations between single nucleotide polymorphisms (SNPs) of the VEGF gene (-2578C/A, -1154G/A, -634G/C, and +936C/T) and the susceptibility of knee OA, as well as to examine the expressions of miR-210 and miR-223 in knee OA patients. The expressions of VEGF mRNA, miR-210, miR-223 were evaluated by real time reverse transcriptase-polymerase chain reaction. The protein levels of VEGF were determined using enzyme-linked immunosorbent assay and immunohistochemistry. The SNPs of VEGF gene were analyzed by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). The VEGF protein was expressed in SF and synovial tissue from OA patients. There was a strong positive correlation between SF VEGF and the disease severity and a weak positive correlation in OA plasma VEGF levels and the disease severity. The expression of VEGF mRNA in OA synovial tissue was not significantly different when compared with non-inflamed anterior cruciate ligament controls. Moreover, the expression of miR-210 and miR-223 in OA synovial tissue were higher than those in controls. However, the expression of VEGF in FLS under hypoxia with IL-1β treatment was remarkably elevated compared to normoxia. The levels of miR-210 were expressed in the same pattern with VEGF mRNA, whereas no significant difference was observed in the expression of miR-223. No associations between genotype distribution and allelic frequency of VEGF SNPs were found in OA compared with those in controls. The stratify analysis according to the severity of OA showed that AA genotype of -2578C/A in early stage was significantly different compared with that in advanced stage. Moreover, plasma VEGF levels were increased in the OA patients carrying the -634CC genotype. These findings indicated that VEGF expression was increased in OA samples and had a positive correlation with the severity of knee OA. The CC genotype of -634G/C was also associated with the VEGF production. The -2578AA was associated with a low risk of OA, therefore the AA genotype may play a protective role against progressive OA. FLS treated with osteoarthritic-like conditions showed elevated VEGF and miR-210 levels. VEGF may be useful for monitoring the OA severity and could play a substantial role in the development and progression of knee OA.

Field of Study: Biomedical Sciences
Academic Year: 2013

Student's Signature .....
Advisor's Signature .....
Co-Advisor's Signature .....

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LIST OF ABBREVIATIONS

3'UTR	3' untranslated region
5' UTR	5' untranslated region
ACL	anterior cruciate ligament
ACR	The American college of Rheumatology
ADAMTS	A Disintegrin And Metalloproteinase with Thrombospondin Motifs
APS	ammonium persulfate
AUC	area under the curve
bFGF	basic fibroblast growth factor
ß–ME	ß–mercaptoethanol
BMI	body mass index
BMPs	bone morphogenetic proteins
Bp mice	brachypodism mice
CAM	chick embryo chorioallantoic membranes
CCL	chronic closed lock
CI	confidence interval
Col2A1	collagen type 2A1
COMP	cartilage oligomeric matrix protein
COPCORD	Community Oriented Program For Control Of Rheumatic Disorders
COX-2	cyclooxygenase-2
CRP	C-reactive protein
CT	calcitonin
DAB	3,3 diaminobenzidine tetrahydrochloride
DEPC	diethylpyrocarbonate
DGCR8	DiGeorge critical region 8
DMEM	Dulbecco's modified eagle medium
DMSO	dimethysufoxide
DVWA	Double von Willebrand factor A domains
ECM	extracellular matrix
ECs	endothelial cells
EDTA	ethylene diamine tetraacetic acid
ELISA	enzyme-linked immunosorbent assay
ER	estrogen receptor
FBS	fetal bovine serum

FGF-2	fibroblast growth factor-2
FLS	fibroblast-like synoviocyte
Flt-1	fms-like tyrosine kinase-1
G-CSF	granulocyte colony stimulating factor
GDF5	growth differentiation factor 5
GM-CSF	granulocyte macrophage-colony stimulating factor
HGF	hepatocyte growth factor
HIF-1	hypoxia inducible factor-1
HLA	human leucocyte antigen
H2O2	hydrogen peroxide
HSCs	hematopoietic stem cells
HWE	Hardy-Weinberg equilibium
I/R	ischemic/reperfusion
IFN-γ	interferon-γ
IGF	insulin-like growth factor
IL-1β	interleukin-1β
IL-6	interleukin-6
IL-8	interleukin-8
IRAK1	interleukin-1 receptor-associated kinase
KCl	potassium chloride
KDR	kinase domain region
KGF	keratinocyte growth factor
$KHCO_3$	potassium bicarbonate
KH_2PO_4	potassium phosphate dibasic
K/L	Kellgren-Lawrence classification
LD	linkage disequilibrium
MCP-1	macrophage chemotactic protein-1
MIA	monoiodoacetate injection
MIG	monokine induced by IFN-γ
miRNAs	microRNAs
MMP	matrix metalloproteinase
MRI	magnetic resonance imaging
MTT	[3-(4,5-dimethylthiazol-2-yl)-2,5-diphenlytetrazolium bromide]
NO	nitric oxide
NOF	neck of femur
Nrp-1	neuropilin-1

NSAIDS	non-steroidal anti-inflammatory drugs
OA	osteoarthritis
OD	optical density
ON	osteonecrosis
ONFH	osteonecrosis of the femoral head
OPN	osteopontin
OR	odd ratio
PCR-RFLP	polymerase chain reaction-restriction fragment length polymorphism
PECAM	Platelet endothelial cell adhesion molecule
PBMC	peripheral blood mononuclear cell
PBS	phosphate buffer saline
PlGF	placental growth factor
Pre-miRNAs	premature miRNAs
Pri-miRNAs	primary miRNAs
PsA	psoriatic arthritis
RA	rheumatoid arthritis
RBCs	red blood cells
RISC	RNA induced silencing complex
SF	synovial fluid
SLE	systemic lupus erythematosus
SNP(s)	single nucleotide polymorphism(s)
Ta	annealing temperature
TEMED	N,N,N',N'-tetramethyl ethylene diamine
TGF-ß	transforming growth factor-ß
TIMPs	tissue inhibitors of metalloproteinases
Tm	melting temperature
TMJ	temporomandibular joint
TNF-α	tumor necrosis factor-α
TRAF6	tumor necrosis factor receptor-associated factor 6
TRAP	tartrate-resistant acid phosphatase
VDR	vitamin D receptor
VEGF	vascular endothelial growth factor
VGIR	visually guided TMJ irrigation
WBCs	white blood cells

CHAPTER I
Introduction

1. Osteoarthritis

Osteoarthritis (OA) is one of the most common chronic, degenerative, painful, and progressive joint diseases. The prevalence of osteoarthritis in the elderly is dramatically increased when compared with that in the young and middle-aged population (Ding *et al.* 2010). The percentages of older population in Thailand are rapidly elevated from 9.4% in 1990 and 14.2% in 2010. When osteoarthritis rates are reported by gender, females are generally found to exhibit higher prevalence rates than males. Like other parts of the world, the older population in developing countries has a higher rate, especially in South-East Asia where relatively large proportions of the population are elderly (Sasat and Bowers 2013). Currently, the conventional treatment for OA is to reduce the symptoms and to improve quality of life. However, a number of OA patients eventually undergo joint replacement surgery. The standard treatments of OA are analgesic agents, non-steroidal anti-inflammatory drugs (NSAIDs) and ultimately total joint replacement surgery (Wieland *et al.* 2005).

Osteoarthritis is characterized by articular cartilage deterioration, joint space narrowing, sclerosis of the subchondral bone, osteophyte formation (bony outgrowth located at the joint margin) and synovial inflammation (synovitis). The clinical features of OA include pain, stiffness, swelling, deformity, and loss of motion leading to limitation in activities and quality of life. This disease frequently affects many joints such as thumb, joints of the fingers, hands, feet, neck and weight-bearing joints including hip and knee joints (Martel-Pelletier and Pelletier 2010). The most affected joint is the knee because the behaviors or life styles leading to stress in joint. The prevalence of knee OA has been estimated approximately 30% in people aged 65 years and over (Galvez-Rosas *et al.* 2010). Factors that enhance the risk of knee osteoarthritis include age, gender, race and ethnicity, obesity, trauma, genetics,

overuse, and nutrition (Benito *et al.* 2005, Krasnokutsky *et al.* 2007, Attur *et al.* 2010). Etiology of osteoarthritis remains largely unclear. However, OA may originate from interactions of genetics, biochemical and environment factors that result in the imbalance between synthesis and degradation of extracellular matrix (Martel-Pelletier and Pelletier 2010). Although most osteoarthritis studies focus on pathology of articular cartilage, the precise cause of this disease is still unknown. The synovium, bone, and cartilage are involved in pathological process leading to progressive joint degeneration. It has been postulated that synovial inflammation and articular cartilage destruction are involved in the pathogenesis of OA. Synovitis can produce and release many factors contributing to secondary cause in the cartilage loss (Martel-Pelletier and Pelletier 2010). Previous studies showed that proinflammatory mediators generated by inflammatory reaction could stimulate chondrocytes and seemed to be important mediators in pathogenesis of OA (Wassilew *et al.* 2010). Moreover, recent investigation demonstrated that synovial tissues from OA patients produced proinflammatory cytokines and mediators of joint damage such as tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and vascular endothelial growth factor (VEGF) (Benito *et al.* 2005).

VEGF is expressed in various tissues such as brain, kidney, liver and spleen. VEGF plays important roles in angiogenesis, proliferation, migration, and tube formation of endothelial cells (Balasubramanian *et al.* 2002, Lingaraj *et al.* 2010). Furthermore, it functions in bone development, osteoblasts and chondrocytes associated with endochondral bone formation by coupling angiogenesis with hypertrophic cartilage remodeling and ossification. Additionally, VEGF regulates proliferation, differentiation, and survival of osteoblasts, osteoclasts and chondrocytes (Maes and Carmeliet 2008, Murata *et al.* 2008). Previous studies have been documented that VEGF was expressed in human osteoarthritis cartilage (Pufe *et al.* 2001, Enomoto *et al.* 2003). Additionally, the roles of VEGF in the pathogenesis of OA have been documented in animal models (Tibesku *et al.* 2011, Ludin *et al.* 2013). Therefore, it is hypothesized that vascular endothelial growth factor was associated with the progression of OA. Although several studies investigated expression of VEGF

in articular cartilage of OA patients, the plasma and synovial VEGF expression in knee OA patients are still unknown.

Although the multifactorial nature of osteoarthritis is well established, genetic factors have been found to be strong determinants of the disease. One of the genetic factors associated with OA is single nucleotide polymorphisms (SNPs: single nucleotide change in the genomic DNA caused DNA sequence variation). Thus far, several SNPs have been demonstrated to be associated with the risk of OA such as growth differentiation factor 5 (*GDF5*), collagen type 2A1 (*Col2A1*), vitamin D receptor (*VDR*), estrogen receptor (*ER*), and matrix metalloproteinase (*MMP*) (Spector and MacGregor 2004, Galvez-Rosas *et al.* 2010, Valdes *et al.* 2011, Honsawek *et al.* 2013). As mentioned above, VEGF plays a possible role in synovitis leading to the development of OA. Many polymorphisms have been described in the *VEGF* gene and have been implicated in various diseases including lung cancer, prostate cancer (Jain *et al.* 2009, Vaziri *et al.* 2010), diabetes mellitus (Vaziri *et al.* 2010), rheumatoid arthritis (RA) (Han *et al.* 2004), and systemic lupus erythematosus (SLE) (Wongpiyabovorn *et al.* 2011). However, there has been no study regarding the *VEGF* SNPs and the susceptibility in knee osteoarthritis.

Another genetic risk factors related with development of osteoarthritis is epigenetic factor. Small non-coding miRNAs, one such genetic defect, had been received an attention to study the association between the level of miRNAs and pathogenesis in osteoarthritis. miRNAs play roles in development and homeostasis of tissue by acting as genetic regulator. miRNAs have specific patterns for tissues and developmental stage of the diseases. A number of miRNA studies had been performed in many diseases such as SLE, psoriasis, RA, osteonecrosis, and especailly OA (Tomankova *et al.* 2011). There are several miRNAs studied in pathogenesis of OA including miR-9, miR-98, miR-146 (Jones *et al.* 2009), miR-140 (Miyaki *et al.* 2009), miR-199a (Lin *et al.* 2009), and miR-675 (Dudek *et al.* 2010). In this study we focused on two miRNAs: miR-210 and miR-223. miR-210 is a regulator of angiogenesis and hypoxia (Yamasaki *et al.* 2012). This miRNA has no evidence indicated the association in osteoarthritis. Meanwhile, miR-223 plays an essential role in osteoclast differentiation. Althrough, previous study determined the expression of miR-223 in

peripheral blood mononuclear cells of OA patients (Okuhara *et al.* 2012), they did not perform in OA synovium tissue and synoviocyte. Thus, it is interesting to study the association between these miRNAs in knee osteoarthritis.

Therefore, the aim of this study was to evaluate the relationship between the expression of VEGF and pathogenesis of OA including expression of VEGF in mRNA and protein levels, VEGF expression in synovial membrane. Moreover, the alterations of *VEGF* gene polymorphisms and miRNAs expression were determined. Finally, *in vitro* study was investigated in order to assess the role of VEGF in pathogenesis of OA. An intensive determination between the correlation of VEGF and knee osteoarthritis may be helpful for better understanding the pathogenic mechanisms of OA and development of biochemical markers for assessing the OA severity.

2. Research questions

a. Is the expression of VEGF associated with disease severity in osteoarthritis patients when compared with controls?
b. Do the SNPs of VEGF associate with an increased susceptibility to knee OA and VEGF expression?
c. Are miRNAs correlated with the pathogenesis of osteoarthritis and VEGF alterations?
d. Are the expressions of VEGF and miRNAs influenced by inflammatory stimulation *in vitro?*

3. Objectives

a. To determine the association between expression of VEGF in plasma, synovial fluid, cartilage, and synovial tissues form OA subjects compared with controls
b. To investigate VEGF SNPs in knee osteoarthritis and healthy population and their relationship with SF and plasma levels
c. To evaluate the expression of miRNAs and their association with knee osteoarthritis and VEGF expression
d. To study the alteration of VEGF and miRNAs expression *in vitro*

4. Hypotheses

a. The expression of VEGF in plasma, synovial fluid, cartilage, and synovial tissues in osteoarthritis will be higher than controls.
b. VEGF SNPs will confer the risk of knee osteoarthritis and VEGF levels.
c. There will be the association between miRNAs in pathogenesis of knee osteoarthritis and alteration of VEGF.
d. The VEGF level and miRNA expression will be influenced by inflammatory conditions.

5. Key words

Knee osteoarthritis, Vascular endothelial growth factor, Single nucleotide polymorphism, Synovial inflammation, Disease severity, miRNA

6. Conceptual framework

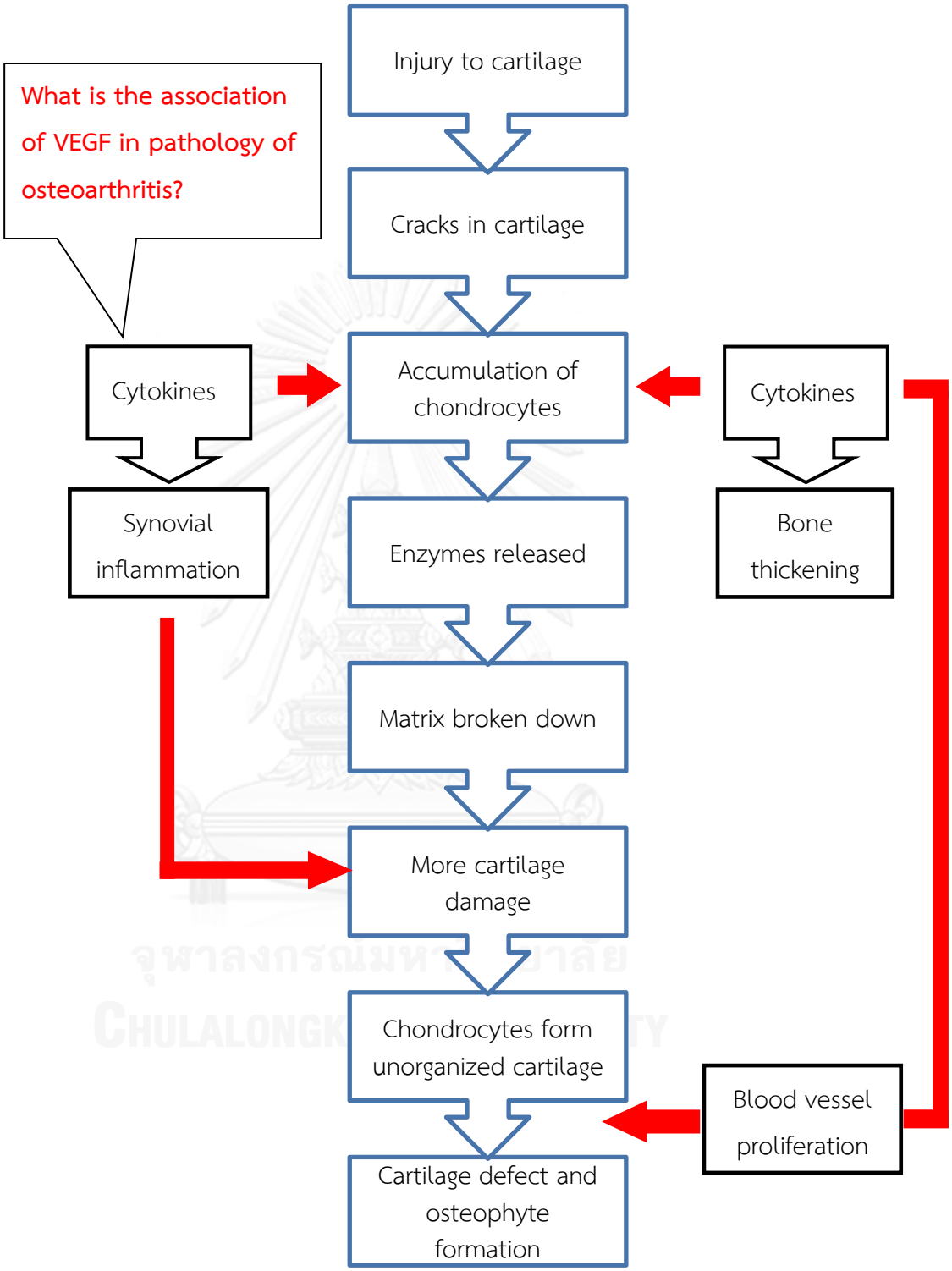
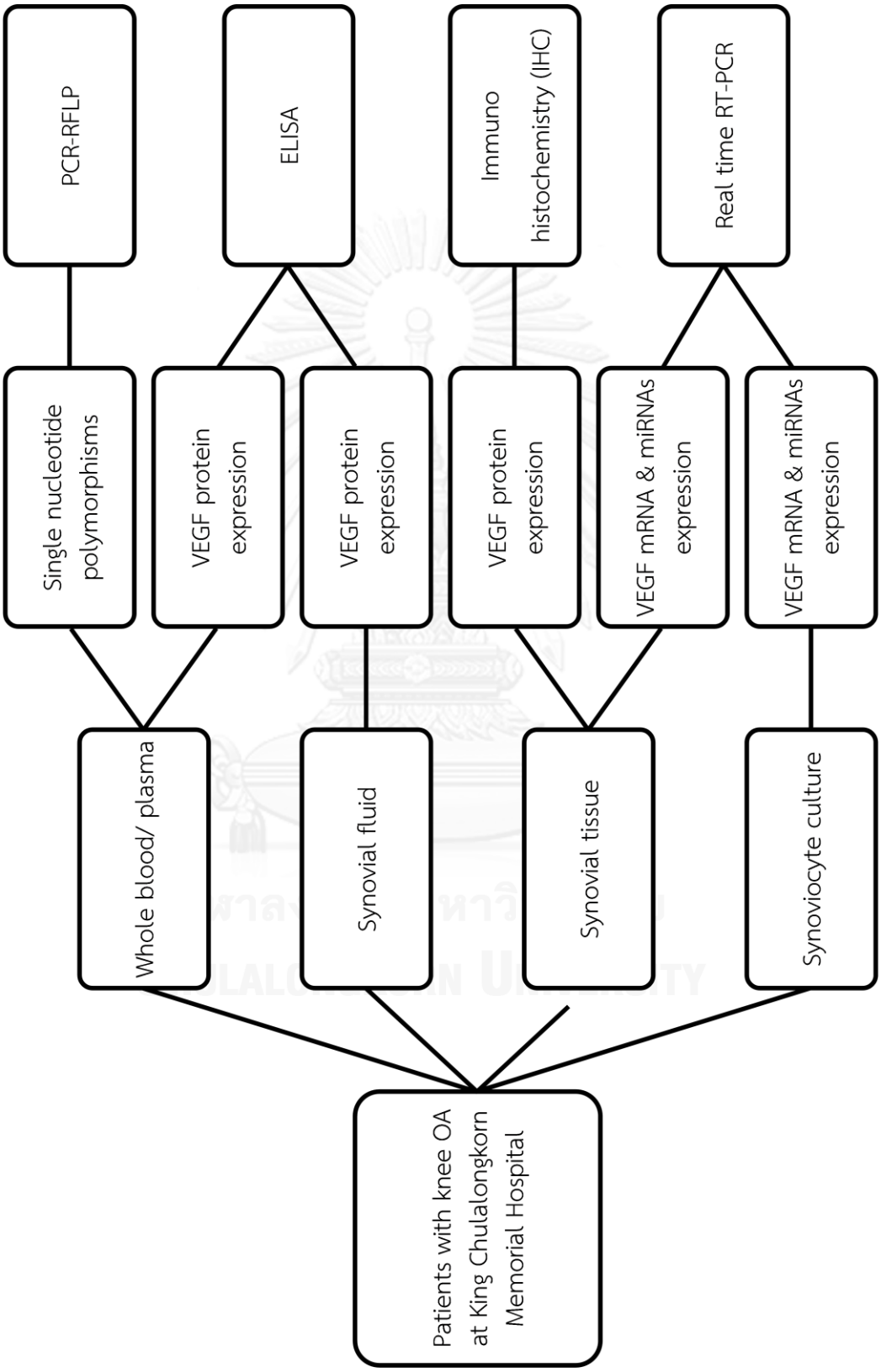


Figure 1.1 Pathology of osteoarthritis (osteoarthritis pathology. [updated 10th June 2009]; Available from: http://www.joint-pain-expert.net/osteoarthritis-pathology.html.)

7. Experimental design



8. Expected benefits and applications

a. Understand the association between the osteoarthritis severity and VEGF expression in knee osteoarthritis.
b. Understand the role of VEGF on inflammatory reaction and correlation between miRNA and osteoarthritis.
c. Understand the association between single nucleotide polymorphisms of VEGF and susceptibility of knee osteoarthritis.
d. Possible use of VEGF as a biochemical marker to assess the severity of knee osteoarthritis.

9. Limitation

Because of human specimens, it is difficult to collect the complete samples in this study. The major obstacle would be the collection of synovial fluid and synovial tissue from control group. It is difficult to obtain the entire data such as weight and height data from control group. Moreover, it is difficult to find the age- and gender-matched controls.

10. Ethical consideration

The sample collection has been performed according to clinical research ethics. The present study was approved by the Institutional Review Board on Human Research of Faculty of Medicine, Chulalongkorn University (IRB. No. 223/54) and was conducted in agreement with the guidelines of the Declaration of Helsinki. Written informed consent was obtained from all participants prior to their participation in the study.

CHAPTER II

Literature Reviews

1. Osteoarthritis

Osteoarthritis (OA) is one of the most common chronic progressive joint diseases occurring in the elderly. As the ratio of the aged-population increases every year, OA is therefore an important influence on quality of life. Data from Epidemiology of Aging supports that populations are aging throughout the globe as shown in **Figure 2.1**. Moreover, the information in an aging study also indicated that arthritis was a commonly chronic disease in persons aged over 65 years old in 2005 (Ferrucci *et al.* 2008)

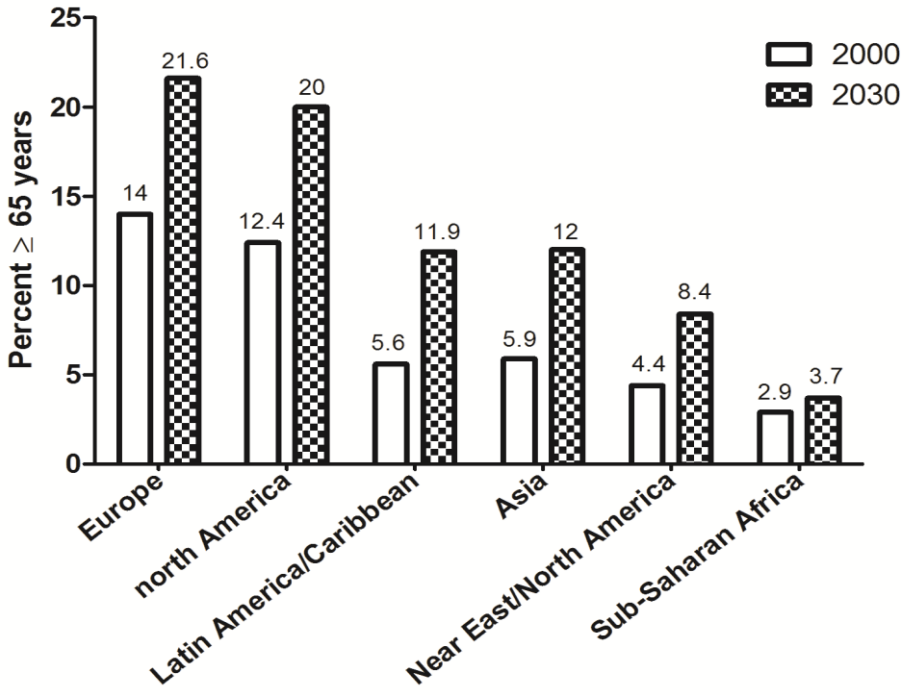


Figure 2.1 Epidemiology of aging modified from (Ferrucci et al. 2008)

Osteoarthritis mostly affects the weight-bearing joints, especially the knee joint. Osteoarthritis can be classified into many subgroups based on the etiology and involved pathologic joint. Classification of OA by articular joint involvement is defined

into monoarticular or polyarticular OA (generalized OA). Moreover, classified by etiology, primary or idiopathic OA is arthritis that has no known cause, mostly related to aging, whereas secondary OA is the occurance of arthritis which resulted from other disorders such as malalignment of joint, joint trauma, or any arthropathic inflammation (Arden and Nevitt 2006). Finger, toe, shoulder, spine, hand, and particularly, knee joints can be affected by OA. The clinical symptoms of OA include pain, stiffness, swelling and deformity, and loss of motion leading to limitation in activities and quality of life (Wieland *et al.* 2005)

1.1 Prevalence and incidence of osteoarthritis

The prevalence of the disease has been studied in many areas around the world because of the rise in people suffering from OA. The data from Community Oriented Program For Control Of Rheumatic Disorders (COPCORD) (Haq and Davatchi 2011) in developing countries showed that knee pain was most common in Asia in areas such as Bangladesh, Shanghai, rural India, the Philippines, Vietnam, and Thailand. The prevalence of OA increased overtime. The incidence of OA in women was higher than that in men and tended to be dramatically elevated with aging. The incidence of OA in the United States was 33.6% in adults over 65 years old and increased with age (Lawrence *et al.* 2008). Furthermore, a population-based report in China showed a number of people in both urban and rural areas were suffering from knee OA. The report also supported that the prevalence of symptomatic knee OA identified by radiographic parameters and knee pain was higher in women when compared with men and tended to be much higher in people aged over 50 years old in both genders (Jiang *et al.* 2012). Another population-based study investigated the incident of knee, hip, and hand osteoarthritis in Spain. This study had a very large sample size of approximately three million participants. The proportion of females and males suffering from OA were 46% and 16%, respectively. The patterns of the prevalence in OA differed between each affected joint. Knee and hip OA prevalences were higher with age in both female and male while incidence of hand OA was the highest in 50-59 years old women (Prieto-Alhambra *et al.* 2013).

1.2 Characteristics and pathophysiology of osteoarthritis

Osteoarthritis is the most common musculoskeletal disease. There are multifactorial factors causing the pathologic process. The etiology of OA is still unclear, however, several risk factors leading to the pathology of OA have been described (Busija *et al.* 2010) (Ding *et al.* 2010). Age is a strong risk factor for the development of OA, especially, indervidiuals of the age of 50 years old and peaking between 70-79 years old (Woolf *et al.* 2012). Female gender is another strong risk factor contributing to the pathology of OA. Various studies (Jiang *et al.* 2012, Prieto-Alhambra *et al.* 2013) found women have a higher risk of osteoarthritis than men in knee and hand joints. Life style and physical activity also seem to be risk factors of OA because the activity of daily life with repetitive or overuse of joints may lead to a higher incident of OA. For example, some of the participants in rural regions have occupations with a physical workload on the joint resulting in knee pain and subsequently OA. Malalignment or abnormal joint shape lead to irregular movement causing cartilage destruction. Genetic factors are also risk factors leading to the pathology of OA; particularly the genetics involved in the synthesis of extracellular matrix molecules (i.e. collagen type II), proteins in bone remodeling, and inflammation pathways. Furthermore, obesity or body mass index (BMI) reflects an increasing risk of osteoarthritis through mechanical-overload of the weight-bearing joints such as the knees and hips. The patients who have high BMI are at more risk of developing knee OA. Finally, an imbalance between synthesis and degradation of extracellular matrix results in the development of OA when the destructive cytokines exceed the synthetic mediators.

The major characteristics of OA are gradual degradation of articular cartilage combined with accumulation of subchondral bone sclerosis. In addition, osteophyte formation and synovial membrane inflammation, or synovitis, also contribute to the pathology of osteoarthritis. Therefore, pathological knee osteoarthritis not only occurs in cartilage but also affects the entire structure of the joint including subchondral bone, synovium, and the joint capsule as shown in **Figure 2.2** (Sutton *et al.* 2009)

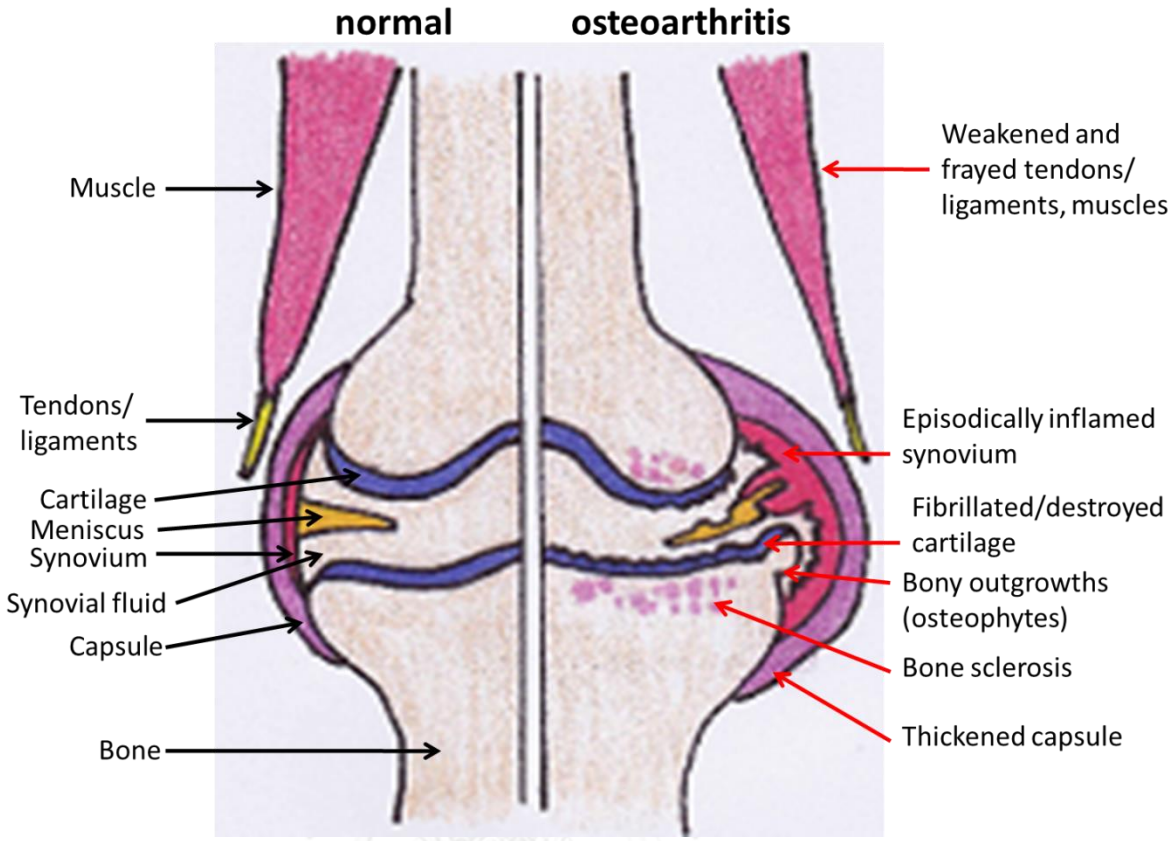


Figure 2.2 Articular cartilage, bone, and synovium are affected in osteoarthritis modified from (Wieland *et al.* 2005)

Cartilage (Krasnokutsky *et al.* 2007, Martel-Pelletier and Pelletier 2010, Loeser *et al.* 2012)

Cartilage is a non-vascular connective tissue organ. It is a smooth-surface cushion which allows for the absorption of mechanical stress between bones. In consequence, cartilage plays a function in preventing biomechanical damage from load-bearing forces. Despite being an avascular tissue, cartilage can obtain nutrition and oxygen from the vascular bed of subchondral bone and from synovial fluid. The articular cartilage is composed of extracellular matrices and chondrocytes which are small and rounded shape cells. An extracellular matrix (ECM) consists mainly of collagen, espectially type II collagen, and proteoglycans including aggrecans. Four zones of cartilage contain the superficial zone, transitional or middle zone, radial or deep zone, and calcified zone. In normal developed cartilage, chondrocytes are in a quiescent stage. In OA, chondrocytes are activated to produce matrix proteins,

matrix degrading enzymes, including matrix metalloproteinases (MMPs), A Disintegrin And Metalloproteinase with Thrombospondin Motifs (ADAMTS), and inflammatory mediators, such as IL-1ß and nitric oxide (NO). During the early stage of disease, cartilage is damaged and its fragments may be released from cartilage into the synovial fluid. Aggrecans can repair the damage initially but in later stages aggrecans are unable to compensate in both articular cartilage and synovial fluid resulting in a loss of matrix. The fragments of cartilage in synovial fluid can feedback to stimulate more cartilage destruction.

