# Association of Estrogen Receptor-alpha Single-nucleotide Polymorphism (Codon 594 G→A) and Thai Patients Affected by Knee Osteoarthritis†

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***Background:*** *Estrogen receptor-alpha single-nucleotide polymorphism (ER-alpha SNP) has previously shown its susceptibility to knee osteoarthritis (OA) but this association cannot be applied to ethnic groups with different genetic backgrounds.*
***Objective:*** *To characterize the genetic association between ER-alpha SNP and knee OA in the Thai*
***Material and Method:*** *A case–control study was conducted at Ramathibodi Hospital from August 2007 to May 2008. Altogether, 104 cases affected by knee OA and 104 controls were included in this study. SNP rs2228480 (codon 594 G!A) on the ER-alpha gene was genotyped by a PCR-RFLP-based technique. Genotype frequencies were analyzed by logistic regression.*
***Results:*** *ER-alpha SNP was normally distributed through the Hardy-Weinberg Equilibrium (HWE). The risk of knee OA was genetically associated to AG and AA genotypes compared with homozygous wild-type GG (OR: 1.02, 95% CI: 0.60–1.80 for AG; OR: 1.27, 95% CI: 0.30–4.90 for AA).*
***Conclusion:*** *Our study showed that these genetic alterations might be associated with knee osteoarthritis in the Thai population. Further investigation on other informative SNPs on the ER-alpha gene should be performed to create a reliable haplotype that might provide a stronger link between genetic profiles and clinical picture.*

***Keywords:*** *Osteoarthritis, OA, Estrogen receptor-alpha, ER-alpha, Single-nucleotide polymorphism, SNP, RFLP, Thai*

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Osteoarthritis (OA) is a common public health problem, particularly in the elderly. The hands, feet, spine, and large weight-bearing joints, *e.g.*, hips and knees, are mainly affected by OA. Clinical manifestations of OA are often associated with significant functional impairment, as well as signs and symptoms of inflammation, *i.e.*, pain, stiffness, and loss of joint mobility[1]. The World Health Organization (WHO) reports that about 10% of patients over 60 years of age have suffered from this particular syndrome, costing more than $60 billion per year on healthcare in the United States[2]. Pathogenesis of this disease is described by the imbalance between the anabolism and catabolism of the cartilage, resulting in the derangement of collagen

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type 2, proteoglycans, and other related matrices. Consequently, joints become stiff and lose elasticity, which leads to a high susceptibility to damage[3,4].

Risk of OA has been modulated by several factors including weight, age, trauma, hormones, mechanical factors, and genetics[1,5,6]. Nevertheless, females are more susceptible to OA, especially in the postmenopausal period. This evidence suggests that the influence of estrogen might underline the protective mechanism of OA in women[7-10]. The estrogen receptor (ER) is expressed and localized in articular sites to regulate the metabolism of cartilages[11,12]. In the past decade, an attempt to predict the genetic risk to OA using gene polymorphisms has been continually carried out and estrogen receptor-alpha single-nucleotide polymorphism (ER-alpha SNP) is one of the potential candidates to elucidate the susceptibility to OA[13-19]. Nevertheless, this association remains controversial and varies across ethnic groups[13,17-19]. ER-alpha SNP has shown a significant link to Japanese and Korean populations, whereas a UK study reported no association to idiopathic OA[13,18,19].

In this study, we investigated the association between the exon 8 G/A polymorphism (NCBI database rs2228480, c. 594 G!A) of the ER-alpha gene and primary knee OA in Thai patients. Due to nucleotide substitution, this SNP creates a restriction site for BtgI that leads to convenient molecular analysis using the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP)-based technique.

## Material and Method

### *Subjects*

This study was approved by the ethics committee of the Faculty of Medicine, Ramathibodi Hospital, Mahidol University, Bangkok, Thailand. Most of the patients recruited in the project were Thai nationals and had families settled in Thailand for at least three generations. In total, 104 knee OA patients and 104 non-knee OA patients from the clinic of the Department of Orthopedics, Ramathibodi Hospital were included. Informed consent was performed after the purpose of the research project was clearly stated to all patients. The diagnosis of knee OA was based on the American College of Rheumatology criteria[20]. Both cases and controls were interviewed to obtain demographic data and general risk factors. Thereafter, standard weight-bearing knee antero-posterior and lateral films were taken in all recruited subjects to confirm the diagnosis of OA using Kellgren and Lawrence scores (KL scores)[21].

