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SHORT COMMUNICATION

Analysis of estrogen receptor alpha gene haplotype in Mexican mestizo patients with primary osteoarthritis of the knee

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Abstract The aim of this work was to test the association between estrogen receptor α gene (*ERα*) polymorphism and primary osteoarthritis (OA) of the knee in Mexican mestizo patients. A case–control study was conducted. Cases were patients >40 years of age, with a body mass index (BMI) ≤ 27 and radiologic score for OA of the knee of ≥2 according to Kellgren–Lawrence scale, and controls were subjects >40 years age with a radiologic score <2. Two restriction fragment length polymorphisms, PvuII (T/C; rs2234693), and XbaI (A/G; rs9340799), of the *ERα* were analyzed. Allelic haplotypes were constructed and non-conditional logistic regression was developed to evaluate risk magnitude through odds ratios (ORs) and 95% Confidence intervals (95% CI). Three different allelic haplotypes were identified: TA; CG, and CA. Unadjusted analysis of the haplotypes did not show significant associations; nonetheless, when data were adjusted for gender, age, and BMI, a significant decrease risk was observed for CG haplotype (OR [95% CI]) = 0.5 (0.3–0.9)] ($P = 0.04$). These results suggest that *ERα* gene CG haplotype could be associated with a reduced risk of primary knee OA in Mexican mestizo population.

Keywords Osteoarthritis · Genetic association · Polymorphism · Estrogen receptor gene · Haplotype

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Introduction

Osteoarthritis (OA) is the most common form of arthritis and a leading cause of disability; it is characterized by progressive degeneration of articular cartilage with joint-space narrowing, osteophyte formation, and subchondral sclerosis, resulting in pain and joint stiffness [1]. It is a multifactorial disorder, and twin-pair and family-risk studies have highlighted the evidence of an important genetic component [2, 3]. To date, it is recognized that multiple genes could be involved in susceptibility to OA, and the candidate genes studied belong to extracellular matrix molecules, the inflammation pathway, and those related with modulation of osteocytes or chondrocyte differentiation, among others [4].

The observation that the prevalence and incidence of OA are higher among postmenopausal women [5], that epidemiologic studies demonstrate a protective effect of estrogens on articular cartilage in women [6], and that estrogen receptors are expressed in human articular chondrocytes and bone cells [7] suggests that estrogens may be involved in the etiology of OA.

The estrogen receptor belongs to a superfamily of steroid hormone receptors [8]. There are two isoforms: *ERα*,

and *ERβ*, which are encoded by separate genes [9]. The *ERα* gene is located on 6q25, is >140 kb in length and contains eight exons [10]. Two common polymorphisms are located in intron 1 near 400 pb upstream of exon 2 and are recognized by PvuII and XbaI restriction fragment length polymorphisms (RFLPs). PvuII detects a T–C substitution at position −397 (−397int1 T/C; rs2234693) before exon 2, while Xba I RFLP detects an A–G substitution at position 351 (−351int A/G; rs9340799) [11].

There are few studies that have searched for an association between *ERα* gene polymorphism and risk of OA in populations of different ethnic backgrounds; however, the results are not consistent. Some reports show increased risk [12–14], while others have not [15, 16], and even a reduced risk has been reported [17]. This prompted us to analyze the association between *ERα* gene polymorphism and primary OA of the knee in Mexican mestizo population.

Patients and Methods

We conducted a case–control study; the study protocol was approved by the Committee for Ethics and Investigation. All patients were Mexican mestizos and were recruited at the Articular Rehabilitation Clinic of the Instituto Nacional de Rehabilitación, a tertiary-care referral center in Mexico City. Cases comprised patients >40 years of age with a clinical diagnosis of OA of the knee and a radiologic score ≥2, with body mass index (BMI, kg/m^2) ≤27, without history of serious knee injuries, and without other articular diseases. Controls were subjects aged >40 years who had undergone treatment for injuries (tendon ruptures, contusions, fractures, etc.) without a clinical diagnosis of OA of the knee and a radiologic score <2. OA was assessed in anteroposterior weight-bearing and lateral radiographs of the knees using a 5-point scale according to the Kellgren–Lawrence radiographic scoring method [18]. Grading was performed by a sole trained observer who was blinded to patients' data. All study subjects were interviewed specifically for this study including evaluation by a clinical rheumatologist and review of the patient's clinical history in order to collect general information, occupational and sports activities, previous knee injuries, and clinical manifestations of OA.

