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Original Article

Association of estrogen receptor alpha gene polymorphisms and its gene-gene interactions with knee osteoarthritis susceptibility in Chinese Han population

Zhen Feng[1,2], Cuiyue Wang[3], Shui Sun[1]

[1]*Department of Joint Surgery, Shandong Provincial Hospital Affiliated to Shandong University, Jinan, Shandong, People's Republic of China; Departments of* [2]*Joint Surgery,* [3]*Digestive, Linyi People's Hospital, Linyi, Shandong, People's Republic of China*

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Abstract: Aims: To investigate the association of estrogen receptor alpha (ESR1) gene polymorphisms and its gene-gene interactions with knee osteoarthritis (OA) susceptibility. Methods: Generalized multifactor dimensionality reduction (GMDR) was used to screen the best interaction combination among SNPs. Logistic regression was performed to investigate association between 3 SNPs within ESR1 gene and knee OA risk and additional gene-gene interaction between rs2228480 and rs9340799. Results: Logistic analysis showed that rs2234693 and rs2228480 within ESR1 gene were both associated with increased knee OA risk in both additive and dominant models. The carriers of homozygous mutant of rs2234693 and rs2228480 polymorphism have higher OA risk than those with wild-type homozygotes, OR (95% CI) were 1.61 (1.22-2.12) and 1.75 (1.40-2.13), respectively. GMDR model shows a significant two-locus model (P=0.0010) involving rs2228480 and rs9340799, the cross-validation consistency of this two-locus model was 9/10, and the testing accuracy was of 60.72%. Participants with rs2228480-TC or CC and rs9340799-AG or GG genotype have the highest knee OA risk, compared to participants with rs2228480-TT and rs9340799-AA genotype, OR (95% CI) was 2.32 (1.52-3.23). Haplotype containing the s2234693-C and rs2228480-A allele were associated with a statistically increased knee OA risk, OR (95% CI)=1.76 (1.38-2.18), *P*<0.001. Conclusions: We found that rs2234693 and rs2228480 within ESR 1 gene, interaction between rs2228480 and rs9340799 and haplotype containing the s2234693-C and rs2228480-A allele were associated with a statistically increased knee OA risk.

Keywords: Estrogen receptor alpha, knee osteoarthritis, SNP, interaction, haplotype

Introduction

Osteoarthritis (OA) is a leading cause of physical disability and is estimated to affect around 40% of people over 70 years of age [1]. In China, radio-imaging has indicated a prevalence of 29.5% in women over age 60, but clinical diagnosis extends that prevalence to ~39% [2]. In all types of OA, Knee OA in particular is a major cause of morbidity and is the primary diagnostic indication for total knee replacement [3]. The mechanism of knee OA susceptibility is multifactorial with various independent risk factors having been identified [4, 5]. Recent years, the role of genetic factors has gained increasing research prominence, and some genes have been reported [6-9]. Study suggested that women were more vulnerable to more severe knee OA especially after menopausal age [10], it could be hypothesized that estrogen may be involved in the onset or progression of knee OA.

The human estrogen receptor has two isoforms: ESR1 and ESR2, which are members of the steroid/thyroid hormone superfamily of nuclear receptors and encoded by separate genes [11]. ESR1 is expressed in chondrocytes, stromal cells, and osteoblasts [12], which potentially indicated that both bone and cartilage can be regulated by ESR1 gene. Recently, some ESR1 gene polymorphisms, such as rs2234693 and

Table 1. Description and primers used for genotyping for 3 SNPs

ID	Chromosome	Functional Consequence	Primer sequences
rs2234693 PvuII	6:151842200	Intron variant	Forward: 5'-CTGCCACCCTATCTGTATCTTTTCCTATTCTCC-3' Reverse: 5'-TCTTTCTCTGCCACCCTGGCGTCGATTATCTGA-3'
rs9340799 XbaI	6:151842246	Intron variant	Forward: 5'-CTGCCACCCTATCTGTATCTTTTCCTATTCTCC-3' Reverse: 5'-TCTTTCTCTGCCACCCTGGCGTCGATTATCTGA-3'
rs2228480 594 (G>A) BtgI	6:152098960	Synonymous codon, utr variant 3 prime	Forward: 5'- GTGGAGGAGACGGACCAAA-3' Reverse: 5'-TGGCCACTCATCTAGAAAGCC-3'

Table 2. Baseline general characteristics of 1562 study participants in cases and controls