Subchondral bone (Martel-Pelletier and Pelletier 2010, Loeser *et al.* 2012)

Subchondral bone consists of two layers; the cortical plate and cancellous bone. The function of subchondral bone is to supply nourishment to cartilage because of its abundant vascularization. Subchondral bone dysfunction has an impact on the degradation of articular cartilage. In OA, subchondral bone becomes thickened, especially the area beneath the pathogenic cartilage. Previous studies revealed that subchondral bone sclerosis has been correlated with osteoarthritis progression. Some evidence indicated that a 20-fold increase in subchondral bone turnover was observed during OA progression. Moreover, subchondral bone from OA patients had enhanced expression of anabolic factors contributed to bone formation (Sharma *et al.* 2013).

Synovial membrane (Krasnokutsky *et al.* 2007, Sutton *et al.* 2009, Martel-Pelletier and Pelletier 2010)

The synovial membrane is a lining-cell capsule that covers all structures within the joint, except the articular cartilage. There are two layers of the synovium, a lining layer called the intima and a supportive layer named the subsynovium or sub-intima. The intima is between the joint space and the outer membrane of the sub-intima combined with the fibrous capsule. The synovium contains two types of cells, namely type A and type B synoviocytes. Type A cells are macrophages functioning in phagocytosis. The type B cells are synovial fibroblasts and have roles in collagen and fibronectin production for synovial fluid. The synovial membrane is extensively vascularized, innervated, and secretes synovial fluid.

Although OA has been described in part as a non-inflammatory disease, various studies have examined the level of inflammatory cytokines in synovia and indicated that synovial inflammation contributed to cartilage and bone destruction leading to the progression of osteoarthritis (Benito *et al.* 2005, Martel-Pelletier and Pelletier 2010, Wassilew *et al.* 2010). Histological features of OA synovia represent hypertrophy and hyperplasia with increasing numbers of lining cells. Synovitis is often restricted to areas of the synovial membrane close to cartilage. The mechanism of synovitis is believed to involve fragments of degraded cartilage. These fragments are released into the synovial fluid and phagocytosed by synovial macrophage cells to maintain inflammation in the synovial membrane by increasing production of inflammatory mediators. In turn, the mediators are secreted from the synovium into the synovial fluid and cartilage leading to more joint destruction and synovitis.

1.3 Diagnostic and therapeutic treatment

(Wieland et al. 2005, Golightly et al. 2011)

Osteoarthritis is a degenerative joint disease characterized by articular cartilage deterioration.. However, radiography is insensitive to detect the alteration in the knee joint. Radiographic tests can diagnose the disease in a symtomatic joint in late stages. Moreover, the results from X-rays cannot represent the symptomatic pain. In some patients, alteration in the joint is not detected by radiographic parameters but they have symptoms of OA-like pain. Recently, magnetic resonance imaging (MRI) has been used to detect changes in all structures in the joint such as cartilage, subchondral bone, and soft tissues in very early stages of OA due to its higher sensitivity. Nonetheless, MRI is still limited because it has no standardized assessment to determine the severity of disease and is more expensive than conventional radiography. Because of the limitations in the sensitivity of diagnostic radiography, biochemical markers have been challenged to reflect the change in molecular processes leading to the development and progression of osteoarthritis. The biomarkers are endogenous products in both local (synovial fluid) and systemic (serum and plasma) sources; therefore, these biomarkers could be more sensitive at

evaluating the early changes in OA joints at the onset stage. At present, there is no curative therapy for OA. The current interventions include the treatment of symptomatic pain by analgesics or anti-inflammatory drugs as represented in **Figure 2.3**. Many treatments including cyclooxygenase-2 (COX-2) inhibitors, non-steroidal anti-inflammatory drugs (NSAIDS) as analgesic agents (pain symptom alleviation) are commonly used in early stage OA. Alternative options are intra-articular injection of hyaluronic and corticosteroids. These techniques can reduced the proinflammatory mediated osteoarthritis; however, all drugs are able to cause side effects to the patient. Finally, joint replacement surgery may be the last option in the treatment of OA.

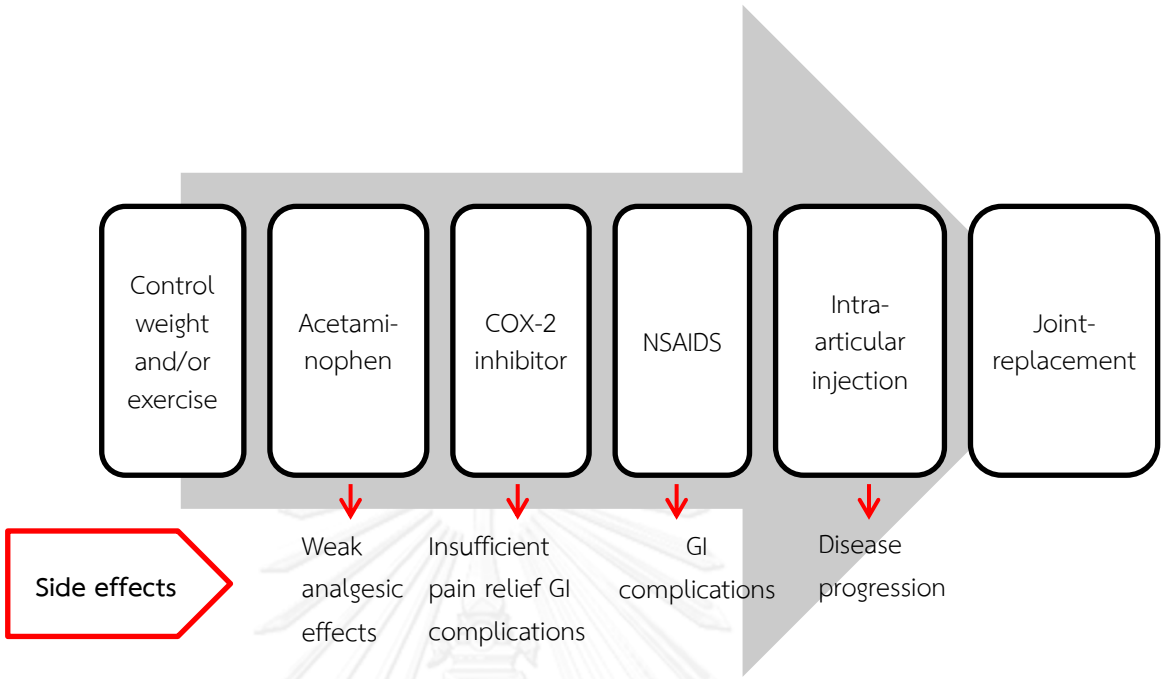


Figure 2.3 Treatment options for osteoarthritis modified from (Wieland *et al.* 2005)

1.4 Osteoarthritis related with cytokine markers (Attur *et al.* 2013)

As mentioned above, the research into biomarkers is becoming popular for identifying, diagnostic or prognostic tools for the osteoarthritis disease. According to the imbalance between anabolic and catabolic mechanisms, the cytokines in both processes combined with inflammatory mediators are considered when investigating the pathology, development, and progression of osteoarthritis. A number of biomarker researches demonstrated the expression of cytokines associated with the pathology of osteoarthritis. The cytokines in matrix synthesis and degradation pathways, such as type II collagen, cartilage oligomeric matrix protein (COMP), were remarkably correlated with progressive OA (Golightly *et al.* 2011). Anabolic factors have been associated with cartilage formation, for instance bone morphogenetic proteins (BMPs), and insulin-like growth factor (IGF). The formation of bony outgrowths at the margin of joints named osteophytes in OA patients was found to be associated with elevated-expression of some cytokines, for example transforming growth factor-ß (TGF-ß) and basic fibroblast growth factor (bFGF) (Wieland *et al.* 2005). With respect to anabolic mediators, there have been controversial data. For

example, some studies found that synovial fibroblasts increased expression of BMPs under stimulation with inflammatory cytokines (Lories *et al.* 2003). On the contrary, some reports have shown that the expression of BMPs was decreased in synovial tissue from OA patients (Bramlage *et al.* 2006). In the other structures in the joint, the cytokine expression was altered after the disease occured. Osteoblasts obtained from sclerotic subchondral bone can stimulate the production of matrix metalloproteinases including MMP-3 and -13. Meanwhile, subchondral OA osteoblasts inhibited the synthesis of cartilage matrix components such as aggrecan. Osteoblasts from sclerotic subchondral bone may lead to pathogenesis of OA (Sanchez *et al.* 2005). Although osteoarthritis is driven by cartilage degradation, synovial membrane inflammation has been expected to correlate with pathology in OA. A previous study demonstrated the different expression of the cytokine profiling of MMPs, ADAMTS, and their inhibitors: the tissue inhibitors of metalloproteinases (TIMPs) families obtained from synovium and cartilage of hip OA compared with fracture to the neck of femur (NOF). MMPs play a role in ECM turnover by cleavage of collagen chains. ADAMTS have an effect on collagen biosynthesis and aggrecan degradation. The results found the expression of MMP-28, ADAMTS-16, ADAMTS-17, and TIMP-2 were signifcantly up-regulated whereas MMP-10 was down-regulated in OA synovia compared with NOF. In cartilage, the levels of MMP-2, MMP-9, MMP-11, and ADAMTS-2 were increased while MMP-1, MMP-3, and TIMP1 were decreased when compared to controls. Moreover, the correlation study showed that there are positive correlations between MMP-9, MMP-10 and ADAMTS-9 in the synovium and cartilage (Davidson *et al.* 2006). Another study determined the effect of ADAMTS-5 in mice models. They found that the cartilage destruction in ADAMTS-5 knockout mice was reduced compared to wild type mice (Glasson *et al.* 2005). MMP-13 (Forsyth *et al.* 2005, Goldring *et al.* 2011) was associated with the pathophysiology of osteoarthritis by increasing catabolic activity in aging human articular chondrocytes at basal level and inflammatory cytokine stimulation. MMP-1 and MMP-3 have been extensively investigated. A prior study indicated that serum levels of MMP-1 and -3 were correlated with cartilage loss and the severity of OA (Pelletier *et al.* 2010). In addition, Wassilew *et al.*(Wassilew et al. 2010) also determined the expression MMPs,

including MMP-1 and MMP-3, in OA synovial tissue. They found that MMP-1 and -3 were expressed in the synovial membrane from OA and traumatic knee disorder. Moreover, there were correlations between inflammatory parameters such as C-reactive protein (CRP) and TNF-α and MMP-1 and MMP-3 mRNA levels. Therefore, MMPs and inflammation play roles in joint deterioration in OA synovial tissue. As mentioned above, the pathology and physiology of OA are related to the change in metabolism pathways of cartilage, subchondral bone, and the synovium.

Not only the production of MMPs, which are collagenase and aggrecanase enzymes, but also the production of other cytokines, including IL-1β, TNF-α, interleukin-6 (IL-6), interleukin-8 (IL-8), and NO (Attur *et al.* 2013), are able to promote progressive of joint destruction. A number of osteoarthritis researches have determined the association between pathogenesis and cytokine expression. Various cytokines have an impact on osteoarthritis development. TNF-α and IL-1β are important proinflammatory cytokines in the catabolic process. These cytokines were produced by mononuclear cells, chondrocytes, and synovia (Kapoor *et al.* 2011). The functions of IL-1β and TNF-α in the pathophysiology of OA are decreasing collagen synthesis and increasing cartilage degradation by inducing the matrix-degrading proteases such as matrix metalloproteinases (Wassilew *et al.* 2010, Kapoor *et al.* 2011). A previous study found that IL-1β and TNF-α increased the production of MMP-13 in human articular chondrocyte cultures. Furthermore, MMP-13 production was reduced by IL-1β and TNF-α inhibitors (Woodell-May *et al.* 2011). Additionally, IL-1β and TNF-α can activate cathepsin K expression. Cathepsin K is a proteolytic enzyme that degrades collagens and other components of the extracellular matrix. Many studies have showed that cathepsin K is expressed in both osteoarthritis cartilage and inflamed synovial tissue (Morko *et al.* 2005). In addition, IL-1β stimulated other cytokines leading to osteoarthritis and it can directly stimulate inflammation in pathologic OA. A study by Attur *et al.* found that increased IL-1β in peripheral blood leukocytes in knee OA patients was associated with a higher risk of osteoarthritis with increased pain scores and reduced joint function (Attur *et al.* 2011). Ning *et al.* examined the expression of inflammatory mediators associated with osteoarthritis progression. The immunohistochemical analysis of synovial tissue

showed that the differential inflammatory expression, such as IL-1β, MMP-1, and COX-2, depended on the severity of knee OA (Ning *et al.* 2010). Moreover, Benito *et al.* studied correlations between the expression of inflammatory mediators and stage of OA by immunohistochemistry in synovial tissue (Benito *et al.* 2005). They found that inflammatory cytokines such as vascular endothelial growth factor (VEGF), TNF-α, and IL-1β were highly up-regulated in early OA more than in the advanced stage.

IL-8 is a chemokine and is considered a potential contributing factor in the pathogenesis of OA. It is an important neutrophil attracting and cytokine-activating agent in OA. IL-8 plays a role in endothelial cell proliferation leading to angiogenesis. It is expressed in monocytes, synoviocytes, chondrocytes, and osteoblasts and can stimulate the release of proinflammatory cytokines leading to cartilage destruction (Hamada *et al.* 2006, Sutton *et al.* 2009).

Interleukin-17 (IL-17) is one of the proinflammatory cytokines produced by T-helper (Th) 17 cells which are a CD4+ Th cell subset. IL-17 has the ability to trigger expression of various cytokines such as IL-1β, TNF-α, interferon-γ (IFN-γ), and IL-8. Depending on the cytokine stimulated, IL-17 can promote bone loss or protection (Gaffen 2004). Synovial fibroblasts from OA patients activated with IL-17 can increase the secretion of angiogenic factors including VEGF, hepatocyte growth factor (HGF), keratinocyte growth factor (KGF) (Honorati *et al.* 2006). Therefore, the inflammation process induced by various cytokines has been closely related with angiogenesis. A study determined the effect of osteoarthritis synovial fluid on proinflammatory cytokines production in chondrocytes. The results found that the levels of inflammatory mediators such as IL-6, IL-8, VEGF increased over time after the chondrocytes were treated with osteoarthritis synovial fluid. IL-6, IL-8, and VEGF are mediators that function in inflammation and angiogenesis. Therefore, these data suggested that osteoarthritis can be progressive inflammatory process (Hoff *et al.* 2013).

IL-6 is produced by synoviocytes, chondrocytes, osteoblasts, and adipose tissue. It is associated with the pathogenesis of OA via the inhibition of proteoglycan synthesis, reduction of chondrocyte differentiation and increase of aggrecanase-

mediated proteoglycan catabolism (Sutton *et al.* 2009). IL-6 can disrupt bone resorption and differentiation of osteoclasts (Kapoor *et al.* 2011). Moreover, IL-6 is believed to be the major activator of C-reactive protein that influences early OA (Bonnet and Walsh 2005). Serum levels of IL-6 were higher in OA patients with active inflammation (swelling, local hyperthermia of one or more joints, and high ESR) compared with healthy controls (Toncheva *et al.* 2009). Sakao *et al*. determined the expression of inflammatory cytokines (IL-6 and IL-8) and protease (MMP-13) which are involved in the pathology of OA in osteoblasts isolated from OA osteophytes. They found that the levels of IL-6 and IL-8 were increased in the osteophytes compared with those in subchondral bone without OA (Sakao *et al.* 2009). The previous study in knee OA was followed up for three years in order to assess the association between inflammatory cytokines such as IL-6 and TNF-α, and cartilage loss. The results sugested that serum levels of IL-6 was able to predict the loss of cartilage volume in both medial and lateral tibial cartilage whereas TNF-α level was associated with joint space narrowing (Stannus *et al.* 2010). Therefore, IL-6 associatied-inflammation may function in knee OA pathogenesis.

Another inflammatory factor involved in osteoarthritis pathological process is NO produced by osteoarthritic cartilage. Nitric oxide plays various roles with respect to its effect on chondrocytes that stimulates cartilage degradation, which, in turn, inhibits collagen synthesis. Moreover, nitric oxide acts as an essential mediator in chondrocyte apoptosis, which is a common feature in OA development (Krasnokutsky *et al.* 2007). Increased amounts of NO and high concentration of nitrites/nitrates were found in synovial fluid and serum of OA patients. NO can promote cartilage catabolism via the induction of synovial inflammation, inhibiting synthesis of cartilage (Martel-Pelletier and Pelletier 2010).

Taken together, many cytokines and growth factors are involved in pathophysiology of OA. One of the most potent cytokines in angiogenesis is VEGF. The main symptom of OA is pain that occurred by interaction between inflammation and other features of disease. Furthermore, angiogenesis and inflammation are closely associated with the pathogenesis of OA (Bonnet and Walsh 2005). Angiogenesis can facilitate inflammation processes and inflammation is capable of

angiogenesis stimulation. VEGF is an important angiogenic factor and associated with inflammatory processes. Therefore, it is reasonable to study the relationship between VEGF expression and OA development.

2. Vascular endothelial growth factor (VEGF)

2.1 Characteristic and functional of VEGF (Tammela *et al.* 2005, Roy *et al.* 2006)

VEGF is a 46-48 kDa glycosylated protein composed of the disulfide-linked subunits (Pufe *et al.* 2005). VEGF is believed to be an essential angiogenic factor facilitating new blood and lymph vessel formation. The VEGF family has at least 7 members including VEGF-A (commonly called VEGF), VEGF-B, VEGF-C, VEGF-D, VEGF-E, VEGF-F, and placental growth factor (PlGF) (Murata *et al.* 2008). VEGF can function by binding with its specific receptors such as VEGFR-1 (fms-like tyrosine kinase-1 (flt-1)) and VEGFR-2 (kinase domain region (KDR)). Each VEGF member has a different function in biological pathways. For example, VEGF-B is abled to bind with VEGFR-1 and neuropilin-1 (Nrp-1). VEGF-B has some partial roles in regeneration of coronary collateral formation. VEGF-C is produced as a premature protein which is developed into its mature form by proteolytic enzymes. The mature VEGF-C protein can activate mitogenesis, migration, and survival of endothelial cells. The effect of lymphangiogenesis is stimulated by VEGF-C/VEGFR-3 while blood permeability is induced by VEGF-C/VEGFR-2. VEGF-D is produced in the vascular endothelium, heart, lungs, and bowel. It is initially a premature protein and matures by a proteolytic process like VEGF-C. The VEGF-D has shown angiogenic and lymphangiogenic properties similar to VEGF-C by binding VEGFR-2 and VEGFR-3. VEGF-E and VEGF-F are different from the previous VEGF proteins. They have been identified from virus and snake (viper) venom, respectively. The PlGF is expressed in the placenta, heart, and lungs. Either PlGF homodimers or PlGF heterodimerized with VEGF act by binding to VEGFR-1 and Nrp-1. It has a function artheriogenesis processes.

Ultimately, the most important of the VEGF family is VEGF-A (named VEGF). It is a major molecule in angiogenesis by binding with VEGFR-1 and VEGFR-2. VEGF has various isoforms occurring by alternative splicing from the single *VEGF* gene situated

on the short arm of chromosome 6. The coding region covers 14 kb and consists of eight exons. Exon 1-5 and 8 are preserved in every isoforms, whereas exons 6a, 6b and 7 differed in each variant (Zygalaki *et al.* 2005). This splicing generates the five different variant with 121, 145, 165, 189 and 206 amino acid residues referred to as $VEGF_{121}$, $VEGF_{145}$, $VEGF_{165}$, $VEGF_{189}$ and $VEGF_{206}$, respectively. These isoforms differ in their biochemical properties such as their angiogenic capability and diffusion potential, their capability to bind to heparin and heparin-sulfate as well as their affinity to react with receptors. $VEGF_{121}$ is the only VEGF isoform that is soluble. $VEGF_{189}$ and $VEGF_{206}$ are strongly bound to the cell surface because of high affinity for heparan sulfate. In addition, $VEGF_{165}$ has a low affinity for heparan sulfate, and therefore it can be secreted into the extracellular matrix and attach to the cell surface. Most VEGF-producing cells seem to preferentially express $VEGF_{121}$, $VEGF_{165}$, and $VEGF_{189}$ whereas $VEGF_{206}$ is a very rare variant (Enomoto *et al.* 2003). There are two factors that regulate VEGF gene expression including low oxygen tension (hypoxia) and inflammatory cytokines such as IL-1β (Ferrara 2004). The VEGF gene promoter region composes of a hypoxia responsive element, therefore, hypoxia inducible factor-1 (HIF-1) can bind and enhanec the transcription of VEGF under hypoxic conditions (Roy *et al.* 2006). VEGF acts not only on endothelial cells (ECs) but also on other cells such as hematopoietic stem cells (HSCs), monocytes, osteoblast, and neuron cells. VEGF can be produced by synovial fibroblasts, hydrotrophic chondrocytes, and activated macrophages. VEGF plays important roles in embryonic vasculogenesis and angiogenesis, wound healing, and tissue remodeling. Moreover, it has a function in pathological conditions such as malignant and inflammatory diseases including osteoarthritis (Tanaka *et al.* 2005, Lingaraj *et al.* 2010). The level of VEGF is detectable in synovial fluid and tissue of patients with RA and OA (Tanaka *et al.* 2005, Honorati *et al.* 2006). Recent studydemonstrated a highly significant association between the levels of MMP-9 (gelatinase property) and MMP-13 (collagenase enzyme) and VEGF concentrations in RA and OA synovial fluid, and that they play an essential role in an angiogenesis (Kim *et al.* 2011).

2.2 VEGF related with arthritic disease

The levels of VEGF have been studied in many types of arthritic diseases. In rheumatoid arthritis, VEGF expression in serum was higher than that in OA and healthy control groups. The levels of synovial fluid VEGF in RA patients were increased compared with OA. This data suggests VEGF plays a role in the pathogenesis of RA (Lee *et al.* 2001). Another study determined the level of VEGF in serum obtained from RA patients. The data found that VEGF levels were higher in RA compared with healthy participants. There is a positive correlation between VEGF concentrations and high-grade RA disease activity (Ozgonenel *et al.* 2010). A study in temporomandibular joint (TMJ) patients with chronic closed lock (CCL) observed the levels of VEGF before and after treatment with visually guided TMJ irrigation (VGIR). They found that the levels of VEGF were remarkably decreased in patients who were in the successful group while adverse VEGF levels were shown in the unsuccessful group (Kumagai *et al.* 2010). Ke *et al*. investigated the expression of VEGF in synovial fibroblasts from TMJ stimulated by hypoxia. They found that VEGF expression was altered by hypoxia. Therefore, elevated VEGF concentrations induced by hypoxia may promote TMJ progression (Ke *et al.* 2007). The expression of VEGF and osteopontin (OPN, the cytokine/extracellular matrix protein) in synovial fluid and matched plasma were evaluated in patients with juvenile idiopathic arthritis. The data illustrated that synovial monocytic cells increased the production of VEGF and OPN mediators. Moreover, the level of VEGF was stimulated under hypoxic conditions and abrogated under reoxygenation (Bosco *et al.* 2009).

2.3 Osteoarthritis and vascular endothelial growth factor (Bonnet and Walsh 2005, Berenbaum 2013)

In the osteoarthritic joint, VEGF plays roles in endochondral bone formation, osteophyte formation, synovitis, and cartilage deterioration. Pufe *et al.* studied the expression of VEGF in OA cartilage. They found that VEGF was not expressed in normal cartilage but VEGF expression was observed in OA cartilage (Pufe *et al.* 2001). On the other hand, Pfander *et al.* found controversial data. They indicated that VEGF was expressed in both normal and osteoarthritic cartilage. A

significant elevation of VEGF was shown in OA cartilage compared with healthy controls (Pfander *et al.* 2001). With regard to endochondral ossification, initiation requires blood vessels to supply nutrients for bone formation. Zelzer *et al.* examined the role of VEGF in bone development by generating VEGF knockout mice. The results indicated that VEGF knockout mice showed delayed blood vessel invasion into the primary ossification center and decreased chondrocytes survival. This study suggested that VEGF supports chondrocyte survival and cartilage vasculogenesis (Zelzer *et al.* 2004). A number of studies from an Italian group have supported the role of VEGF in the pathogenesis of OA. Osteoblasts isolated from OA, osteoporotic, and healthy subjects were cultured and the expression of VEGF in both mRNA and protein measured. They found the expression of VEGF mRNA and protein were higher in osteoarthritic osteoblasts compared to osteoblasts from osteoporosis patients and healthy controls (Corrado *et al.* 2013). In agreement with the previous data, Neve *et al*. found VEGF expressions were elevated in osteoblastic osteoarthritis compared with those in osteoblasts from osteoporosis patients. Moreover, culture media from pathological OA and OP osteoblast can induce the formation of blood vessels in chick embryo chorioallantoic membranes (CAM) (Neve *et al.* 2013). Inflammatory proteins in the serum of osteoarhtritis patients, for example IL-1β, IL-1Ra, IL-6, MCP-1 (macrophage chemotactic protein-1), MIG (monokine induced by IFN-γ), VEGF, and GM-CSF (granulocyte macrophage-colony stimulating factor), were increased when compared with those in healthy controls (Sohn *et al.* 2012).

There is some evidence linking VEGF and the pathogenesis of OA using *in vivo* models. P.I Mapp and his colleagues determined osteochondral and synovial angiogenesis in rats with induced-OA by medial meniscal transection (Mapp *et al.* 2008). The data found that the induced-OA rats had significantly higher vascular density and more blood vessels in both their osteochondral and synovial membranes compared to sham operation rats. Therefore, angiogenesis is one of the features in knee osteoarthritis. VEGF expression in cartilage from three different OA models in rodents found: medial meniscectomy, monoiodoacetate injection (MIA), and age-assoicated spontaneous joint destruction demonstrated increased VEGF levels concurrently with the severity of cartilage destruction. These results suggest

that VEGF may play a role in progressive of OA (Yamairi *et al.* 2011). Furthermore, there are studies in mice (Ludin *et al.* 2013) and rabbits (Tibesku *et al.* 2011) to confirm the effects of VEGF in OA. The joint of healthy mice injected with VEGF initiated a full-range of osteoarthritic processes such as subchondral bone alteration, cartilage degradation, and synovial membrane hyperplasia. The study in rabbits, which operated on anterior cruciate ligaments to create osteoarthritis, reported increasing VEGF production in chondrocytes over time when compared with sham operations.

3. MicroRNAs (miRNAs)

The first miRNA is *lin-4* and was found in *Caenorhabditis elegans* in 1993 (Lee *et al.* 1993) and the first human miRNA, *let-7,* was discovered in 2001 (Tomankova *et al.* 2011). In the last decade, miRNAs have rapidly advanced investigation. There are some associations between miRNAs as regulators of transcriptional process and pathology of diseases. More evidence suggests that small non-coding miRNAs result in the pathogenesis of OA especially in articular cartilage.

3.1 Characteristic of miRNA (Tomankova *et al.* 2011, Bernardo *et al.* 2012)

miRNAs are small non-coding nucleotides (approximately 22 bp). They regulate the expression of target mRNA by binding a sequence in the 3' untranslated region (3'UTR) leading to degradation or inhibition of mRNA. miRNAs play functions in most biological pathways. They involve in cell differentiation, proliferation, apoptosis andregulate inflammationand malignancy. When the balance of miRNA is impaired, it may contribute to the pathogenesis and development of disease. Biogenesis of miRNAs is illustrated in **Figure 2.4**. The processes is initiated by miRNA transcription from the genome are called primary miRNAs (pri-miRNAs). In the nucleus, approximately 1,000 nucleotide pri-miRNA chains are cleaved by a microprocessor complex containing Drosha, an RNase III enzyme, and DiGeorge critical region 8 (DGCR8) in order to produce roughly 60 nucleotide hair-pin loop precursor miRNAs (pre-miRNAs). Pre-miRNAs are then exported to the cytoplasm by exportin 5 and processed by Dicer, a microRNA processing enzyme, to generate short sequence duplexs of miRNA: miRNA*. miRNA*, called passenger strand, is degraded while

miRNA becomes mature miRNA. Then miRNA or guide strands essemble with RNA induced silencing complex (RISC). The miRNA-RISC complex functions via base-pairing with sequences within the mRNA target. The complex miRNA-RISC which is a perfect binding target for mRNA results in mRNA degradation. The partially complementary mRNA and miRNA-RISC leads to translational repression. Each miRNA can suppress various target mRNAs and mRNA is able to be the target of multiple miRNAs.

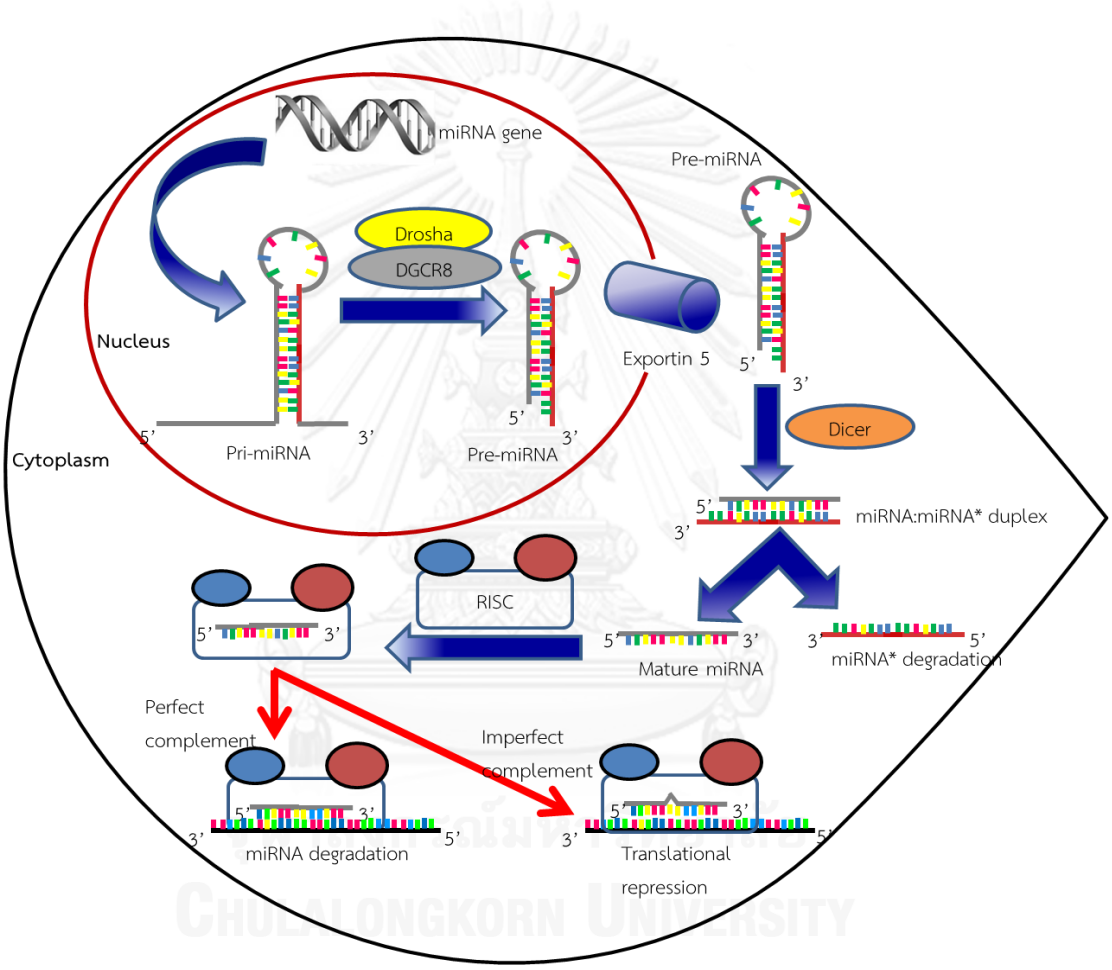


Figure 2.4 Biological mechanism of miRNAs modified formv(Winter *et al.* 2009, Staszel *et al.* 2011, Bernardo *et al.* 2012)

3.2 miRNAs in arthritis diseases

miRNAs act as negative regulators of gene expression in the development and pathology of diseases. A number of miRNAs studies were performed in many diseases such as SLE, psoriasis, rheumatoid arthritis, osteonecrosis (ON), and especially osteoarthritis (Tomankova *et al.* 2011). Previous studies have reported that miR146a

plays a role as a regulator of acute inflammatory responses. This miRNA is capable of complementary binding with the tumor necrosis factor receptor-associated factor 6 (TRAF6) and interleukin-1 receptor-associated kinase (IRAK1) genes which are associated with the NF-kB pathway (Taganov *et al.* 2006). The expression of miR-146a was up-regulated in the superficial and sublining layers of RA synovial tissue when compared with those in OA synovial tissue and healthy controls. Moreover, miR-146a expression was remarkably increased in rheumatoid arthritis synovial fibroblasts stimulated with TNF-α and IL-1ß (Nakasa *et al.* 2008). Yamasaki K. and his colleagues also determined the expression pattern of miR-146a in osteoarthritis cartilage. They found increased expression of miR-146a in mild OA cartilage when compared with moderate and severe groups. In progressive OA (high severity), the expression of miR-146a was decreased. Furthermore, the miR-146a expression in chondrocytes was also induced by IL-1ß stimulation (Yamasaki *et al.* 2009). Previous studies reported that the expression of miR-155 and miR-146 were elevated in RA synovial tissue, synovial fluid, and synoviocytes. miR-155 may induce the proinflammatory cytokine production which supports arthritis inflammation (Stanczyk *et al.* 2008, Kurowska-Stolarska *et al.* 2011). Some miRNAs can function by up-regulation, however, other miRNAs have a down-regulatory role in the development stage of RA, such as miR-132. Murata K *et al.* studied the differentiation patterns of miRNAs between plasma and synovial fluid. The results suggested that plasma miR-132 expression was lower in RA and OA than in healthy controls (Murata *et al.* 2010). Therefore, the expression of miRNAs are specific to cell-developmental, tissue, and developmental stages of disease. Additionally, they can play roles as pathogenic and protective substances in biological process.