Only after obtaining signed informed consent, a 5-ml blood sample was drawn from each patient into tubes containing EDTA. Peripheral blood mononuclear cells were isolated, and DNA was extracted utilizing a salting-out method. The ratio of absorbance at 260 and 280 nm ($A_{260/280}$) was utilized to assess DNA purity. PvuII (T/C, rs2234693) and XbaI (A/G, rs9340799) polymorphic sites were amplified by polymerase chain reaction (PCR) using the primer pair listed previously [12]. A three-temperature thermal profile was optimized for a thermocycler as follows: 94°C for 5 min, followed by 35 cycles at 94°C for 30 s, at 63°C for 30 s, and at 72°C for 1:10 min. Subsequently, one microgram of the PCR product was digested with an excess of XbaI or PvuII endonucleases under conditions specified by the supplier (New England Biolabs, Inc., Beverly, MA, USA). Additionally, to verify the correct site digestion, two plasmids containing the restriction site were used: pTXB1 and PBR 322 (New England Biolabs, Inc.). After digestion, the amplification products were run in 1% agarose gel; a 1,374-bp band was observed if the amplified product did not include the restriction sites. When sites were present, digestion rendered two bands of 936 and 438 bp for PvuII (rs2234693) and of 981 and 393 for XbaI (rs9340799). Because the two polymorphisms are linked (<1.3 kb), haplotypes may be constructed for the 2 loci; then, four possible allelic haplotypes exist: CG, CA, TG, and TA, which could form nine possible genotypes (11). Haplotype pattern analysis, allele frequencies, and Hardy–Weinberg equilibrium (H–WE) were assessed with Haplo View 4.0. For statistical analysis, comparisons of continuous variables were tested by Student's *t*-test, and corrected chi-squared statistics were applied for categorical variables. Uni- and multivariate non-conditional logistic regression analyses were conducted to estimate probability for developing OA comparing allelic haplotypes as main effect; odds ratios (ORs) and 95% Confidence intervals (95% CI) were reported. Alpha level was 0.05, and the STATA ver.10.0 statistical software package was utilized for calculations.

Results

We recruited 115 cases and 117 control subjects; their characteristics are shown in Table 1. Mean age, BMI, and previous sports activities differed significantly among the groups ($P = 0.0001$, 0.03, and 0.01, respectively).

Both PvuII; rs2234693, and XbaI; rs9340799, polymorphisms fell within H–WE ($P = 0.49$ and 0.69, respectively). Among the four possible allelic haplotypes, TG was not observed in the study population, and the TA allele haplotype was the most frequent in cases and controls; this tendency was conserved when data were stratified according to gender, although it did not reach statistical significance. A slight decrease in CG allele in cases was observed; nonetheless, there were no significant differences (Table 2). According to the three allelic haplotypes, six genotypes were found and their frequencies are shown in Table 3. There were no statistical significant differences among all six genotypes ($P > 0.05$).

Unadjusted OR exhibited no significant association among the different alleles with OA in the overall sample.

