CARTILAGE
2022, Vol. 13(4) 66–76
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DOI: 10.1177/19476035221132260
journals.sagepub.com/home/CAR
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Association between Common Variants in *VEGFA* Gene and the Susceptibility of Primary Knee Osteoarthritis

Natthaphon Saetan[1], Sittisak Honsawek[2,3], Aree Tanavalee[3], Srihatach Ngarmukos[3], Pongsak Yuktanandana[3], and Yong Poovorawan[4]

Abstract

Objectives. This study aimed to analyze the relationship between vascular endothelial growth factor A (*VEGFA*) gene polymorphisms, plasma VEGFA, and the susceptibility of knee osteoarthritis (OA). *Design.* A total of 404 subjects, 202 knee OA subjects and 202 healthy volunteers, were enrolled into the study. Four distinct polymorphisms of the *VEGFA* gene were evaluated using polymerase chain reaction-restriction fragment length polymorphism: −2578C/A (rs699947), −1154G/A (rs1570360), −634C/G (rs2010963), and +936C/T (rs3025039). Plasma VEGFA levels were analyzed using enzyme-linked immunosorbent assay. *Results.* The most common nucleotides in both knee OA subjects and healthy controls were CC for −2578C/A, GG for −1154G/A, CG for −634C/G, and CC for +936C/T in the *VEGFA* gene. Genotype distribution and allele frequencies of *VEGFA* −2578C/A, −1154G/A, −634C/G, and +936C/T single nucleotide polymorphisms did not differ between OA patients and the controls. Plasma VEGFA levels showed no difference between OA patients and the controls. In contrast, plasma VEGFA levels of −634C/C genotype were significantly higher in OA patients than in the controls ($P = 0.035$). According to the −2578A/A genotype, patients with early stage OA had a higher odds ratio than those with advanced stage OA ($P = 0.023$). *Conclusions. VEGFA* −2578C/A (rs699947), −1154G/A (rs1570360), −634C/G (rs2010963), and +936C/T (rs3025039) polymorphisms may not be responsible for OA susceptibility in the Thai population. However, the OA patients with A/A genotype at the −2578C/A seemed to have a lower potential risk of developing severe OA than those with the C/A and C/C genotypes. These findings would help elucidate and facilitate a better understanding of the genetic fundamentals of OA.

Keywords

osteoarthritis, polymorphisms, plasma, vascular endothelial growth factor-A, *VEGFA*

Introduction

Osteoarthritis (OA) is a highly prevalent age-related joint disorder affecting the elderly population. The most clinically prominent sites of primary OA involve the apophyseal joints of the cervical and lumbar spine, interphalangeal joints of the hand, the thumb base, the first metatarsophalangeal joint, the hips, and the knees. It is characterized by articular cartilage deterioration, narrowing of the joint space, subchondral bone sclerosis, osteophyte formation, and synovial inflammation.[1] The clinical features of OA are pain, joint swelling, short-lasting stiffness, deformity, and restricted joint mobility leading to limitation in the activities and poorer quality of life.[2] The definite etiopathogenesis of OA still remains under intense exploration; however, a vast array of plausible factors including environment, biomechanics, biochemical processes, and/or genetics have been highlighted to exert integral roles in OA development.[3]

Angiogenesis, the generation of new blood vessels, plays a consequential part in the pathogenesis of a wide variety of human disorders. Among the known angiogenic factors, vascular endothelial growth factor A (VEGFA), which can also be referred to as VEGF, is a heparin-binding growth factor and has emerged as a pivotal modulator of the angiogenic process in physiological and pathological changes.[4] VEGFA is a tyrosine kinase glycoprotein that exerts a fundamental role as a prime mediator of migration, proliferation, tubal formation of endothelial cells, and angiogenesis.[5] Furthermore, it is involved in bone development, osteoblasts, and osteoclasts, which are associated with endochondral bone formation by coupling angiogenesis with hypertrophic cartilage remodeling and ossification.[4] Previous studies have documented that

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VEGF is expressed in human OA articular cartilage,[6,7] and the roles of VEGF in the pathogenesis of OA have been reported in animal models.[8,9]

Although the elevation of VEGF expression in OA has been previously documented,[10-12] and the association is convincing, yet the distinct aspect of *VEGFA* remains equivocal. As a result of this, we studied the relationship between *VEGFA* polymorphisms and the susceptibility of developing knee OA. VEGFA is a major angiogenic factor and a prime regulator of endothelial cell proliferation.[13] *VEGF* generates several different isoforms that occur when there is an alternative mRNA splicing from the single *VEGFA* gene which is situated on the short arm of chromosome 6 and its gene contains a 14-kb coding region with 8 exons interrupted by 7 introns.[14] The *VEGFA* gene is highly polymorphic with multiple typical functional single nucleotide polymorphisms (*SNPs*) in the promoter, 5′ untranslated region and 3′ untranslated region that could modulate *VEGFA* expression: −2578C/A (rs699947), −1154G/A (rs1570360), −634C/G (rs2010963), and +936C/T (rs3025039).[15-17]

Three of these polymorphisms reside within the promoter region at −2578, −1154, and −634 relative to the translation start site. The −2578A, −1154A, and −634G alleles are all involved with down-regulation of *VEGFA* expression.[15,16] Besides the promoter region polymorphisms, the T allele of the common +936C/T polymorphism in the 3′-untranslated region (3′-UTR) is also related with significant decline of circulating VEGFA levels.[17] In recent years, evidences have suggested that *VEGFA* SNPs are associated with the development and prognosis of a variety of rheumatological abnormalities.[18-20] However, little information exists regarding the association of *VEGFA* polymorphisms with genetic susceptibility to developing knee OA in the Thai population.