Recently, numerous researches have discusssed the importance of miRNAs in the pathology of cartilage in osteoarthritis. Various miRNAs have been differently expressed in normal and pathological OA cartilage (Reynard and Loughlin 2012). For example, miR140 which has a cartilage specific expression pattern was increased during chondrogenesis parallel with collagen 2A1 expression. Meanwhile down-regulation of miR-14 was observed in human articular cartilage obtained from OA patients including OA chondrocytes treated with IL-1ß. The results indicated that

decreased miR-140 in OA chondrocytes may be associated with the pathology of the disease (Miyaki *et al.* 2009). More mice lacking miR-140 developed osteoarthritis than wild type mice related with age. Furthermore, up-regulated miR-140 by transfection could protect the deterioration of mice cartilage (Miyaki *et al.* 2009). The pattern of miR-146 expression in OA was distinct from RA because the levels of the miR-146 were decreased according to the severity of OA (Yamasaki *et al.* 2009). miR-199a* was defined as a suppressor of chondrogenesis in the early stages via imperfect binding with Smad1 (downstream of the BMP-2 pathway) leading to the inhibition of Smad1 expression and controling chondrogenic growth (Lin *et al.* 2009). Additionally, miR-199a regulated the expression of COX-2 which is associated with inflammation and leads to pain in OA. A previous study found that decreased-expression of miR-199a could elevate COX-2 expression and that there were negative correlations between miR-199a and COX-2 protein levels in OA chondrocytes (Akhtar and Haqqi 2012). Besides miRNAs research in chondrocytes, there are some studies in osteoblasts. Several miRNAs have been defined to osteoblastogenesis regulators, such as miR-29, miR-141, miR-206, and miR-210 (Kapinas and Delany 2011).

In this study, we focus on the expression profile of miR-210 and miR-223. Only limited liturature has evaluated the association between the roles of both miRNAs and osteoarthritis.

miR-210 is involved in angiogenesis which is increased under hypoxic conditions. A previous study in ON or avascular necrosis patients found that miR-210, VEGF, MMP-2, MMP-7, and hypoxia-inducible factor-1α expressions were elevated in ON. MiR-210 and VEGF levels were especially expressed in endothelial cells around necrotic areas. Therefore, miR-210 may be associated with hypoxia resulting in ON (Yamasaki *et al.* 2012). Moreover, miR-210 is able to stimulate blood vessel formation and angiogenesis in ischemic diseases such as ischemic/reperfusion (I/R) renal injury. Over expression of miR-210 in HUVEC cells can stimulate tube formation and also activate VEGF expression. I/R renal injuries in mouse models showed the level of miR-210 was increased in the injuried renal group compared with the sham group (Liu *et al.* 2012). Another *in vivo* experiment by Shoji *et al.* studied anterior cruciate ligament (ACL) healing. Intra-articular injections of miR-210 can promote ACL healing

via VEGF and fibroblast growth factor-2 (FGF-2) enhancing angiogenesis (Shoji *et al.* 2012). In addition to its angiogenesis role, miR-210 can act as a positive fine tuner of osteoblastic differentiation. The study by Mizuno and his colleagues demonstrated that miR-210 repressed the activin A receptor type 1B gene in TGF-β /activin signaling resulting in elevated osteoblastic differentiation (Mizuno *et al.* 2009).

miR-223 is a regulator in osteoclast differentiation processes (Sugatani and Hruska 2007). It also plays roles in the immune system, regulation of granulocytic differentiation and maturation, and inflammation. Many kinds of cells can express this miRNA such as macrophages, monocytes, osteoclasts, and fibroblast-like synoviocytes (Shibuya *et al.* 2013). A previous investigation demonstrated the expression of miR-223 in rheumatoid arthritis. miR-223 was expressed at higher levels in the superficial and sublining layers of synvovium in RA than those in OA. Moreover, an osteoclastogenesis model was performed by coculturing RA synovial fibroblasts and peripheral blood mononuclear cells (PBMC) combind with receptor activator of nuclear factor kappa-β (RANKL) or TNF-α stimulation. The cells were then transfected with miR-223. Overexpression of miR-223 blocked osteoclasogenesis by reducing tartrate-resistant acid phosphatase (TRAP) positive cells (Shibuya *et al.* 2013). Therefore, miR-223 suppresses osteoclastogenesis resulting in a new gene therapy to treat bone damage in RA.

In the recent years, Fulci *et al.* investigated the role of miRNA in RA, especially in T-lymphocytes. They suggested that miR-223 was significantly increased in RA T-lymphocytes, particularly naive T-lymphocytes while it was not expressed in naive T-lymphocytes from healthy controls (Fulci *et al.* 2010).

Interestingly, Okuhara A and colleagues examined the expression of miRNAs in PBMC of OA patients. The expression patterns of miR-146a, -155, -181a and -223, which have functions in immunity and inflammation, were up-regulated in the disease group when compared with the control group. The expression of miR-146a and miR-223 were highly expressed in early OA while miR-155 was increased in late stage OA (Okuhara *et al.* 2012).

Although miR-210 and miR-223 were studied in arthritic disseases, there is little evidence to verify the correlation of these miRNAs and VEGF expression resulting in the pathogenesis of osteoarthritis.

4 Polymorphism in osteoarthritis

(Balasubramanian et al. 2002, Wu and Jiang 2013)

4.1 Characteristics of single nucleotide polymorphisms

Single nucleotide polymorphisms (SNPs) are the most frequent polymorphisms occuring at DNA sequence of central dogma. SNPs have a frequency of at least 1% resulting in a single base mutation substituting one nucleotide for another. It means SNPs can occur approximately every 300 basepairs in the genome. Therefore, there are an estimated 10 milion SNPs in the entire human DNA sequence. Most of the SNPs are in non-coding regions (99% not in genes). Some SNPs affect the regulation of gene expression and the production of proteins.

4.2 Single nucleotide polymorphisms in arthritis diseases

SNPs have been studied not only in many diseases but also in many positions in the genome. A SNP which strongly increased the susceptiblity in osteoarthritis is the rs143383C/T SNP in 5'UTR of growth differentiation factor 5 (*GDF5)*. This gene has functions as an extracellular molecule regulating the development, maintenance, and repair of cartilage, bone, and synovial tissue in joints. The T allele is associated with the reduction of the *GDF5* mRNA transcription factor compared to the C allele. The results indicated that the T allele rs143383 increased the risk of osteoarthritis. There are many OA researches studing the GDF5 C/T SNP in various ethnic groups including Japanese, European, and Asian (Southam *et al.* 2007, Chapman *et al.* 2008, Valdes *et al.* 2011). A mice model was used to comfirm the functional of the *GDF5* polymorphism by Harada M *et al*. They investigated the characteristics of knee joints in brachypodism mice (bp mice) which lack of the *GDF5* gene. The knee joint of bp mice exhibited the failure of chondrogenesis and lack of intra-articular ligament (Harada *et al.* 2007).

Besides the *GDF5* polymorphism, variations in collagen type 2A1 (COL2A1), calcitonin, and the MMP family were also determined in osteoarthritis. The polymorphism of COL2A1 in the Mexican Mestizo cohort found no association of COL2A1 among P and p alleles in OA and controls. However, there was association with p allele and knee OA grade 4 (Galvez-Rosas *et al.* 2010). While the study in the Han Chinese population showed the AA genotype of the G4006A SNP was significantly elevated in OA patients compared to controls (Xu *et al.* 2011). Calcitonin (CT) is a hormone that possesses an anabolic effect in cartilage and plays a chondroprotective role. The genetic variation in the *CT* gene illustrated that G-706T (GT genotype and G allele) and C-788T (CC genotype and C allele) polymorphisms increased supceptibility in knee OA patients (Magana *et al.* 2013). For *MMP* gene promoter polymorphisms, the genotype 1G/1G and 1G/2G of MMP-1 were significantly elevated in knee OA patients when compared with controls. No association between genotype distribution and allelic frequency of MMP-2 and MMP-9 were found in OA and healthy controls (Barlas *et al.* 2009). In addition, the MMP-3 (-1612 5A/6A) polymorphism was not associated with the development of knee osteoarthritis (Honsawek *et al.* 2013). Nevertheless, the MMP-3 5A allele was remarkedly higher in RA patients compared with healthy subjects and MMP-1 and MMP-9 were not significantly different in RA and controls (Scherer *et al.* 2010).

4.3 Vascular endothelial growth factor single nucleotide polymorphisms in arthritic diseases

VEGF plays roles in the proliferation, migration, and tube formation of endothelial cells and vascular permeablility regulation. Polymorphisms of the *VEGF* gene have been correlated with VEGF protein production and susceptibility to various diseases in which angiogenesis may be important to the pathology. The consensus sequence of *VEGF* gene is shown in **Figure 2.5**. The *VEGF* gene is located on chromosome 6p12 and consists of eight exons. There are numerous VEGF SNPs that have been studied including polymorphisms in the promoter, 5'untranslated region (5'UTR), and 3'UTR. The most important SNPs which are widely investigated in those positions are rs699947 (-2578C/A), rs1570360 (-1154G/A), rs2010963 (-634G/C), and

rs3025039 (+936C/T). The variations in 5'and 3'UTR are associated with a high variability of VEGF production including VEGF protein and circulating plasma concentrations (Jain *et al.* 2009).

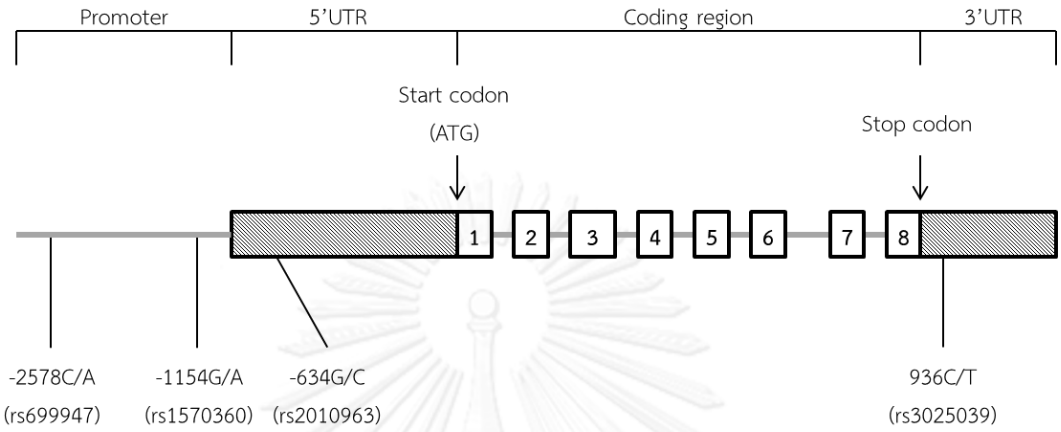


Figure 2.5 Consensus sequence of *VEGF* gene modified from (Etienne-Grimaldi *et al.* 2011)

VEGF polymorphisms influence many cancers. For example, a case-control study in breast cancer examined the VEGF 936C>T SNP. It found that participants with the 936T allele had a reduced risk of breast cancer because this variant correlated with lower VEGF production in plasma. Moreover, subjects with the -2578C and -634C haplotype had more progressive tumors (Jain *et al.* 2009). VEGF polymorphisms have been shown to be associated with cancer-specific survival. In addition, -1154 G>A was correlated with an increasing risk of invasive breast cancer (Vaziri *et al.* 2010). Furthermore, there is evidence of a relationship between VEGF SNPs and SLE. Wongpiyabovorn *et al*. reported that the polymorphisms within the *VEGF* gene (-460C>T and +405C>G) were not different between SLE patients and healthy controls. However, the +405GG was significantly increased in lupus nephritis patients with low VEGF mRNA expression in the kidneys when compared to those patients with high VEGF mRNA expression in the kidneys (Wongpiyabovorn *et al.* 2011). A study in psoriasis, chronic inflammation in skin keratinocytes, also demonstrated the SNP in the *VEGF* gene at 405G/C (rs2010963) position. The results showed that the GG genotype was observed more in psoriasis cases than controls. The G allele was also associated with increased-VEGF production (Wu *et al.* 2010).

Moreover, Butt *et al.* examined genetic variants of the *VEGF* genes in psoriatic arthritis (PsA), which is an interrelated disorder with psoriasis but for which PsA is a unique epidemiologic and genetic feature. They observed that the T allele of the 936C/T SNP was increased in controls when compared with PsA patients. They suggested that the 936T allele has a protective effect in PsA (Butt *et al.* 2007). On the other hand, Han *et al.* studied VEGF SNPs in rheumatoid arthritis such as -2578C>A, -1154G>A, -634C>G, and 936C>T in Korean patients. They found that only the T allele of 936C>T was more frequent in RA compared with controls while other SNPs were not associated with the pathology of RA. The data indicated the 936T allele provides an increased risk of RA (Han *et al.* 2004). Zhang *et al.* conducted a study of RA in a Chinese population. They performed analysis of the VEGF rs69947C/A (-2578C/A), rs2010963G/C (-634G/C), and rs3025039C/T (+936C/T) gene polymorphisms. No SNPs were associated with a risk of RA susceptibility. However, the dominant CC/AA genetic model reduced the risk of RA in older patients when compared with the genotype CC (Zhang *et al.* 2013). Moreover, Rueda *et al*. investigated a Spanish caucacian population and also found that the single nucleotide polymorphisms at -1154 and -634 positions were not correlated with RA susceptibility (Rueda *et al.* 2005). Interestingly, there is a significant polymorphism of VEGF -634G/C of osteonecrosis of the femoral head (ONFH) in a Korean population. The results demonstrated the C dominant (GC+CC) and C allele in case were significantly associated with an increased risk of ONFH (Kim *et al.* 2008).

According to all mentions above, these findings suggest that different diseases and ethnicities can exhibit various SNPs. Therefore, we may predict the risk of disease development using SNPs. However, there is no evidence of VEGF SNPs associated with the risk of knee osteoarthritis. Thus, this study proposed to determine the VEGF SNPs in knee osteoarthritis in a Thai population.

CHAPTER III

Materials and methods

1. Raw materials, chemicals, and reagents

1.1 Raw materials

1. EDTA blood collection tube (Vacuette, USA)
2. Illustra blood genomicPrep Mini Spin Kit (GE Healthcare, UK)
3. RNeasy mini kit (Qiagen, USA)
4. mirVana™ miRNA isolation kit (AmbionInc, USA)
5. Bio-Plex Pro™ Assays Human Angiogenesis 9-plex Panel (Bio-Rad Laboratories Inc., USA)
6. Human VEGF Quantikine ELISA kit (R&D systems Inc., USA)
7. *Taq* DNA polymerase (Thermo Scientific, Germany)
8. *Taq*man® reverse transcription reagents (Applied biosystems, USA)
9. Quantitect® Multiplex PCR NoROX kit (Qiagen, USA)
10. Primary antibody: Rabbit polyclonal anti-human VEGF antibody (A-20):sc-152 (Santa Cruz biotechnology, Inc, USA)
11. Secondary antibody: Goat polyclonal anti-rabbit immunoglobulin (Dako an agilenttechnologies company, Denmark)
12. Recombinant human IL-1β, CF (R&D systems Inc., USA)
13. Pierce™ BCA protein assay kit (Thermo Scientific, Germany)
14. *Taq*Man® MicroRNA Reverse Transcription kit (Applied biosystems, USA)
15. *Taq*Man® MicroRNA Assays: primer for RT reaction and primer and probe for real time PCR (Applied biosystems, USA)
16. *Taq*Man® Universal PCR master mix (Applied biosystems, USA)

1.2 Chemicals and reagents

1. RNA*later*™ RNA stabilization reagent (Qiagen, USA)
2. Chemicals for red blood cells lysis buffer
    a. Potassium bicarbonate ($KHCO_3$)
    b. Ammonium chloride (NH_4Cl)

   c. Ethylenediaminetetraacetic acid (EDTA)
3. Absolute ethanol
4. Liquid nitrogen
5. 10% formalin
6. Xylene
7. Phosphate buffer saline (PBS)
   a. Sodium phosphate dibasic (Na_2HPO_4)
   b. Potassium phosphate dibasic (KH_2PO_4)
   c. Sodium chloride (NaCl)
   d. Potassium chloride (KCl)
8. Proteinase K ready to use (Dako an agilent technologies company, Denmark)
9. 3,3 diaminobenzidine tetrahydrochloride (DAB)
10. Hydrogen peroxide (H_2O_2)
11. Dulbecco's modified Eagle medium (DMEM) (Hyclone, USA)
12. Fetal bovine serum (FBS) (Hyclone, USA)
13. 0.25% Trypsin with EDTA·4Na (Hyclone, USA)
14. 100 U/ml Penicillin/streptomycin antibiotic (Hyclone, USA)
15. Tween 20
16. Dimethylsulfoxide (DMSO) (Sigma-Aldrich, USA)
17. Reagents for polymerase chain reaction (PCR)in single nucleotide polymorphism (SNP) experiment (Thermo Scientific, Germany)
   a. 10X *Taq* buffer with $(NH_4)_2SO_4$
   b. 25 mM $MgCl_2$
   c. 2 mMdNTP Mix
   d. 5 U/µl *Taq* DNA polymerase
   e. 10 µM forward primer and 10 µM reverse primer (Bio Basic Inc. Canada)
18. Reagents for restriction fragment length polymorphism (RFLP) (New England Biolabs, Inc., USA)
   a. 1X NEBuffer

b. Restriction enzyme: *BstYI, MnlI, BsmFI,* and *NlaIII*

19. Trizma® base, primary standard and buffer ($C_4H_{11}NO_3$)
20. Glacial acetic acid ($C_2H_4O_2$)
21. Boric acid (H_3BO_3)
22. Agarose molecular biology grade (Research Organics, USA)
23. Reagents for preparing polyacrylamide gel
    a. 5X TBE buffer
    b. 30% acrylamide (40% acrylamide + N,N'-methylenebis-acrylamide (Bis-acrylamide))
    c. 10% ammonium persulfate (APS)
    d. N,N,N',N'-tetramethylethylenediamine (TEMED)
24. DNA ladder 100 bp + 1.5 Kb (SibEnzyme ltd., Russia)
25. GeneRuler™ ultra low range DNA ladder (Thermo Scientific, Germany)
26. Ethidium bromide

1.3 Equipment

1. Surgical blade stainless steel no. 11 and 23 (Feather, Japan)
2. Petri dish (Sterilin, UK)
3. Microcentrifuge tube: size 1.5 ml, 0.2 ml, 0.1 ml
4. Centrifuge 5804R (Eppendorf, USA)
5. Centrifuge: Spectrafuge 24D (Labnet international, Inc., USA)
   : H-103N series (KokusanEnshinki co., ltd, Japan)
6. Heat block: Accublock™ digital dry bath (Labnet international, Inc., USA)
7. Horizontal shaker: Rocker NB-104 (N-Biotek, Inc. Belgium)
8. Vortex mixer (IKA-Labortechnik, China)
9. Nanodrop 2000 spectrophotometer(Thermo Scientific, Germany)
10. Mortar and pestle
11. 20-gauge and syringe (Nipro, Belgium)
12. Spin down centrifuge: Force mini (Liolab international co., ltd, Thailand)
13. Microplate reader

: BiochromAnthos 2010 microplate reader (Biochrom, UK)

: Biorad model 680 microplate reader (Bio-Rad Laboratories Inc., USA)

14. Glass slide: SuperFrost® Plus (Thermo Scientific, Germany)
15. Microscope cover slip 22 X 22 mm (Thermo Scientific, Germany)
16. Microscope and cooled color camera (Nikon eclipse 50i and Nikon DS-Fi1, Japan)
17. Thermal cycler (PCR): Mastercycler personal (Eppendorf, USA)
18. Electrophoresis chamber set (Bio-Rad Laboratories Inc., USA)
19. Vertical electrophoresis: Mini-PROTEAN® Tetra System (Bio-Rad Laboratories Inc., USA)
20. StepOnePlus™ Real-Time PCR Systems (Applied Biosystems, USA)
21. Water bath: Memmert WB 45 (Memmert, German)
22. 25 cm^2 cell culture flask (T25 flask) and 75 cm^2 cell culture flask (T75 flask) (Corning, USA)
23. 15 ml and 50 ml centrifuge tube (Corning, USA)
24. 6 well cell culture cluster plate (Corning, USA)
25. Accu-jet® pro Pipette Controller (BrandTech scientific, Inc. USA)
26. Sero pipette 1, 5, 10 ml (Henneberg-Sander GmbH(HBG), Germany)
27. Cell culture incubator: Forma Seried II water jacketed CO_2 incubator (Thermo Scientific, Germany)

    : Napco Automatic three-gas CO_2 incubator (Thermo Scientific, Germany)
28. Class II biological safety cabinet:model BH-120 (GelmanSciences, Australia)

    : ESCO (ESCO Technologies, Inc., Singapore)
29. Power supply: PowerPac™ HC (Bio-Rad Laboratories Inc., USA)
30. Bio-Rad Gel doc™ XR (Bio-Rad Laboratories Inc., USA)
31. UV transilluminator 2000 (Bio-Rad Laboratories Inc., USA)
32. Multichannel pipette
33. Autopipette

2. Experimental procedures

We divided the experiments into four separate parts in order to determine the role of vascular endothelial growth factor in the pathogenesis of osteoarthritis. The first part was to study the association of VEGF protein levels in samples obtained from knee OA patients. The second part was to evaluate the single nucleotide polymorphisms (SNPs) of the *VEGF* gene in OA compared with healthy controls. The third part was to determine the expression of VEGF mRNA, miR-210, and miR-223 in synovial tissues from the OA patients and to examine the results of VEGF and microRNA *in vitro* under inflammatory and hypoxic stimulation. All participants in this study were recruited with the criteria as shown in **section 2.1**. Informed consent was obtained from all subjects according to protocols approved by the Institutional Review Board on Human Research of the Faculty of Medicine, Chulalongkorn University (IRB. No. 223/54). The details of each part are described as follows.

2.1 Population in the study

Osteoarthritis patients

Patients with knee osteoarthritis underwent total knee replacement surgery at King Chulalongkorn Memorial Hospital. The patients aged from 50 to 90 years were recruited in this study. Diagnosis of OA was based on clinical and radiographic parameters. The American college of Rheumatology (ACR) criteria of knee OA as described by Altman *et al*. was used to diagnose the osteoarthritis (Altman *et al.* 1986). According to clinical knee OA, the patients should have knee pain and at least three out of six of the following criteria: age > 50 years, bony tenderness, stiffness < 30 minutes, bony enlargement, crepitus, or no palpable warmth (Belo *et al.* 2009). The radiographic severity of OA patients were graded by Kellgren-Lawrence (K/L) classification and divided into 5 groups (grade 0-4) in accordance with changing in radiographic features of the knee joint such as formation of osteophyte or narrowing of joint space. The subjects were classified as grade 0 of osteoarthritis (normal): no radiographic findings, grade 1 (doubtful): possible osteophyte formation with doubtful narrowing of joint space, grade 2 (mild): definite osteophyte with possible narrowing of joint space, grade 3 (moderate): moderate osteophyte with definite narrowing of

joint space, some sclerosis and possible deformity of bone, grade 4 (severe): large osteophyte with marked narrowing of joint space, more sclerosis, and deformity of bone. The OA patients defined as having pathologic of osteoarthritis of K/L grade $\geq$ 2 in at least one knee were including in this study. The radiograph representing knee osteoarthritis classification was shown in **Figure 3.1** (Kellgren and Lawrence 1957, Kijowski *et al.* 2006). All participants gave written inform consent. The number of synovial tissues from registered participants was 44 according to sample size equation shown as below:

$$n = \frac{2\left(Z_{\alpha/2} + Z_{\beta}\right)^2 \sigma^2}{(\bar{x}_1 - \bar{x}_2)^2}$$

$$\alpha = 0.05 \text{ , } z_{\alpha/2} = 1.96$$

$$\beta = 0.1 \text{ , } z_{\beta} = 1.28$$

($\overline{X_1}, \overline{X_2}$, and σ^2 obtained from preliminary experiment)

The number of subjects giving whole blood which brought to use in single nucleotide polymorphism assay was approximately 100 according to sample size equation shown as below:

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

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

$$n = \frac{(1.96)^2}{4(0.1)^2} = 96.04 \approx 100$$

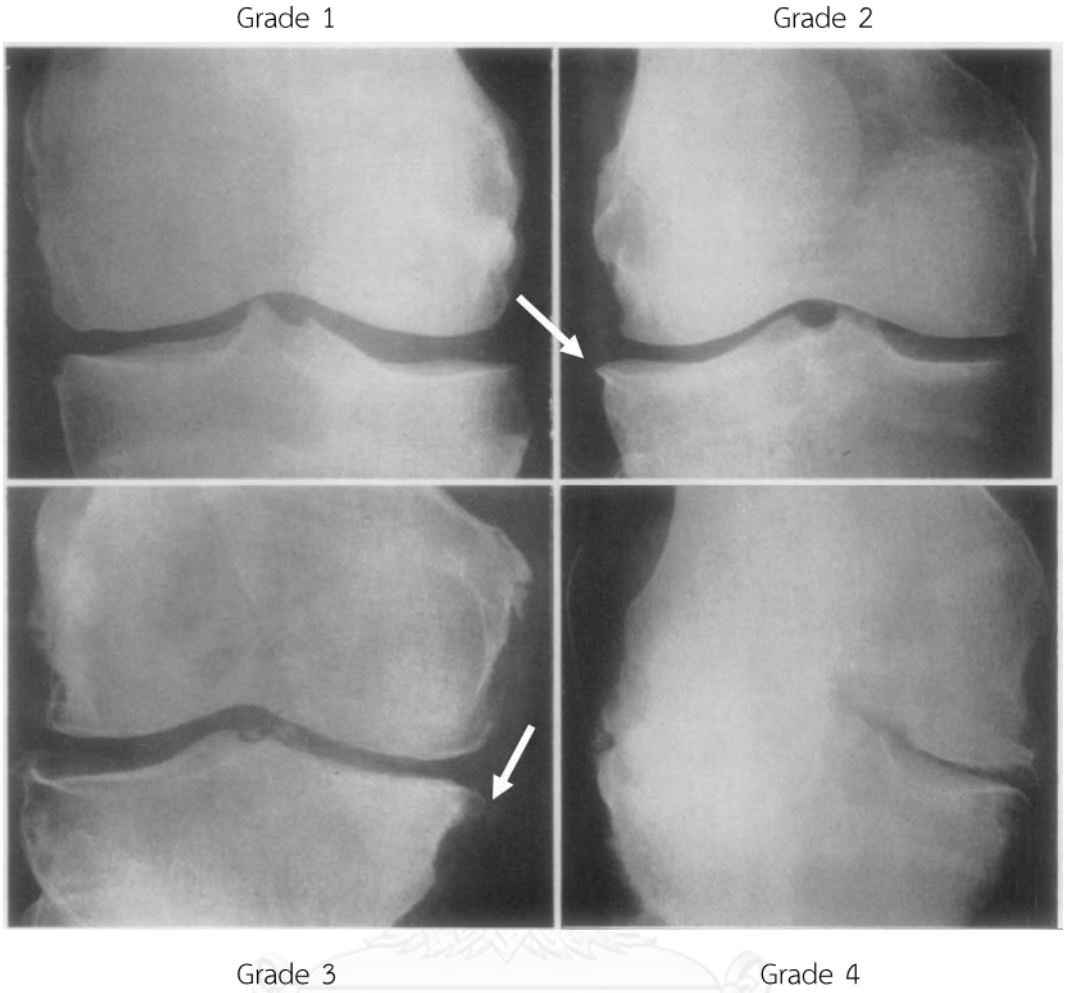


Figure 3.1 Classification of the severity of knee osteoarthritis using X-ray film, arrows indicate osteophyte formation (Kellgren and Lawrence 1957)

Healthy controls

Forty to eighty years old participants with knee trauma who underwent knee surgery or arthroscopy by orthopedic surgeons at King Chulalongkorn Memorial Hospital were recruited in this study as control group. Controls also were defined as having no clinical and no radiographic knee OA as indicated by K/L classification of 0. The subjects with arthropathy resulting from gout, pseudogout, rheumatoid arthritis, psoriasis, previous joint infection were excluded from the study (Honsawek *et al.* 2009). Moreover, the healthy volunteers who registered in this research were healthy aging volunteers without knee pain at Lumpini Park and the elderly subjects who visited the hospital for an annual checkup.

2.2 Part I: Association of vascular endothelial growth factor protein in pathogenesis of knee osteoarthritis

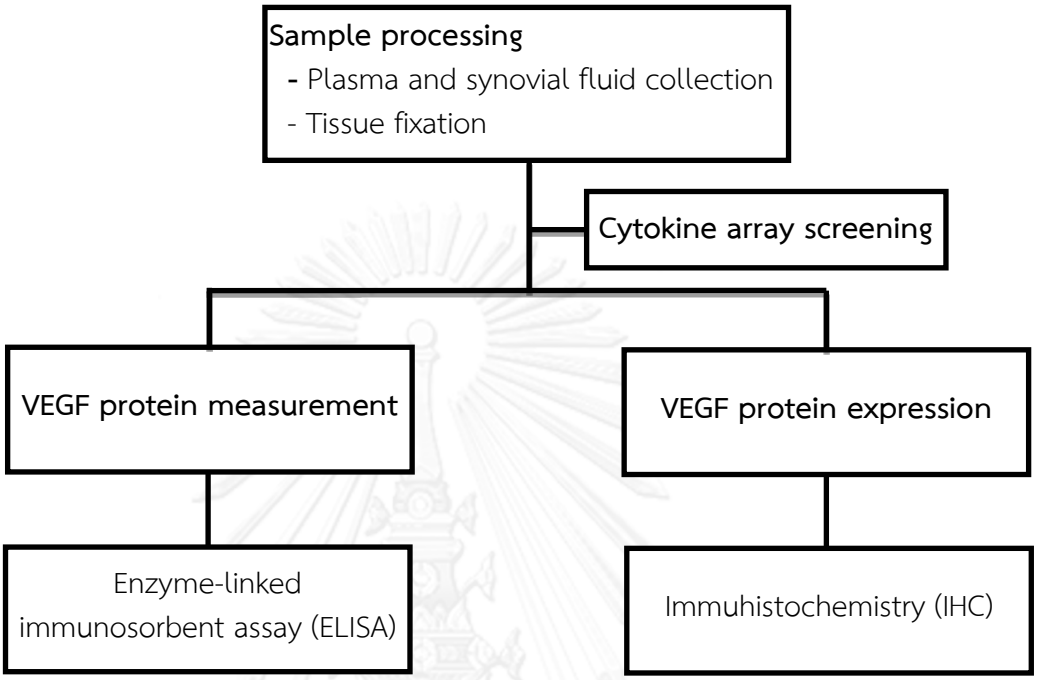


Figure 3.2 Flow chart of part I experiment

2.2.1 Sample processing

We collected all samples including synovial tissues, articular cartilage, synovial fluid, and whole blood. The synovial tissues, cartilage, and fluid were obtained when the OA patients underwent knee operations. The synovial tissues from the control group were collected while they were receiving arthroscopic surgery of the knee. However, we could not collect cartilage and synovial fluid from controls. Whole blood samples were obtained from OA and control groups.

