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The correlation between estrogen receptor gene polymorphism and osteoporosis in Han Chinese women

D. XIANG, J. HE, T. JIANG

Orthopedic Department, The First Affiliated Hospital of Chengdu Medical College, Chengdu, Sichuan, China

Deng Xiang and *Jun He* contribute equally to this research and should be considered as co-first author

Abstract. – OBJECTIVE: To uncover the role of estrogen receptor gene polymorphism in the onset of osteoporosis in Han Chinese women.

PATIENTS AND METHODS: A total of 122 osteoporosis woman patients who were admitted to this hospital between April 2016 and April 2017 were enrolled in this study as the case group, and during the same period, 106 healthy counterparts who took physical examination as the control group. With the genetic samples collected from subjects in two groups, we detected the polymorphisms of Pvu II and Xba I in the estrogen receptor alpha (ERα) gene and the Rsa-I and Aiu-I polymorphisms in the ERβ gene by Restriction Fragment Length Polymorphism (RFLP), and the related-alleles frequency in subjects carrying the genotype of Pvu-1I and Xba-I polymorphisms in the ERα gene or the genotype of Rsa-I and Alu-I polymorphisms in the ERβ gene in the two groups.

RESULTS: Comparison of genotype frequencies pp, Pp, and PP of ERα Pvu-II polymorphisms between the case group and the control group showed the differences were statistically significant ($p < 0.05$), in which the P allele in the case group had a higher frequency than that in the control group ($p < 0.05$). However, comparisons of the genotype frequencies of xx, Xx, and XX of ERα Xba-I polymorphisms between the case group and the control group showed no statistically significant differences ($p > 0.05$), and similar results were also found in comparison of the genotype frequencies of rr, Rr, and RR of Rsa-I polymorphisms ($p > 0.05$). By the comparison of genotype frequencies of ERβ Alu-I and Rsa-I polymorphisms in the case group with those in the control group, and by the comparison of genotype frequencies aa, Aa, and AA of ERβ Alu-I polymorphisms in the case group with those in the control group, all the differences were statistically significant ($p < 0.05$).

CONCLUSIONS: In Han Chinese women, susceptibility to osteoporosis may be affected by ERα Pvu-II polymorphisms and ERβ Alu-I polymorphisms; those carrying genotypes containing A and P alleles may have a higher risk in osteoporosis.

Key Words:
Osteoporosis, Estrogen receptor (ER), Gene polymorphism, Han nationality.

Introduction

Osteoporosis is a metabolic bone disease[1] characterized by bone loss and bone microstructural destruction, leading to decreased bone strength, increased brittleness and easy fracture. With the aging of the population and the extension of life expectancy, the incidence of osteoporosis has increased year by year, and has become one of the major diseases affecting the quality of life of our elderly[2]. Osteoporosis is a common pathological state in the elderly, characterized by bone mineralization, bone loss and normal bone microstructure changes, which is caused by increased bone resorption and decreased bone formation[3]. As the incidence of osteoporosis is mainly affected by the role of genes, a large number of clinical and experimental studies at home and abroad are trying to find the genes related to the incidence of osteoporosis, and to prevent and treat osteoporosis from the level of genes[4,5]. A major factor of osteoporosis in postmenopausal women is estrogen deficiency. Relevant data show that lower estrogen levels can increase the bone turnover rate, and accelerate bone loss, resulting in increased bone fra-

Corresponding Author: Tao Jiang, MD; e-mail: zzzxsh@163.com

gility, easily lead to the occurrence of fractures[6]. Estrogen receptor (ER) gene has been attracting more and more attention in recent years. ER is a group of nuclear receptor superfamily members with the role of transcription factor in the nucleus. There are two types of estrogen receptors, i.e., ERα and ERβ. The estrogen receptor gene is an early gene that is found to be associated with osteoporosis, which is located on the long arm of chromosome 6, consisting of 8 exons and 7 introns, about 140 kB in length[7]. Some data show[8] that, ER gene mutation can affect the body ER expression level and its function, thus affecting the biological effects of estrogen *in vivo*. At present, there is little research on the correlation between ER gene polymorphisms and osteoporosis. The aim of this study is to investigate the relationship between ER gene polymorphisms and osteoporosis in order to establish early screening of osteoporotic markers, thus achieving early prevention and early intervention of osteoporosis, and reducing the incidence of osteoporosis rate.