In this case-control study, our primary hypothesis focused on potential differences in the genotype distribution and allele frequency of *VEGFA* polymorphisms between OA patients and the controls. We further hypothesized that the *VEGFA* polymorphisms would contribute to the susceptibility of knee OA. Therefore, the purpose of this study was to explore the frequencies of the *VEGFA* −2578C/A, −1154G/A, −634C/G, and +936C/T polymorphisms in Thai subjects with knee OA. This study also examined whether the risk of developing knee OA was associated with these 4 polymorphisms.

Materials and Methods

Study Population

This case–control study was approved by the Institutional Review Board on Human Research of our institute. The current protocol was carried out in accordance with the tenets of the Declaration of Helsinki. Written informed consent was obtained from all subjects prior to participating in the study.

All patients and controls were recruited from the orthopaedic outpatient clinics, King Chulalongkorn Memorial Hospital. Our earlier studies have documented the genetic studies of interleukin-6, ADAM12, ADAMTS14, adiponectin, and matrix metalloproteinase-3 in knee OA patients.[1,21-24] As per prior studies, we have increased our sample sizes to a total of 404 unrelated individuals: 202 knee OA patients and 202 healthy controls. The participants from both groups were matched by age, sex, and ethnic origin. There were 169 females and 33 males with knee OA (average age = 68.8 ± 7.8 years, range = 49-85 years). The diagnosis of knee OA was based on the criteria set forth by the American College of Rheumatology (ACR), which included primary OA with any clinical symptoms and radiographic features of OA corresponding to the Kellgren-Lawrence (KL) grading system.[25] Clinical knee OA was defined as persistence of knee pain due to daily activities, morning stiffness <30 minutes, crepitus, bony tenderness, and no palpable warmth. We excluded individuals who suffered from additional chronic inflammatory disorders, immunological disturbances, prior joint infection, precedent knee injury, or surgical operation. The KL classification system was assigned to define the severity of the knee OA into KL grade 1, 2, 3, or 4 based on the radiographic examination.[25] According to the KL classification, 55 patients had KL grade 2, whereas 71 patients had KL grade 3, and 76 patients had KL grade 4 OA. Subsequently, the knee OA patients were categorized into 2 groups: early OA (KL grade 2, $n = 126$) and advanced OA (KL grades 3 and 4, $n = 76$).

[1]Biomedical Science Program, Faculty of Graduate School, Chulalongkorn University, Bangkok, Thailand
[2]Department of Biochemistry, Center of Excellence in Osteoarthritis and Musculoskeleton, Faculty of Medicine and King Chulalongkorn Memorial Hospital, Thai Red Cross Society, Chulalongkorn University, Bangkok, Thailand
[3]Department of Orthopaedics, Vinai Parkpian Orthopaedic Research Center, Faculty of Medicine, Chulalongkorn University and King Chulalongkorn Memorial Hospital, Thai Red Cross Society, Bangkok, Thailand
[4]Center of Excellence in Clinical Virology, Department of Pediatrics, Faculty of Medicine, Chulalongkorn University and King Chulalongkorn Memorial Hospital, Thai Red Cross Society, Bangkok, Thailand

Corresponding Author:
Sittisak Honsawek, Department of Biochemistry, Center of Excellence in Osteoarthritis and Musculoskeleton, Faculty of Medicine and King Chulalongkorn Memorial Hospital, Thai Red Cross Society, Chulalongkorn University, 1873 Rama IV Road, Patumwan, Bangkok 10330, Thailand.
Email: sittisak.h@chula.ac.th

Table 1. Sequences of the PCR Primers, Restriction Enzymes, and Fragment Sizes after Digestion.

SNPs	PCR primers	Product size (bp)	Restriction endonucleases	Fragment size (bp)
−2578C/A (rs699947)	F: 5'-GGCCTTAGGACACCATACC-3' R: 5'-CACAGCTTCTCCCCTATCC-3'	455	BstY I	CC: 455 AA: 207, 248
−1154G/A (rs1570360)	F: 5'-TCCTGCTCCCTCCTCGCCAATG-3' R: 5'-GGCGGGGACAGGCGAGCCTC-3'	207	Mnl I	GG: 3, 19, 34, 150 AA: 3, 19, 184
−634C/G (rs2010963)	F: 5'-CGACGGCTTGGGGAGATTGC-3' R: 5'-GGGCGGTGTCTGTCTGTCTG-3'	274	BsmF I	CC: 274 GG: 118, 156
+936C/T (rs3025039)	F: 5'-AGGGTTTCGGGAACCAGATC-3' R: 5'-CTCGGTGATTTAGCAGCAAG-3'	266	Nla III	CC: 266 TT: 55, 211

Moreover, 202 healthy controls who visited the hospital for their annual health checkup during the same period and were the patients' friend or relatives were enrolled into the study. These healthy controls did not have clinical or radiographic evidence of knee OA (average age 66.2 ± 7.3 years, range = 45-80 years, 160 females and 42 males). The healthy controls reported that they currently did not have any knee pain or history of knee pain.