Table 1 Characteristics of the study population

	Cases ($n = 115$)	Controls ($n = 117$)	P
Females (n %)	92 (80.70)	97 (82.91)	0.6
Age (mean ± SD, years)	57.38 ± 9.20	51.84 ± 8.92	0.0001
BMI (mean ± SD, kg/m^2)	26.55 ± 2.8	25.66 ± 3.4	0.03
Smoking (n %)	17 (14.78)	11 (9.40)	0.2
Alcoholism (n %)	19 (16.52)	10 (8.55)	0.07
Menopause (n %)	37 (40.22)	22 (22.68)	0.01
Current occupational activity (n %)*	8 (6.9)	10 (8.5)	0.6
Previous occupational activity (n %)*	15 (23.8)	13 (17.3)	0.3
Current sports activity (n %)	20 (17.39)	30 (25.64)	0.1
Previous sports activity (n %)	58 (50.43)	78 (66.67)	0.01
Kellgren-Lawrence grading			
Grade 0 (n %)	0 (0.0)	73 (62.39)	
Grade 1 (n %)	0 (0.0)	44 (37.61)	
Grade 2 (n %)	43 (37.39)	0 (0.0)	
Grade 3 (n %)	47 (40.87)	0 (0.0)	
Grade 4 (n %)	25 (21.74)	0 (0.0)	

* Kneeling, squatting, and lifting. *SD* Standard deviation, *BMI* body mass index

Table 2 Frequencies of *ERα* allelic haplotypes

Alleles		All subjects			Women			Men		
PvuII (rs2234693)	XbaI (rs9340799)	Cases ($n = 115$)	Controls ($n = 117$)	P	Cases ($n = 93$)	Controls ($n = 97$)	P	Cases ($n = 22$)	Controls ($n = 20$)	P
T	A	153 (66.5)	152 (65.0)	0.7	125 (67.2)	130 (67.0)	0.6	28 (63.6)	22 (55.0)	0.7
C	G	49 (21.3)	63 (26.9)	0.2	41 (22.0)	48 (24.7)	0.6	8 (18.2)	15 (37.5)	0.07
C	A	28 (12.2)	19 (8.1)	0.2	20 (10.8)	16 (8.2)	0.4	8 (18.2)	3 (7.5)	0.3

Table 3 Frequencies of *ERα* genotypes

Genotypes		All subjects			Women			Men		
PvuII (rs2234693)	XbaI (rs9340799)	Cases ($n = 115$)	Controls ($n = 117$)	P	Cases ($n = 93$)	Controls ($n = 97$)	P	Cases ($n = 22$)	Controls ($n = 20$)	P
T T	A A	52 (45.2)	51 (43.6)	0.8	42 (45.2)	46 (47.4)	0.7	10 (45.4)	5 (25.0)	0.17
C T	G A	34 (29.6)	40 (34.2)	0.3	28 (30.1)	30 (30.9)	0.8	6 (27.3)	10 (50.0)	0.07
C T	A A	15 (13.0)	10 (8.5)	0.3	13 (14.0)	8 (8.2)	0.2	2 (9.1)	2 (10.0)	0.9
C C	G G	4 (3.5)	8 (6.8)	0.2	4 (4.3)	6 (6.2)	0.6	0	2 (10.0)	
C C	G A	7 (6.1)	7 (6.0)	0.9	5 (5.4)	6 (6.2)	0.8	2 (9.1)	1 (5.0)	0.6
C C	A A	3 (2.6)	1 (0.9)	0.3	1 (1.1)	1 (1.03)	0.9	2 (9.1)	0	

When ORs were adjusted for gender, age, and BMI, a decreased risk was observed in CG allele [OR (95% CI) = 0.5 (0.30.9)] ($P = 0.04$), and after data were stratified by gender and ORs were adjusted by age and BMI, this association was maintained only in males [OR (95% CI) = 0.2 (0.05–0.9)] ($P = 0.04$) (Fig. 1). There was no modification of OR (95% CI) when data were adjusted for other different significant variables, such as previous sport activities (data not shown).