- **Plasma and synovial fluid collection**

Plasma samples were collected from knee OA and controls. To obtain plasma, whole blood was drawn into tubes containing EDTA. Then the EDTA-blood was centrifuged at 4000 rpm for 10 minutes and plasma supernatant was aspirated. Pack red blood cells were collected for the experiment in **Part II (*VEGF* single nucleotide polymorphisms)**. Synovial fluid samples from OA patients were also centrifuged to aggregate red blood cells and other debris. The supernatant was collected. All of the samples were kept at -20°C until analysis.

- **Tissue fixation**

The synovial tissues and cartilage were cut into 0.3 X 0.3 centimeters, soaked in 10% formalin and embedded in paraffin. The tissue fixation was performed and subjected to immunohistochemical staining.

2.2.2 Angiogenesis cytokine array screening

A magnetic-bead based multiplex angiogenesis assay kit (Bio-Plex Pro, Bio-Rad Laboratories Inc., USA) was used to analyze 9 angiogenic proteins simultaneously on 96-well plates. A Bio Plex-200 array reader was used to analyze the samples. The proteins under investigation and their detection levels were: Angiopoietin-2 (≤500 pg/ml), follistatin (≤100 pg/ml), granulocyte-colony stimulating factor (G-CSF) (≤2 pg/ml), hepatocyte growth factor (HPF) (≤100 pg/ml), interleukin (IL)-8 (≤2 pg/ml), leptin (≤100 pg/ml), platelet-derived growth factor-BB (PDGF-BB) (≤100 pg/ml), platelet endothelial cell adhesion molecule (PECAM)-1 (≤1000 pg/ml), and vascular endothelial growth factor (VEGF) (≤2 pg/ml). The procedure was performed in accordance with the manufactures protocol (Bio-Plex Pro™ Assays Human Angiogenesis 9-plex Panel (Bio-Rad Laboratories Inc., USA).

2.2.3 VEGF protein measurement in plasma and synovial fluid by enzyme-linked immunosorbent assay (ELISA)

In this research, we used the human VEGF Quantikine ELISA kit (R&D systems Inc., USA) to measure the concentration of VEGF in synovial fluid and plasma

in OA patients as well as plasma in healthy controls. Quantitative protein analysis was performed in accordance with the manufacturer's recommendation **(Figure 3.3)**. The samples kept at-20°C were thawed at room temperature and centrifuged for a short time before used. All reagents were placed at the ambient room temperature. The VEGF standard reconstitute was prepared by mixing VEGF standard with 1 ml of calibrator dilute RD6U (an animal serum with preservative buffer). After that the standard stock solution was used with serial dilutions from concentration 2000 to 0 pg/ml. Following this, 100 µl of diluents RD1W was added to each well in a 96 well microtiterplate and then 200 µl per well of the standards, controls, and samples were added and incubated for two hours at room temperature. The wells were washed with 400 µl of wash buffer for a total of three washes. After the last wash, the plate was inverted and blotted against clean paper towels to remove any remaining wash buffer. After three washes, 200 µl VEGF conjugate was added and the VEGF plate was incubated for two hours at room temperature. After a further three washes, 200 µl of substrate solution was added to each well and incubated for 25 minutes at room temperature in dark. Finally, the reaction was stop by the stop solution and the concentration of VEGF was determined within 30 minutes using a microplate reader set to 450 nm, and subtract readings at 540 nm or 570 nm. The absorbance or optical density (OD) from the microplate reader was calculated for the VEGF concentration using standard curve.

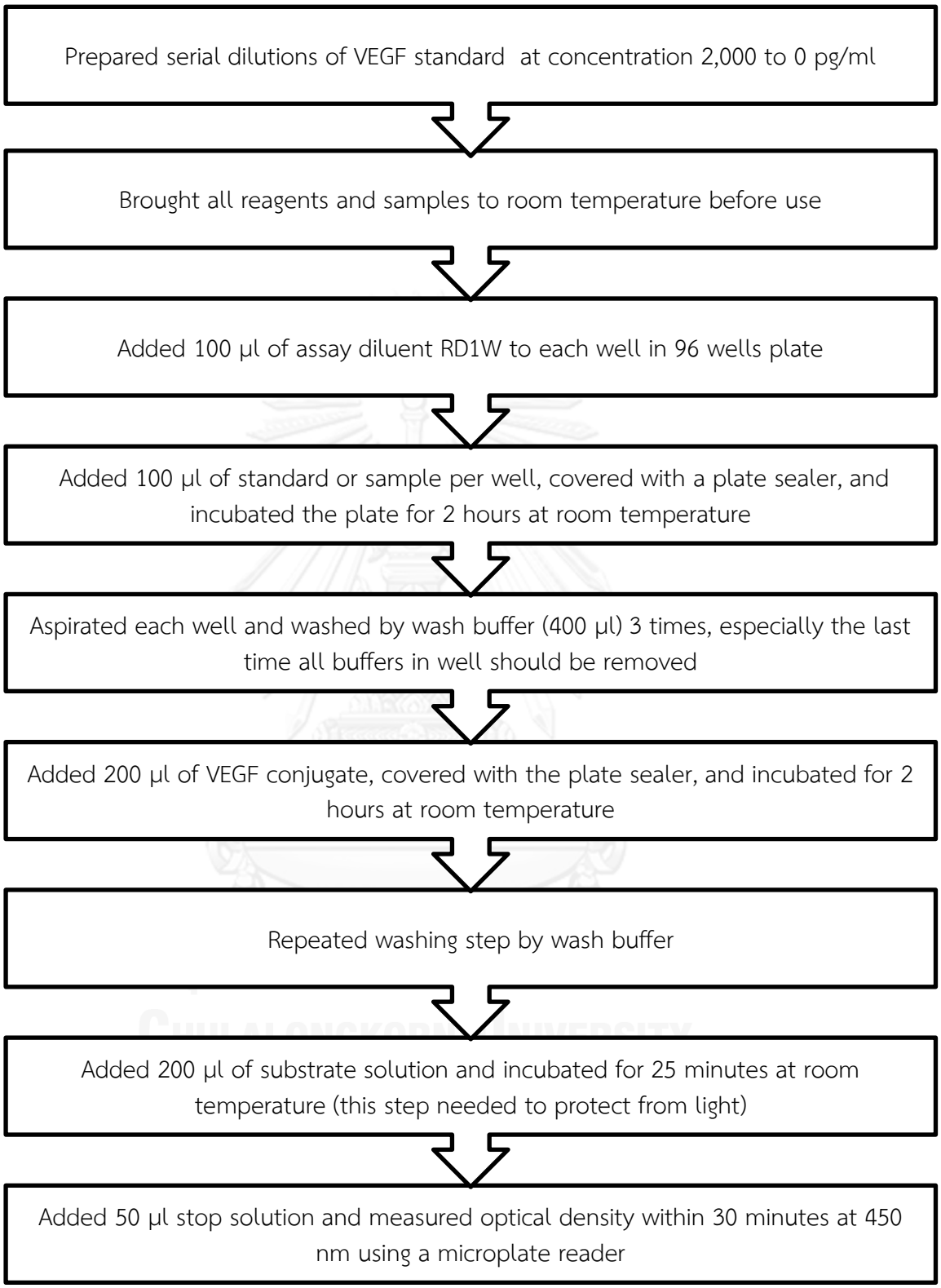


Figure 3.3 Diagram of enzyme-linked immunosorbent assay

2.2.4 VEGF protein expression in synovial tissues and articular cartilage by immunohistochemistry

The expression of VEGF was examined in cartilage and synovial tissues from OA knee patients using an immunohistochemical assay. The tissues embedded in paraffin were de-waxed, rehydrated, and peroxidase blocked. Serial sections of paraffin-embedded tissue were cut in 5 μm thickness and processed for VEGF staining. Sections were deparaffinized in xylene and rehydrated through gradient alcohol, and distilled water. For antigen retrieval, tissue sections were microwave heated in 10 mmol/L citrate buffer for 5 minutes. Endogenous peroxidase activity was blocked with 3% H_2O_2 for 5 minutes. Nonspecific binding was blocked for 20 minutes with 3% normal horse serum (DAKO, Glostrup, Denmark), followed by incubation with primary antibody (rabbit polyclonal anti-human VEGF antibody, 1:100; Santa Cruz Biotech, Santa Cruz, USA) in Tris-buffered saline containing 2% rabbit serum and 1% bovine serum albumin for 1 hour. Tissues were incubated with the same buffer without the antibody to serve as negative controls. Sections were subsequently stained with biotinylated goat anti-rabbit immunoglobulins (1:400; DAKO) and streptavidin/horseradish peroxidase complex (1:400; DAKO) and incubated at room temperature for 30 minutes. Reaction products were visualized using diaminobenzidine (Sigma-Aldrich, St. Louis, USA) as the chromogen. The VEGF positive staining was presented in brown color under light microscope.

2.3 Part II: SNPs of *VEGF* gene in knee osteoarthritis patients determination

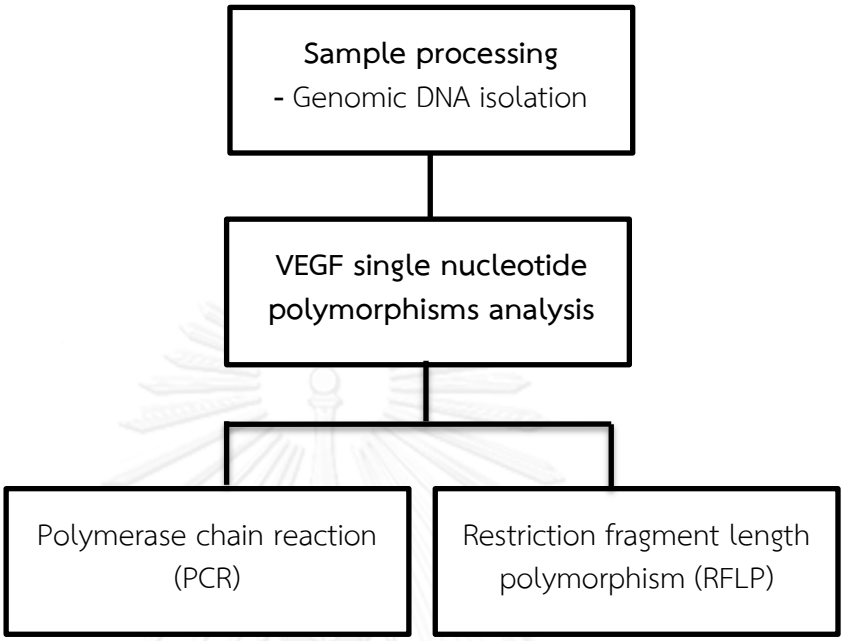


Figure 3.4 Flow chart of part IV experiment

2.3.1 Sample processing

The whole blood samples were collected from OA patients undergoing total knee replacement. The blood samples form healthy controls were obtained at annual check-ups. EDTA-blood samples were centrifuged at 4,000 rpm for 10 minutes and the supernatant (plasma) aspirated. The pack red blood cells were kept for DNA extraction and single nucleotide polymorphism (SNP) analysis.

- **Genomic DNA isolation**

DNA was extracted from peripheral blood mononuclear cells obtained from osteoarthritis patients and healthy controls by illustra blood genomic Prep Mini Spin Kit (GE Healthcare, UK) **(Figure 3.5)**. A volume of pack red cells were mixed with 3 folds the blood sample volume of red blood cells (RBCs) lysis buffer, incubated and inverted at room temperature for 5 minutes in order to break red blood cells. The lysed-RBCs were centrifuged at 4,000 rpm for 5 minutes to precipitate the white blood cells (WBCs). After centrifugation, the supernatant was discarded and the pellet re-suspended with 100-150 µl of PBS to take volumes up to 200 µl of

suspension (if the volume of suspension was 200 µl, PBS buffer would not be added). Then the cell suspension was transferred to a fresh 1.5 ml microcentrifuge tube containing 20 µl of proteinase K and mixed with 400 µl of lysis buffer type 10. The WBCs suspension was incubated at room temperature for 10 minutes with intermittent vortex in order to lysed WBCs (at the end of this step, the solution was changed to dark brown color). Next process was the genomic DNA binding step using a mini column insert into the collection tube. The complete lysate was loaded 600 µl into the mini column and spun for 1 minute at 11,000 g. Then the flow-through in the collection tube was removed and placed the column back into the collection tube. However, if the lysate was more than 600 µl, it was loaded and centrifuged again until completely. After that, 500 µl of lysis buffer type 10 was added into the column, centrifuged for 1 minute at 11,000 g to ensure complete cell lysis and denaturation of proteins. The flow-through was discarded. Then 500 µl wash buffer type 6 was added to the column, spun for 3 minutes at 11,000 g and removed the flow-through. The column was re-centrifuged to get rid of ethanol which is component of wash buffer (at the end of this step, the genomic DNA was trapped on the silica matrix in column). The column was transferred to the new 1.5 ml microcentrifuge tube. The 70°C pre-heated elution buffer type 5 was then added 200 µl in the center of the silica membrane in the column and incubated at room temperature for 1 minute. The column was centrifuged for 1 minute at 11,000 g to recover the DNA. The concentration of genomic DNA was measured using Nanodrop 2000 spectrophotometer (Thermo Scientific, Germany). The acceptable purity ratio of purified DNA (ratio 260/280) of this kit is 1.7 to 1.9. The genomic DNA was stored at -20°C for use in single nucleotide polymorphism analysis.

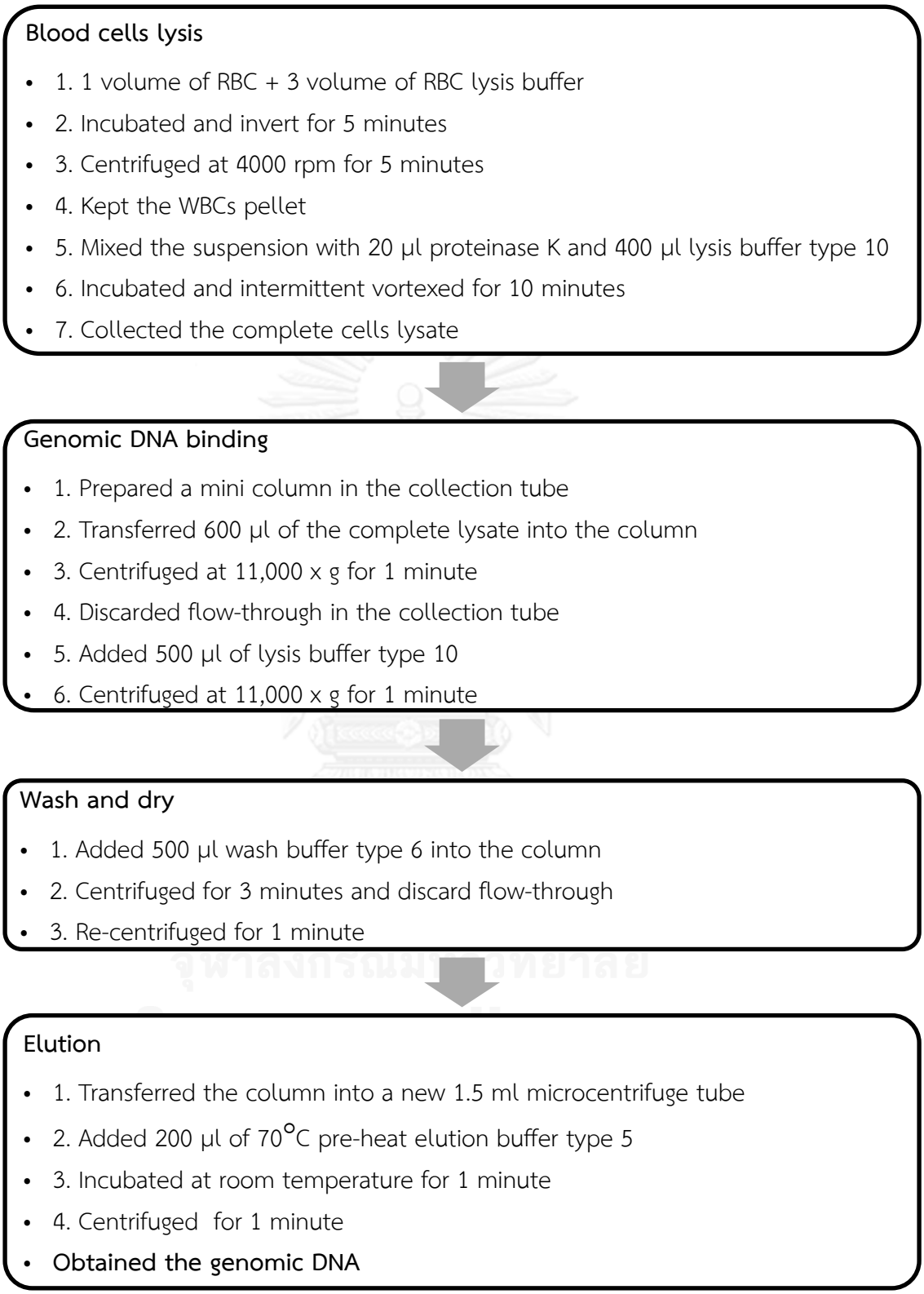


Figure 3.5 Diagram of genomic DNA isolation procedure

2.3.2 VEGF single nucleotide polymorphisms (SNPs) analysis using polymerase chain reaction- restriction fragment length polymorphism (PCR-RFLP)

The PCR-RFLP method used two steps in order to analyze the specific pattern of DNA fragments represented distribution of genotype and allelic frequency. The principle of this technique is that a specific position of DNA is cut with a restriction enzyme and illustrated a particular pattern. In this study, we performed analysis four single nucleotide polymorphisms (SNPs) of *VEGF* gene, -2578C/A, -1154G/A, -634G/C, and +936C/T.

- **Polymerase chain reaction (PCR)**

Genomic DNAs from 202 knee OA patients and 202 healthy controls were evaluated for the polymorphisms in the *VEGF* gene. Four SNPs including -2578C/A and-1154G/A located in the promoter, -634G/C in 5' untranslated region (UTR) and 936C/T situated in 3' UTR were evaluated in the study. There were specific primers for these SNPs as described in **Table 3.1**.

Table 3.1 List of primers

SNP genes	Primers	Reference
-2578C/A	F: 5'-GGCCTTAGGACACCATACC-3'	(Han *et al.* 2004)
(rs699947)	R: 5'-CACAGCTTCTCCCCTATCC-3'	
-1154G/A	F: 5'-TCCTGCTCCCTCCTCGCCAATG-3'	(Li *et al.* 2010)
(rs1570360)	R: 5'-GGCGGGGACAGGCGAGCCTC-3'	
-634G/C	F: 5'-CGACGGCTTGGGGAGATTGC -3'	(Han *et al.* 2004)
(rs2010963)	R: 5'-GGGCGGTGTCTGTCTGTCTG-3'	
+936C/T	F: 5'-AGGGTTTCGGGAACCAGATC-3'	(Han *et al.* 2004)
(rs3025039)	R: 5'-CTCGGTGATTTAGCAGCAAG-3'	

PCR was performed in 25 µl reaction volume containing 10X *Taq* buffer 2.5 µl (Thermo Scientific, USA), 25mM $MgCl_2$ 1-4 mM (1-4 µl), 2 mM dNTP mix 2.5 µl, 10 mM forward 1 µl, 10 mM reverse primer 1 µl, enzyme *Taq* polymerase 1.25 U (0.2 µl), and water to 25 µl as shown in **Table 3.2**. The thermal cycling condition for PCR was

illustrated in **Table 3.3**. The initial denaturation step was performed at 95°C for 5 minutes then followed by 35 cycles of denaturation at 95°C 1 minute, annealing at (62°C for -2578C/A), (67°C for -1154G/A), (71°C for -634G/C), (62°C for +936C/T) for 30 seconds, and extension at 72°C for 45 seconds. Final extension was set at 72°C for 7 minutes (Mastercycler personal, Eppendorf, USA).The PCR product was confirmed by 2% agarose gel electrophoresis. The size of PCR amplification fragments were 455 bp for -2578C/A, 207 bp for -1154G/A, 274bp -634G/C, and 266 bp for +936C/T.

Table 3.2 Component of master mix for PCR reaction

Chemicals	**Volume (µl)**	**Final concentration**
10X *Taq* buffer	2.5	1X
25 mM $MgCl_2$	2.5	2.5 mM
2 mM dNTP	2.5	0.2 mM of each
10 µM Forward primer	1	0.4 µM
10 µM Reverse primer	1	0.4 µM
Taq polymerase	0.2	1 U/µl
Water	12.3	
Template DNA	3	
Total volume	25	

Table 3.3 PCR thermal cycling condition

Step	Temperature (°C)	Time	Number of cycles
Initial denaturation	95	5 minutes	1
Denaturation	95	1 minute	35
Annealing *	depend on primer	30 seconds	
• -2578C/A	62		
• -1154G/A	67		
• -634G/C	71		
• +936C/T	62		
Extension	72	45 seconds	
Final extension	72	7 minutes	1

Note*: the optimal annealing temperature (Ta) is melting temperature (Tm) minus with 5 degree Celsius (Tm-5). If Ta is higher than optimal point, the primer is not suitable work and cause low yield. However, Ta is lower than optimal temperature; the primer is able to bind the template with non-specificity.

- **Restriction fragment length polymorphism (RFLP)**

When PCR had been performed, genotyping was examined by restriction fragment length polymorphism (RFLP). The condition of each enzyme was based on the manufacturer's protocol. The reaction used 1.5 µl 10X NEBuffer for each enzyme. Then the buffer was mixed with 1µl restriction enzyme, 8 µl of PCR product and water up to 15 µl **(Table 3.4)**. After that the -2578C/A amplified sequence mixture was digested with *BstYI* at 60°C for 1 hour and heat inactivate the enzyme at 80°C for 20 minutes. The -1154G/A and +936C/T enzymatic mixture were digested with *MnlI* and *NlaIII*, respectively at 37°C for 1 hour and enzyme was inactivated by incubation at 65°C for 20 minutes. Finally, the -634G/C PCR product mixture was digested with *BsmFI* at 65°C for 1 hour and inactivated enzyme activity at 80°C for 20 minutes. **Table 3.5** demonstrates the characteristics of the restriction enzymes for the 4 SNP positions. Subsequently, the digested-PCR products were separated by 2% agarose gel electrophoresis except -1154G/A which was distinguished by 12% native

polyacrylamide gel electrophoresis. The DNA fragments were stained with ethidium bromide fluorescent nucleic acid dye and visualized by a Bio-Rad Gel Doc™ XR gel documentation system.

Table 3.4 Component of master mix for restriction fragment length polymorphism reaction

Chemicals	**Volume (µl)**
1X NEBuffer	1.5
Water	4.5
Restriction enzyme	1
PCR product	8
Total volume	15

Table 3.5 Characteristics of restriction enzymes

Restriction enzyme	**Recognition size**	**Activated temp. (°C)**	**Inactivated temp. (°C)**	**Manufacturer**
BstYI (10,000U/ml)	5'...R▼G A T C Y...3'*	60	80	New England Biolabs Inc., USA
MnlI (5,000U/ml)	5'...C C T C $(N)_7$▼...3'	37	65	
BsmFI (2,000U/ml)	5'...G GG A C $(N)_{10}$▼...3'	65	80	
NlaIII (10,000U/ml)	5'...C A T G▼...3'	37	65	

Note*: ▼ represent the cleavage site of restriction enzyme

: R = A or G and Y = C or T

The results from electrophoresis of VEGF genotype restriction pattern were shown in **Table 3.6**. For -2578C/A variation, the fragment of 455 bp represented

CC genotype. The fragments of 248 and 207 bp indicated the homozygote genotype (AA). All of fragments 455, 248, and 207 bp represented heterozygote genotype (CA). For -1154G/A polymorphism, the presence of fragments 3, 19, and 184 bp represented A/A genotype, 3, 19, 34, and 150 bp fragments illustrated G/G genotype, and all of the five fragments including 3, 19, 34, 150, and 184 denoted G/A genotype. For -634G/C, the original 274 bp fragment was homozygous individual for the VEGF C allele, lacking the *BsmFI* site. Two fragments of 156 and 118 bp were analyzed as homozygous individual for VEGF G variant. Three fragments of 274, 156, and 118 bp represented heterozygous individual (G/C) genotype. For the last SNPs, +936C/T, fragments digested into two (55 and 211 bp) fragments and represented T allele while a single 266 bp fragment was shown as C allele. The C/T polymorphism was visible in three fragments of 266, 211, and 55 bp.

Table 3.6 Patterns of 4 VEGF single nucleotide polymorphisms

SNPs	PCR amplification fragment (bp)	Region	Restriction enzyme	Genotype (bp)
-2578C/A (rs699947)	455	Promoter	*BstYI*	CC: 455 AA: 207, 248
-1154G/A (rs1570360)	207	Promoter	*MnlI*	GG: 3, 19, 34, 150 AA: 3, 19, 184
-634G/C (rs2010963)	274	5'UTR	*BsmFI*	CC: 274 GG: 118, 156
+936C/T (rs3025039)	266	3'UTR	*NlaIII*	CC: 266 TT: 55, 211

2.4 <u>Part III:</u> (A) Determination of VEGF mRNA, miR-210, and miR-223 in knee OA patients and *in vitro* model (B) fibroblast-like synoviocyte (FLS) cultured under hypoxia and interleukin-1ß stimulated condition

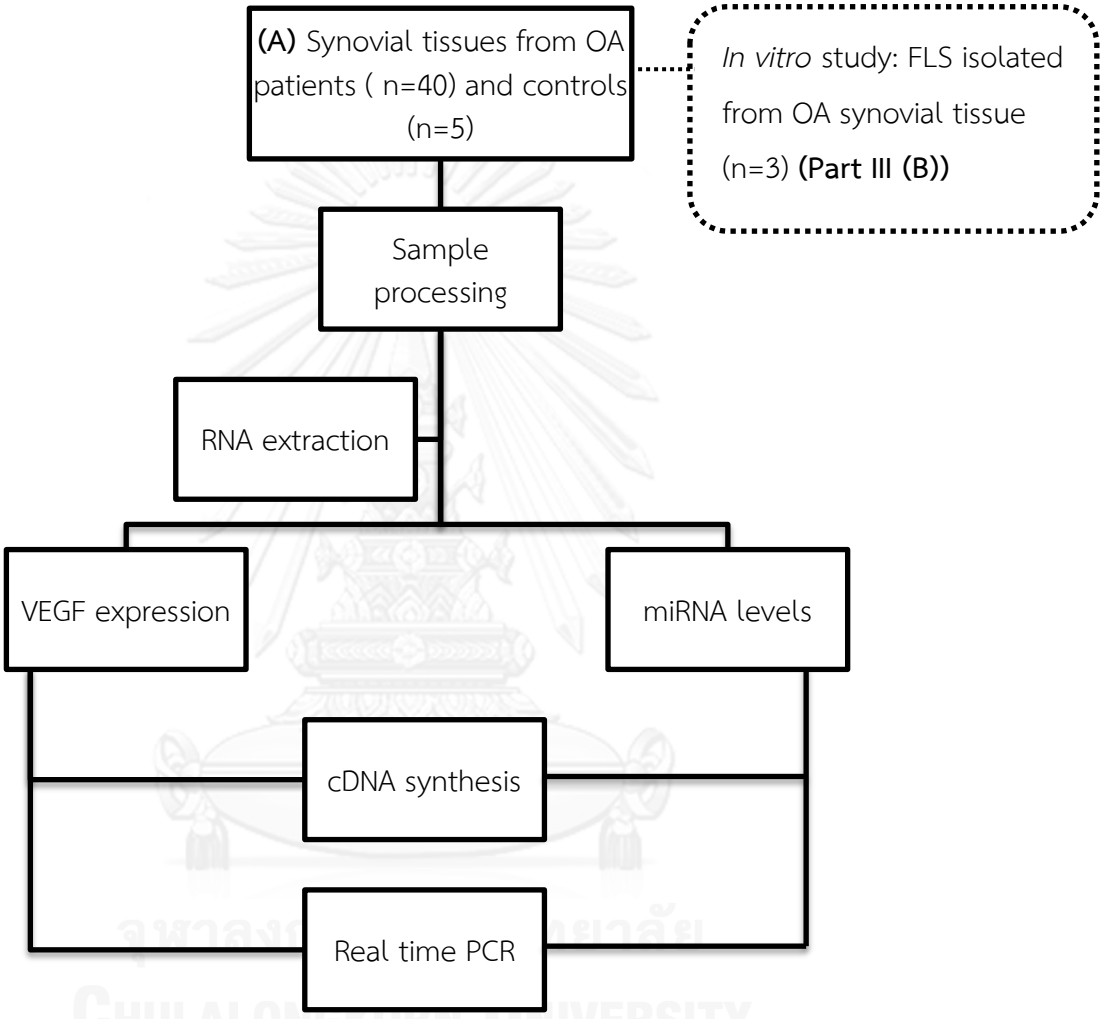


Figure 3.6 Flow chart of part III (A) experiment

(A) VEGF mRNA, miR-210 and miR-223 in synovial tissues determination

Synovial tissue samples were obtained from 40 OA patients and 5 controls from anterior cruciate ligament injury patients. The samples were prepared in order to detect the VEGF and microRNA expression.

2.4.1 Sample processing

The synovial tissues obtaining from OA patients and control groups were cut approximately 0.3 X 0.3 centimeters (approximately 30 mg) in size and immersed in RNA*later* (RNA preserved solution for RNA extraction) as soon as possible. The tissues were stored at -80°C until analysis**.** RNA was extracted from the frozen tissues for the real-time PCR experiment.

- **RNA extraction**

The total RNAs from synovial tissues were extracted by mirVana™ miRNA isolation kit (Ambion Inc, USA) **(Figure 3.7)**. The RNA obtained from the kit is able to be used for VEGF gene and miRNA expression analysis. First of all steps, wash buffer solutions were prepared by adding 100% ethanol according to manufacturer recommendation. The 30 mg of frozen tissue was immediately ground to a powder with liquid nitrogen in a prechilled mortar and pestle. The tissue powder was mixed with 10 volumes of lysis/binding buffer per tissue mass (e.g. if tissue mass is 30 mg, lysis/binding buffer 300 µl is added). Then, the mixture was passed through a 20-gauge needle to homogenize the lysate. The next step was organic extraction. A one in ten (1/10) volume of miRNA homogenate additive was added to the lysate and vortexed several times (for example, if the lysate is 300 µl, 30 µl of the miRNA homogenate additive is added). The lysate was placed on ice for 10 minutes. Then 300 µl of acid-phenol: chloroform (a volume of acid-phenol :chloroform is equal to the lysate volume before addition the miRNA homogenate additive) was added to the lysate, vortexed for 30 to 60 seconds and centrifuged for 5 minutes at 10,000 x g at room temperature to separate the aqueous and organic phase. The aqueous phase was transferred to a new microcentrifuge tube. The elution solution was preheated at 95°C for use in eluting the RNA. The 1.25 volumes of 100% ethanol were mixed with the aqueous phase. The 700 µl of mixture was then transferred to the filter cartridge and centrifuged for 15 seconds at 10,000 x g. The flow-through was discarded and repeated until all of the mixture was through the filter. Next, 700 µl of miRNA wash solution 1 was applied to the filter cartridge, centrifuged for 5 to 10 seconds at 10,000 x g and the flow-through discarded. After that, 500 µl of wash solution 2/3

was added to the cartridge, centrifuged at 10,000 x g for 5 to 10 seconds and repeated with a second 500 µl of wash solution 2/3. After discarding the flow-through from the last wash, the column was spun again for 1 minute to remove residual fluid from the filter. Then, the collection tube was changed and 50 µl of preheat elution buffer was added, placed for 1 minute and centrifuged at 13,000 x g for 20 to 30 seconds. The concentration of total RNA was determined by Nanodrop 2000 spectrophotometer (Thermo Scientific, Germany). The RNA was kept at -80°C or below until real time RT-PCR analysis.