DNA Extraction and SNP Genotyping

Three milliliters of ethylenediaminetetraacetic acid-peripheral venous blood samples were collected from all participants by the standard venipuncture procedure. The genomic DNA was extracted by Illustra Blood GenomicPrep Mini Spin Kit (GE Healthcare, Buckinghamshire, UK) and was stored at −20 °C until measured. The *VEGFA* gene polymorphisms were determined using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) technique. Polymerase chain reaction was conducted for −2578C/A, −1154G/A, −634C/G, and +936C/T SNPs using the specific primer sets as shown in **Table 1**.[26,27]

The PCR conditions for all SNPs were performed using the following conditions: initial denaturation at 95°C for 5 minutes, followed by 35 cycles of 1 minutes of denaturation at 95°C, annealing at 62°C, 67°C, 71°C, and 62°C for SNPs −2578C/A, −1154G/A, −634C/G, and +936C/T, respectively, for 45 seconds and extension at 72°C for 1 minutes, and a cycle of final extension at 72°C for 5 minutes. Afterward, the PCR products were digested with restriction enzymes according to the manufacturer's recommendation: BstY I for −2578C/A, Mnl I for −1154G/A, BsmF I for −634C/G, and Nla III for +936C/T. Finally, the patterns of DNA fragments of −2578C/A, −634C/G, and +936C/T were detected by 2% agarose gel electrophoresis. The variation of −1154G/A polymorphism was evaluated using 12% polyacrylamide gel electrophoresis. The DNA fragments were visualized by ethidium bromide fluorescent nucleic acid dye staining. The electrophoresis *VEGFA* genotype restriction patterns are shown in **Table 1**.

Assessment of Plasma VEGFA by ELISA

After a 12-hour overnight fasting period, peripheral venous blood specimens were collected from every subject 1 day before surgery. The blood was centrifuged to remove cells and debris, and immediately stored at −20 °C for subsequent measurement. Double-blind quantitative determination of plasma VEGFA level was performed using a commercially available enzyme-linked immunosorbent assay (ELISA; Quantikine R&D Systems, Minneapolis, MN, USA). According to the manufacturer's protocol, 100 µl of recombinant human VEGFA standards and plasma samples were pipetted into 96-well microtitre plates precoated with mouse monoclonal antibody against human VEGFA and incubated for 2 hours at room temperature. The wells were then washed 3 times with washing buffer and incubated for 2 hours at room temperature with a horseradish peroxidase-conjugated monoclonal antibody specific for VEGFA. After 3 washes, substrate solution was added to each well, and the plate was incubated for 25 minutes at room temperature in the dark. Finally, the reaction was stopped with the stop solution, and the optical density was measured at 450 nm using an automated microplate reader. A standard optical density-concentration curve (range = 31.2–2,000 pg/ml) was drawn to determine the VEGFA level. The primary antibody used was a human VEGF 165 antibody that recognizes $VEGF_{165}$, $VEGF_{121}$, and $VEGF_{165b}$ and does not cross-react with placenta growth factor (PLGF), VEGFB, C, or D, or the VEGF receptor ligands VEGF-R1 or R2 below concentrations of 50 ng/ml. The manufacturer-reported precision was 4.5% to 6.7% (intra-assay) and 6.2% to 8.8% (inter-assay). The assay sensitivity of VEGFA was 3.25 pg/ml.

Statistical Analysis

Statistical analyses were conducted using Statistical Package for Social Sciences (SPSS) software version 22.0 (SPSS Inc., Chicago, IL, USA). Genotype frequencies and deviations of genotype distributions from the Hardy-Weinberg equilibrium (HWE) for each polymorphism were

Table 2. Genotype and Allele Frequency Distributions of the *VEGFA* Polymorphisms in Knee OA Patients and the Healthy Controls.

SNPs	Genotype or allele frequency	OA patients ($n = 202$) n (%)	Controls ($n = 202$) n (%)	P value
−2578	CC	96 (48)	110 (55)	
	CA	86 (42)	77 (38)	0.284
	AA	20 (10)	15 (7)	0.332
	C	278 (69)	297 (74)	
	A	126 (31)	107 (26)	0.162
−1154	GG	134 (66)	138 (68)	
	GA	63 (32)	58 (29)	0.687
	AA	5 (2)	6 (3)	0.952
	G	331 (82)	334 (83)	
	A	73 (18)	70 (17)	0.854
−634	GG	67 (33)	75 (37)	
	CG	95 (47)	82 (41)	0.298
	CC	40 (20)	45 (22)	0.905
	G	229 (57)	232 (57)	
	C	175 (43)	172 (43)	0.887
+936	CC	133 (66)	146 (72)	
	CT	59 (29)	50 (25)	0.303
	TT	10 (5)	6 (3)	0.37
	C	325 (80)	342 (85)	
	T	79 (20)	62 (15)	0.138

assessed using χ^2 test or Fisher's exact test. Moreover, the strength of the association between the observed genotype and allele frequencies in OA patients and healthy controls was evaluated by the χ^2 test. Odd ratios (OR) and 95% confidence intervals (CIs) of genotypes and alleles were calculated using StatCalc program (AcaStat Software, Leesburg, VA, USA). Their haplotypes and linkage disequilibrium (LD), coefficients (D'), and r^2 were assessed by using the Haploview software version 4.2 (Broad Institute Cambridge, MA, USA). Plasma VEGFA levels were expressed in pg/ml and presented as a mean ± standard error of the mean (SEM). Unpaired Student's t test and 1-way analysis of variance were utilized to analyze quantitative data of 2 and more than 2 independent groups. P value < 0.05 was considered statistically significant.