### *PCR-RFLP for BtgI restriction site*

First, 5 ml peripheral blood samples were collected from all subjects using ethylenediamine tetraacetic acid as an anticoagulant and processed for SNP analysis. Genomic DNA was extracted from buffy coat leukocytes using the standard phenol-chloroform method. PCR primers to amplify exon 8 of the ER-alpha gene were designed by the Primer-3 web-based tool[22]: GTGGAGGAGACGGACCAAA (forward) and TGGCCACTCATCTAGAAAGCC (reverse).

The 50 μl PCR mixture contained 100 ng of genomic DNA, 20 pmol of each primer, 0.2 μM of each dNTP, 1 unit of Taq DNA polymerase (AmpliTaq®, Applied Biosystem, Foster City, CA), 3.0 mM $MgCl_2$ in 10x PCR buffer containing 10 mmol of Tri-HCl pH 9.0, 10 mmol KCl and 0.1% Triton X-100 (Invitrogen, Carlsbad, CA). PCR started with an initial denaturation at 95□C for 5 min, followed by 35 cycles of amplification in a thermocycler (PCR Sprint, Thermofisher, Waltham, MA) with denaturation at 94°C for 1 min, annealing at 58°C for 1 min, extension at 72°C for 1 min, and final extension at 72°C for 10 min. Then, 10 μL of PCR product was incubated at 37°C with 3 units of BtgI for 4 h under the manufacturer's recommended conditions (New England Biolabs, Ipswich, MA). The digested product was electrophoresed on 2% agarose gel with ethidium bromide staining before being visualized on a UV transilluminator. The expected fragment length was 101 and 146 bp in GG, 101, 146, and 247 bp in AG, and 247 bp in AA genotypes.

### *Statistical analyses*

Analysis of demographic data was performed by SPSS 16.0 (Chicago, IL). For continuous data, the unpaired t-test was applied, and Chi-square was used for categorical data. Allele frequencies were estimated by the gene counting method. By creating a 2 x 2 table, the odd ratios (OR), 95% confidence interval (CI) and risk stratification were calculated using CDC Epi Info software (Atlanta, GA). Subgroup analysis of each genotype was performed by logistic regression to obtain OR and 95% CI as estimated relative risks. The probability for Hardy-Weinberg Equilibrium was analyzed by Stata (College Station, TX). $p < 0.05$ was considered significant.

## Results

The demographic data of cases and controls are shown in Table 1. Mean age and BMI in knee OA subjects were substantially higher than in the control group ($p < 0.01$). In OA affected subjects, mean age of

**Table 1.** Subject characteristics

| Variables | Cases (n = 104) | Control (n = 104) | p-value |
|---|---|---|---|
| Age (years), mean (SD) | 66.5 (8.9) | 59.4 (8.0) | <0.01** |
| Age of onset (years), mean (SD) | 57.93 (8.63) | N/A | N/A |
| WOMAC scores | 59.14 (8.43) | N/A | N/A |
| Female (%) | 91 (87.5) | 92 (88.4) | 1.00 |
| BMI (kg/m$^2$), mean (SD) | 27.5 (4.4) | 24.6 (4.3) | <0.01** |
| Labor work (%) | 31 (29.0) | 31 (29.0) | 1.00 |
| Kneeling (%) | 52 (50.0) | 48 (46.2) | 0.15 |
| Exercise (%) | 11 (10.5) | 13 (12.5) | 0.95 |
| Walking upstairs (times/day)(SD) | 3.04 (1.8) | 3.38 (1.8) | 0.18 |
| History of fracture (%) | 10 (9.6) | 13 (12.5) | 0.25 |
| Smoking (%) | 2 (1.9) | 4 (3.8) | 0.66 |

** Statistically significant, SD = standard deviation, N/A = not applicable

**Table 2.** Association between ER-alpha c.594 BtgI SNP and susceptibility to knee OA

| Genotypes | Cases (n =104) | Controls (n = 104) | Odd ratios (95% CI) | p-value |
|---|---|---|---|---|
| GG | 62 | 63 | 1 | - |
| AG | 37 | 37 | 1.02 (0.57-1.80) | 0.96 |
| AA | 5 | 4 | 1.27 (0.32-4.95) | 0.73 |

onset of knee OA was 57.93 $\pm$ 8.63 years old and mean Western Ontario and McMaster Universities (WOMAC) index of OA was 59.14 $\pm$ 8.43. The other collectable risk factors (daily activities, exercise, and walking upstairs) were not different between the two groups.

