The Knee 21 (2014) 242–246

Contents lists available at ScienceDirect

The Knee

The single nucleotide polymorphism (SNP) of the *estrogen receptor-β* gene, rs1256049, is associated with knee osteoarthritis in Korean population

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ARTICLE INFO

Article history:
Received 12 July 2012
Received in revised form 21 December 2012
Accepted 26 December 2012

Keywords:
Single nucleotide polymorphism
Genetic susceptibility
Osteoarthritis
ER-β
Rsa polymorphism

ABSTRACT

Background: Estrogens affect articular cartilage metabolism via estrogen receptors (ER) in chondrocytes and are believed to play an important role in the pathophysiology of osteoarthritis (OA). The aim of this study is to determine whether the single nucleotide polymorphism (SNP) of the *estrogen receptor-β* (*ER-β*) is associated with an increased susceptibility to knee OA.
Methods: The possible influence of the SNP of the *ER-β* was investigated in 286 OA patients and 294 healthy subjects as controls. A polymerase chain reaction (PCR)-restriction fragment length polymorphism (RFLP) assay and a PCR-single strand conformation polymorphism (SSCP) assay were used to identify the *Rsa polymorphism* genotype among healthy controls and OA patients, respectively.
Results: For rs1256049 (Rsa), frequencies of genotypes GG, GA, and AA were 49.0% (144/294), 43.5% (128/294), and 7.5% (22/294) in healthy controls, and 35.3% (101/286), 45.5% (130/286), and 19.2% (55/286) in OA patients. Frequencies of alleles G and A among healthy controls were 70.7% (416/588) and 29.3% (172/588); whereas those among OA patients were 58.0% (332/572) and 42.0% (240/572). Statistically significant differences in allele and genotype frequencies of rs1256049 were observed between OA patients and controls ($P<0.0001$). In particular, the risk of OA was significantly increased in carriers with the rs1256049A allele and rs1256049 AA homozygotes.
Conclusions: These results suggest a close association of *rs1256049 ER-β* polymorphisms with susceptibility to OA in the Korean population.
Clinical relevance: The rs1256049 polymorphism of the estrogen receptor-β gene can potentially be used to identify genetically high-risk subgroup of osteoarthritis in advance and to understand pathogenesis of osteoarthritis.

1. Introduction

Osteoarthritis (OA) is a slowly progressive degenerative joint disease of the articular cartilage that generally occurs in weight-bearing joints and fingers of elderly individuals. In addition, it is the most common form of arthritis and leading cause of musculoskeletal disability in developed countries [1,2]. It is characterized by degeneration and progressive loss of articular cartilage and changes in bone, synovium and other soft tissues in the involved joints [3]. Clinical manifestations of OA may include joint pain, swelling, stiffness, and even loss of function. It is well known that development of OA is a multifactorial process, which is associated with a variety of risk factors, including genetic predisposition, aging, obesity, inflammation, and excessive mechanical loading. In Korea, OA patients are estimated to afflict 10.2% of the general population, with 5.9% males and 12.9% females. Its incidence increases with advancing age and is estimated to be present in 33.8% of the general population of people older than 60 years of age [4]. In addition, the prevalence of knee OA is 37.3% for radiological disease and is 24.2% for symptomatic disease in Korea which are significantly higher in women than in men [5]. In addition to the observation that the prevalence and incidence of OA are higher among postmenopausal women compared to men at the same age, a beneficial role of estrogen in OA has been suggested on the basis of the observation that postmenopausal women under estrogen-replacement therapy (ERT) present reduced severity and prevalence of OA and osteoporosis [6,7]. These observations have led to the hypothesis that sex hormones, especially estrogen, may be involved in the etiology of osteoarthritis [8].