Results

Characteristics of the Subjects

To examine the relationship of *VEGFA* gene polymorphism and the risk for developing knee OA, we evaluated the genotype of the 4 polymorphisms (−2578C/A, −1154G/A, −634C/G, and +936C/T), 2 (−2578C/A and −1154G/A) in the promoter region, 1 (−634 C/G) in the 5′-UTR and 1 (+936C/T) in the 3′-UTR of the *VEGFA* gene, by PCR-RFLP analysis. The genotype frequencies between knee OA subjects and healthy volunteers were comparable.

There were no statistically significant differences between the groups in terms of age, sex, and body mass index (BMI). In the OA subjects, the average age was 68.8 ± 7.8 years. In the healthy controls, the average age was 66.2 ± 7.3 years ($P = 0.4$). The female/male ratio was 169/33 in the knee OA subjects and 160/42 in the controls ($P = 0.2$). In addition, there were no significant differences in mean BMI between the 2 groups, 27.0 ± 3.7 kg/m^2 in the knee OA subjects and 25.6 ± 3.7 kg/m^2 in the healthy volunteers ($P = 0.1$).

Genotype distribution and allelic frequencies of the VEGFA *polymorphisms*

The genotype distribution and allele frequencies for *VEGFA* −2578C/A (rs699947), −1154G/A (rs1570360), −634C/G (rs2010963), and +936C/T (rs3025039) polymorphisms are shown in **Table 2**. There was no statistical differences in the genotype and allele frequencies for any of the polymorphisms between the OA patients and the controls (**Table 2**). There was no significant deviations from HWE for all loci.

Association between VEGFA *Polymorphisms and OA Severity*

In the stratified analysis, we subsequently compared the genotype distributions and allele frequencies between the knee OA subjects and the controls of the same sex for the 4 selected *VEGFA* polymorphisms. There were no significant

Table 3. Genotype and Allele Frequency Distributions of *VEGFA* Polymorphisms in OA Patients and Healthy Controls Stratified by Sex.

Variables	Genotype or allele frequency	−2578C/A OA patients, *n* (%)	−2578C/A Controls, *n* (%)	−2578C/A *P* value	Genotype or allele frequency	−1154G/A OA patients, *n* (%)	−1154G/A Controls, *n* (%)	−1154G/A *P* value
Sex								
Female	CC	81 (40)	76 (38)	1	GG	114 (56)	88 (43)	1
	CA	70 (35)	46 (23)	0.188	GA	51 (25)	40 (20)	0.948
	AA	18 (9)	11 (5)	0.403	AA	4 (2)	5 (2)	0.354
	C	232 (58)	198 (49)	1	G	279 (69)	216 (54)	1
	A	106 (26)	68 (16)	0.118	A	59 (15)	50 (12)	0.670
Male	CC	15 (8)	34 (17)	1	GG	20 (10)	50 (25)	1
	CA	16 (7)	31 (15)	0.888	GA	12 (6)	18 (9)	0.374
	AA	2 (1)	4 (2)	0.610	AA	1 (1)	1 (1)	0.501
	C	46 (11)	99 (25)	1	G	52 (13)	118 (29)	1
	A	20 (5)	39 (10)	0.763	A	14 (3)	20 (5)	0.228

Variables	Genotype or allele frequency	−634C/G OA patients, *n* (%)	−634C/G Controls, *n* (%)	−634C/G *P* value	Genotype or allele frequency	+936C/T OA patients, *n* (%)	+936C/T Controls, *n* (%)	+936C/T *P* value
Sex								
Female	GG	57 (28))	51 (25)	1	CC	112 (56)	94 (46)	1
	CG	79 (39)	54 (27)	0.368	CT	48 (24)	37 (18)	0.843
	CC	33 (16)	28 (14)	0.996	TT	9 (4)	2 (1)	0.067
	G	193 (48)	156 (38)	1	C	272 (67)	225 (56)	1
	C	145 (36)	110 (27)	0.702	T	66 (17)	41 (10)	0.188
Male	GG	10 (5)	24 (12)	1	CC	21 (10)	52 (26)	1
	CG	16 (8)	28 (14)	0.686	CT	11 (5)	13 (7)	0.196
	CC	7 (4)	17 (8)	0.785	TT	1 (1)	4 (2)	0.564
	G	36 (9)	76 (19)	1	C	53 (13)	117 (29)	1
	C	30 (7)	62 (16)	0.943	T	13 (3)	21 (5)	0.421

differences in the genotype and allele frequencies of the knee OA subjects and the controls when the data were stratified by sex (**Table 3**). In addition, the knee OA patients were divided into 2 groups: early OA (KL grade 2) and advanced OA (KL grades 3 and 4). According to the −2578A/A genotype, the patients with early stage OA had a greater odds ratio than those with advanced stage OA ($P = 0.023$) (**Table 4**). However, there were no notable differences in the genotype and allelic frequencies for −1154G/A, −634C/G, and +936C/T polymorphisms (**Table 4**).