The association between genotypes and knee OA is shown in Table 2. The prevalence of alleles A and G in the control group was 22% and 78% respectively, and codon 594 SNP (G/A) was suggested to be distributed according to Hardy-Weinberg Equilibrium (p = 0.78) (data not shown). The OR of AA and AG compared with wild-type GG genotypes was 1.27 (95% CI: 0.30-4.90) and 1.02 (95% CI: 0.60-1.80) respectively. Since OR appeared to be increased by non-dominant homozygous AA, and age and BMI between the two groups were different, those two factors were stratified using a statistical program. The stratified OR for homozygous AA was 1.15 (95% CI: 0.27-4.90).

## Discussion

Several risk factors, *i.e.*, activities, BMI, age, preexisting injury and genetic background, have been described to modulate the aggravation and progression of OA. Many studies have demonstrated the association between polymorphisms and mutations in genes that encode protein structures for extracellular matrices and signaling molecules in the metabolism of cartilages and their susceptibility to OA[5,13-19,23]. The ER is one of the candidate mediators that might be involved the modulation of this phenomenon. The incidence of OA in postmenopausal women is higher than in men. This observation has led to the hypothesis that female sex hormones may be involved in the etiology of OA. Particular studies have confirmed that estrogen has a protective effect on the development of OA in women[7-9].

ER-alpha, encoded by the ESR1 gene on chromosome 6q25.1, is a subtype of the ER found in the articular surface of cartilages[11,12]. Few studies have reported that ER-alpha SNP might increase the susceptibility of OA. In a case-control study of 383 Japanese women, Ushiyama et al. reported the association between PvuII and XbaI SNPs in intron 1 of the ER-alpha gene and an increase in the prevalence of generalized OA[18]. Moreover, work by Jin et al revealed that ER-alpha codon 594 G!A promoted the

risk of knee OA in the Korean population[19]. On the other hand, Loughlin et al studied 740 subjects (371 patients who had undergone total hip and/or knee replacement surgery for idiopathic OA and 369 controls) and found no supportive evidence that ER-alpha SNP was a risk factor[13]. Regarding our results, although this study could not demonstrate significance in the association of this particular SNP and knee OA, it suggests the tendency of ER-alpha SNP to be a susceptible factor in the Thai population affected by this particular syndrome. Noticeably, only Asian populations have shown the link between the disease and ER-alpha SNP. The differing results of studies can be explained by the analysis of the heterogeneity of the study populations, different OA end points, and differences in statistical power.

Since Thailand has a population of migrants from the Indo-China peninsula, we attempted to reduce the heterogeneity of study subjects by confirming their Thai nationality and selecting only patients whose grandparents were of Thai origin. Moreover, we identified OA cases and controls using reliable methods such as the American College of Rheumatology criteria, which has an excellent sensitivity and specificity. The diagnosis of all cases and controls was confirmed by radiographic studies of the knee and classified by KL scores. However, there were some limitations in this study, including age and BMI, which play a role as risk factors for OA and these data were substantially different between the two groups. We attempted to adjust the OR of homozygous AA by stratifying the risk from age and BMI, and the stratified result continued to show the trend that the A allele might be a risk factor for OA and G was implied as a protective allele. There are reasons for our data not showing significance. First, other SNPs and microsatellites on either the ER-alpha or other related genes may be confounders of this study because OA is a multi-factorial disease and we have only worked with one polymorphism from one gene. Haplotype studies from other SNPs on the ER-alpha gene might help obtain stronger and more reliable associations. Another issue is that our sample size was too small compared with previous studies. To increase the power of the research, an expanded study group would be required.