Tissue disruption

- 1. 30 mg of frozen tissue was ground in a mortar and pestle using liquid nitrogen and the tissue powder decanted into tubes
- 2. 300 µl of lysis/binging buffer was added and the lysate homogenized with 20-gauge needles and syringes

Organic extraction

- 1. 30 µl of miRNA homogenate additive was added, mixed by vortexing
- 2. Placed on ice for 10 minutes
- 3. 300 µl of acid-phenol: chloroform was added, vortexed for 30-60 seconds
- 4. Centrifuged at 10,000 x g for 5 minutes to separate aqueous and organic phase

Elution

- 1. Mixed 1.25 volumes 100% ethanol to the aqueous phase
- 2. Transferred 700 µl of the mixture into a filter cartridge, centrifuged at 10,000 rpm for 15 seconds, and threw away the flow-through. If the sample exceeded 700 µl , repeat this step again
- 3. 700 µl of miRNA wash solution 1 was added, centrifuged at 10,000 rpm for 5-10 seconds and the flow-through discarded
- 4. 500 µl of wash solution 2/3 was added, spun at 10,000 rpm for 5-10 seconds and repeated with a second 500 µl of wash solution 2/3
- 5. The column was spun for 1 minute at 10,000 x g and changed to a new collection tube
- 6. 50 µl of preheated elution buffer was applied, placed the column for 1 minute, and centrifuged 13,000 x g for 20-30 seconds
- Obtained a total RNA and kept at -80°C or below until analysis

Figure 3.7 Diagram of total RNA extraction using mirVana™ miRNA isolation kit

2.4.2 VEGF and miRNA determination using two-step RT-PCR

VEGF mRNA

The expression of VEGF was detected in OA synovial tissue. We detected the expression of the VEGF gene by real time PCR and 18s rRNA were used as a control to normalize differences in total RNA levels between samples. The real time RT-PCR reaction was conducted in accordance with each pair of oligonucleotide primers, **VEGF:**5'-CAAATGCTTTCTCCGCTCTGA-3' (forward), 5' CCTACAGCACAACAAATG TGAATG-3' (reverse) and 5'-FAM-CAAGACAAGAAAATCCCTGTGGGCCT-TAMRA-3' (probe), **18s rRNA:**5'-GCCCGAAGCGTTTACTTTGA-3' (forward), 5'-TCCATTATTCCTAGCTGCGGTA TC-3' (reverse) and 5'-FAM-AAAGCAGGCCCGAGCCGCC-TAMRA-3' (probe) (Avihingsanon *et al.* 2009).

miRNA: miR-210 and miR-223

The expressions of both miRNAs were determined using specific primers: has-miR-210 and has-miR-223 (Applied biosystems, USA). The RNU6B was used as an internal control for miRNA expression in different samples. The RNA was reverse transcribed to produce complementary DNA (cDNA) that had specificity with gene and sample (*Taq*Man® MicroRNA Reverse Transcription kit, Applied biosystems, USA). The real time PCR was then performed using *Taq*Man® Universal PCR master mix (Applied biosystems, USA).

- **cDNA synthesis (reverse transcription (RT) step)**

VEGF mRNA

One hundred nanograms of total RNA was reverse transcribed using *Taq*man® Reverse Transcription Reagents (Applied biosystems, USA) according to the manufacturer's protocol. The master mix of a RT is shown in **Table 3.7**. First, 2 µl of 10X *Taq*man RT buffer were combined with 4.4 µl 25 mM Magnesium chloride, 0.4 µl of 100 mM dNTP, 1 µl Random hexamer, 0.4 µl RNase inhibitor, and 0.5 of 50U/ µl of Multiscribe™ reverse transcriptase. The volume of RNase-free water is 11.3 – RNA sample in a 20-µl reaction. The RT thermal cycling was: incubation at 25°C for 10

minutes, RT at 48°C for 30 minutes, and reverse transcriptase inactivation at 95°C for 5 minutes as shown in **Table 3.8**.

Table 3.7 Component of master mix for VEGF reverse transcription reaction

Chemicals	**Master mix volume per 20 µl reaction* (µl)**
10X *Taq*man RT buffer	2
25 mM Magnesium chloride	4.4
100 mM dNTP	0.4
Random hexamer (50 µM)	1
RNase inhibitor, 20 U/µl	0.4
Multiscribe™ reverse transcriptase, 50 U/µl	0.5
RNase-free water + 100 ng total RNA template	11.3
Total volume	20

Table 3.8 VEGF reverse transcription thermal cycling condition

Step	**Temperature (°C)**	**Time**
Incubation	25	10 minutes
RT	48	30 minutes
Reverse Transcriptase inactivation	95	5 minutes

miRNA: miR-210 and miR-223

The first strand cDNA was synthesized from 10 ng of total RNA. The master mix of a RT reaction comprised of 10x reverse transcription buffer 1.5 µl, 0.15 µl of 100 mM dNTP (with dTTP), 0.19 µl of RNase inhibitor, 20 U/µl, 1 µl of Multiscribe™ reverse transcriptase, 50 U/µl, and RNase-free water 4.16 µl. The total volume of master mix was 7 µl and combined with 3 µl 5x RT primer and 5 µl of RNA sample. In this study,

we used 3 paired RT primers for hsa-miRNA-210, hsa-miRNA-223, and RNU6B (as internal control) *Taq*Man® MicroRNA Assays (Applied biosystems, USA). Moreover, the concentration of each RNA sample was 5 ng/ml. Therefore, only 2 µl of RNA sample was sufficient and 3 µl more RNase-free water was added. The **Table 3.9** shows the components of the reaction mix for reverse transcription. After incubating the RT reaction on ice for 5 minutes, the reaction was loaded into the thermal cycler. The temperature settings for RT reaction illustrated in **Table 3.10** were a hold I at 16°C for 30 minutes followed by hold II 42°C for 30 minutes and hold III 85°C for 5 minutes. After that cDNA product was kept at -20°C until real time PCR analysis.

Table 3.9 Component of master mix for miRNA reverse transcription reaction

Chemicals	**Master mix volume per 10 µl reaction* (µl)**
10X Reverse transcription buffer	1.5
100 mM dNTP (with dTTP)	0.15
RNase inhibitor, 20 U/µl	0.19
Multiscribe™ reverse transcriptase, 50 U/µl	1
5X primer (each gene)	3
RNase-free water	7.16
RNA sample*	2
Total volume	15

Note*: each 15 µl RT reaction consists of 10 µl master mix, 3 µl of 5X primer, and 2 µl of 5 ng/µl RNA sample

Table 3.10 miRNA reverse transcription thermal cycling condition

Step	Temperature (°C)	Time
Hold I	16	30 minutes
Hold II	42	30 minutes
Hold III	85	5 minutes
Hold	4	∞

- **real time PCR**

VEGF mRNA and 18s rRNA

The cDNA product was examined for the level of VEGF mRNA by QuantiTect® Multiplex PCR NoROX kit (Qiagen, USA). The components of master mix for VEGF and 18s real time PCR are presented in **Table 3.11**. All chemicals were mixed as follows: 10 µl of 2X QuantiTect Multiplex PCR Master Mix, 1 µl of 10 µM forward primer, 1µl of 1 µM reverse primer, 0.4 µl of probe, and RNase-free water 5.6 µl. The 2 µl template cDNA was added and the real-time PCR cycler was program according to **Table 3.12**.

Table 3.11 Component of master mix for real time PCR

Chemicals	Volume per 20 µl reaction (µl)
2X QuantiTect Multiple PCR Master Mix	10
10 µM Forward primer	1
10 µM Reverse primer	1
10 µM Probe	0.4
RNase-free water	5.6
cDNA product	2
Total volume	20

Table 3.12 Real time PCR thermal cycling condition

Step	Temperature (°C)	Time
PCR initial activation	95	15 minutes
PCR cycling 40 cycles		
Denature	95	1 minute
Anneal/extend*	60	1 minute

Note: * fluorescence data collection

miRNA: miR-210 and miR-223

The cDNA product was assessed for the expression of miR-210 and miR-223 using real time PCR with *Taq*Man® Universal PCR master mix (Applied biosystems, USA). Primers combined with probes and cDNA were thawed on ice while the master mix reagent was thawed at room temperature. The 20 µl real time PCR reaction shown in **Table 3.13** contained with 1 µl *Taq*Man® MicroRNA Assays (primers and probes for each microR-210, microR-223, and RNU6B as a quantitative internal control), 1.33 µl of product from RT reaction, 10 µl *Taq*Man® Universal PCR master mix, and 7.67 µl RNase-free water. Then the thermal cycling conditions were set at 50°C for 2 minutes, 95°C for 10 minutes followed by 40 cycles of 95°C for 15 seconds, and 60°C for 60 seconds as presented in **Table 3.14**.

Table 3.13 Component of master mix for miRNA real time PCR

Chemicals	Volume per reaction (µl)
*Taq*man® universal PCR master mix	10
RNase-free water	7.67
*Taq*man® microRNA assays (primer and probe)	1
cDNA product	1.33
Total volume	20

Table 3.14 Real time PCR thermal cycling condition

Step	Temperature (°C)	Time
Hold I	50	2 minutes
Hold II	95	10 minutes
PCR cycling 40 cycles		
Denature	95	15 seconds
Anneal/extend*	60	60 seconds
Hold	4	∞

Note: * fluorescence data collection

The threshold cycle (C_t) values from VEGF mRNA and miRNA expression were determined automatically by the instrument: StepOnePlus™ Real-Time PCR Systems (Applied Biosystems, USA). The C_t value was defined as the fractional cycle number for the fluorescence to reach a specific threshold level of detection. The Ct value was inversely correlated with the amount of template present in the reaction. The fold changes of expression level of each gene were calculated by the equation $2^{-\Delta\Delta Ct}$.

The gene expression ratio was evaluated by the relative quantification (ΔC_t). The ratio was calculated with the equation as follows:

$$R = 2^{-\Delta\,(\Delta Ct\ sample\ -\ \Delta Ct\ control)} = 2^{-\Delta\Delta Ct}$$

$$\left[\begin{array}{c} \Delta C_t \text{ sample} = C_{t\text{VEGF of OA}} - C_{t\text{18s rRNA of OA}} \\ \Delta C_t \text{ control} = C_{t\text{VEGF of control}} - C_{t\text{18s rRNA of control}} \end{array}\right]$$

(B) *In vitro* study: VEGF mRNA, miR-210, and miR-223 in FLS isolate from OA synovial tissues

Fibroblast-like synoviocyte (FLS) cells were isolated from synovial tissue samples to determine the expression and the effect of VEGF, miR-210 and miR-223 after treating the cells with various experimental conditions. The 3 biological replications were performed (n=3).

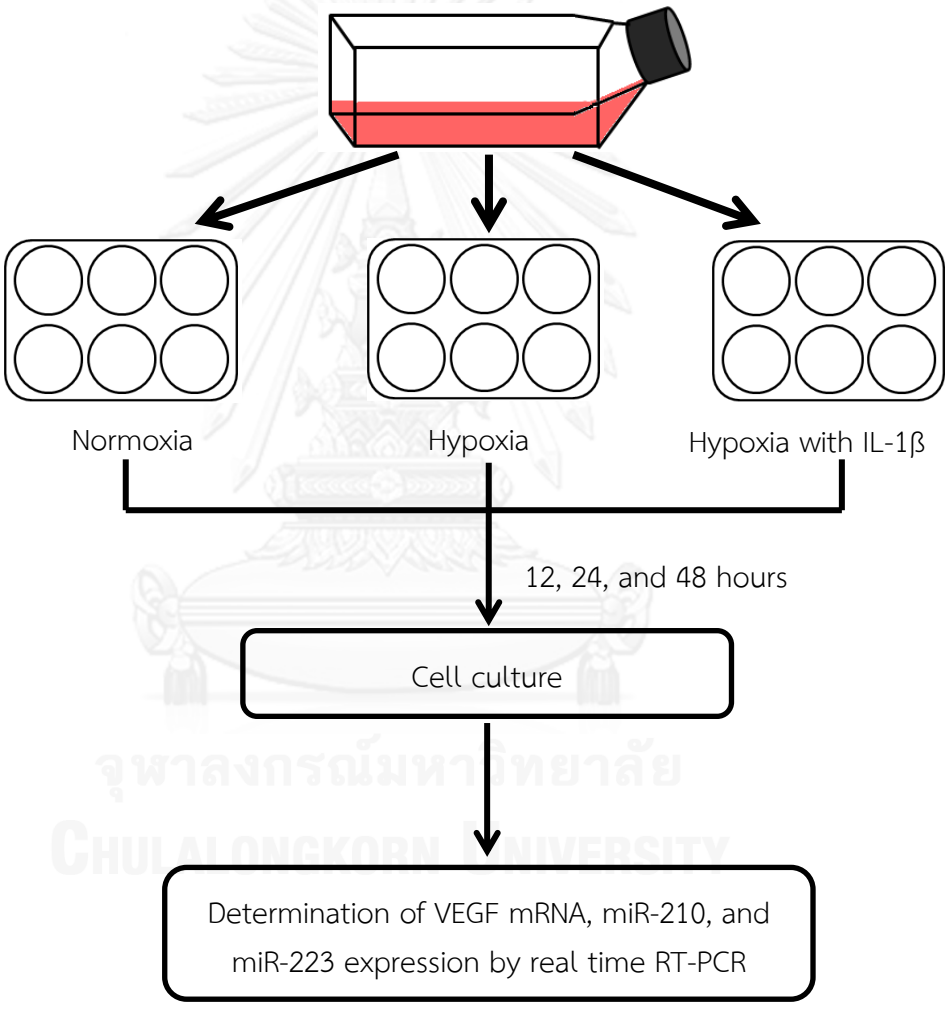


Figure 3.8 Flow chart of part III (B) experiment

2.4.3 Cell culture

2.4.3.1 Sample processing

The fresh tissues obtained from OA patients were washed in normal saline and cut into many small pieces. The minced tissues were attached to T25 flasks containing Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 200 IU/ml penicillin/ streptomycin. Cells were allowed to grow for 1 to 2 weeks at 37°C in a humidified atmosphere of 5% CO_2. The tissue pieces were removed and the cells were continued to be grown until they had reached confluence. The confluent cells were trypsinized with 0.25% trypsin with EDTA·4Na. After removing the media, 3 ml of 0.25% trypsin with EDTA was added. The cells were incubated in the incubator for 5 - 10 minutes. Afterward, if the round cells were observed under microscopy, equal amount of media was added into the flask in order to inhibit activity of trypsin. The cells were transferred to 15 ml centrifuge tube and centrifuged at 1500 rpm for 10 minutes. The pellet was suspended with new DMEM supplemented with 10% FBS, 100 IU/ml penicillin/ streptomycin and then transferred to new T25 culture flasks (1:3) or T75 culture flask (1:1). The cells after trypsinization are the first passage (P1). The media was changed every 2 days. The cells after passage 3 appeared to be homogenous FLS cells. The FLS cells from passage 3 to7 were processed in the experiment (Park *et al.* 2012).

2.4.3.2 Culture of FLS in hypoxia and interleukin-1beta (IL-1β)

The cells were seeded into six-well cell culture plates at a density of 1 x 10^5 cells/ 2 ml of DMEM containing 5% FBS and 100 IU/ml penicillin/ streptomycin for 24 hours to reduce the effect of FBS on microRNAs and VEGF induction. Then, the cells were incubated with hypoxia (1% O_2, 99% N_2), and with or without IL-1β (10 ng/ml) for 12, 24, or 48 hours in medium containing 1% serum under an atmosphere 5% CO_2 at 37°C. The cells incubated under normoxia conditions were set as a control. Finally, microRNA expression, and VEGF mRNA and

protein expression of the treated FLS cells were investigated by Western blotting analysis and immunocytochemistry (Demasi *et al.* 2004, Kawaguchi *et al.* 2004, Nakasa *et al.* 2008, Miyake *et al.* 2009).

2.4.4 VEGF and miRNA determination using two-step real time PCR

2.4.4.1 RNA extraction

The RNeasy mini kit (Qiagen, USA) was used to extract RNA from the FLS cell culture. This kit was divided into two steps: tissue disruption and homogenization, and purification of total RNA, as shown in **Figure 3.9**. The cell disruption was performed by washing the cells with PBS. Then, the cells were immediately lysed by adding 700 µl of lysis buffer (RLT buffer 1ml adding with 10 µl of β-ME[*1]). The cell lysate was pipetted into tubes and homogenized using 20-gauge needles. Subsequently, 500 µl of 70% ethanol was added and mixed immediately by pipetting. The lysate was centrifuged for 3 minutes at 13,000 rpm. For purification of total RNA, 700 µl of the sample was transferred into an RNeasy spin column placed in a 2 ml collection tube with the lid closed, centrifuged at 10,000 rpm for 1 minute, and the flow-through discarded. If the sample volume exceeded 700 µl, the steps were repeated until complete. Next, 700 µ of buffer RW1 was added, incubated at room temperature for 5 minutes, and centrifuged at 10,000 rpm for 1 minute. Following this, 500 µl of RPE buffer was added, spun for 1 minute at 10,000 rpm, and the flow-through thrown out. After that, 500 µl of RPE buffer was added again and spun for 2 minutes at 13,000 rpm. The columns were changed with a new 2 ml collection tube and the carryover of ethanol eliminated by spinning for 2 minutes at 13,000 rpm. RNeasy spin columns were placed in a new 1.5 ml microcentrifuge tube, 32 µl of RNase-free water added directly to the center of silica membrane of the spin column, and set at the room temperature for 2 minutes. The sample was eluted by centrifuging at 13,000 rpm for 2 minutes. Finally, the concentration of RNA was

determined using Nanodrop 2000 spectrophotometer (Thermo Scientific, Germany). The RNA was kept at -80°C until real time RT-PCR analysis.

Note*[1]: β-mercaptoethanol (β-ME) should perform in a fume hood and protective gloves worn.

Tissue disruption and homogenization step

- 1. Frozen-tissues were ground in a mortar and pestle using liquid nitrogen and the issue powder was decanted into tubes
- 2. 700 µl of lysis buffer RLT (mixture of 1 ml RLT per 10 µl β-ME) was added and the lysate homogenized with 20-gauge needles and syringes
- 3. 500 µl of 70% ethanol was added, mixed by pipetting and centrifuged at 13,000 rpm for 3 minutes
- 4. Ready for purification of total RNA step

Purification of total RNA step

- 1. 700 µl of the sample from previous step was transfered into an RNeasy spin column, centrifuged at 10,000 rpm for 1 minute, and the flow-through thrown away. If the sample exceeded 700 µl, repeat this step again
- 2. 700 µl of RW1 was added, placed at room temperature for 5 minutes, and centrifuged at 10,000 rpm for 1 minute and the flow-through discarded
- 3. 500 µl of RPE was added, spun at 10,000 rpm for 1 minute and RPE repeatedly added, and centrifuged at 13,000 rpm for 2 minutes
- 4.Collection tubes changed to new tubes and centrifuged to dry ethanol on the membrane at 13,000 rpm for 2 minutes
- 5. Placed the column into a new microcentrifuge tube, added 32 µl RNase-free water, stood for 2 minutes, and centrifuged at 13,000 rpm for 2 minutes.
- 6. Total RNA was obtained and kept at -80^{o}C until analysis

Figure 3.9 Diagram of RNA extraction procedure from tissues

2.4.4.2 RT and real-time PCR

The VEGF mRNA, miR-210, and miR-223 expressions in FLS cultured under hypoxia and IL-1β stimulation were evaluated using two-step real time PCR as mentioned in **Topic 2.4.2**.

3. Statistical analysis

Statistical analysis was performed using the statistical package for social sciences (SPSS) software, version 16.0 for Windows (SPSS Inc., Chicago, USA). Student's unpaired *t*-test was used to compare the means of VEGF mRNA and protein as well as microRNA expression of two independent groups**.** The correlation between VEGF expression and disease severity was calculated using Spearman's correlation coefficient (*r*). To set a cut-off point in the OA patients, the areas under the curve (AUC) of ROC curves were calculated. The best equilibrium between sensitivity and specificity was determined for each cut-off point. Data were expressed as mean ± standard error of the mean (SEM). $P<0.05$ was considered to be statistically significant for differences and correlations.

The *in vitro* experimental data are expressed as the mean ± SEM of three different donors. Difference was determined by paired *t*-test or one-way analysis of variance. $P<0.05$ was considered to be statistically significant.

In the single nucleotide polymorphism study, the demographic data of patients and controls were analyzed by the Chi-square test and Student's t test. Differences in genotype distributions and allele frequencies between patients and control subjects were compared for statistical analysis by the Chi-square test with Yates correction. Odds ratios (ORs) with a 95% confidence interval (CI) were calculated using StatCalc program (AcaStat Software, VA, USA). The wild-type genotype/allele served as a reference category. Allele and genotype proportions were evaluated for Hardy–Weinberg equilibrium (HWE) by the χ^2 test. 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). A *P*-value less than 0.05 were regarded as indicating statistical significance.

CHAPTER IV

Results

Part I: Association of vascular endothelial growth factor (VEGF) protein in the pathogenesis of knee osteoarthritis

In order to screen which angiogenic cytokine specifically expressed in OA patients, the cytokine array were performed in synovial fluid (SF) and plasma OA patients compared with plasma controls. Thirty-one knee OA patients and fifteen healthy controls were included in angiogenic cytokine array study. The results are shown in **Table 4.1** and **Figure 4.1**. The results demonstrated that PECAM-1, HGF, angiopoietin-2, follistatin, and IL-8 levels in plasma OA patients were higher than those found in healthy controls. Particularly, VEGF is one of the most potent angiogenic factors and it is only one angiogenic factor that significantly elevated in OA synovial fluid when compared with paired plasma. VEGF concentration was detectable in almost all SF and plasma samples of knee OA patients. According to the cytokine assay study, VEGF was selected and subsequently determined for the association of VEGF expression and pathogenesis of OA.

Table 4.1 The expression of angiogenesis cytokines in OA patients and healthy controls

Cytokine		n	Mean (pg/ml)	SD (pg/ml)	Median (pg/ml)	IQR (pg/ml)	Min (pg/ml)	Max (pg/ml)
G-CSF	Plasma control	15	50.4	20.5	48.4	32.3-62.4	26.5	82.6
	Plasma OA	29	95.7	69.4	86.8	42.7-136.3	0.4	328.1
	SF OA	10	3.8	4.5	2.9	1.0-4.1	0.4	15.4
PECAM	Plasma control	9	160.5	87.9	159.7	116.0-168.8	23.6	323.1
	Plasma OA	29	644.0	520.3	501.6	319.6-714.7	4.3	2427.4
	SF OA	10	1256.1	1998.8	436.1	132.5-1090.7	4.3	6261.1
HGF	Plasma control	15	74.2	37.9	69.0	46.8-100.4	25.1	146.2
	Plasma OA	28	251.6	176.6	262.5	124.2-307.8	10.4	800.8
	SF OA	17	338.2	966.9	57.7	25.9-153.9	5.5	4055.0
VEGF	Plasma control	15	7.8	3.2	7.6	5.78-9.57	4.3	13.9
	Plasma OA	29	18.5	9.8	18.4	13.2-23.6	1.4	47.2
	SF OA	31	95.2	174.8	53.6	26.1-90.6	7.9	999.6
Leptin	Plasma control	5	800.0	666.6	1185.1	88.8-1265.1	60.7	1400.4
	Plasma OA	26	2509.3	2576.7	1610.4	915.2-3010.4	345.5	10572.8
	SF OA	17	2959.2	3796.2	1647.4	997.3-4435.7	372.7	15930.9
PDGF-BB	Plasma control	1	20	-	20.0	20.0-20.0	20.0	20.0
	Plasma OA	21	34.3	20.6	32.0	19.6-48.6	2.3	66.2
	SF OA	0	-	-	-	-	-	-

Cytokine		n	Mean (pg/ml)	SD (pg/ml)	Median (pg/ml)	IQR (pg/ml)	Min (pg/ml)	Max (pg/ml)
Angiopoietin-2	Plasma control	15	167.9	49.9	173.7	132.4-198.5	73.6	261.1
	Plasma OA	30	403.7	195.5	367.6	267.7-471.0	190.8	1072.2
	SF OA	14	539.7	1412.1	87.4	31.6-130.3	12.4	5355.8
Follistatin	Plasma control	15	87.4	29.7	80.5	67.4-94.8	50.1	171.5
	Plasma OA	28	153.6	73.5	134.3	106.4-166.2	76.6	369.7
	SF OA	9	13.6	16.0	2.0	2.01-2.9	1.4	48.6
IL-8	Plasma control	15	3.6	1.7	3.8	2.1-4.8	1.2	7.2
	Plasma OA	29	36.7	138.7	11.0	8.0-13.3	1.7	757.3
	SF OA	29	75.8	242.5	10.7	4.5-29.5	0.6	1292.7

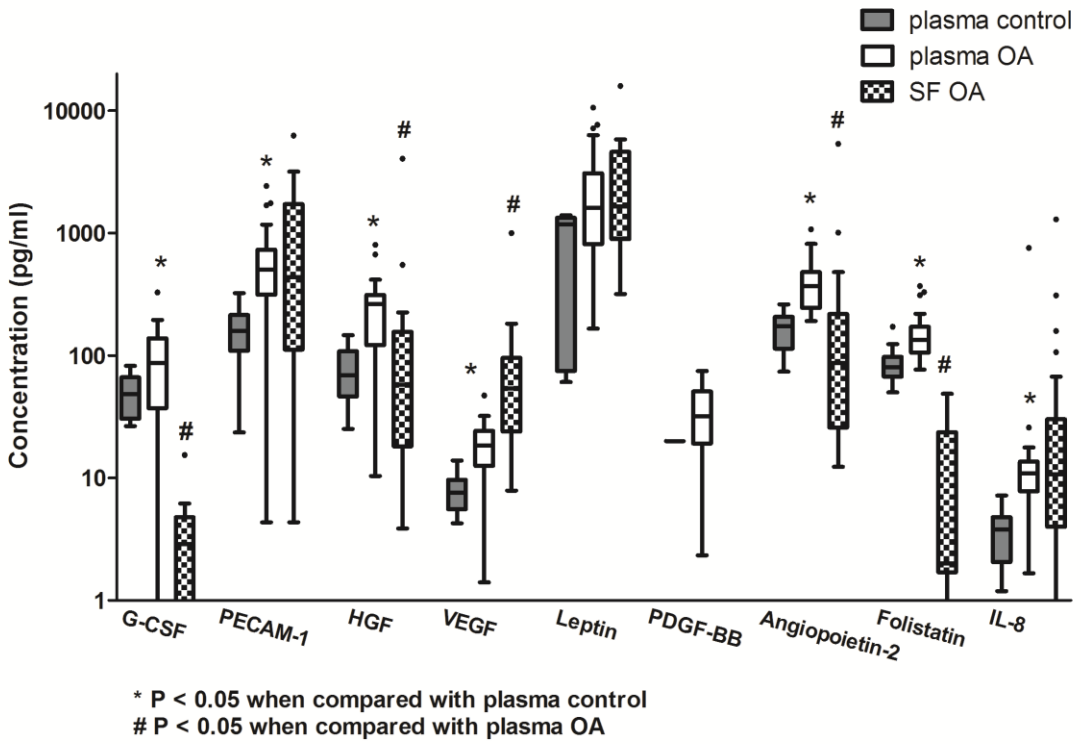


Figure 4.1 Concentrations of angiogenic cytokines

1.1 Characteristic of participants

This study was approved by the Institutional Review Board on Human Research of the Faculty of Medicine, Chulalongkorn University. Osteoarthritis patients who underwent total knee replacement surgery at King Chulalongkorn Memorial hospital were recruited in this research. All patients were diagnosed with an osteoarthritic disease using clinical American College of Rheumatology criteria of knee OA combined with radiographic features classified into Kellgren-Lawrence grade 2, 3, and 4. Additionally, the healthy control group consisted of participants who were aged over 50 years old and came into the hospital for an annual check-up and had no joint disorders.

1.2 Concentration of VEGF protein in SF and plasma

The demographic data of all participants in **part I** are shown in **Table 4.2**. Eighty knee OA patients and twenty healthy controls were acquired for determining the concentration of VEGF. There were no significant differences in age between OA and controls.

Table 4.2 Demographic and characteristic of patients suffering from osteoarthritis and normal controls.

	Normal controls (%)	**Osteoarthritis patients (%)**	**K/L grade 2 (mild)**	**K/L grade 3 (moderate)**	**K/L grade 4 (severe)**
N	20	80	29	27	24
Age (years)	68.2 ± 1.1	69.8 ± 0.9	70.1 ± 1.3	68.4 ± 1.3	71.1 ± 2.1
Range	56-79	49-91			
Gender (M/F)	12/8	17/63	6/23	6/21	5/19
BMI; kg/m^2	NA	26.73 ± 0.46	26.5 ± 0.7	26.4 ± 0.8	27.5 ± 0.8

Data show as mean ± SEM (BMI= body mass index)

The concentrations of VEGF in plasma and SF of OA patients and healthy controls were determined by ELISA. In this experiment, we detected the level of VEGF by choosing samples from the 80 OA patients who had both SF and plasma and 20 normal controls. The levels of VEGF are shown in **Figure 4.2**. The average VEGF levels in plasma of OA was lower than that found in control plasma but there was no significant difference between the two groups (163.5 ± 13.7 vs 195.4 ± 26.7 pg/ml, $P > 0.05$). Moreover, the levels of SF VEGF obtained from OA patients was 10 fold higher than that in plasma (1,614.3 ± 64.5 vs 163.5 ± 13.7 pg/ml, $P < 0.001$).

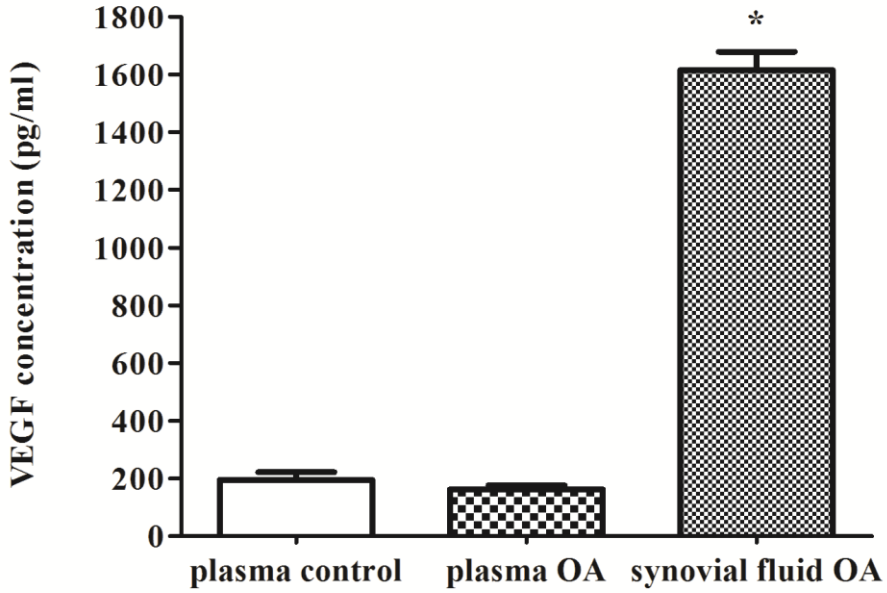


Figure 4.2 VEGF concentration in plasma and SF of OA patients and healthy controls Data show as mean ± SEM (* $P < 0.05$ when compared with plasma OA)

1.3 Correlation between VEGF expression and severity of knee osteoarthritis

Regarding Kellgren-Lawrence (K/L) classification, the knee OA patients were classified into 3 groups: grade 2, mild (n = 29); grade 3, moderate (n = 27); and grade 4, severe (n = 24). Age and BMI had no significant differences between OA groups ($P = 0.498$ and $P = 0.587$, respectively).The synovial fluid levels of VEGF were compared in relation to radiological K/L grading of OA. The Synovial fluid levels of VEGF from K/L grade 2, 3, and 4 were 1,153.5 ± 76.1 pg/ml, 1,602.5 ± 76.4 pg/ml, and 2,184.6 ± 80.1 pg/ml, respectively. The results indicated that synovial fluid VEGF levels had a strong positive correlation with severity of OA (r = 0.727, $P < 0.001$) (**Figure 4.3**). Furthermore, the levels of plasma VEGF from K/L grade 2, 3, and 4 were 100.5 ± 10.1, 166.2 ± 19.2, and 236.6±32.9 pg/ml, respectively. The correlation analysis between plasma VEGF and OA grading found that plasma VEGF was positively correlated with radiographic severity of knee OA (r = 0.454, $P < 0.001$) (**Figure 4.4**). Therefore, with higher VEGF, greater disease severity is expected, especially the level of VEGF in synovial fluid.