Association of Plasma VEGFA Levels and Significant VEGFA *Polymorphisms*

Subsequent measurement of VEGFA levels showed that circulating VEGFA values did not differ between OA subjects and the healthy participants (78.8 ± 7.2 pg/ml *vs*. 70.6 ± 7.8 pg/ml, $P = 0.44$). The functional relevance of −2578C/A, −1154G/A, −634C/G, and +936C/T polymorphisms on plasma VEGFA was assessed; the association between each genotype distribution of these polymorphisms and plasma VEGFA levels are illustrated in **Figure 1.** Our findings showed that plasma VEGFA of the −634C/C genotype were significantly higher in knee OA subjects compared with the controls ($P = 0.035$). However, plasma VEGFA levels of −634 G/G and G/C genotypes were not significantly different between OA patients and the controls. We also compared OA with the controls by determining the association between −2578C/A, −1154G/A, and +936C/T polymorphisms and corresponding plasma VEGFA levels. There were no significant differences between OA and control groups with respect to their genotype distribution (**Fig. 1**).

VEGFA *Haplotypes and Risk of OA*

To further determine whether haplotypes of *VEGFA* were associated with OA, the frequencies of LD and haplotype were estimated for 4 identified polymorphisms in the *VEGFA* gene. The pattern of linkage disequilibrium (LD) in the *VEGFA* locus was measured by D' and r^2 score. The 3 polymorphisms −2578C/A, −1154G/A, and −634 G/C were in linkage disequilibrium, while the +936C/T was visibly

Table 4. Genotype and Allele Frequency Distributions of the *VEGFA* Polymorphisms in Knee OA Patients Stratified by Severity of the Disease.

SNPs	Genotype or allele frequency	Early OA ($n = 126$) *n* (%)	Advanced OA ($n = 76$) *n* (%)	OR (95% CI)	*P* value
−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)	5.9 (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	
	A	205 (81)	126 (83)	1.11 (0.64-1.95)	0.797
−634	GG	26 (21)	14 (18)	1	
	CG	54 (43)	41 (54)	0.85 (0.34-2.11)	0.859
	CC	46 (36)	21 (28)	0.60 (0.30-1.22)	0.174
	G	106 (32)	69 (45)	1	
	C	146 (58)	83 (55)	0.87 (0.57-1.34)	0.582
+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

OR (95% CI): odd ratio (95% confidence interval).

physically far from, and had a low LD with the other 3 markers in the gene. Exact values of the paired D' and r^2 in OA patients and the controls are demonstrated in **Figure 2**. Briefly, pairwise LD in case-control study was observed for the first 2 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 SNPs (D' = 0.70 and $r^2 = 0.15$). The relative low values of r^2 indicated that none of the 3 markers were redundant in an association study.

In this study, the *VEGFA* marker +936C/T (rs3025039) was not in the linkage disequilibrium with the other 3 markers. Therefore, only −2578C/A, −1154G/A, and −634 G/C were considered for further haplotype analysis. Haplotype distributions of −2578C/A, −1154G/A, and −634G/C *VEGF* gene polymorphisms are shown in **Table 5**. The most frequent haplotype observed in OA patients and the controls was CGC (35.6 and 39.6%, respectively). The frequencies of the CAC haplotypes displayed a significant difference in distribution between OA patients and the controls, OR (95%CI) = 6.96 (1.46-33.01), $P = 0.005$.

Discussion

OA is a complex condition with multiple etiologies, affecting all joint compartments, and has an important genetic component. Although the multivariable character of OA is widely established, genetic factors have been unveiled to be robust determinants of this degenerative disease. In recent years, it has been delineated that a number of candidate genes are greatly associated with the risk of developing knee OA.[1,21,22,28-31]

The VEGF family of angiogenic growth factors are VEGFA, PLGF, VEGFB, VEGFC, VEGFD, VEGFE, and VEGFF.[4] There is an emerging evidence that VEGF significantly contributes to the pathogenesis of OA and has a distinctive role in rheumatic disorders.[4] The genetic variability in the promoter region, 5′UTR, and 3′ UTR of the *VEGFA* gene affects the activity and expression of VEGFA.[32] The plasma and synovial VEGFA expressions have been previously documented in knee OA patients.[10] A greater comprehension of the factors influencing the production of VEGFA may allow us to accurately determine which individuals are at risk for developing degenerative joint disease. According to the reports, polymorphisms in the regulatory region of the *VEGFA* gene have been investigated in a diverse range of diseases, including rheumatoid arthritis.[26] It is unclear whether the VEGFA genetic polymorphisms can influence the susceptibility of developing knee OA. To address this issue, we determined the impact of *VEGFA* −2578C/A, −1154G/A, −634C/G, and +936C/T SNPs on the susceptibility of knee OA in the Thai population. In this case–control study, our results uncovered that the percentage of the