Apart from its susceptibility to OA, Ongphiphadhanakul et al reported ER-alpha SNP as a risk factor in Thai postmenopausal women affected by osteoporosis[24]. Few studies have demonstrated the association between OA and osteoporosis but the correlation remains controversial and needs further investigation[25,26].

## Conclusion

ER-alpha SNP may be associated with OA in Asian ethnic groups. Further haplotype studies must be undertaken before this concept becomes a biomarker for the evaluation and risk prediction of patients affected by OA. Since genetic susceptibility varies among different ethnic groups, we should intensively create our own genetic data. High-throughput microarray-based technology could provide convenient SNPs analysis.

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## ความสัมพันธ์ระหว่างการเปลี่ยนแปลงของลำดับเบสโคดอนที่ 594 G→A บนยีนตัวรับสัญญาณเอสโตรเจนอัลฟากับผู้ป่วยไทยที่มารับการรักษาโรคข้อเข่าเสื่อมแบบปฐมภูมิ

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***ภูมิหลัง:*** *ก่อนหน้านี้มีการแสดงให้เห็นถึงความหลากหลายทางพันธุกรรมของยีนตัวรับสัญญาณเอสโตรเจนอัลฟามีผลต่อความเสี่ยงในการก่อโรคข้อเข่าเสื่อมแบบปฐมภูมิ แต่ความสัมพันธ์นี้จะแตกต่างกันไปในแต่ละเชื้อชาติเนื่องจากมีพื้นฐานทางพันธุกรรมที่แตกต่างกัน*

***วัตถุประสงค์:*** *เพื่อศึกษาหาความสัมพันธ์ของการเปลี่ยนแปลงลำดับเบสของยีนตัวรับสัญญาณเอสโตรเจนอัลฟาในผู้ป่วยไทยที่มารับการรักษาโรคข้อเข่าเสื่อมแบบปฐมภูมิ*

***วัสดุและวิธีการ:*** *การศึกษาแบบ case-control ได้ดำเนินการในโรงพยาบาลรามาธิบดี ตั้งแต่เดือนสิงหาคม 2550 ถึงเดือนพฤษภาคม 2551 มีผู้ป่วยที่เป็นโรคข้อเข่าเสื่อมแบบปฐมภูมิเข้าร่วมการวิจัยจำนวน 104 ราย และกลุ่มผู้ป่วยที่ไม่ได้เป็นโรคอีกจำนวน 104 ราย โดนทำการตรวจหาจีโนไทป์ของยีนตัวรับสัญญาณเอสโตรเจนอัลฟา ตามข้อมูลการเปลี่ยนแปลงลำดับเบสของมนุษย์หมายเลข rs2228480 ด้วยวิธีการ PCR-RFLP การแจกแจงความถี่ของการเปลี่ยนแปลงของเบสวิเคราะห์โดยวิธี logistic regression*

***ผลการศึกษา:*** *การกระจายตัวของชนิดเบสในโคดอนนี้เป็นไปตามกฎสมดุลของ Hardy-Weinberg ลักษณะของจีโนไทป์ที่สัมพันธ์กับโรคข้อเข่าเสื่อมมีลักษณะดังนี้ เมื่อเปรียบเทียบ AG และ AA กับ GG ซึ่งเป็นลักษณะปกติ odds ratios (OR) ของ AG มีค่า 1.02 (95% CI 0.60-1.80) และ OR ของ AA มีค่า 1.27 (95% CI 0.30-4.90)*

***สรุป:*** *ความหลากหลายทางพันธุกรรมยีนตัวรับสัญญาณเอสโตรเจนอัลฟาที่บริเวณดังกล่าว มีแนวโน้มต่อการเกิดโรคข้อเสื่อมในกลุ่มคนไทย การศึกษาถึงการเปลี่ยนแปลงลำดับเบสที่บริเวณอื่น ๆ ของยีนนี้ควรได้รับการศึกษาต่อไปเพื่อสร้างชนิด haplotype ที่จำเพาะของประชากรไทยเพื่อนำมาใช้เป็นตัวพยากรณ์โรคทางคลินิกต่อไป*