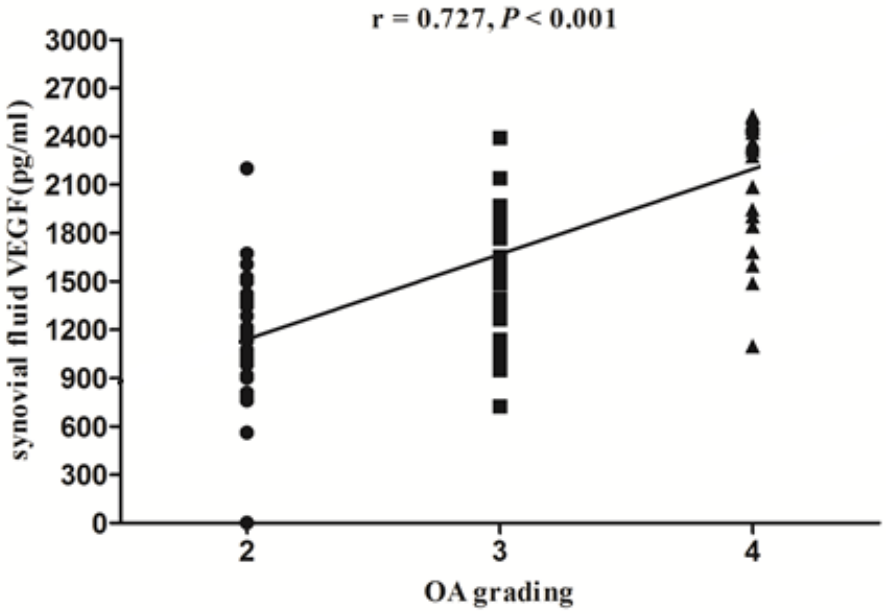


Figure 4.3 Correlation of synovial fluid VEGF concentration and OA severity

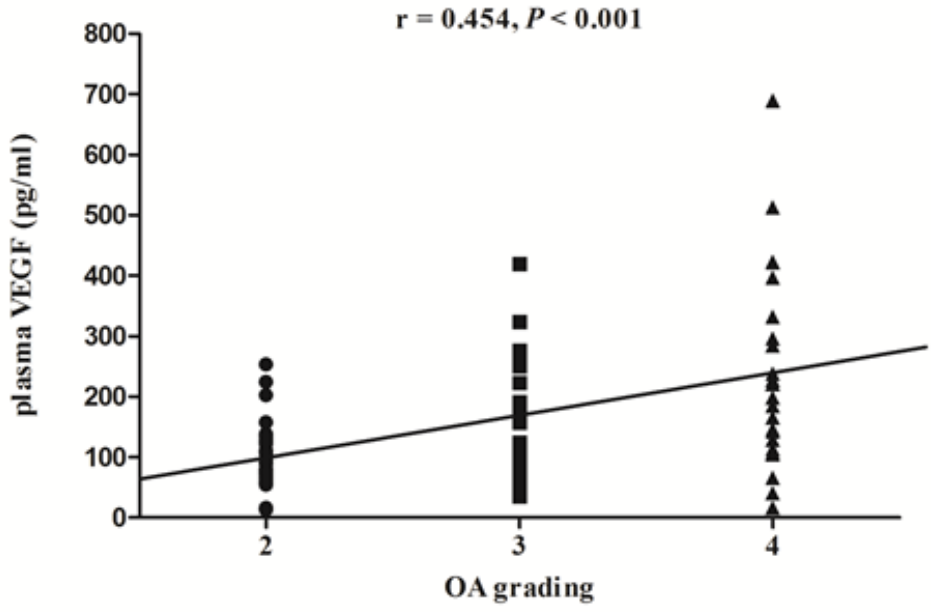


Figure 4.4 Correlation of plasma VEGF concentration and OA severity

1.4 VEGF expression in synovium tissue

To examine the source of VEGF production, we performed immunohistochemical staining. The synovium and articular cartilage were stained with primary VEGF antibodies and the VEGF protein expression visualized under a microscope. The result of the immunohistochemical staining is shown in **Figure 4.5**. The VEGF protein was expressed in synovial lining cells and chondrocytes in synovial membrane and cartilage tissues of knee OA patients, respectively. The data suggested that VEGF may be produced in many tissues in the knee joint including the synovium and articular cartilage.

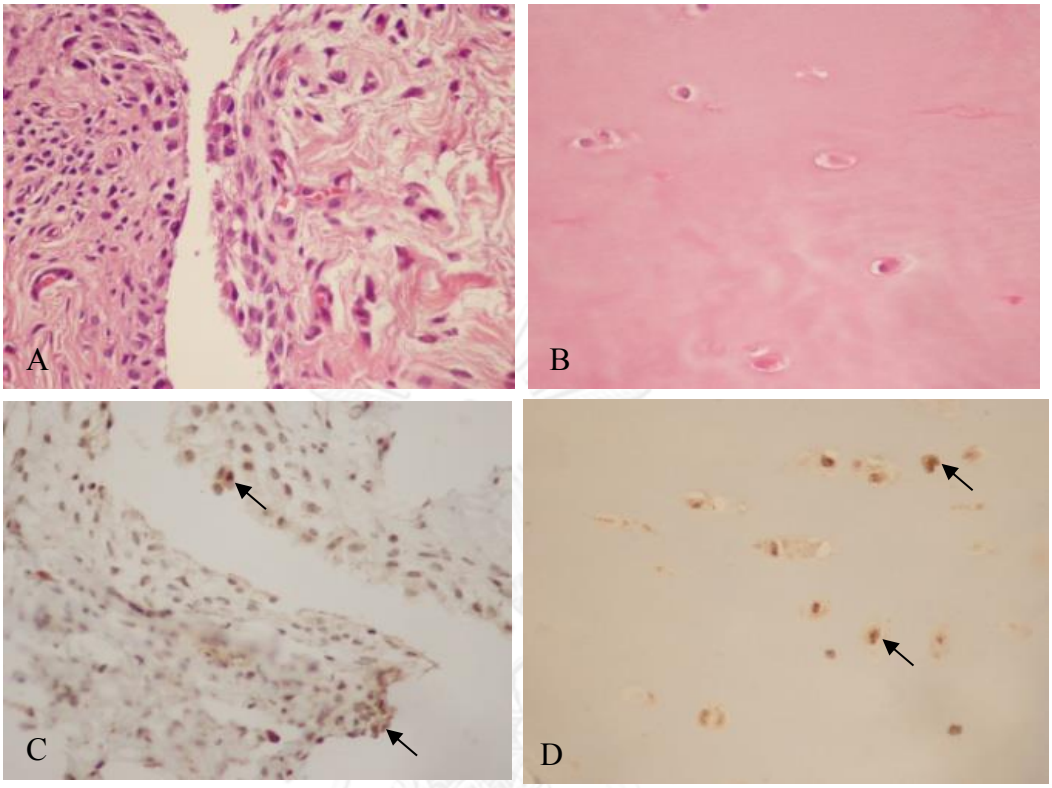


Figure 4.5 Expression of VEGF in osteoarthritis synovium and articular cartilage. (A, B) photomicrographs of hematoxylin and eosin (H&E) staining. The histology of synovial lining cells and chondrocytes were evaluated in synovium and cartilage, respectively. (C, D) VEGF immunohistochemistry analysis demonstrated the presence of VEGF antigen in synovial lining cells and chondrocytes (arrow, stained brown) (original magnification X40).

1.5 Receiver operating characteristic (ROC) curve

The synovial fluid VEGF was significant positive correlation with severity of OA. Classification of patients based on the severity of OA related with synovial fluid VEGF concentration was performed using ROC curve analysis. The first cut-off point was calculated in the VEGF levels to differentiate patients with mild group, and moderate and severe combined group. The cut-off point was set at 1,453.80 pg/ml, with a sensitivity of 0.80 and a specificity of 0.79 (**Figure. 4.6A**) as well as the area under the curve (AUC) of 0.87 (95%CI 0.79-0.95). The second cut-off point was determined in the VEGF concentrations to differentiate patients with mild and moderate combined group, and severe group. The cut-off point was set as 1,837.80 pg/ml, with a sensivity of 0.83, a specificity of 0.86, and an AUC of 0.91 (95%CI 0.83-0.98) (**Figure. 4.6B**). Taking into account these cut-off points, in the VEGF levels, the severity of OA patients may be divided into: grade 2 (mild): < 1,453.80 pg/ml, grade 3 (moderate): 1,453.80 – 1,837.80 pg/ml, and grade 4 (severe): ≥ 1,837.8 pg/ml.

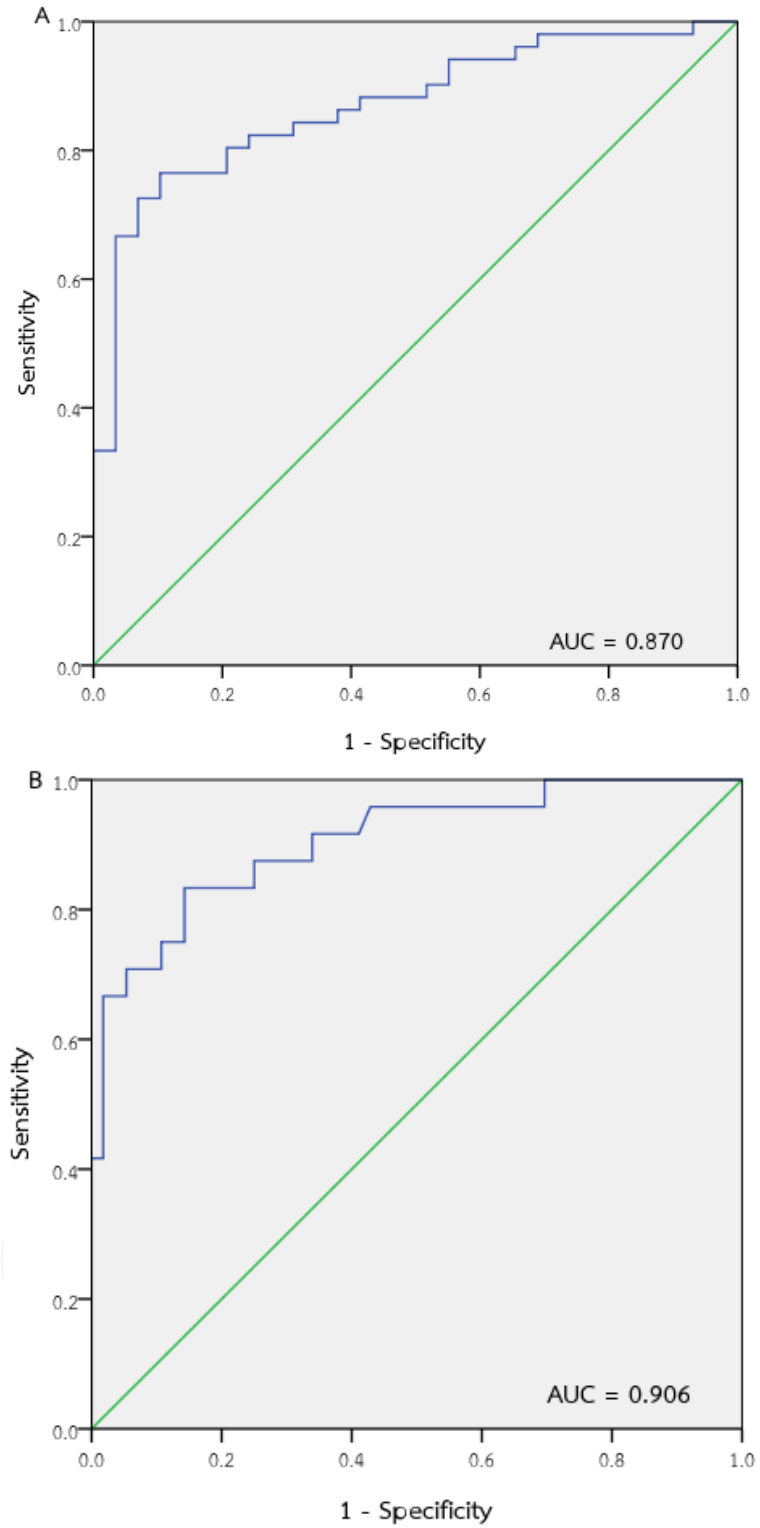


Figure 4.6 ROC curve testing the ability of VEGF to predict the severity in OA patients (A) difference between mild group, and moderate combined with severe group (B) difference between mild and moderate group, and severe OA group

<u>Part II</u>: Single nucleotide polymorphism (SNPs) of *VEGF* gene in knee osteoarthritis and their effect on VEGF levels

2.1. Characteristic of participants

Characteristics of the population studied and the number of individuals in each group are summarized in **Table 4.3**. In this study, VEGF −2578, −1154, -634, and +936 polymorphisms were analyzed in 202 patients with OA and 202 healthy controls. In terms of mean age, gender, and BMI of cases and controls, there were significant differences between the two groups. The average age in the OA group was 68.8 ± 7.8 years old while the mean age in the control group was 58.8 ± 6.3 years old ($P < 0.05$). The age range of OA patients and healthy controls were 49 to 91 and 40 to 80 years old, respectively. The male/female ratio was 33/169 in the patients with knee OA and 69/133 in controls ($P < 0.05$). Moreover, the mean BMI value was significantly different between OA patients (27.0 ± 3.7 kg/m^2) and controls (24.5 ± 3.8 kg/m^2). However, BMI data of some of the participants was lost. According to severity classification, the patients were divided into 3 groups: 55 mild, 71 moderate and 76 severe. In an analysis of subsets of OA patients, each individual was classified according to the severity of disease: $\leq$ grade 3 (early stage) or > grade 3 (advanced stage).

Table 4.3 Demographic data of all participants

Characteristics	Controls	OA patients	*P-value*
N	202	202	
Age ± SD (years)	58.8 ± 6.3	68.8 ± 7.8	<0.05
Age range (years)	40 – 80	49 - 91	
Gender (F/M)	133/69	169/33	<0.05
BMI (kg/m^2)*	24.5 ± 3.8	27.0 ± 3.7	<0.05
KL classification			
Grade 2		55	
Grade 3		71	
Grade 4		76	

*controls: n=106 and OA patients: n=192. Data expressed as mean ± standard deviation (SD)

2.2. VEGF SNPs by PCR-RFLP technique

In this study, we determined the four different single nucleotide polymorphisms of the *VEGF* gene including -2578C/A, -1154G/A, -634G/C, +936C/T. The *VEGF* polymorphisms were evaluated with agarose and polyacrylamide gel electrophoresis and visualized with ethidium bromide staining. An example of -2578C/A polymorphism patterns after PCR product digestion with *BstYI* is illustrated in **Figure 4.7**. The CC genotype was shown in a 455-bp PCR fragment. The AA genotype was shown in the presence of 248 and 207 bp fragments. The heterozygous CA genotype was shown in the presence of 455, 248, and 207 bp fragments.

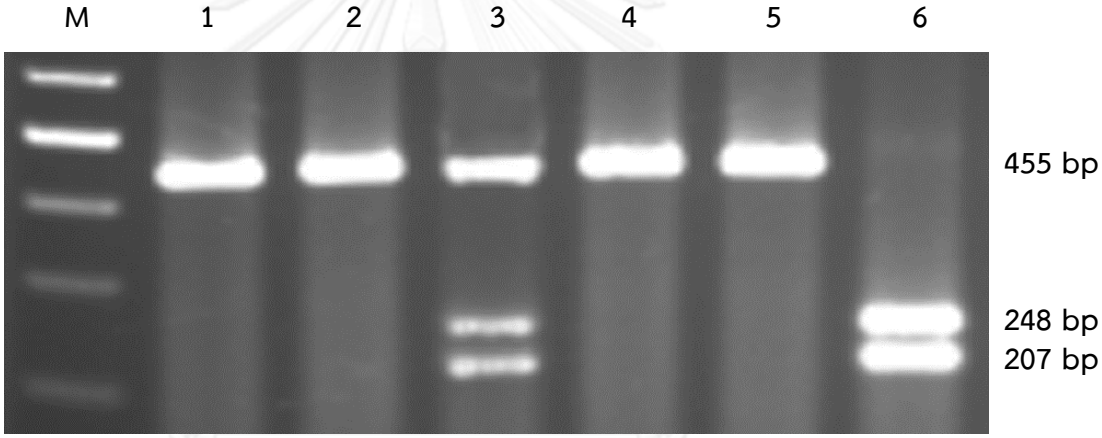


Figure 4.7 Example of genotypic pattern of the *VEGF* -2578C/A polymorphism. *Lane* M: DNA ladder (100 bp + 1.5 Kb (SibEnzyme ltd., Russia)), *Lane1-2 and 4-5*: 455 bp fragment represent CC genotype, *Lane3*: 455, 248, and 207 bp fragments represent CA genotype, *Lane6*: 248 and 207 bp fragments represent AA genotype.

Examples of -1154G/A SNP genotypic patterns on polyacrylamide gel electrophoresis are illustrated in **Figure 4.8**. The GG genotype was presented as four fragments of 3, 19, 34, and 150 bp. The genotype of AA was observed as three fragments of 3, 19, and 184 bp. Because the very small sized bands were not observed on the gel, only two different fragments were able to characterize the genotypes of the -1154G/A SNP.

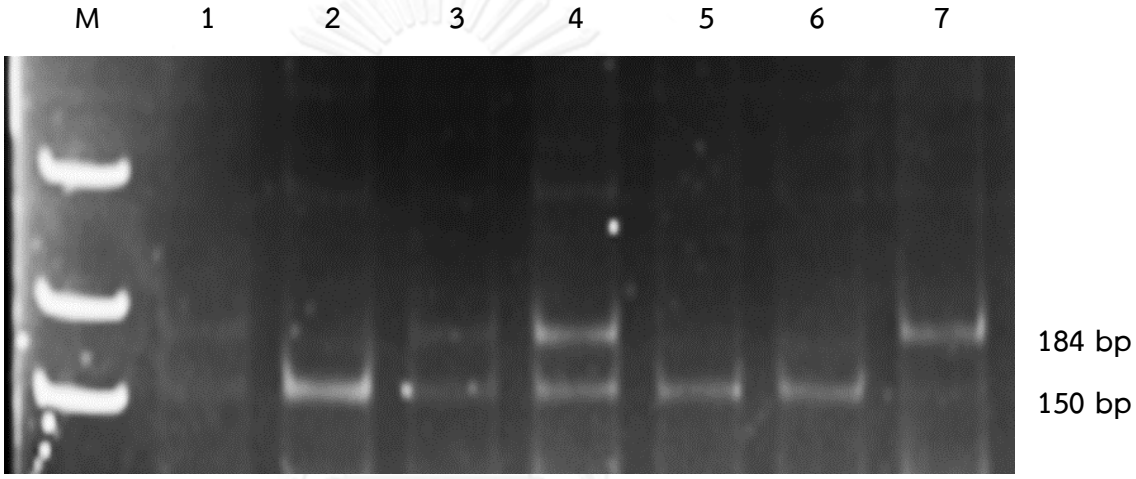


Figure 4.8 Example of genotypic pattern of the *VEGF* -1154G/A polymorphism.*Lane* M: GeneRuler™ ultra low range DNA ladder (10 to 300 bp) (Thermo Scientific, Germany), *Lane1,3, and 4*: 184 and 150 bp fragments represent GA genotype, *Lane2, 5, and 6*: 150 bp fragment represent GG genotype, *Lane7*: 184 bp fragment represent AA genotype

Moreover, the patterns of the *VEGF* -634G/C polymorphism after cutting the PCR product with *BsmFI* are showed in **Figure 4.9**. The CC homozygous is illustrated in a 274 bp fragment. The GG homozygous is represented by the two fragments that are 118 and 156 bp in size. The GC heterozygous is demonstrated by 118, 156, and 274 bp fragments.

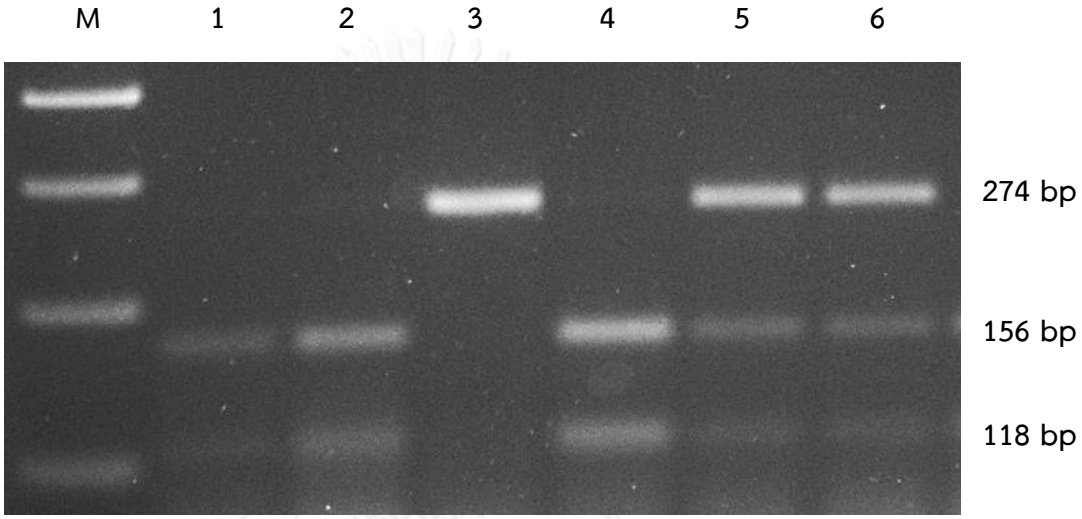


Figure 4.9 Example of genotypic pattern of the *VEGF* -634G/C polymorphism. *Lane* M: DNA ladder (100 bp + 1.5 Kb (SibEnzyme ltd., Russia)), *Lane1-2, 4*: 118 and 156 bp fragments represent GG genotype, *Lane3*: 274 bp fragment represent CC genotype, *Lane5-6*: 118, 156, and 274 bp fragments represent GC genotype.

The genotypic pattern of the last SNPs in this study is the +936C/T polymorphism as shown in **Figure 4.10**. The CC genotype was represented in a single band at 266 bp in size. The TT genotype was presented in 55 and 211 bp fragments. The CT genotype corresponded to the presence of 55, 211, and 266 bp fragments.

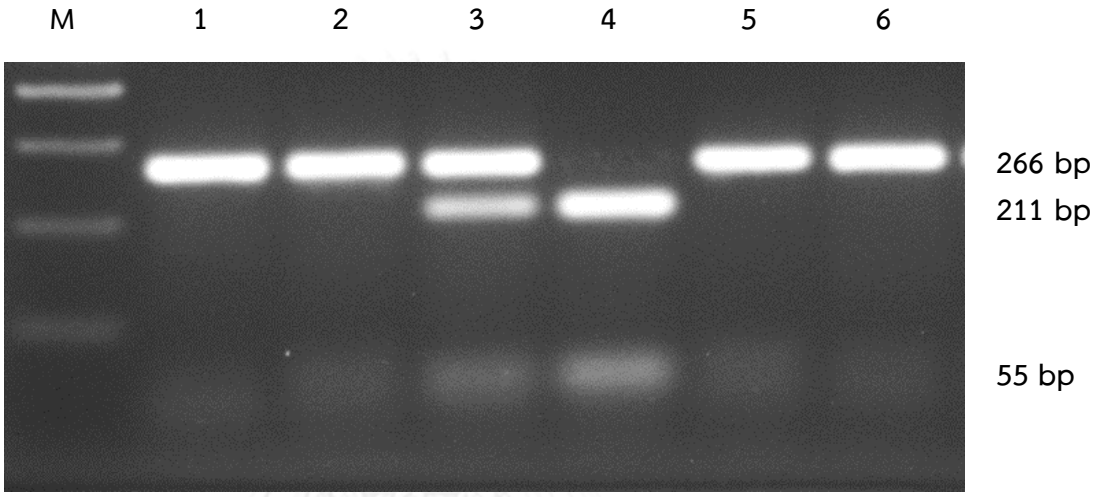


Figure 4.10 Example of genotypic pattern of the *VEGF* +936C/T polymorphism.*Lane* M: DNA ladder (100 bp + 1.5 Kb (SibEnzyme ltd., Russia)), *Lane1-2* and *5-6*: 266 bp fragment represent CC genotype, *Lane3*: 55, 211 and 266 bp fragment represent CT genotype, *Lane4*: 55 and 211 bp fragments represent TT genotype.

The genotype distributions of all polymorphisms were in accordance with Hardy–Weinberg equilibrium (HWE) among the cases and controls (HWE OA and controls of -2578C/A: χ^2 0.013 and 0.09, *P-value* 0.908 and 0.764, HWE OA and controls of -1154G/A: χ^2 0.575 and 0.001, *P-value* 0.448 and 0.975, HWE OA and controls of -634G/C: χ^2 0.361 and 5.824, *P-value* 0.548 and 0.016, and HWE OA and controls of +936C/T: χ^2 1.036 and 0.453, *P-value* 0.309 and 0.501). However, the HWE for *VEGF* -634G/C was not respected in healthy controls. The genotype distribution and allelic frequency of *VEGF* single nucleotide polymorphisms for cases and controls is shown in **Table 4.4**. There were no significant differences between the two groups with respect to both genotype distribution and allelic frequency.

Table 4.4 Genotype distribution and allelic frequency of the *VEGF* polymorphisms in OA patients and healthy controls

SNP	Genotypic or Allelic frequency	OA patients (n=202) n (%)	Controls (n=202) n (%)	OR (95% CI)	*P*-value
-2578	CC*	96 (48)	110 (55)	1	
(rs699947)	CA	86 (42)	77 (38)	1.28 (0.83-1.97)	0.284
	AA	20 (10)	15 (7)	1.53 (0.70-3.35)	0.332
	C*	278 (69)	297 (74)	1	
	A	126 (31)	107 (26)	1.26 (0.92-1.73)	0.162
-1154	GG*	134 (66)	138 (68)	1	
(rs1570360)	GA	63 (32)	58 (29)	1.12 (0.71-1.76)	0.687
	AA	5 (2)	6 (3)	0.86 (0.22-3.27)	0.952
	G*	331 (82)	334 (83)	1	
	A	73 (18)	70 (17)	1.05 (0.72-1.53)	0.854
-634	GG*	67 (33)	75 (37)	1	
(rs2010963)	CG	95 (47)	82 (41)	1.30 (0.81-2.07)	0.298
	CC	40 (20)	45 (22)	1.00 (0.56-1.77)	0.905
	G*	229 (57)	232 (57)	1	
	C	175 (43)	172 (43)	1.03 (0.77-1.38)	0.887
+936	CC*	133 (66)	146 (72)	1	
(rs3025039)	CT	59 (29)	50 (25)	1.30 (0.81-2.07)	0.303
	TT	10 (5)	6 (3)	1.83 (0.59-5.84)	0.37
	C*	325 (80)	342 (85)	1	
	T	79 (20)	62 (15)	1.34 (0.92-1.96)	0.138

***Reference genotype and allele**

In subset analysis of osteoarthritis patients classified by gender and severity of disease (early or advanced stage), there was no statistically significant difference between female and male groups (**Table 4.5.**). According to the -2578AA genotype, the patients with early stage had higher odd ratio than that in the patients who are in advanced stage (*P* = 0.023). However, no notable differences were evaluated in genotypic and allelic frequencies for -1154G/A, -634G/C, and +936C/T (**Table 4.6**).

Table 4.5 Genotype distribution and allelic frequencies of the *VEGF* polymorphisms in OA patients and healthy controls stratified by gender

Variable	Statistic	2578C/A (case/control)			1154G/A (case/control)		
		CC	CA	AA	GG	GA	AA
Gender							
Female		81/76	70/46	18/11	114/88	51/40	4/5
	OR (95% CI)	1	1.43 (0.85-2.39)	1.54 (0.64-3.74)	1	0.98 (0.58-1.67)	0.62 (0.13-2.75)
	P-value		0.188	0.403		0.948	0.354
Male		15/34	16/31	2/4	20/50	12/18	1/1
	OR (95% CI)	1	1.17 (0.46-3.01)	1.13 (0.13-8.53)	1	1.67 (0.62-4.48)	2.50 (0.0-97.21)
	P-value		0.888	0.61		0.374	0.501

Variable	Statistic	634G/C (case/control)			936C/T (case/control)		
		CC	CG	GG	CC	CT	TT
Gender							
Female		57/51	79/54	33/28	112/94	48/37	9/2
	OR (95% CI)	1	1.31 (0.76-2.26)	1.05 (0.54-2.08)	1	1.09 (0.63-.187)	3.78 (0.74-25.98)
	P-value		0.368	0.996		0.843	0.067
Male		10/24	16/28	7/17	21/52	11/13	1/4
	OR (95% CI)	1	1.37 (0.47-4.00)	0.99 (0.27-3.60)	1	2.10 (0.73-6.01)	0.62 (0.02-6.55)
	P-value		0.686	0.785		0.196	0.564

Table 4.6 Genotype distribution and allelic frequencies of the *VEGF* polymorphisms in OA patients and healthy controls stratified by severity

SNP	Genotypic or Allelic frequency	OA patients		OR (95% CI)	*P*-value
		Early stage (n=126) n (%)	Advanced stage (n=76) n (%)		
2578	CC	58 (46)	38 (50)	1	
	CA	50 (40)	36 (47)	0.91 (0.48-1.72)	0.872
	AA	18 (14)	2 (3)	5.90 (1.21-39.09)	**0.023**
	C	166 (66)	112 (74)	1	
	A	86 (34)	40 (26)	1.45 (0.91-2.32)	0.126
1154	GG	83 (66)	51 (67)	1	
	GA	39 (31)	24 (32)	1.00 (0.52-1.94)	0.879
	AA	4 (3)	1 (1)	2.46 (0.25-59.39)	0.38
	G	47 (19)	26 (17)	1.11 (0.64-1.95)	0.797
	A	205 (81)	126 (83)	1	

SNP	Genotypic or Allelic frequency	OA patients		OR (95% CI)	*P*-value
		Early stage (n=126) n (%)	Advanced stage (n=76) n (%)		
634	GG	26 (21)	14 (18)	0.85 (0.34-2.11)	0.859
	CG	54 (43)	41 (54)	0.60 (0.30-1.22)	0.174
	CC	46 (36)	21 (28)	1	
	G	106 (42)	69 (45)	0.87 (0.57-1.34)	0.582
	C	146 (58)	83 (55)	1	
936	CC	85 (67)	48 (63)	1	
	CT	36 (29)	23 (30)	0.88 (0.45-1.75)	0.825
	TT	5 (4)	5 (7)	0.56 (0.13-2.40)	0.29
	C	206 (82)	119 (78)	1	
	T	46 (18)	33 (22)	0.81 (0.47-1.37)	0.472

2.3. Linkage Disequilibrium test of *VEGF* gene polymorphisms by Haploview analysis

The linkage disequilibrium between -2578C/A, -1154G/A, -634G/C, and +936C/T of VEGF polymorphisms is shown in **Figure 4.11**. The data revealed that there was high linkage disequilibrium across the SNP including -2578, -1154, and -634, but excluding +936. Briefly, pairwise LD in case-control study was observed for the first two SNPs (D' = 0.69 and r^2 = 0.25), for the second and third SNPs (D' = 0.74 and r^2 = 0.08), and for the first and third (D' = 0.70 and r^2 = 0.15). The relatively low values r^2 indicate that none of the three markers can be considered redundant in an association study.

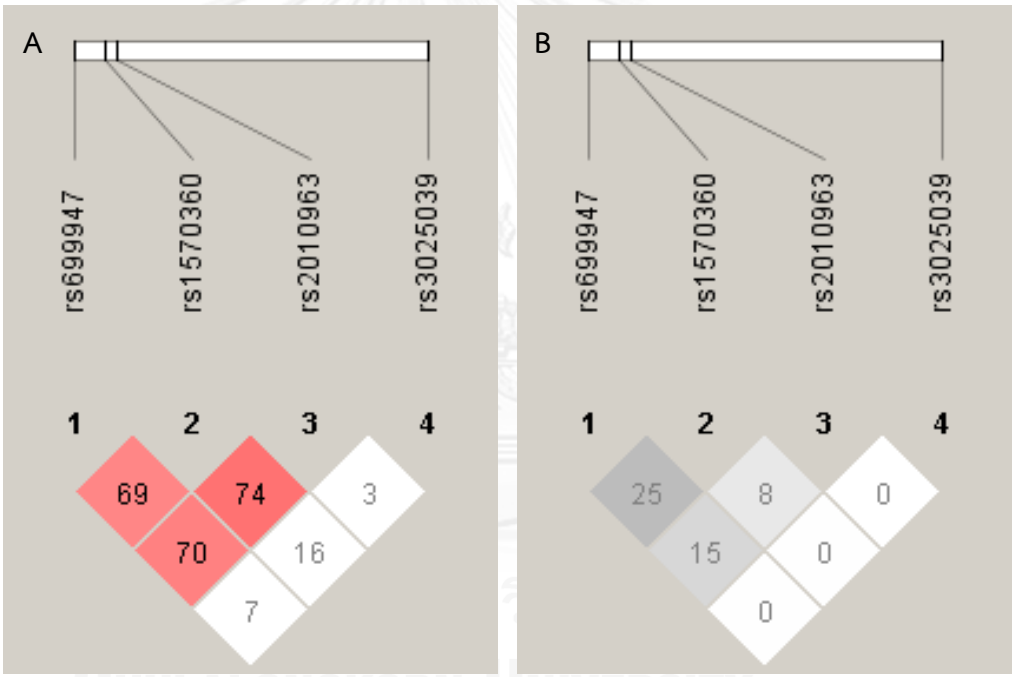


Figure 4.11 Linkage disequilibrium plot of -2578C/A (rs699947), -1154G/A (rs1570360), -634G/C (rs2010963), and +936C/T (rs3025039) of VEGF polymorphisms (A) D' (shown in percentages) between the SNPs. The pink: D' < 1; Red: D' = 1; White: D' = 0 (B) r^2. The gray shaded boxes correspond to the paired r^2 between the single nucleotide polymorphisms (SNPs). White: r^2 = 0; Shades of gray: 0 < r^2 < 1; Black: r^2 = 1

To determine whether any specific haplotypes with 3 VEGF polymorphisms (-2578C/A, -1154G/A, and -634G/C) were associated with OA, haplotype analysis was performed. Haplotype distributions of -2578C/A, -1154G/A, and -634G/C *VEGF* gene polymorphisms is illustrated in **Table 4.7**. The most frequent haplotype found in OA patients and controls was C-G-C (36% and 40%, respectively). The frequencies of the C-A-C haplotypes displayed a significant difference in distribution between OA patients and controls (OR (95%CI) = 6.96 (1.46-33.01), *P* = 0.005).