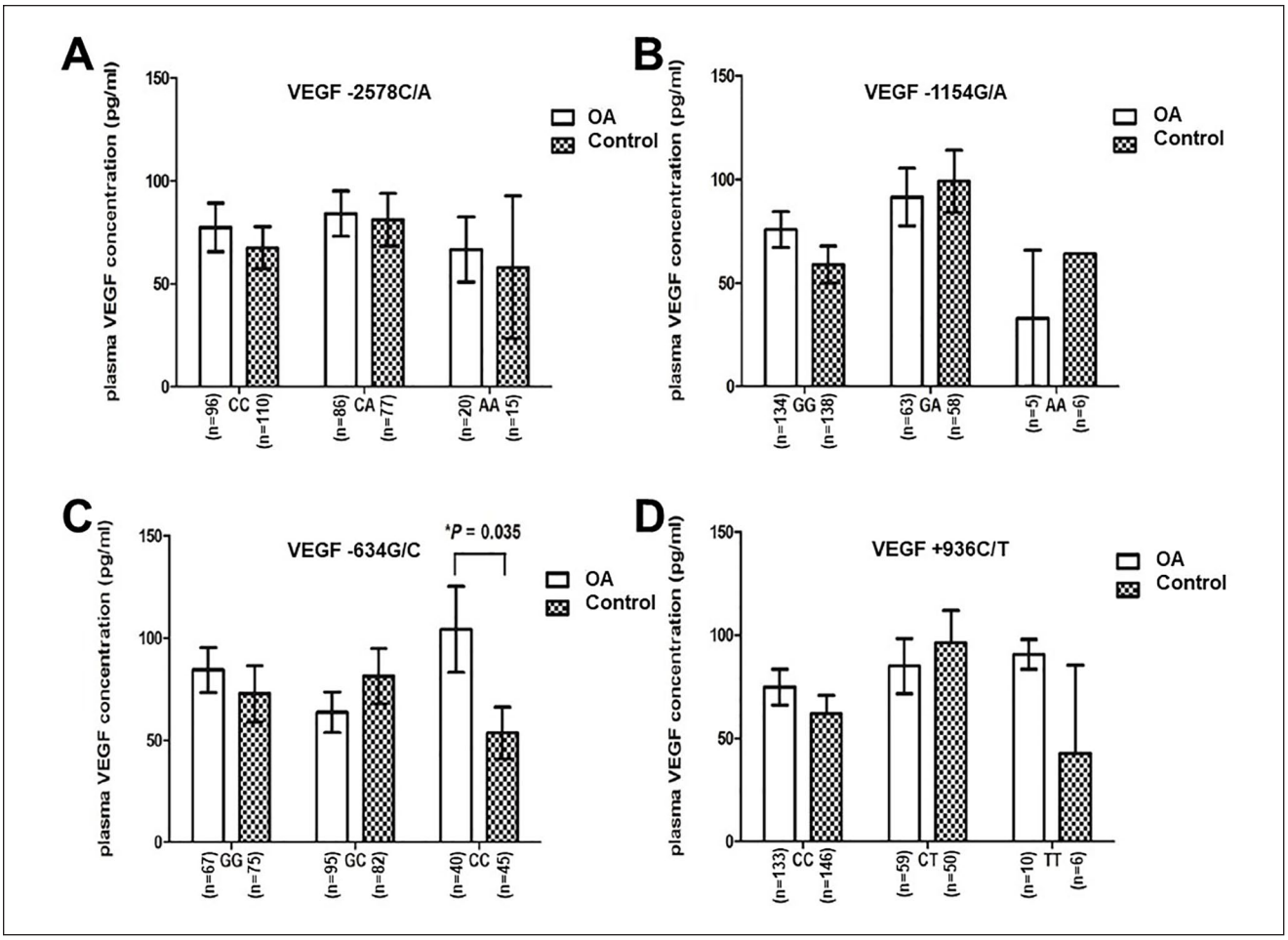

Figure 1. Plasma VEGFA concentration between knee OA patients and the controls for each genotypic group: (A) −2578C/A (rs699947), (B) −1154G/A (rs1570360), (C) −634C/G (rs2010963), and (D) +936C/T (rs3025039) *VEGFA* polymorphisms. Data are presented as pg/ml and mean ± SEM.

VEGFA polymorphism allele and the distribution of genotypes were not significantly different between knee OA subjects and the healthy volunteers.