Variables	Cases (n=520)	Controls (n=1042)	*P*-values
Age (year)	62.4±16.9	63.1±16.2	0.428
Males, N (%)	201 (38.6)	417 (40.0)	0.603
BMI (kg/m^2) (mean ± SD)	24.4±6.9	23.1±6.2	<0.001
Age of symptom onset (years)	58.4±12.3	N/A	0.302
Smoking, N (%)	177 (34.0)	324 (31.1)	0.240
Kellgren-Lawrence grading			
Grade 2, N (%)	266 (51.2)		
Grade 3, N (%)	120 (23.1)		
Grade 4, N (%)	134 (25.8)		

rs9340799, have been reported associations with knee OA in different populations, but the findings were controversial [13, 14]. In addition, to date, no study focused on the impact of gene-gene interaction in ESR1 gene on knee OA risk, so the aim of this study was to examine the genetic association of the ESR1 gene polymorphisms, and additional gene-gene interaction with knee OA in the Chinese Han population.

Materials and methods

Subjects

All participants were consecutively recruited between June 2011 and June 2015 from the Provincial Hospital Affiliated to Shandong University. The case group included 520 subjects with knee OA, who were diagnosed according to American College of Rheumatology criteria, which include primary OA with any symptom and/or sign of OA, positive finding on radiographs according to the Kellgren-Lawrence grading [15], and no evidence of arthritis due to other disease. Healthy controls were randomly selected from volunteers in the same regions and approximate 1:2 matched to knee OA cases on the basis of age (±3 years) and sex. A total of 1562 participants (618 males, 944 females) were selected, including 520 knee OA patients and 1042 normal controls. The mean age of all participants was 62.9±15.8 years. Data on demographic and clinical information for all participants was obtained by questionnaire investigation. Only after obtaining signed informed consent, a 5-ml blood sample was drawn from each patient into tubes containing EDTA. The protocol of this study was approved by the Ethics Committee of Shandong University.

Genomic DNA extraction and genotyping

Genomic DNA was prepared from whole blood samples using the DNA Blood Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions and stored at 20°C until use. The polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method was used for genotyping of ESR1 gene. The primers for the selected SNPs were shown in **Table 1**. The reaction volume was 25 mL: 5 mL DNA at 100 ng/mL, 15.0 mL Dream-Taq Green PCR master mix (lot 00141171; Fermentas, Germany), 0.5 mL of each primer (25 pmol/mL) and 4.0 mL H_2O. Thermal cycling conditions were 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, PvuII or BtgI endonucleases under conditions specified by the supplier (New England Biolabs, Inc., Beverly, MA, USA). Genotyping results were confirmed by randomly assaying 10% of the original specimens for replication to exclude genotyping errors. There were no discrepancies between genotypes determined in duplicate.

Statistical analysis

In this study, SPSS 22.0 software package (SPSS Inc, Chicago) for Windows 7 (Microsoft

Table 3. Genotype and allele frequencies and analysis on association between 3 SNPs and knee OA risks

SNP	Genotypes and Alleles	Frequencies N (%)		OR (95% CI)*	HWE test for controls
		Controls (n=1042)	Cases (n=520)		
rs2234693 PvuII	Additive				
	TT	665 (63.8)	260 (50.0)	1.00	0.234
	TC	327 (31.4)	209 (40.2)	1.54 (1.20-1.91)	
	CC	50 (4.8)	51 (9.8)	1.82 (1.26-2.43)	
	Dominant				
	TT	665 (63.8)	260 (50.0)	1.00	
	TC+CC	377 (36.2)	260 (50.0)	1.61 (1.22-2.12)	
	Allele, C (%)	427 (20.5)	311 (29.9)		
rs9340799 XbaI	Additive				
	AA	618 (59.3)	276 (53.1)	1.00	0.694
	AG	366 (35.1)	201 (38.6)	1.23 (0.95-1.57)	
	GG	58 (5.6)	43 (8.3)	1.45 (0.89-2.08)	
	Dominant				
	AA	618 (59.3)	276 (53.1)	1.00	
	AG+GG	424 (40.7)	244 (46.9)	1.28 (0.93-1.76)	
	Allele, G (%)	482 (23.1)	287 (27.6)		
rs2228480/594 (G>A), BtgI	Additive				
	GG	684 (65.6)	269 (51.7)	1.00	0.812
	GA	319 (30.6)	213 (41.0)	1.70 (1.43-2.06)	
	AA	39 (3.8)	38 (7.3)	1.84 (1.32-2.42)	
	Dominant				
	GG	684 (65.6)	269 (51.7)	1.00	
	GA+AA	358 (34.4)	251 (48.3)	1.75 (1.40-2.13)	
	Allele, A (%)	397 (19.0)	289 (27.8)		

*Adjusted for gender, age, BMI and smoking.