Discussion

In this case–control study, in Mexican mestizo population, significantly decreased risk to knee OA of up to 50% was found in the presence of allelic haplotype CG; formerly named PX, of the *ERα* gene polymorphism after adjustment for age, gender, and BMI. Previously, Lian et al. [17] demonstrated a decreased prevalence of hip OA in presence of C allele. Our results are in line with those of Lian

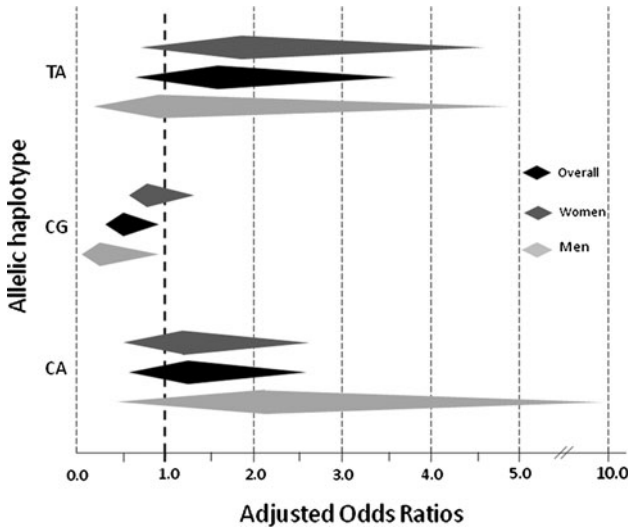

Fig. 1 Association between *ERα* gene polymorphism and knee osteoarthritis. *Diamonds* indicate adjusted odds ratios (ORs) and their 95% Confidence intervals [OR (95%CI)]. ORs for overall sample were adjusted by gender, age, and body mass index (BMI), and for women and men, these were adjusted for age and BMI. For the unstratified sample, the allele CG shows a significant relationship with a decreased risk of OA, which was conserved by males when data were stratified by gender

and indicate that some polymorphisms could confer a reduced risk on OA development.

ERα gene polymorphisms described here are located in intron 1; however, it is not clear how intronic polymorphisms could affect cartilage metabolism. There is recent evidence that in human genome, intronic and exonic portions have been subjected to the same degree of selective pressure, and that the intronic portion possesses a similar level of functional importance to that of the exonic [19]. Therefore, intronic polymorphisms could have functional consequences in OA. In fact, functional analysis of intronic polymorphism D6S1276 of bone morphogenetic protein 5 (*BMP5*), previously related to OA, showed an important effect on transcriptional activity of the gene's promoter, probably due to a differential affinity for nuclear factors [20]. ERα is an important mediator in the signal transduction pathway [9]; it is possible that specific estrogen receptor genotypes may affect expression in chondrocytes through transcriptional regulation resulting in increasing cartilage formation protecting against OA development. The polymorphisms studied here are located in a non-coding region, which usually contains several regulatory sequences that could interfere with transcription. Then, a possible functional consequence is a change of *ESR1* gene expression because of alternative splicing or because of an alteration of transcription factors binding site. Three isoforms of *ERα* of 66, 46, and 36 kDa have been described which are due to alternative splicing of the gene and with transcriptional and functional differences since 66 kDa isoform lacks the transcriptional activation domains AF-1 and AF-2, but it retains the DNA-binding domain [21]. On the other hand, in breast cancer has been noted that the C allele of the PvuII (rs2234693) produces a functional myb binding site that, in the presence of B-myb, is capable of augmenting transcription up to 10 folds [22].

As in other similar studies allele TG was not found [12, 13, 15], which confirms that this is a very uncommon polymorphism. However, in terms of risk magnitude, our results show an important difference with those in which an increased risk was reported for OA conferred by the CG haplotype. Such differences could be due to several reasons: ethnic differences, sample size, false positive results, and OA subsets.

Although certain factors are shared by all humans, each ethnic group possesses their own set of genetic and environmental factors that contribute differentially to disease risks [23]. Ethnic differences could modify allelic frequencies, affecting the ability to detect a susceptibility allele, and possibly for that reason, several association studies on candidate OA genes have demonstrated differences across populations. As an example, analysis of asporin gene (*ASPN*) has exhibited wide variation among different studies. A meta-analysis demonstrated a very significant association between the knee OA and the D14 allele in Asians ($P = 0.0000013$; OR = 1.95), but not in Europeans ($P = 0.2$; OR = 1.14). This clearly shows the effect of ethnic background on allelic distribution and on risk magnitude and, as the authors noted, this heterogeneity could be due to gene–gene or gene–environmental interaction [24].