Several genotypes of VEGF polymorphisms influence VEGF production and are associated with susceptibility or severity of many diseases, including *arthritis*.[33] According to *VEGFA* SNP studies, the common *VEGFA* SNPs are located in the promoter, 5'UTR, and 3'UTR regions. Nevertheless, there are scarce data on the effects of *VEGFA* polymorphisms may have on the risk of developing knee OA. There are controversial evidences in functional *VEGFA* polymorphisms. To the best of our knowledge, this is the first study to determine the susceptibility of knee OA based on the *VEGFA* gene polymorphisms at the promoter, 5'UTR, and 3'UTR regions. The genotype distributions and allele frequencies of all 4 *VEGFA* SNPs (−2578C/A, −1154G/A, −634C/G, and +936C/T) were not significantly different between knee OA patients and the healthy individuals. Moreover, we analyzed the variations of *VEGFA* polymorphisms in accordance with the sex of the participants. Our findings showed that the polymorphisms in the females and males from both case and control groups were not significantly different in genotype and allele frequencies. Previous studies have not reported whether the polymorphisms in the promoter (−2578C/A and −1154G/A) and in 3'UTR (+936C/T) regions of the *VEGFA* gene are involved in knee OA. Sanchez-Enriquez *et al.*[34] examined the correlation between polymorphisms of *VEGFA* gene in knee OA patients, and their findings were similar to our results. They demonstrated that the −460T/C and +405C/G *VEGFA* polymorphisms were not significantly associated with the susceptibility of OA. Likewise, Seo *et al*.[35] showed that there were no significant differences in genotype distribution and allele frequencies in VEGFA polymorphisms of −2578C/A, −1154G/A, and −634C/G between ankylosing spondylitis patients and the controls.[35] However, Rodriguez-Fontenla *et al.*[31] investigated the candidate genes for hip OA in a large meta-analysis of genome-wide

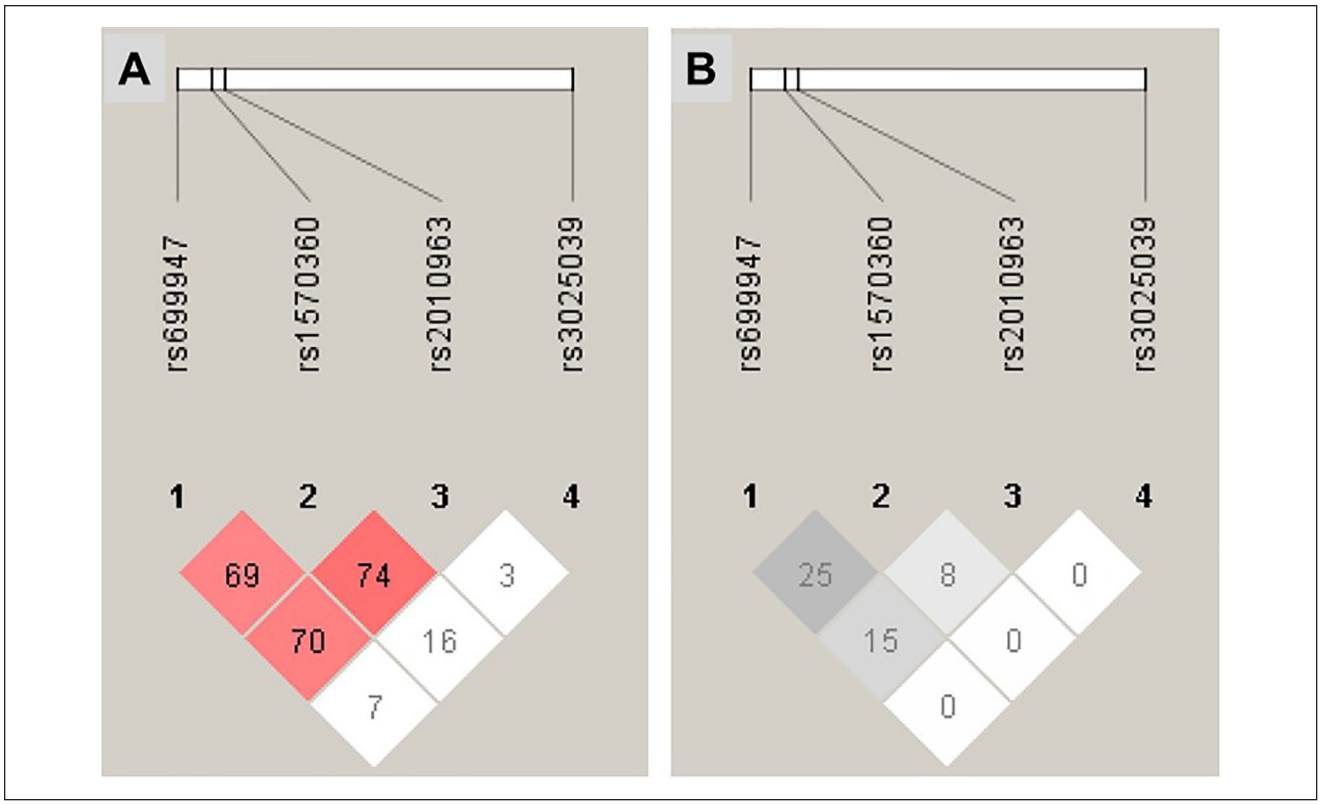

Figure 2. Linkage disequilibrium (LD) plot of −2578C/A (rs699947), −1154G/A (rs1570360), −634G/C (rs2010963), and +936C/T (rs3025039) of *VEGFA* polymorphisms (A) D' (shown as percentages) between the single nucleotide polymorphisms (SNPs). The pink: D' < 1; Red: D' = 1; White: D' = 0 (B) r^2. The gray-shaded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

Table 5. Haplotype Analysis of the *VEGFA* Polymorphisms in Knee OA Patients and the Controls.