Estrogens are the primary female sex hormones and natural estrogens readily diffuse across the cell membrane like all steroid hormones. Once they bind to and activate estrogen receptors, which serve as nuclear receptors, the ER complex binds to specific DNA sequences called a

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http://dx.doi.org/10.1016/j.knee.2012.12.011

hormone response element to activate the transcription of some 137 ER-regulated genes [9]. Estrogen carries out its functions through binding to either estrogen receptor α or β, which are encoded by a separate gene, ESR1 and ESR2 on the chromosome 6q25.1 and 14q23.2, respectively [10,11]. With regard to OA, estrogens affect articular cartilage metabolism directly, via estrogen receptors (ER) α and β in chondrocytes [12]. Identification of the two receptors *ER-α* and *ER-β* in chondrocytes from osteoarthritic hips and knees provided further evidence that the cartilage is sensitive to estrogens [12]. Several in vitro studies and in vivo animal experiments confirmed that chondrocytes respond to sex steroid hormones and provide insight into the mechanisms by which these hormones may influence chondrocyte metabolism [13].

Single nucleotide polymorphisms (SNPs) include some differences or variations in the genome between individuals. Some of these polymorphic variants are functionally expressed, suggesting that SNP is one of the factors associated with susceptibility to disease. Recently, it has been reported that rs1256409 is SNP, Guanine to Adenine (G2809A), which exists in exon 5 of *ER-β* gene. It is also known as *Rsa* polymorphism because the polymorphism is detected when using Rsa I restriction enzyme [14]. SNP on *ER-β* may suppress transcriptional repression of pro-inflammatory genes, which in turn, increases the expression of pro-inflammatory genes contributing to OA and other *ER-β*-related diseases [14]. Two studies have reported an association between SNPs of the *ER-α* gene and OA [15,16]. Additionally, several studies regarding the *ER-α* polymorphism and bone mineral density were reported [17–20]. Some of these SNPs have shown correlation with OA and others not, suggesting the involvement of racial difference and/or environmental factors [21].

Sato et al. studied one of the polymorphisms of the *ER-β* gene, rs1256409, also known as *Rsa*, on patients with rheumatoid arthritis (RA) and control subjects with osteoarthritis (OA), and they found statistically significant correlation between the *Rsa* polymorphism and occurrence of RA [14]. In addition, Karlson et al. suggested that hormone-related genes including *ER-β* haplotype analysis of selected five SNPs, which have been identified to capture the genetic variation in Caucasians; however, those SNPs are not reported frequently in Japanese except for rs1256049 (Rsa) and rs944459 [22,23]. The impact, however, of the *ER-β Rs1256409* polymorphism of the development of OA has not been examined in patients with OA and healthy controls. Thus, we investigated whether the above polymorphism is associated with increased risk of OA in Korean population.

In this study, we analyzed the genotype and allele frequencies of the *ER-β* rs1256049 (Rsa) polymorphism using polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) in 294 healthy individuals and in 286 patients with OA. The *ER-β* rs1256049 (Rsa) polymorphism was further examined by polymerase chain reaction-single strand conformation polymorphism (PCR-SSCP) and direct sequencing.

2. Materials and methods

2.1. Tissue samples

Degenerative articular cartilage, meniscus and ligament tissue specimens were obtained from 286 OA patients who had undergone total knee arthroplasty at St. Mary's hospital, the Catholic University of Korea, in Seoul, between 2004 and 2005. All patients were confirmed by radiology and pathology to have OA. The 286 cases included 51 men (17.8%) and 235 women (82.2%) with a mean age of 61.3 years at initial diagnosis. Since patients who had undergone total knee arthroplasty were included in this study, our specimens were derived from patients with Kellgren and Lawrence grade IV, or joint space narrowing grade four or higher OA. In this study, we excluded patients with RA, polyarthritis associated autoimmune disease, post-traumatic OA, and infection-induced OA. In addition, patients who had clinical and radiographic findings suggestive of skeletal dysplasia were also excluded from the study. Exclusion criteria included other malignant disease such as bone tumor, secondary metastasis, alcohol or drug abuse, hepatic failure, and renal failure. The healthy control group consisted of 134 females and 160 males, with a mean age of 43.9 years. We excluded healthy individuals with symptoms of joint pain, limp, and limitation of joint movement, as well as radiographic signs of joint space narrowing and formation of osteophytes. Due to these strict criteria that fit and excluded more women than men, the resulting healthy control group was composed of more men than women. Both controls and patients with OA belong to the same ethnicity and geographical area. This study was approved by the institutional review board (IRB) of the Catholic University of Korea, College of Medicine (IRB approval number CUMC11U180).