Table 4.7 Haplotype analysis of VEGF gene polymorphisms in OA patients and controls

Haplotype	OA patients (%)	Controls (%)	OR (95% CI)	*P*-value
VEGF -2578C/A / -1154G/A / -634G/C				
A-A-G	12.6	14.5	0.85 (0.56-1.27)	0.422
A-G-C	4	2.4	1.70 (0.76-3.82)	0.194
A-G-G	13.9	9.6	1.53 (0.99-2.37)	0.053
C-A-C	3.1	0.5	6.96 (1.46-33.01)	0.005
C-A-G	1.8	2.4	-	-
C-G-C	35.6	39.7	0.84 (0.63-1.12)	0.223
C-G-G	28.4	31	0.88 (0.65-1.20)	0.426
A-A-C	0.7	0	-	-

2.4. Association of *VEGF* polymorphisms with SF and plasma levels of VEGF

The levels of VEGF were determined in 79 synovial fluid OA, 109 plasma OA and 99 plasma healthy controls by ELISA. The concentration of SF VEGF was remarkably higher in OA patients than that in plasma OA (626.53 ± 25.26 and 78.84 ± 7.23 pg/ml, respectively, P < 0.001). VEGF concentration in plasma did not significantly differ between OA patients and healthy participants (78.84 ± 7.23 and 70.62 ± 7.81 pg/ml, respectively, P = 0.440). These data supported the results from **part I** that VEGF levels in SF were higher expressed in OA compared with paired plasma and there were no significant differences in plasma VEGF levels of OA compared to that of controls. To assess the functional relevance of the -2578C/A,

-1154G/A, -634G/C, and +936C/T polymorphisms on synovial fluid and plasma VEGF levels, the association between polymorphisms and VEGF levels were investigated. SF levels of VEGF in each genotype distribution of the 4 SNPs are illustrated in **Figure 4.12**. There were no associations between the levels of SF VEGF and genotype distribution in each SNP in OA patients.

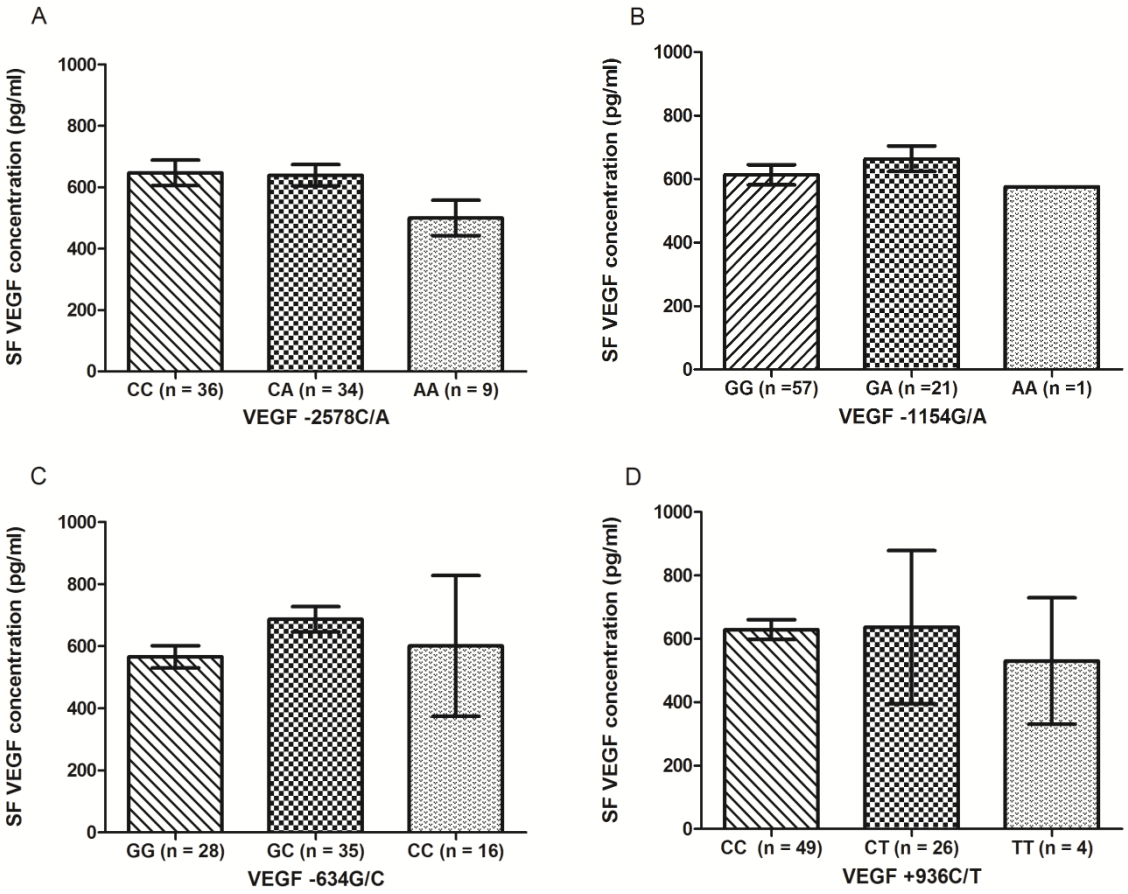


Figure 4.12 The concentrations of SF VEGF from knee OA patients in each genotypic group**.** (A) VEGF -2578C/A, (B) VEGF -1154G/A, (C) VEGF -634G/C, and (D) VEGF +936C/T

In addition, the concentrations of plasma VEGF in OA patients were no significant difference in each genotypic group of all SNPs as shown in **Figure 4.13**.

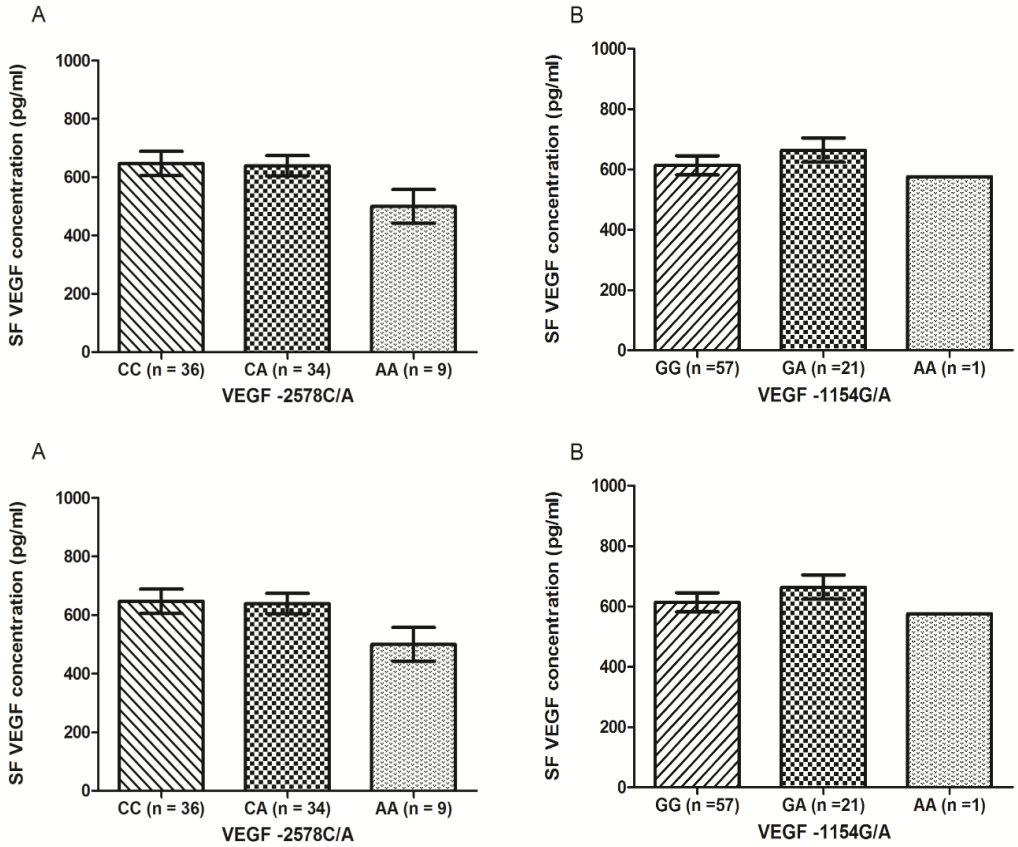


Figure 4.13 The concentrations of plasma VEGF from knee OA patients in each genotypic group. (A) VEGF -2578C/A, (B) VEGF -1154G/A, (C) VEGF -634G/C, and (D) VEGF +936C/T

In healthy subjects, plasma VEGF levels were not significantly associated with genotype distribution in all SNPs shown in **Figure 4.14**.

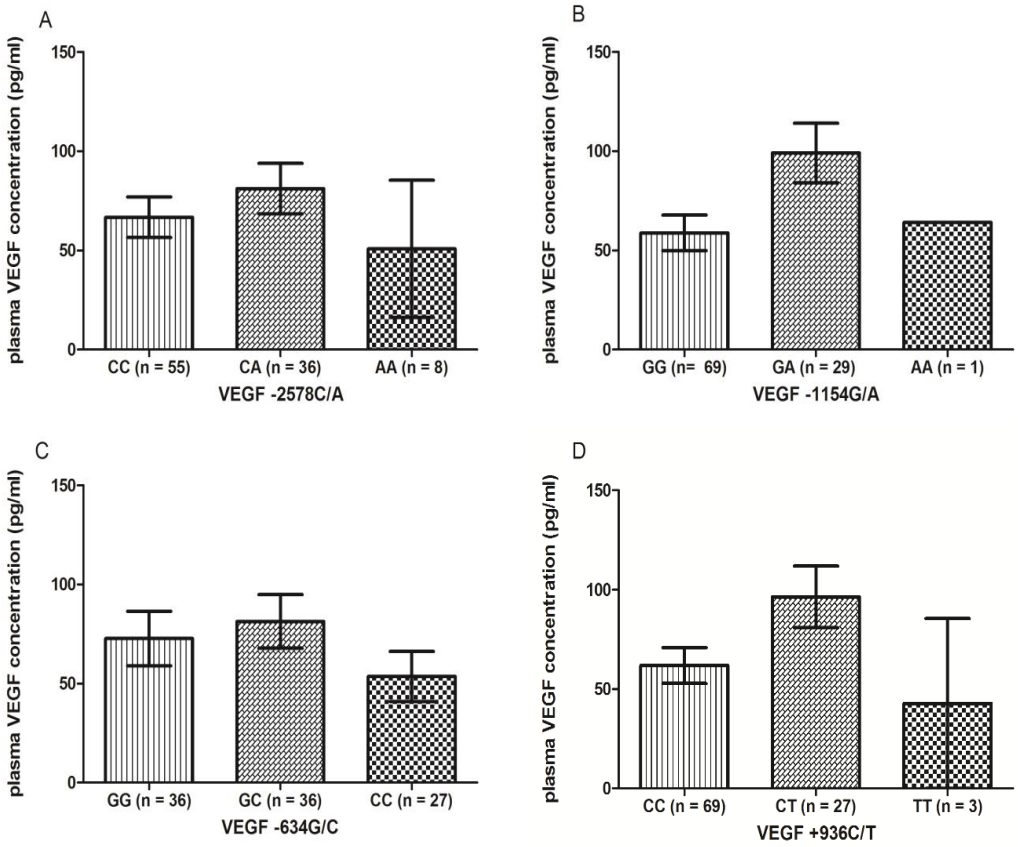


Figure 4.14 The concentrations of plasma VEGF from healthy controls in each genotypic group**.** (A) VEGF -2578C/A, (B) VEGF -1154G/A, (C) VEGF -634G/C, and (D) VEGF +936C/T

Interestingly, plasma VEGF levels of the -634CC genotype were significantly greater in knee OA patients than those in controls (P = 0.035) (**Figure 4.15 C**). However, plasma VEGF levels of -634GG and GC genotypes were not significant difference when compared between OA patients and controls. We also compared OA to controls by determining the association between -2578C/A, -1154G/A, and +936C/T polymorphisms and corresponding plasma VEGF levels. The levels of plasma VEGF were no significant difference between the two groups with respect to their genotype distribution (**Figure 4.15 A, B, and D**).

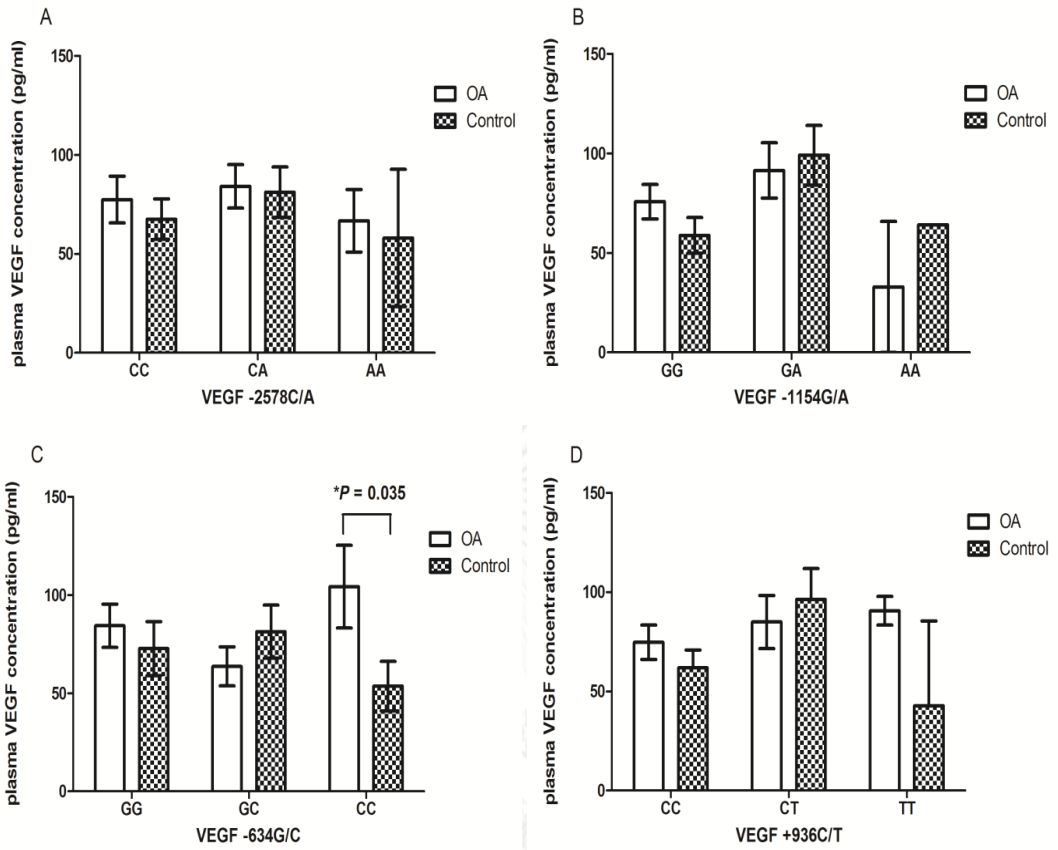


Figure 4.15 The concentrations of plasma VEGF between knee OA patients and healthy controls in each genotypic group. (A) VEGF -2578C/A, (B) VEGF -1154G/A, (C) VEGF -634G/C, and (D) VEGF +936C/T

<u>Part III</u>: Determination of VEGF mRNA, miR-210, miR-223 in knee OA synovial tissues and *in vitro* model

3.1 Characteristics of participants

Forty synovial tissues samples obtained from knee OA patients and five synovium controls without inflammation gained from anterior cruciate ligament (ACL) injury patients were used in the extraction of total RNA and performed real time RT-PCR for VEGF mRNA determination. Meanwhile the expressions of miR-210 and miR-223 were measured in 15 synovial tissue from OA patients and 5 from controls. The expression of VEGF, miR-210, and miR-223 were examined and the associations between these factors were evaluated. The median age of OA patients was 71 years old (range 47 to 82 years old). The median age of controls was 45 years old (range age 21 to 75 years old). There was a significant difference with respect to age ($P < 0.001$) and gender ($P = 0.030$).

All *in vitro* experiments (miR-210, miR-223, and VEGF expression) were carried out with fibroblast-like synoviocytes (FLS) derived from three individual OA patients at passage 3-7. The FLS were grown under hypoxia with or without IL-1β for 12, 24, and 48 hours.

3.2 The expression of VEGF mRNA, miR-210, and miR-223 in knee osteoarthritis synovial membrane

According to the elevated VEGF in OA synovial fluid and positive staining in synovial tissue, we attempted to clarify the expression of VEGF in mRNA level in OA synovial membranes compared with controls by real time RT-PCR using 18s rRNA as an internal control. The mean value of each control sample was set at 1 and then this was used to calculate the relative expression of target genes. In this study, the level of VEGF mRNA was expressed in both OA and control groups. The results demonstrated that the VEGF level in OA and controls were not significant difference as shown in **Figure 4.16**.

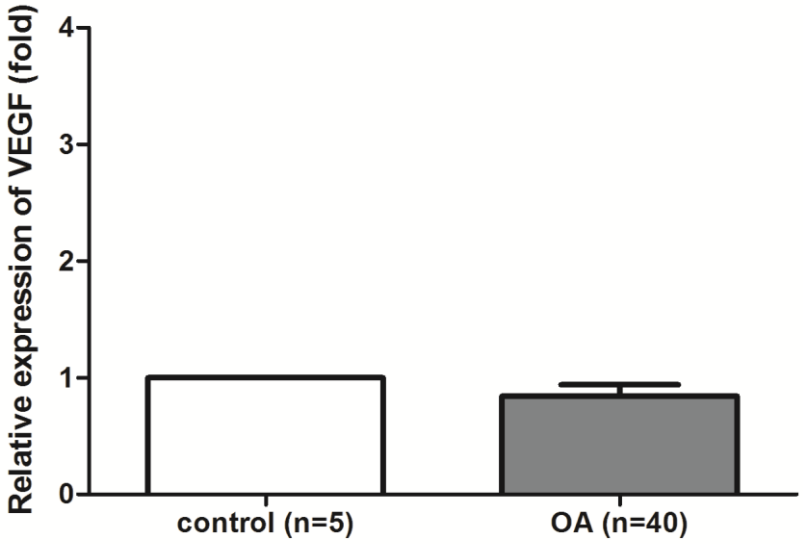


Figure 4.16 Relative expression level of VEGF in synovial tissues from OA patients and controls

Moreover, we investigated whether miR-210 and miR-223 are expressed in OA synovial tissues. The expression of miR-210 and miR-223 were analyzed by real time RT-PCR and normalized with RNU6B. The average relative expression levels of miR-210 and miR-223 were 7.9 and 5.5 fold in OA synovial tissue compared with levels in controls (**Figure 4.17** and **Figure 4.18**). The miR-210 and miR-223 levels were significantly higher in OA patients than in controls (miR-210: $P = 0.029$, miR-223: $P \leq 0.01$).

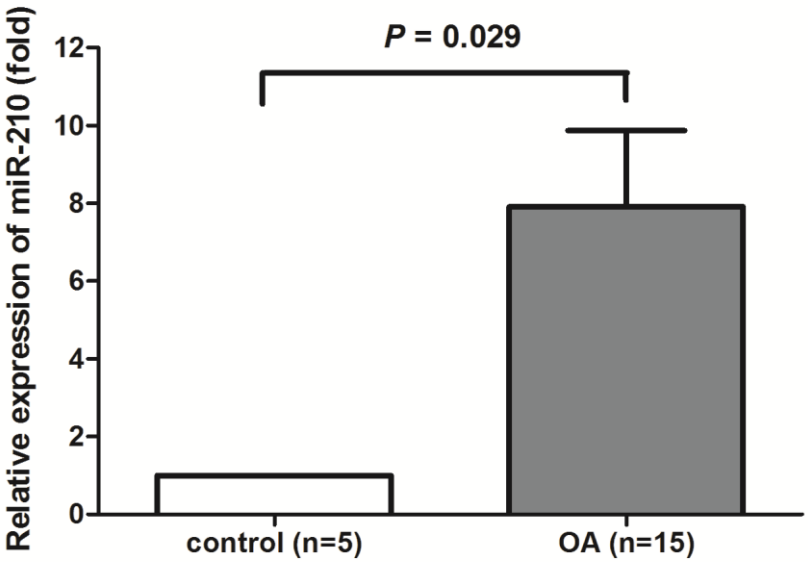


Figure 4.17 Relative expression level of miR-210 in synovial tissues from OA patients and controls

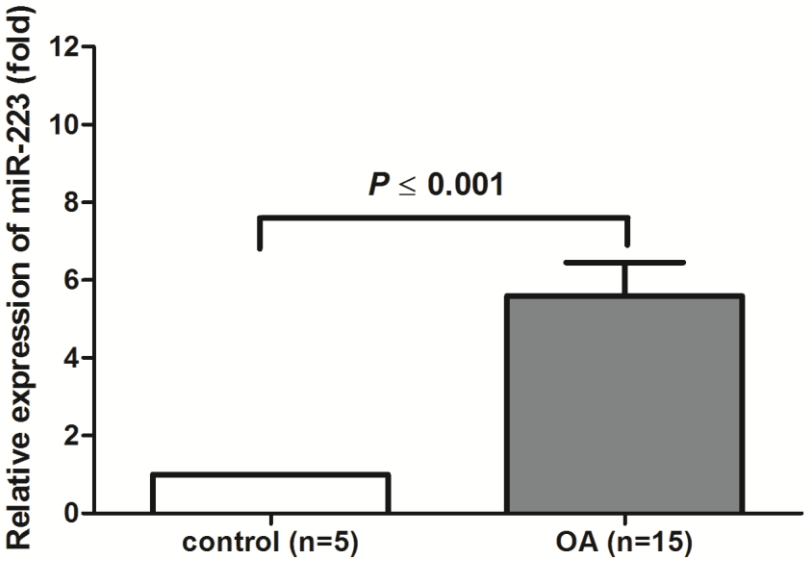


Figure 4.18 Relative expression level of miR-223 in synovial tissues from OA patients and controls

There was no association between VEGF mRNA level and miR-210 and miR-223 expressions in OA synovial tissues. In addition, age and gender did not associate with the expression of the VEGF and miRNAs.

3.3 *In vitro* study: the expression of VEGF mRNA, miR-210, and miR-223 in OA FLS cultured under hypoxia and interleukin-1β (IL-1β) stimulated conditions

To assess the effects of osteoarthritic-like conditions on FLS VEGF, miR-210, and miR-223 expression, FLS were cultured under hypoxia with or without IL-1β (10 ng/ml) treatment. The data revealed that at 12 hours, VEGF expressions were not significantly different between each group. The exposure of FLS to hypoxia combined with IL-1β resulted in the highest increase in VEGF levels at 24 and 48 hours compared with hypoxia (P = 0.025 and P = 0.029, respectively) and normoxia (P = 0.04 and P = 0.09, respectively). The expression of VEGF tended to be up-regulated under hypoxia conditions compared to normoxia; however, there was no significant difference (P = 0.178 at 24 hours, P = 0.264 at 48 hours). Moreover, the osteoarthritic-like conditions stimulated VEGF mRNA in a time-dependent manner **(Figure 4.19)**.

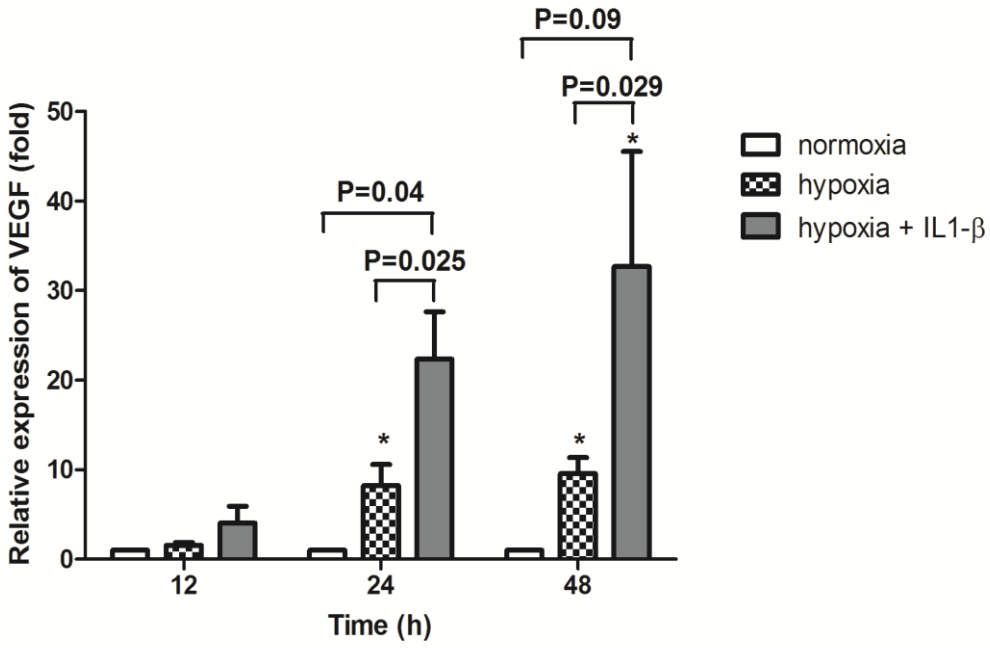


Figure 4.19 Relative expression level of VEGF in FLS under hypoxia and interleukin-1β treatment (* P < 0.05 when compared with corresponding group at 12 hours)

The expressions of miR-210 in the stimulated FLS at 12, 24, and 48 hours are illustrated in **Figure 4.20**. The expression of miR-210 was gradually increased in human FLS under hypoxia condition and the presence or absence of IL-1ß treatment over a 48–hour time course. There was a significant difference between cells treated with hypoxia and IL-1ß and those without treatment (normoxia). The levels of miR-210 responded to hypoxia, although the increase was not statistically significant difference ($P > 0.05$). Additionally, VEGF and miR-210 levels in the stimulated groups had a similar expression pattern. They were induced by hypoxia and hypoxia with IL-1ß. The data indicated that VEGF and miR-210 were associated with the pathogenesis of OA *in vitro*.

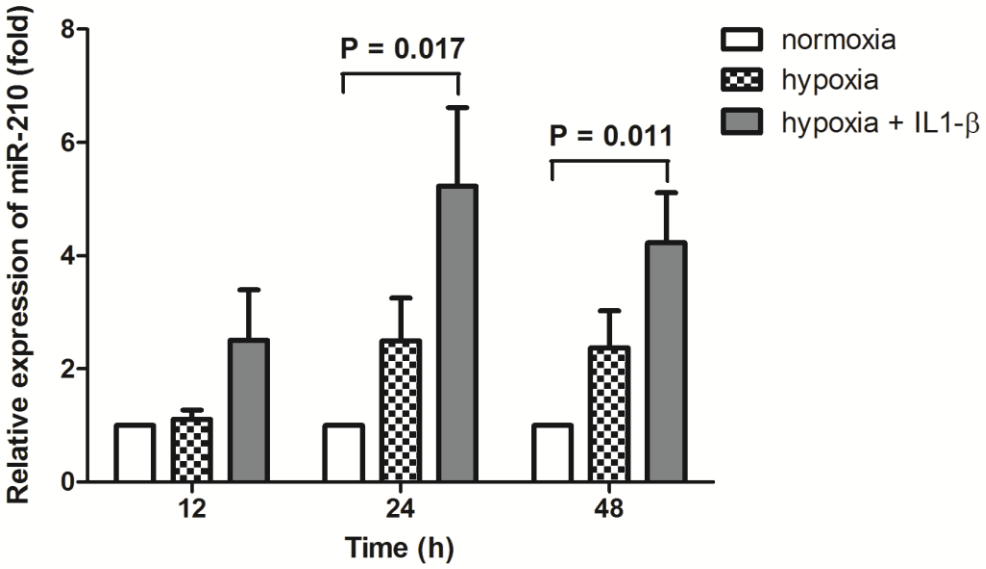


Figure 4.20 Relative expression level of miR-210 in FLS under hypoxia and interleukin-1ß treatment

The expression of miR-223 was examined in FLS under normoxia or hypoxia with or without IL-1β treatment. The data are shown in **Fig. 4.21**. FLS under hypoxia with or without IL-1β treatment did not significantly affect miR-223 expression at each time point.

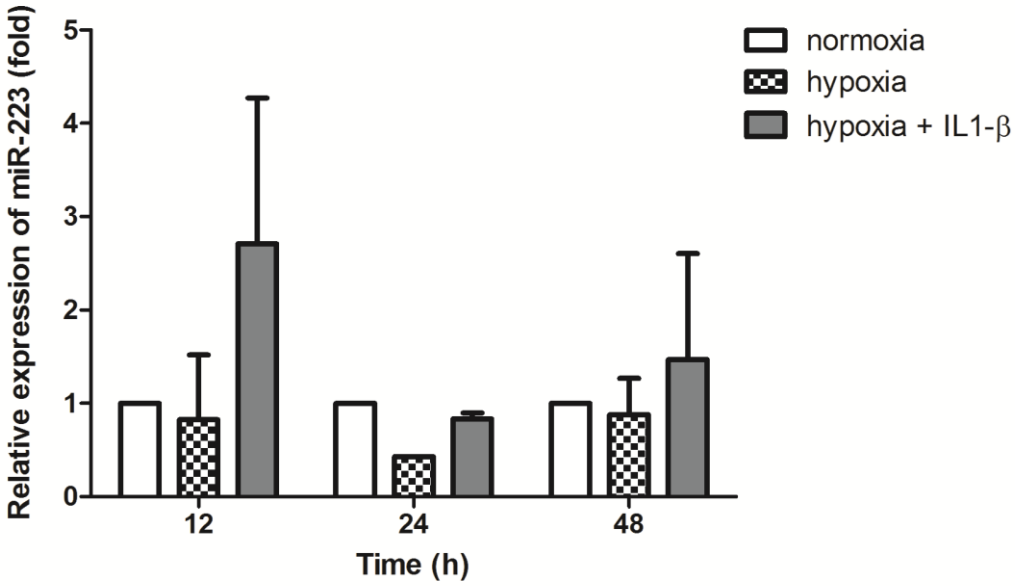


Figure 4.21 Relative expression level of miR-223 in FLS under hypoxia and interleukin-1β treatment

CHAPTER IV

Discussion

Osteoarthritis is considered as a degenerative, chronic, or age-related joint disorder. The cause of the disease remains poorly understood. OA affects multiple joints and periarticular tissues such as articular cartilage, subchondral bone, and synovium. The etiology of OA is may be due to injury on the surface of articular cartilage. The products of cartilage break down release into the synovial fluid. Considered foreign bodies, synovial cells in the inflamed synovium (macrophage and synoviocyte) phagocytose and synthesize inflammatory mediators secreted in the synovial fluid. These mediators can directly trigger chondrocyte to produce catabolic mediators leading to an increase of cartilage degradation. The mediators can induce angiogenesis of synovial tissues and stimulate the inflammatory process, creating a vicious circle (Berenbaum 2013, Henrotin *et al.* 2014). Inflammation is increasingly recognized as an essential feature in OA particularly, inflammation in synovial membrane (synovitis). Previous report showed that the indices of angiogenesis including endothelial cell fractional areas and proliferation were positively correlated with inflammation grade in OA synovium. They suggested that inflammation and angiogenesis are associated with OA (Haywood *et al.* 2003). The histological experiment was performed in inflammatory synovial tissue (I) compared with normal/reactive tissue (N/R) to determine the correlation of inflammation and angiogenesis in OA. The results showed that OA blood vessel density and vessel size were increased in I area compared to N/R. Moreover, the productions of proinflammatory mediators including IL-6, IL-8, and VEGF were elevated in the cells isolated from I area. The VEGF concentration was positively correlated with IL-6 and IL-8. These data suggested that inflammation was associated with angiogenesis (Henrotin *et al.* 2014). Not only synovial angiogenesis, but also osteochondral angiogenesis were observed in OA. New blood vessel formation across the osteochondral junction was correlated with cartilage calcification or ossification in OA (Walsh *et al.* 2007).