Haplotype	Knee OA ($n = 202$) *n* (%)	Controls ($n = 202$) *n* (%)	OR (95% CI)	*P* value
***VEGFA* −2578C/A**				
−1154G/A				
−634C/G				
AAG	25 (12.4)	29 (14.3)	0.85 (0.56-1.27)	0.422
AGC	8 (4.0)	5 (2.5)	1.70 (0.76-3.82)	0.194
AGG	28 (13.8)	19 (9.4)	1.53 (0.99-2.37)	0.053
CAC	6 (3.0)	1 (0.5)	6.96 (1.46-33.01)	0.005
CAG	4 (2.0)	5 (2.5)	—	—
CGC	72 (35.6)	80 (39.6)	0.84 (0.63-1.12)	0.223
CGG	57 (28.2)	63 (31.2)	0.88 (0.65-1.20)	0.426
AAC	2 (1.0)	0 (0.0)	—	—

OR (95% CI): odd ratio (95% confidence interval).

association studies. They highlighted that *VEGF* SNP rs833058 was associated with hip OA in men.

In the present study, the *VEGFA* polymorphism at −2578C/A was found to be significantly different between early and advanced stage OA patients. The A/A genotype of −2578C/A polymorphism was remarkably expressed in early stage OA subjects when compared with advanced stage OA subjects. A previous study demonstrated that the *VEGFA* −2578 A/A genotype was associated with lower circulating VEGFA level.[36] This finding suggests that A/A genotype of −2578 SNP in early stage OA could play a protective role in the progression of knee OA. In addition,

−634C/C genotype in OA patients had an increased plasma VEGFA levels compared with the controls. However, there was no association in plasma VEGFA between OA and the control groups for −634C/G and −634G/G genotypes. In addition, the analysis of *VEGFA* polymorphisms in rheumatoid arthritis (RA) in a Spanish cohort showed that −1154G/A and −634C/G variants were not associated with the risk for RA.[37] Furthermore, a study conducted in the Chinese population showed that there was no association between −2578C/A, −1154G/A, and −634C/G *VEGFA* polymorphisms and RA development.[38] In contrast, Zhang *et al*.[39] stratified the participants according to age and sex and disclosed that the −2578C/A genotype in older RA patients was associated with lower risk for developing RA. Furthermore, Han *et al*.[26] demonstrated that the +936T allele was significantly higher in RA patients. Moreover, the T allele of +936C/T *VEGFA* polymorphism acts as a protective allele in the progression of psoriatic arthritis.[40] The discrepancies between the previous studies and our findings may be attributed to the sample size, diverse genetic polymorphisms in the various ethnic populations, cohort heterogeneity, and confounding factors attributed to the population stratification.

In addition, 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. Only 3 SNPs (−2578C/A, −1154G/A, and −634G/C) were conducted for haplotype distribution. This study showed that the risk for developing OA was 7-fold higher in patients with CAC haplotype when compared with the controls. Thus, the CAC haplotype might confer susceptibility to knee OA in the Thai population.

A number of caveats need to be acknowledged with regard to the current study. Due to the small sample size of our study, the finding cannot be generalized to other populations. The results from this study cannot conclusively be formally drawn with respect to the analyzed polymorphisms. Large-scale investigations in populations with different ethnicities are warranted to validate the results presented in this study. Moreover, the heterogeneity in clinical characteristics, confounding factors, and limited clinical data provided by the study populations could have adversely affected the findings. Another limitation is the lack of information regarding synovial VEGFA levels that are genetically determined by the functional polymorphisms in the *VEGFA* gene. Since OA is genetically heterogeneous, it could potentially be studied in combination with loci to predict the risk of developing OA. In future studies, haplotype patterns may be analyzed to corroborate the function and relationship of variants responsible for OA susceptibility.

Conclusions

The *VEGFA* polymorphisms −2578C/A (rs699947), −1154G/A (rs1570360), −634C/G (rs2010963), and +936C/T (rs3025039) may not be responsible for the susceptibility of developing primary knee OA. However, the OA patients with A/A genotype at the −2578C/A seemed to have a lower potential risk in developing severe OA compared with those with C/A and C/C genotypes. The CAC haplotype may be associated with an increased risk of knee OA in the Thai population. Further researches on the expression of the *VEGFA* gene and the correlation of its polymorphisms might elucidate the role of *VEGFA* and its impact in developing primary knee OA.

Acknowledgments and Funding

This research has been facilitated by the Office of the Higher Education Commission, Ministry of Education through the grant, "Strategic Scholarships for Frontier Research Networks" for the PhD Program, and the 90th Year Chulalongkorn Scholarship. This study was also supported by the Fundamental Fund (CUFRB65_hea(18)_025_30_06), Ratchadapiseksompotch Fund (CUGR 63953002), Center of Excellence in Osteoarthritis and Musculoskeleton (GCE 6507230031-1), Chulalongkorn University. We also thank Ms. June Ohata and Mr. Paul Hatton for reviewing and proofreading the manuscript.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Ethical Approval

This study has been approved by the Institutional Review Board on Human Research of Faculty of Medicine, Chulalongkorn University, and complied with the ethical requirements of the Declaration of Helsinki. Informed consent was obtained from all individual participants included in the study

Informed Consent

Written informed consent for publication was obtained from all participants prior to their enrolment in the study.

Trial Registration

Not applicable.

ORCID iDs

Sittisak Honsawek https://orcid.org/0000-0003-3852-9092
Pongsak Yuktanandana https://orcid.org/0000-0002-8532-7867

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