Patients and Methods

Patients

A total of 122 female patients with osteoporosis of Han nationality and whose parents are also Han people admitted to our hospital from April 20 to April 2017 were selected as the case group. The patients were aged 60-78 years old, with an average of (68.3 ± 2.1) years old. The body weight was 47-58 kg, with an average of (50.4 ± 1.7) kg. Inclusion criteria: local Han women, and their parents are local Han people; no heart, liver and other vital organs diseases; willing to participate in this study, and signed informed consent. Exclusion criteria: Patients who took drugs that have affected bone metabolism; whose osteoporosis caused by rheumatoid arthritis, hyperthyroidism, glucococcal disease, osteoarticular tuberculosis and severe liver and kidney disease; and who had incomplete clinical data. At the same time, 106 cases of healthy volunteers of Han nationality who underwent physical examination in our hospital and whose parents are Han people were selected as the control group. The volunteers were aged 60-79, with an average of (68.5 ± 2.3) years old; the weight was 46-57 kg, with an average of (50.2 ± 1.4) kg. There was no significant difference in the age and weight of the two groups ($p > 0.05$).

Methods

Main Reagents and Instruments

DNA extraction kit (Tiangen Biotech (Beijing) Co., Ltd.); dNTP Mix, 10X × Ex TaqBuffer, Ex Taq enzyme (Shanghai Yeasen Biotechnology Co., Ltd.); agarose (Beijing Wobisen Technology Co., Ltd.); restriction enzymes Pvu-II, Xba-I, A1u-I, and RsA-I (Beijing Biorad Life Science Development Co., Ltd.). PCR Gene Amplifier (Esco Shanghai Trading Co., Ltd.), electrophoresis instrument (Beijing Baygene Biotech Co., Ltd.), UV gel imaging analyzer (Shanghai Chitang Electronics Co., Ltd.).

Blood Sample Collection and Genomic DNA Extraction

The subjects were sacrificed 5 mL of fasting cubital venous blood, anticoagulated with ethylenediaminetetraacetic acid and placed under the condition of -80°C. DNA extraction was carried out by genomic DNA extraction kit, and DNA extraction was carried out on blood samples. The experimental operation was carried out strictly according to the kit instructions.

Genotyping Tests of ER α and β

The Cleaved Amplified Polymorphisms of ERα Rva-II and Xba-I, and ERβ Rsa-I and A1u-I in the two groups of subjects were analyzed by PCR restriction fragment length polymorphism. The design and synthesis of ERα and ERβ gene primers, respectively, refer to reference[9] and[10], of which, the forward gene primer of ERα Pvu-II and Xba-I was 5'-CTGCCACCCTATCTGTATCTTTTCCTATTCTCC-3', the primer length was 25 bp, and the length of the amplified product was 1.3kb; the reverse primer was 5'-TCTTTCTCTGCCACCCTGGCGTCGATTATCT GA-3, the primer length was 25 bp and the length of the amplified product was 1.3 kb. The forward gene primer of ERβ Alu-I was 5'-TTTTTGTCCCCATAGTAACA-3', the length of the primer was 20 bp, and the length of the amplified product was 310 bp; the reverse primer was 5'-AATGAGGGACCACAGCA-3', the length of the primer was 20 bp, and the length of the amplified product was 310 bp. The forward gene primer of ERβ RSA-I was 5'-TCTTGCTTTCCCCAGGCTTT-3', the length of the primer was 20bp, and the length of the amplified product was 200 bp; the reverse primer was 5'-ACCTGTCCAGAACAAGATCT-3', the length of the primer was 20 bp, and the length of the amplified