We are aware that sample size could be an important limitation resulting in erroneous conclusions. Because this was a hospital-based study, it was difficult to achieve a larger sample; nonetheless, in order to assess only primary knee OA, variables significantly associated with the development of secondary OA [25] were strictly controlled, including only patients with BMI < 27 and no history of knee injuries. Moreover, regarding other OA-related variables such as occupational and sport activities [25], the former did not differ among groups, and although the latter demonstrated significant differences, multivariate analysis showed no effect of this variable on the results. Therefore, we believe that despite the sample size, our cases are properly selected as primary OA because environmental variables associated with OA development were properly controlled; therefore, the results reflect a real genetic association in our population.

The possibility of non-differential genotyping error exists, resulting in false positives and biasing the results. However, in order to control this potential error, quality control in laboratory measures was carried out by means of

DNA rating purity and integrity, properly quantifying the manner in which the PCR product was digested, and through restriction tests employing internal controls. Additionally, the alleles analyzed were in H–WE, and it has been suggested that departures from equilibrium may be indicative of genotyping errors [26]. Thus, we think that if errors in genotyping exist, these are minimal.

Genetic factors may vary with disease pattern, disease severity, and according to patients' characteristics such as gender and age. Recently, Herrero-Beaumont et al. [27] proposed three different interrelated subsets of primary OA that exhibit their own clinical and etiological characteristics: type I or genetically determined, type II or hormone-dependent, and type III or aging related. Our patients were younger compared with those of other reports and probably were more symptomatic than those of population studies. Therefore, we possibly studied a form that begins earlier, with more symptomatology, and that requires care before other forms of OA do.

Finally, in case–control studies of chronic diseases as OA the choice of control group is important and frequently difficult. Ideally, they should be matched for confounding variables such as age and other potential confounders to avoid bias. When there is not matching sufficient adjustment for variables in the analysis which could have a strong effect on OA must be done [28]. In our study, as in many others about genetic association in OA, matching was complicated; nevertheless, adjustment of potential confounders in analysis was properly realized and we not found modification of results as is indicated above. In conclusion, we found that the *ERα* gene CG haplotype could be associated with reduced risk to primary knee OA in Mexican mestizo population. This datum could be affected or modified by the previously mentioned factors; notwithstanding this, and principally because of sample size, we are unable to discard the possibility of a type I error. Therefore, additional studies are required to confirm the association.

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Conflict of interest The authors declare that they have no conflict of interest.