Corp, Redmond, Wash) was used for statistical analyses. Mean and SD were calculated and compared between cases and controls for normally distributed continuous variables using Student's t test, and percentages were calculated and compared for categorical variable between cases and controls using χ^2 test. Hardy-Weinberg equilibrium test and Pairwise LD analysis were also conducted for genotype frequencies using SNPstats (http://bioinfo.iconcologia.net/SNPstats). Generalized multifactor dimensionality reduction (GMDR) was used to screen the best interaction combination among SNPs. Logistic regression was performed to investigate association between 3 SNPs within ESR1 gene and knee OA risk and additional gene-gene interaction between rs2228480 and rs9340799. All reported *P*-values were two-tailed, and those less than 0.05 were considered statistically significant.

Results

Baseline characteristics of all the subjects were shown in **Table 2**. A total of 1562 participants (618 males, 944 females) were selected, including 520 knee OA patients and 1042 normal controls. The mean age of all participants was 62.9±15.8 years. The mean of age and the distribution of gender and smoking were not significantly different between cases and controls. The mean of BMI is higher in cases than that in controls.

Table 3 shows the frequencies of genotype and allele for 3 SNPs and analysis on association between SNPs and knee OA risk. Logistic analysis showed that rs2234693 and rs2228480 within ESR1 gene were both associated with increased knee OA risk in both additive and dominant models, after adjustment for gender, age, BMI and smoking. The carriers of homozy-

Table 4. GMDR analysis on the best gene-gene interaction models

Locus no.	Best combination		Cross-validation consistency	Testing accuracy	*P-values*[*]
Gene-gene interactions[*]					
2	rs2228480	rs9340799	9/10	0.6072	0.0010
3	rs2228480 rs2234693	rs9340799	8/10	0.5399	0.1719

[*]Adjusted for gender, age, BMI and smoking.

Table 5. Interaction analysis for rs2234693 and rs9340799 by using logistic regression

rs2234693	rs9340799	OR (95% CI)[*]	*P-values*
TT	AA	1.00	-
TC or CC	AA	1.38 (1.02-1.84)	0.032
TT	AG or GG	1.45 (1.13-1.92)	0.002
TC or CC	AG or GG	2.32 (1.52-3.23)	<0.001

[*]Adjusted for gender, age, BMI and smoking.

gous mutant of rs2234693 and rs2228480 polymorphism have higher OA risk than those with wild-type homozygotes, OR (95% CI) were 1.61 (1.22-2.12) and 1.75 (1.40-2.13), respectively. We did not find any relation of rs9340799 with knee OA risk in two models.

We also investigate the impact of the interaction among 3 SNPs within ESR1 gene on knee OA risk by using GMDR model. **Table 4** shows a significant two-locus model (P=0.0010) involving rs2228480 and rs9340799, indicating a potential gene-gene interaction between rs2228480 and rs9340799 on knee OA risk, Overall, the cross-validation consistency of this two-locus model was 9/10, and the testing accuracy was of 60.72%. In order to investigate the interaction effect between the significant model, we also conducted an interaction analysis by using logistic regression (**Table 5**), to obtain the odds ratios and 95% CI for the joint effects. We found that participants with rs2228480-TC or CC and rs9340799-AG or GG genotype have the highest knee OA risk, compared to participants with rs2228480-TT and rs9340799-AA genotype, OR (95% CI) was 2.32 (1.52-3.23).

Pairwise LD analysis between the 3 SNPs was measured, and just D'value between rs2234693 and rs2228480 was more than 0.8 (0.847) (**Table 6**). The most common haplotype was rs2234693-T and rs2228480-G haplotype, the frequency of which was 0.4328 and 0.4805 in the case and control group respectively. Haplotype containing the s2234693-C and rs2228480-A allele were associated with a statistically increased knee OA risk, OR (95% CI)=1.76 (1.38-2.18), *P*<0.001) (**Table 6**).