The close interdependence of inflammation and angiogenesis lead to OA progression and pain by triggering the imbalance between cartilage matrix degradation and synthesis. VEGF is a potent angiogenic factor and contributed to inflammation via plasma extravasation. It functions in developmental stage, normal and disease situation. VEGF plays roles in bone remodeling, bone fracture repairing, and supplying the nutrient and oxygen from subchondral bone to the cartilage via subchondral-cartilage interface. The change in vascularization may lead to pathology of osteoarthritis (Chim *et al.* 2013). Several researches have been shown the association of VEGF and pathogenesis of OA (Pufe *et al.* 2001, Enomoto *et al.* 2003, Tanaka *et al.* 2005). In OA, VEGF expression is induced by many factors such as inflammatory cytokines (IL-1β, TNF-α), hypoxic condition (hypoxia inducible factor (HIF)-1α), and mechanical stress (Ashraf and Walsh 2008). VEGF expression seems to be the crucial molecular mechanism, exacerbating both osteoarthritic angiogenesis and inflammation.

In this study, we focused on the role of VEGF in pathogenesis of knee OA to better understand the assocation of VEGF and progression of disease.

<u>Part I</u>: Association of VEGF protein in the pathogenesis of knee osteoarthritis

In angiogenic cytokine array study, a number of cytokines showed significantly different levels in OA patients and controls. Notably, Angiopoietin-2, follistatin, VEGF, granulocyte-colony stimulating factor(G-CSF), hepatocyte growth factor (HGF), plaste/endothelial cell adhesion molecule-1 (PECAM-1), and interleukin-8 (IL-8) all presented higher levels in OA plasma samples than control plasma samples. G-CSF stimulates neutrophil survival and function. PECAM-1 involves in white blood cell migration. The follistatin, HGF, angiopoietin-2, IL-8, and especially VEGF play roles in angiogenesis and inflammation. All those angiogenic cytokines may play a role in pathogenesis of OA. The high VEGF expression in synovial fluid and plasma OA compared with plasma controls. The SF concentration of VEGF was correlated with the severity of OA. Herein, the VEGF expression was investigated to determine the relationship of VEGF with pathogenesis of OA.

Vascular endothelial growth factor, a heparin-binding dimeric glycoprotein can act not only on endothelial cells but also on many cell types such as hematopoietic stem cells, monocytes, megakaryocytes, osteoblasts and chondrocytes (Tammela *et al.* 2005, Lingaraj *et al.* 2010). It has essential functions in embryonic vasculature, tissue remodeling, wound healing, malignant, cancer and endochondral ossification (Tanaka *et al.* 2005, Roy *et al.* 2006). VEGF and its receptors have been shown to be expressed in articular cartilage of OA patients compared to healthy controls (Enomoto *et al.* 2003). Furthermore, VEGF levels were highly expressed in the induced-OA rabbit than that in sham operation group (Jansen *et al.* 2012). These results were in agreement with Yamairi's study showing that VEGF was detected by immunohistochemical study and was increased simultaneously with the activity of cartilage degradation (Yamairi *et al.* 2011). Corrado *et al*. reported that VEGF played an important role in bone formation via acting on osteoblasts. VEGF may stimulate angiogenesis resulting in osteophyte formation and subchondral bone sclerosis in OA (Corrado *et al.* 2013). Taken together, these studies revealed that VEGF might be associated with pathogenesis of osteoarthritis.

Recent studies showed that several cytokines in plasma and SF were associated with the radiographic severity of knee osteoarthritis (Honsawek *et al.* 2009, Honsawek *et al.* 2011, Honsawek *et al.* 2012, Mabey *et al.* 2014). To our knowledge, there is no study contributing with relationship of VEGF in SF and plasma and severity of OA. The current study is the first to demonstrate that the level of VEGF concentrations in both plasma and SF were determined and VEGF expression was positively correlated with the severity of OA.

These findings revealed a marked increase of VEGF levels in SF of knee OA patients compared to the paired plasma levels. Concurrently, the elevated levels of VEGF in SF as compared to paired plasma samples had been found in juvenile idiopathic arthritis (Bosco *et al.* 2009). However, this study did not compare the SF level of VEGF between the OA patients and healthy controls. Fay and colleagues (Fay *et al.* 2006) found that the concentration of VEGF in SF obtained from OA patients was strongly increased compared to the control. The expression of VEGF in SF from knee OA patients was significantly elevated when compared with that in

plasma. The explanation for these findings might be that local synovial milieu had more recruited for VEGF production (Bosco *et al.* 2009). Additionally, the increased VEGF levels in SF may be possibly attributed to either the release of VEGF residing in extracellular matrix, the production of the cells in synovial membrane and/or articular cartilage, or both processes. The source of VEGF in the SF is presumably originated from the local tissues (such as the synovium and articular cartilage). The immunohistochemical study showed VEGF expression in synovial lining cells, fibroblasts in synovium, and articular chondrocytes. This result was in accordance with various researches in OA model (Yamairi *et al.* 2011, Jansen *et al.* 2012) which demonstrated a VEGF positive staining in chondrocytes of OA articular cartilage. Synovitis and degenerative articular cartilage are likely to be facilitating factors in the release of VEGF into the SF.

We observed that VEGF levels in plasma and SF of OA patients were positively correlated with the radiographic severity. The results showed that SF and circulating VEGF concentration were higher in end-stage knee OA patients (KL grade 4) when compared with early knee OA (KL grade 2), in agreement with Rübenhagen R study. Recently, Rübenhagen R and colleagues have found that the increased concentration of VEGF in SF was positively correlated with the radiographic OA grading (Rübenhagen *et al.* 2012). More osteophyte formation and subchondral bone sclerosis in severe OA may result from up-regulated VEGF production. Angiogenesis stimulates invasive subchondral bone and activates proliferative and bone formation (Corrado *et al.* 2013). As a consequence, VEGF may be released from pathologic lesion into the joint. Therefore, severe pathology of OA in the late stage causes the elevation of VEGF production.

However, in contrast with our observations, Benito MJ *et al.* found the expression of inflammatory mediators such as TNF-α, IL-1ß and VEGF were greater in early OA than those in the late OA. These conflicting results may be related to the differences in population, tissue collection and method of quantification (Benito *et al.* 2005). This previous study used the immunohistochemical staining whereas out study used the ELISA for quantification the expression of VEGF.

In this regard, we observed that synovial fluid VEGF levels illustrated a good discriminating power to predict the severity of knee OA with a cut-off point value of less than 1,453.80 pg/ml for grade 2 knee OA patients and more than 1,837.80 pg/ml for grade 4 knee OA patients. The cut-off points had good accuracy as a diagnostic test at AUC 0.87 and 0.91, respectively. These findings indicate that synovial fluid VEGF concentration could be used as a predictive marker for ongoing severity of knee osteoarthritis.

The current study inevitably has some limitations. First, sample size was small. Therefore, further study should include more subjects. Second, both groups in control and OA subjects were not matched in age and gender. OA is commonly found in female with aging, thus it is difficult to obtain aged-match and sex-match in samples. Third, only VEGF production has been examined in plasma and SF, other cytokines in inflammation or angiogenesis may be added to prove association between both cytokine expression and the severity of OA. Lastly, we could not collect SF from healthy controls because of ethical reason. More researches will be required to determine whether VEGF correlates with angiogenesis or inflammation in the development of OA. However, our research provides evidences for the association of VEGF concentration and the severity of knee OA. Further studies with large population and VEGF mRNA expression are needed to gain the precise role of VEGF on the pathogenesis of OA.

In conclusion, synovial fluid VEGF levels were remarkably higher than those in matched plasma. The concentration of VEGF in both plasma and SF were positively correlated with the radiographic severity in knee OA patients. Therefore, these findings suggested that VEGF may play a role in pathogenesis of OA. Several researches are warranted to get a better understanding of the potential role of VEGF as a biochemical marker of OA development.

Part II: Single nucleotide polymorphisms (SNPs) of *VEGF* gene in knee osteoarthritis

The cause of OA is not entirely understood. However, it was well described that the OA is caused by complex processes between environmental factors and genetic factors. Genome-wide association study (GWAS) was performed on large cohorts and with replication in OA. There are two associations from reports of Asian descents, and twelve associations from European ancestry in OA (Panoutsopoulou and Zeggini 2013). The robustly identified 15 OA associated variants with GWAS was found in European and Asian ethnicity. For example, GDF5 (growth differentiation factor 5 or bone morphogenetic protein 14, a member of transforming growth factor family) was a strong candidate gene in both knee and hip OA (Valdes *et al.* 2011, Panoutsopoulou and Zeggini 2013). Double von Willebrand factor A domains (DVWA) is correlated with knee OA in Japanese combined with Chinese cohort (Gonzalez 2013). Moreover, human leucocyte antigen (HLA) class II/ III was another strong candidate variant associated with knee OA in Japanese and Caucasian descents (Nakajima *et al.* 2010). GWAS indicated that the large linkage disequilibrium (LD) block consisted of six genes harbored on Chromosome 7q22 locus was a susceptibility locus for knee OA in Caucasian population (Evangelou *et al.* 2011). MCF2L established with GWAS was associated with knee and hip OA in Europeans. It was implicated in pain and nerve growth factor. GWAS data found that OA is a polygenic disease. There was no information in the association between the genes related with angiogenesis and OA in genome-wide association study.

VEGF is a polymorphic gene with several genotypes. According to *VEGF* single nucleotide polymorphism studies, the common *VEGF* SNPs have been described as located in promoter, 5'untranslated region (UTR), and 3'UTR. These polymorphisms have been associated with differential expression of VEGF protein. Nonetheless, there is a little information on VEGF polymorphism in the risk of osteoarthritis. There are controversial evidences in functional *VEGF* polymorphisms.

To the best our knowledge, this is the first study to demonstrate the association between *VEGF* gene polymorphisms at the promoter, 5'UTR, and 3'UTR and the susceptibility of OA. Almost all polymorphisms satisfied Hardy-Weinberg

equilibrium in case and control set tested separately except -634G/C polymorphism in only control group. However, the same participant population was performed in all SNPs. Therefore, the results from -634G/C variation retained for further analysis.

The genotypic and allelic frequencies of all four VEGF SNPs (-2578C/A: rs699947, -1154G/A: rs1570360, -634G/C: rs2010963, and +936C/T: rs3025039) were not significantly different between OA patients and healthy controls. These data are in line with previous studies. Sanchez-Enriquez *et al.* (Sanchez-Enriquez *et al.* 2008) had examined the correlation between polymorphism of *VEGF* gene in knee OA. They demonstrated that the -460T/C and +405G/C *VEGF* polymorphisms were not significant difference between OA patients and healthy controls. In agreement with this study, Seo JS *et al*. showed that there were no significant differences in genotypic distribution and alleleic frequencies in *VEGF* polymorphisms of -2578C/A, -1154G/A, and -634G/C between ankylosing spondylitis (AS) patients and controls (Seo *et al.* 2005). Additionally, the genetic polymorphism of *VEGF* in osteonecrosis of the femoral head was studied in Korean population. The results showed that neither -2578C/A nor +936C/T was associated with risk of osteonecrosis. However, -634G/C genotype was significantly associated with the susceptibilityof the osteonecrosis in Korean population (Kim *et al.* 2008). Analysis of VEGF polymorphisms in rheumatoid arthritis (RA) in Spain cohort showed that -1154G/A and -634G/C variants were not associated with risk of RA (Rueda *et al.* 2005).

Our results are inconsistent with the reports from Han *et al.* demonstrating that the +936T allele in rheumatoid arthritis was significantly increased in RA patients (Han *et al.* 2004). Whereas T allele of +936C/T *VEGF* polymorphism acted as protective allele in the progression of psoriatic arthritis (Butt *et al.* 2007). The discrepancies between previous studies and our findings may be explained by the difference in genetic backgrounds of the populations, or methodology. The significant difference of genotype distribution and allelic frequency differed between diseases such as OA, RA, and psoriatic arthritis, between ethnicity (Caucasian, Mongolian) or between techniques such as a sequenom chip-based matrix-assisted laser desorption/ionization- time of flight (MALDI-TOF), amplification refractory mutation system (ARMS) PCR, and PCR-RFLP.

The effects associated with a single SNP are aimed to validation of their associations; however, association of more than one SNP within a haplotype is more informative association study. Therefore, haplotype analysis was constructed. Our linkage disequilibrium and haplotype analysis data indicated that -2578C/A, -1154G/A, and -634G/C were in LD whereas +936C/T was not included in this LD. Because +936C/T located in 3' UTR was far from the others. Only 3 SNPs (-2578C/A, -1154G/A, and -634G/C) were conducted the haplotype distribution. The C-A-C haplotype (-2578C/A / -1154G/A / -634G/C) was significantly 7-folded higher risk in OA patients when compared with controls. Thus, the C-A-C haplotype might confer risk for knee OA. Previous study was conducted haplotype analysis of these three SNPs (-2578C/A, -1154G/A, and -634G/C) in oral squamous cell carcinoma patients. They revealed C-G-G haplotype was decreased the risk of oral cancer whereas C-A-G haplotype was associated with the increased risk of this disease (Supic *et al.* 2012). The 3 VEGF SNPs (-2578C/A / -1154G/A / -634G/C) haplotype study in ankylosing spondylitis were shown that the patients with A-G-G haplotype had significantly higher in the frequency of cervical spine involvement (Seo *et al.* 2005). Therefore, various diseases might be associated with the different haplotype patterns. In OA, C-A-C haplotype was associated with the increased risk of disease.

According to influence of gender on OA, the recent data showed the polymorphisms in female and male gender from both case and control groups were not significant difference in genotypic and allelic frequencies. Moreover, stratification assessment with the severity of OA was evaluated. Interestingly, the VEGF polymorphism at -2578C/A was found to be significantly different between early and advanced stage OA patients. The AA genotype of -2578C/A polymorphism was remarkably significant expressed in the early stage OA patients when compared with advanced stage OA patients. The A allele of the -2578C/A was associated with the decreased VEGF level. Chen Y and colleagues revealed the -2578AA genotype was associated with the decrease activity of RA in Caucasian cohort. In addition, they found the patients with the AA genotype of -2578 *VEGF* SNPs had a lower concentration of serum VEGF (Chen *et al.* 2012). These data supported our results that the early stage of OA patients (grade 2 and 3) had a lower VEGF concentration

(the report from **part I**). This might explain that AA genotype of -2578 SNP in early stage could play a protective role in the progression of OA.

We had investigated the association between the polymorphisms and VEGF concentration in SF and plasma. The data showed that there were no significant difference between genotypic distribution in each SNP and the levels of VEGF in both SF and plasma of OA and plasma controls. Surprisingly, -634CC genotype in OA patients had an increased VEGF plasma levels compared with controls. However, no association in the concentration of VEGF plasma between case and controls was observed in the -634GG and -634GC genotypes. This finding is in line with the study from Awata *et al*. in type 2 diabetes. They found the serum VEGF levels were higher in subjects with -634CC genotype (Awata *et al.* 2002). In addition, -634CC genotype demonstrated the high VEGF expression in peripheral blood mononuclear cells (PBMCs) (Young *et al.* 2006).

In contrast to Young study, Watson *et al.* (Watson *et al.* 2000) found that -634GG genotype had a higher VEGF production in peripheral blood mononuclear cell stimulated with lipopolysaccharide (LPS) and lower in the CC genotype. In breast cancer, the GG genotype of -634G/C SNPs was association with the high level of VEGF (James *et al.* 2014). The discrepancy between these results may be differences in genetic background, racial, disease, and methodology.

Some limitations are recognized in this study. The study had been performed with small cohort. Therefore, it could not represent the population in the whole country. Moreover, because of the small sample size, there was lack of accuracy in genotype distribution and allelic frequency stratified into subgroups according to gender and OA severity. A larger sample size is required to verify the present results. This study is unable to match age and gender between knee OA patients and controls. Since OA is genetic heterogeneity, additional studies might be needed in the combination of loci to predict the risk of OA. Because of the high polymorphism of *VEGF* gene, only four SNPs might not enough to verify the association of *VEGF* gene on OA susceptibility.

In conclusion, we found no association between the genotypic distributions and allelic frequencies of -2578C/A, -1154G/A, -634G/C, and +936C/T *VEGF* polymorphisms and the risk of osteoarthritis. The -2578C/A, -1154G/A, and -634G/C were in linkage disequilibrium and the C-A-C haplotype of -2578C/A, -1154G/A, and -634G/C SNPs might be the risk for OA. Furthermore, the -2578AA genotype was approximately six folds significantly increased in the early stage OA patients when compared with the advanced stage OA patients. Therefore, the AA genotype of -2578C/A *VEGF* polymorphisms in early OA may reduce the risk of OA severity in Thai population. Moreover, there was association between the elevated VEGF production in the carrier CC genotype of -634G/C SNPs.

Part III: The expression of VEGF mRNA, miR-210, miR-223 in knee osteoarthritis synovial membrane and their expression *in vitro*

1. The expression of VEGF in OA synovial membrane and FLS cell culture

The data from **part I** indicated that VEGF was elevated in OA SF compared with paired plasma. The positive VEGF staining in synovial tissue and articular cartilage were observed. To confirm the elevated VEGF expression in OA patients, VEGF mRNA level was determined in the synovial tissues. Previous study demonstrated that higher VEGF expression were found in OA cartilage than in healthy cartilage (Pfander *et al.* 2001). Not only VEGF but also its receptors were reported in the OA articular cartilage (Enomoto *et al.* 2003). There was limited information reported the expression of VEGF in synovial tissue. The results of this study showed that VEGF were expressed in the synovial tissue of knee OA subjects. Unfortunately, we could not find the significant difference between the VEGF mRNA expressions in synovial tissue obtained from the knee OA patients compared with controls (synovium from the patients with anterior cruciate ligament (ACL) injury). The discrepancy between the VEGF protein and mRNA levels may be explained by the Bigoni study (Bigoni *et al.* 2013). They measured the levels of proinflammatory cytokines in synovial fluid from ACL patients. The results found that there were high levels of IL-1β, IL-6, and IL-8 proteins in ACL groups. Proinflammatory cytokines such as IL-1β and IL-6 can activate angiogenesis by stimulating VEGF production and maintaining a chronic inflammatory reaction in the ACL. The chronic inflammation in ACL leads to an increased risk of post traumatic OA. The synovium obtained from ACL patients may not be a perfect control tissue sample. However, there was an ethical limitation to obtain the synovial tissue from healthy individual. Another limitation was lack of VEGF protein determination in synovial tissues. As a result, no direct examination between the expression of VEGF mRNA and VEGF protein was measured in the synovial tissues.

We attempted to verify the association between the expression of VEGF and pathogenesis of OA using *in vitro* model. In synovitis of OA, the oxygen consumption was increased leading to insufficient perfusion (hypoxia) and new blood vessel

formation (angiogenesis). Furthermore, articular cartilage is known as avascular tissue contributing to low oxygen level. Inflammation in OA results in the decreased oxygen tension in the synovial fluid and may lead to lower oxygen supply to the articular cartilage. In the current study, the fibroblast-like synoviocytes (FLS) isolated from OA synovial tissue were incubated under hypoxic condition with or without IL-1ß addition to mimic osteoarthritic –like condition. There was no document on hypoxia and IL-1ß induced VEGF expression in OA synoviocytes. The present study is the first to demonstrate that the increased VEGF expression was observed in FLS under hypoxic condition with or without IL-1ß treatment. The expressions of VEGF were markedly induced in the hypoxia with IL-1ß at 24 and 48 hours compared with normoxia and hypoxia alone. These results are inconsistent with previous reports showing that hypoxia and IL-1ß enhanced production of VEGF at 24 hours after stimulation (Murata *et al.* 2006). Murata *et al*. investigated the VEGF expression in OA chondrocyte under hypoxic (95% N_2 and 5% $CO_{2)}$ condition in the presence or absence of IL-1ß (10 ng/ml) treatment. They found that hypoxia exposure induced the expression of VEGF in chondrocytes. Hypoxia and IL-1ß stimulated the expression of VEGF via distinct pathways. Hypoxia induced VEGF secretion through p38MAPK whereas IL-1ß induced VEGF expression by JNK pathway (Murata *et al.* 2006). The chondrocytes and synoviocytes from RA, OA, and post-traumatic OA patients expressed VEGF and matrix metalloproteinase (MMP)-3 by interleukin-1ß induction (Inoue *et al.* 2005). The expression of VEGF by FLS from RA patients showed VEGF mRNA expression was significantly up-regulated by IL-1ß addition under hypoxic condition (Miyake *et al.* 2009). On the other hand, El Awad *et al.* (El Awad *et al.* 2000) reported that VEGF mRNA expression in proximal tubular epithelial cells were not significant difference between normoxia and hypoxia after 24 hours. The reason for the discrepancy is unclear. A possible explanation may be attributed to the different cell-type (chondrocytes, FLS, and proximal tubular epithelial cells) and mRNA examination technique. Limitation of VEGF mRNA expression *in vitro* model was small amount of individual replication (n=3). From these results, we suggested that VEGF expression was stimulated by IL-1ß addition under hypoxic condition.

Hypoxia may acts alone or in conjuction with various proinflammatory cytokines resulting in the pathogenesis of OA.

2. The expression of miR-210 and miR-223 in synovial membrane and FLS cell culture

miRNAs are a small non-coding RNA regulating gene expression by posttanscriptional gene repression. The miRNAs control gene expression by binding the 3'UTR of target genes (Bernardo *et al.* 2012). Several miRNAs play roles in tissue-specific or developmental stage specific expression associated with various diseases including osteonecrosis (Yamasaki *et al.* 2012), RA (Duroux-Richard *et al.* 2011), and OA (Okuhara *et al.* 2012, Le *et al.* 2013). miRNAs can disrupt the expression network of the target genes in homeostasis, cell differentiation, cell death, cell proliferation, and angiogenesis pathways (Miyaki and Asahara 2012). A number of miRNAs involved in the pathogenesis of osteoarthritis have been documented. Yamasaki *et al*. determined the expression of miR-146a in osteoarthritic cartilage and compared with articular cartilage grading. The results suggested that miR-146a was decreased according to Mankin cartilage grading and miR-146a was stimulated with IL-1β (Yamasaki *et al.* 2009). Another report demonstrated that the relative expression levels of miR-146a, miR-155, and miR-181a were significantly higher in PBMCs of OA patients than those in PBMCs of healthy controls (Okuhara *et al.* 2012). miR-146a level decreased when the K/L grading increased. The expression of miR-155 was increased when the K/L classification increased. However, no significant difference was found between the levels of miR-181a in the various K/L grading (Okuhara *et al.* 2012). Previous study revealed that the expressions of miR-16, miR-132, miR-146a, miR-155 in plasma were markedly higher than those in synovial fluid (Murata *et al.* 2010). The synovial fluid miRNA expression patterns were similar to those patterns in synovial tissues (Murata *et al.* 2010). These data represent tissue-specific difference in osteoarthritis. Therefore, we investigated the role of expressions of miR-210 and miR-223 in the development of OA.

Previous studies characterized the expression of miR-210 and miR-223 in knee osteoarthritis. Iliopoulos and colleagues determined miRNA profiling in osteoarthritic

cartilage compared with normal cartilage. They found that nine miRNAs were up-regulated including miR-483, miR-22, miR-377, miR-103, miR-16, miR-223, miR-30b, miR-23b, and miR-509 whereas seven miRNAs were down-regulated such as miR-29a, miR140, miR-25, miR-337, miR-210, miR-26a, and miR-373 (Iliopoulos *et al.* 2008). However, the current study is the first to focus on the patterns of miR-210 and miR-223 expression in both synovium and synoviocyte cell culture of knee osteoarthritis patients.

miR-210 plays various biological roles such as apoptosis (Iliopoulos *et al.* 2008), osteoblastogenesis (Mizuno *et al.* 2009), and angiogenesis (Sen *et al.* 2009). miR-210 is a potential player in cell response to hypoxia (Shoji *et al.* 2012). Both VEGF expression and hypoxic condition are associated with the pathogenesis of OA. Therefore, the miR-210 expression was examined in the synovium of knee OA patients and *in vitro* under osteoarthritic-like stimulation. Our data found that the level of miR-210 was increased in OA synovium compared with controls. Moreover, the miR-210 expression was significantly higher in the cell culture under hypoxia combined with IL-1β than that under normoxia condition. The pattern of miR-210 expression *in vitro* was similar to the expression pattern of VEGF. Previous study in renal angiogenesis under ischemia/perfusion (I/P) injury showed that miR-210 was up-regulated in the kidney I/P injury. Overexpression of miR-210 stimulated VEGF and VEGFR-2 expressions. They suggested that miR-210 might be associated with the VEGF production related to angiogenesis in renal I/P injury (Liu *et al.* 2012). Yamasaki *et al.* (Yamasaki *et al.* 2012) identified the expression pattern of miR-210 in bone specimens from osteonecrosis patients. They found that expression levels of miR-210 and VEGF were remarkably higher in osteonecrosis group compared to OA and control group. They also indicated that miR-210 expression was correlated with hypoxia and angiogenesis. In addition, bone biopsy from osteosarcoma patients had a higher miR-210 expression when compared with corresponding noncancerous bone (Cai *et al.* 2013). This report indicated an association of miR-210 and VEGF expression responded to hypoxia. The association between hypoxia, angiogenesis, and miR-210 expression in pathogenesis of OA may be supported by Yamasaki's study. During progressive OA, hypoxia in the joint stimulated the secretion of angiogenic factors

including VEGF to adjacent blood vessels. Inflammatory cells were recruited into the hypoxic area and stimulated cell proliferation. These cells produced more inflammatory cytokines including matrix metalloproteinases to degrade cartilage. Hypoxia directly binds to a hypoxia responsive element on the miR-210 promoter and *VEGF* gene promoter, which in turn increases the transcription of miR-210 and VEGF leading to angiogenesis and development of OA (Roy *et al.* 2006, Yamasaki *et al.* 2012). miR-210 and VEGF levels were co-expressed and were associated with the development of OA.

The expressions of miR-223 have been documented in SF, plasma, PBMCs, synovial tissue, and FLS (Murata *et al.* 2010, Chatzikyriakidou *et al.* 2012, Okuhara *et al.* 2012). The expression of miR-223 was increased in PBMCs of OA patients compared with healthy controls. Furthermore, the expressions of miR-223 were decreased when K/L grading increased (Okuhara *et al.* 2012). Although the miR-223 expression was revealed in synovial tissue from OA, there is a little information in miR-223 expression from OA synovial tissue compared with nonarthritic controls. There is no information of the expression of miR-223 in FLS cultured under osteoarthritic-like condition. Our results showed that the expression of miR-223 was significantly increased in synovial tissue obtained from OA compared with controls. This result is in line with the Shibuya study. They showed the expression pattern of miR-223 in RA synovial tissue compared with OA patients and non-inflammatory ACL controls. They found that the miR-223 level was significantly higher in RA synovium compared with that in OA. However, the expression of miR-223 in OA was four fold higher than controls (Shibuya *et al.* 2013). Herein, the recent data *in vitro* study revealed that the relative expression of miR-223 in FLS treated with hypoxia and with or without IL-1ß were not significant difference when compared to normoxia. This is the first report studied in the expression of miR-223 in FLS in the presence or absence of IL-1ß under hypoxia. The miR-223 plays a key role in osteoclast differentiation and osteoclastogenesis (Sugatani and Hruska 2007, Shibuya *et al.* 2013). The miR-223 expression may not respond to hypoxia or inflammatory stimulation. Therefore, the expression of miR-223 might stimulate osteoclastogenesis leading to cartilage degradation and the development of OA. The limitation in miRNA

expression in synovial tissue and FLS *in vitro* study is small sample size leading to lack of association between miR-210 and miR-223 expression and expression of VEGF. Further study on the target genes and function of miRNAs would be required in order to better understand the relationship of miRNAs in the pathogenesis of OA.

In conclusion, the expression of VEGF was elevated in SF of knee OA patients. There were positively correlation between VEGF levels in synovial fluid and plasma and the severity of OA. VEGF were also expressed in OA synovial tissue and articular cartilage. We confirmed that the increased VEGF level was correspond with the elevated VEGF mRNA expression. The expression of VEGF mRNA was determined in synovial tissue and FLS *in vitro* cell culture.

Unfortunately, there was no significant difference between VEGF expression in OA synovial tissue compared with that in controls. However, the VEGF expressions in FLS with or without IL-1β treatment under hypoxia were significantly increased when compared to normoxia. Therefore, the hypoxia and inflammatory stimuli are associated with VEGF expression in the pathogenesis of OA. The elevated VEGF levels in OA may be due to genetic alteration in VEGF gene. Therefore, the VEGF SNPs in the regulatory position including promoter (-2578C/A, -1154G/A), 5'UTR (-634G/C), and 3'UTR (+936C/T) were investigated in OA patients compared with healthy controls.

Although, no association with genotype distribution and allele frequency between OA participants and controls were observed, the AA genotype of -2578C/A in early stage of OA may be protective effect against progressive OA. Moreover, the -634CC genotype was correlated with higher VEGF production compared with controls. Recently, there is evidence that miRNAs can also result in the pathogenesis of disease. Thus, the miR-210 and miR-223 levels were examined in the synovial tissues and FLS cell culture. The results found that both miR-210 and miR-223 were induced in OA synovial tissue compared with controls. The hypoxia and IL-1β treated FLS increased the miR-210 expression whereas miR-223 expression was not different under those conditions. The miR-210 and VEGF expression had a similar pattern and both expressions may be associated with the pathogenesis of OA. Taken together, we

indicated that VEGF in synovial fluid may use as a biochemical marker associated with the OA severity. The VEGF expression may play a possible role in the development of knee OA.

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APPENDIX

Appendix

Buffer preparation

1. DNA extraction
    a. Red blood cell lysis buffer
        - Components

10 mM $KHCO_3$	0.5 g
155 mM NH_4Cl	4.15 g
0.1 mM EDTA	0.019 g

        - Add water 500 ml
        - Filter by 0.2 micron filter

2. ELISA experiment
    a. Wash buffer
        - Components

Na_2HPO_4	1.44 g
KH_2PO_4	0.204 g
NaCl	8 g
KCl	0.2 g

        - Add water 1,000 ml
        - Adjust to pH 7.4
        - Autoclave at 121°C for 15 minutes

3. PCR-RFLP experiment
    a. TE buffer (for diluted primer)
        - Components

1.0 M Tris-HCl, pH 8.0	1,000 µl
0.5 M EDTA (disodium salt), pH 8.0	200 µl

        - Make up to volume with water. Total volume: 1,000 ml
        - Autoclave at 121°C for 15 minutes

b. 10X TAE buffer (for gel electrophoresis)

- Components

Tris-base	48.4 g
0.5 M EDTA, pH 8.0	20 ml
Glacial acetic acid	11.42 ml

- Make up volume with water. Total volume: 1,000 ml
- Adjust to pH 7.6-7.8

c. 10X TBE buffer (for polyacrylamide gel electrophoresis)

- Components

Tris-base	121.1 g
$Na_2EDTA \cdot 2H_2O$	3.7 g
Boric acid, anhydrous	55.6 g

- Add water 1,000 ml
- Adjust to pH 8.3

d. 30% acrylamide

- Mix 75 ml of acrylamide IEF (40%), with 8 g of methylene bis-acrylamide dissolved in 25 ml of water

e. 10% ammonium persulfate (10% APS)

- Component

Ammonium persulfate	0.1 g

- Add water 1 ml

Cell culture experiment

1. Calculate the number of total cells

   After trypsinization, the cells were counted with trypan blue solution staining. The cell pellet was suspended in 1 ml media. Then 20 µl of cell suspension was mixed with 20 µl trypan blue solution to make a dilution factor of 2. The stained cells were added to a counting Chamber /Haemacytometer and the viable cells, which were unstained, counted under a microscope. The number of cells was calculated using the following equation:

   The number of cells = average cells x 10^4 x dilution factor

2. Calculate the amount of seeded cells

   From the total cell number, the volume of seeded cell solution was calculated using the relationship below:

$$\frac{n_{\text{total cell}}}{V_{\text{total cell}}} = \frac{n_{\text{seeded cell}}}{V_{\text{seeded cell}}}$$

VITA

Miss Natthphon Saetan was born in Songkhla province, Thailand on the 8th December 1985. She is a daughter of Mr. Suekiang Saetan, Mrs. Muaimai Saelao with two younger sisters. She graduated her Bachelor's Degree of Science (Medical Technology) with the 2nd class honors from Chulalongkorn University in 2007. Some parts of her thesis were accepted for poster presentation at the 2012 Annual Meeting of the Orthopaedic Reseacrh Society (ORS) at San Francisco, California, United State of America

Additionally, Miss Natthaphon presented her work on the associations between vascular endothelial growth factor and knee osteoarthritis at a number of academic conferences including the Commission of Higher Education Congress II – V in Pattaya, Chonburi, and Joint Conference in Medical Sciences 2009 (JCMS 2009) in Bangkok, Thailand. She later published the results in the International Orthopaedics

Moreover, she was appointed as oral presenter for the 35th Annual Meeting of the Royal College of Orthopaedic Surgeons of Thailand (RCOST).

Publications

Saetan N, Honsawek S, Tanavalee A, Yuktanandana P, Meknavin S, Ngarmukos S, Tanpowpong T, Parkpian V. Relationship of plasma and synovial fluid vascular endothelial growth factor with radiographic severity in primary knee osteoarthritis. Int Orthop. 2013. DOI 10.1007/s00264-013-2192-y.