References

1. Felson DT (2009) Developments in the clinical understanding of osteoarthritis. Arthritis Res Ther 11:203–213
2. Spector TD, Cicuttini F, Baker J et al (1996) Genetic influences on osteoarthritis in women: a twin study. Br Med J 312:940–943
3. Chitnavis J, Sinsheimer JS, Clipsham K et al (1997) Genetic influences in end-stage osteoarthritis. Sibling risks of hip and knee replacement for idiopathic osteoarthritis. J Bone Joint Surg 79:660–664
4. Valdes AM, Spector TD (2008) The contribution of genes to osteoarthritis. Rheum Dis Clin North Am 34:581–603
5. Spector TD, Campion GD (1989) Generalized osteoarthritis: a hormonally mediated disease. Ann Rheum Dis 48:523–527
6. Zhang Y, McAlindon TE, Hannan MT et al (1998) Estrogen replacement therapy and worsening of radiographic knee osteoarthritis: the Framingham Study. Arthritis Rheum 41:1867–1873
7. Richette P, Corvol M, Bardin T (2003) Estrogens, cartilage, and osteoarthritis. Joint Bone Spine 70:257–262
8. Thornton JW (2001) Evolution of vertebrate steroid receptors from an ancestral estrogen receptor by ligand exploitation and serial genome expansions. PNAS 98:5671–5676
9. Cheung E, Schwabish MA, Kraus WL (2003) Chromatin exposes intrinsic differences in the transcriptional activities of estrogen receptors α and β. EMBO J 22:600–611
10. Ponglikitmongkol M, Green S, Chambon P (1988) Genomic organization of the human oestrogen receptor gene. EMBO J 7:3385–3388
11. Kobayashi S, Inoue S, Hosoi T et al (1996) Association of bone mineral density with polymorphism of the estrogen receptor gene. J Bone Miner Res 11:306–311
12. Ushiyama T, Ueyama H, Inoue K et al (1998) Estrogen receptor gene polymorphism and generalized osteoarthritis. J Rheumatol 25:134–137
13. Bergink AP, van Meurs JB, Loughlin J et al (2003) Estrogen receptor alpha gene haplotype is associated with radiographic osteoarthritis of the knee in elderly men and women. Arthritis Rheum 48:1913–1922
14. Jin SY, Hong S, Yang H et al (2004) Estrogen receptors-α gene haplotypes is associated with primary knee osteoarthritis in Korean population. Arthritis Res Ther 6:415–421
15. Loughlin J, Sinsheimer JS, Mustafa Z et al (2000) Association analysis of the vitamin D receptor gene, the type I collagen gene COL1A1, and the estrogen receptor gene in idiopathic osteoarthritis. J Rheumatol 27:779–784
16. Fytili P, Giannatou E, Papanikolaou V et al (2005) Association of repeat polymorphisms in the estrogen receptors alpha, beta, and androgen receptor genes with knee osteoarthritis. Clin Genet 68:268–277
17. Lian K, Lui L, Zmuda JM et al (2007) Estrogen receptor alpha genotype is associated with a reduced prevalence of radiographic hip osteoarthritis in elderly Caucasian women. Osteoarthritis Cartilage 15:972–978
18. Kellgren JH, Lawrence JS (1957) Radiological assessment of osteo-arthrosis. Ann Rheum Dis 16:494–502
19. Davidson S, Starkey A, MacKenzie A (2009) Evidence of uneven selective pressure on different subsets of the conserved human genome; implications for the significance of intronic and intergenic DNA. BMC Genomics 10:614–620
20. Wilkins JM, Southam L, Mustafa Z et al (2009) Association of a functional microsatellite within intron 1 of the BMP5 gene with susceptibility to osteoarthritis (Abstract). BMC Medical Genetics 10:141
21. Wang ZY, Zhang XT, Shen P et al (2005) Identification, cloning, and expression of human estrogen receptor-α36, a novel variant of human estrogen receptor-α66. Biochem Biophys Res Commun 336:1023–1027
22. Herrington DM, Howard TD, Brosnihan KB et al (2009) Common estrogen receptor polymorphism augments effects of hormone replacement therapy on E-selectin but not C-reactive protein. Circulation 23(105):1879–1882
23. Hines LM, Risendal B, Slattery ML et al (2010) Comparative analysis of breast cancer risk factors among Hispanic and non-Hispanic white women. Cancer 116:3215–3223

24. Nakamura T, Shi D, Tzetis M et al (2007) Meta-analysis of association between the ASPN D-repeat and osteoarthritis. Hum Mol Genet 16:1676–1681
25. Cimmino MA, Parodi M (2005) Risk factors for osteoarthritis. Semin Arthritis Rheum 34(Suppl 2):29–34
26. Hosking L, Lumsden S, Lewis K et al (2004) Detection of genotyping errors by Hardy-Weinberg equilibrium testing. Eur J Hum Genet 12:395–399
27. Herrero-Beaumont G, Román-Blás JA, Castañeda S et al (2009) Primary osteoarthritis no longer primary: three subsets with distinct etiological, clinical, and therapeutic characteristics. Semin Arthritis Rheum 39:71–80
28. Spector TD, Hochberg MC (1994) Methodological problems in the epidemiological study of osteoarthritis. Ann Rheum Dis 53:143–146