Discussion

In this study, we found that both rs2234693 and rs2228480 within ESR1 gene were both associated with increased knee OA risk in Chinese population. The carriers of homozygous mutant of rs2234693 and rs2228480 polymorphism have higher OA risk than those with wild-type homozygotes. We did not find any relation of rs9340799 with knee OA risk in two models. Although our study is not the first case-control study to investigate the association between the human ESR1 gene polymorphisms and knee OA risk, previously several studies were conducted to investigate this relationship, however, considering the inconsistent results from previous studies, perhaps substantially different results [7, 13, 14, 16-20], we think the results obtained from this study is necessary and meaningful for understanding the mechanism of knee OA, particularly for susceptibility lead by ESR1 gene polymorphisms. Hu et al [19] conducted a meta-analysis and indicated that ESR1 rs2234693 (T/C) polymorphism may be associated with a reduced OA risk among Chinese and the rs9340799 (A/G) polymorphism may not be associated with OA risk. Another meta-analysis [20] also suggested a weak relationship between the ESR1 rs9340799 polymorphism and OA in Europeans but not Asians, and that the ESR1 rs2234693 polymorphism was not associated with OA in either population. Ma et al [21] indicated that rs9340799 and rs2228480 rather than rs2234693 polymorphisms are associated with the decreased incidence of OA. A recent study [22] for Mexican mestizo population suggested that ESR1 gene CG haplotype could be associated with a reduced risk of primary knee OA. Jin et al [18] conducted a case-control study and sug-

Table 6. Haplotype analysis on association between ESR1 gene and knee OA risk

Haplotypes	rs2234693	rs2228480	Frequencies		OR (95% CI)	*P*-values*
			Case group	Control group		
H1	T	G	0.4328	0.4805	1.00	--
H2	C	G	0.2909	0.2788	1.31 (0.95-1.72)	0.421
H3	T	A	0.1955	0.1958	1.42 (0.89-2.05)	0.126
H4	C	A	0.0808	0.0449	1.76 (1.38-2.18)	<0.001

*Adjusted for gender, age, BMI and smoking.

gested that rs2228480 minor allele was associated increased knee OA risk. However, another meta-analysis by Wang et al [23] indicated that the rs2234693 polymorphism is associated with an increased knee OA risk. A Chinese study [7] found that the presence of two rs9340799 restriction sites (xx) in the ESR1 gene is associated with increased susceptibility to OA in Han Chinese females. These findings were consistent with results obtained from studies for other ethnic groups [22, 24]. Another study for 1,483 older Dutch patients with OA showed that polymorphisms of the ER gene were correlated with OA, and the PX allele was associated with a markedly increased prevalence of radiographic knee osteoarthritis [25], thus, they think that rs2228480 and rs2234693 genotypes of ESR1 appear to contribute to the pathogenesis of OA in a variety of populations. A recent meta-study including 8,792 subjects indicated that the rs9340799 polymorphism might be associated with increased knee OA risk, but they also think that the results should be interpreted with caution because of the publication bias, so the results should be confirmed by further case-control studies with large sample size.

Besides of the inconsistent results reported in previous studies, the multiple diseases, including knee OA, was a result of many gene polymorphism and gene-gene interactions, hence, if we want to know the potential mechanism on knee OA susceptibility, we need to investigate the impact of ESR1 gene-gene interactions on knee OA risk, which have not been reported previously. In this study, we found a significant gene-gene interaction between rs2228480 and rs9340799 on knee OA risk, participants with rs2228480-TC or CC and rs9340799-AG or GG genotype have the highest knee OA risk, compared to participants with rs2228480-TT and rs9340799-AA genotype. Loci that are located nearby on the same chromosome may be in linkage disequilibrium (LD). This means that alleles at these loci are not inherited in an independent manner but certain allele combinations occur more often than expected by random segregation. We found a significant haplotype containing the s2234693-C and rs2228480-A allele, which was associated with a statistically increased knee OA risk. In addition, the SNPs in the interaction model were different with SNPs in the haplotype combination, so the significant interaction found in our study was not because of linkage effect between SNPs.

Our study has several limitations. Firstly, the sample in present study was relatively small, study with large sample size are needed to confirm the results in the future. Secondly, the number of SNP was limited, more SNPs within ESR1 gene should been included in the study. Thirdly, gene-environment interaction should be investigated in the future studies.

In conclusion, we found that rs2234693 and rs2228480 within ESR 1 gene, interaction between rs2228480 and rs9340799 and haplotype containing the s2234693-C and rs2228480-A allele were associated with a statistically increased knee OA risk.

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Disclosure of conflict of interest

None.

Address correspondence to: Shui Sun, Department of Joint Surgery, Shandong Provincial Hospital Affiliated to Shandong University, 324 Jing Wu Wei Qi

Road, Jinan 250021, Shandong, People's Republic of China. Tel: +86- 539-8078275; E-mail: sunshuihhf57@163.com

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