2.2. DNA extraction

DNA templates were extracted from paraffin-embedded knee joint tissues of OA patients. Paraffin-embedded tissues were cut into 4 μm thickness slices; paraffin was dissolved with xylene, followed by washing with 100% ethanol, suspension in ice-cold Nonidet P-40 lysis buffer, and treatment with proteinase K. DNA was extracted by phenol–chloroform–isoamylalcohol and ethanol precipitation, as previously described [24]. For the healthy control population, a leukocyte cell pellet from each blood sample was obtained from the Buffy coat by centrifugation of 2 mL of whole blood. The cell pellet was used for DNA extraction. The Qiagen DNA Blood Mini Kit (Qiagen, Valencia, CA, USA) was used according to the manufacturer's instructions to obtain genomic DNA. DNA purity and concentration were determined using a Nanodrop® ND-1000 spectrophotometer (Nanodrop technologies, Wilmington, DE, USA).

2.3. PCR-RFLP for ER-β in control

A polymerase chain reaction (PCR)-restriction fragment length polymorphism (PCR-RFLP) assay was used to identify the *ER-β* genotype with primers of sense 5′-GTAAAACGACGGCCAGTTCTCACCGCCTC TTGCTTTCCCCAG-3′ and antisense 5′-AAGGGAACAAAAGCTGGAGAAA CACAATGTATTTTTTCTCAC-3′. Each PCR procedure was performed under standard conditions in a 10 μL PCR mixture containing 1 μL of template DNA, 0.5 μM of each primer, 0.2 μM of each deoxynucleotide triphosphate, 1.5 mM $MgCl_2$, a 0.4 unit of AmpliTaq gold polymerase (Perkin-Elmer, Foster City, CA, USA), and 1 μL of 10× buffer. The reaction mixture was denatured for 12 min at 94 °C and incubated for 40 cycles (denaturing for 30 s at 94 °C, annealing for 30 s at 60 °C, and extension for 30 s at 72 °C). The final extension was continued for 5 min at 72 °C. After amplification, the PCR products were digested with 5 U of the restriction enzyme, Rsa I, at 37 °C for 4 h. The digested product was separated on a 3% agarose gel with ethidium bromide, and photographed using an Ultra Violet Product Image Store system (Fig. 1A). To ensure the reliability of the RFLP results, sequencing of PCR products was carried out using the fluorescent dideoxy chain termination method with an ABI 3730XL Analyzer (Applied Biosystems, Foster City, CA, USA), according to the manufacturer's instructions (Fig. 1C).

2.4. PCR-SSCP for ER-β in OA patients

A PCR-single strand conformation polymorphism (SSCP) assay was used for identification of the *ER-β* genotype with primers of sense 5′-ACACACAGGGAGCTGAGGAG-3′ and antisense 5′-CCAGAACAAGA TCTGGAGCA-3′. Each PCR procedure was performed under standard conditions in a 10 μL PCR mixture containing 1 μL of template DNA, 0.5 μM of each primer, 0.2 μM of each deoxynucleotide triphosphate, 1.5 mM $MgCl_2$, 0.4 unit of AmpliTaq gold polymerase (Perkin-Elmer, Foster City, CA, USA), 0.5 μCi of [^{32}P]dCTP (Amersham, Buckinghamshire, UK), and 1 μL of 10× buffer. The reaction mixture was denatured for 12 min at 95 °C and incubated for 40 cycles (denaturing for 30 s at 95 °C, annealing for 30 s at 56 °C, and extension for 30 s at 72 °C). The