product was 200 bp. PCR reaction conditions: at 94°C pre-denaturated for 5 min, 1 cycle; At 94°C denaturated for 30 sec, ERα Pvu-II, Xba-I and ERβ Alu-I, RSA-I were annealed at 68°C, 55°C, and 60°C for 30 s, at 72°C extended for 30 s, a total of 35 cycles; in the last one cycle, at 72°C extended for 7 min. PCR amplification products were stored at -20°C. 10 μL of each PCR amplification product was taken and used for electrophoresis with 2% agarose gel, and the electrophoresis voltage was 120 V for 30 min. If the DNA band appeared at the corresponding position in the electrophoretic result, it was determined that the amplification was successful. 10 μL of each PCR amplification product was taken for enzymatic reaction with restriction enzyme Pvu-II (10 U), Xba-I (10 U), Alu-I (8 U) and RSA-I (10 U), respectively. The products of enzymatic reactions were used for electrophoresis with 2% agarose gel, and the electrophoresis voltage was 120 V for 30 min. After electrophoresis, the electrophoretic results were observed by UV gel imaging analyzer to determine ERα and ERβ genotyping.

Statistical Analysis

The SPSS 13.0 statistical software package (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. Genotype, allele frequency and other count data were expressed by rate (%). The genotype and allele frequencies of the two groups were calculated by direct counting method. After the Hardy–Weinberg equilibrium test, X^2-test was used to compare the genotype and allele frequencies of the two groups. Tukey's HSD (honestly significant difference) test is used in conjunction with an ANOVA to find means that are significantly different from each other. The difference was statistically significant with $p < 0.05$.

Results

Comparison of Gene Polymorphisms of ERα Pvu-II and Xba-I

ERα gene was digested with restriction enzyme Pvu-II, which could distinguish three genotypes: one band of PP type, with the length of 1.3 kb; three bands of Pp type, with the lengths of 1.3 kb, 850 bp, and 450 bp, and two bands of pp type, with the lengths of 850 bp and 450 bp (Figure 1). ERα gene was digested with restriction enzyme Xba-I, which could distinguish three genotypes: one band of XX type, with the length of 1.3 kb; three bands of Xx type, with the lengths of 1.3 kb, 910 bp, and 390 bp, and two bands of xx type, with the lengths of 910 bp and 390 bp (Figure 2).

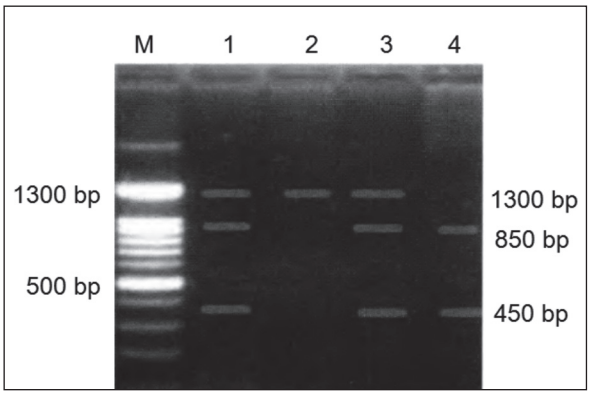

Figure 1. ERα Pvu-II enzyme electrophoresis (M: DNA molecular weight markers, 1, 3: Pp genotype, 2: PP genotype, 4: pp genotype).

The genotype frequencies of ERα Pvu-II and Xba-I polymorphisms in the case group and the control group were consistent with Hardy-Weinberg equilibrium law, and featured with group representation. There were no significant differences ($p > 0.05$). By the comparison of genotype frequencies pp, Pp, and PP of ERα Pvu-II polymorphisms in the case group with those in the control group, the difference was statistically significant ($p < 0.05$). The frequency of P allele in the case group was significantly higher than that in the control group, the difference was also statistically significant ($p < 0.05$) (Table I). By the comparison of genotype frequencies xx, Xx, and XX of ERαXba-I polymorphisms in the case group and in the control group with

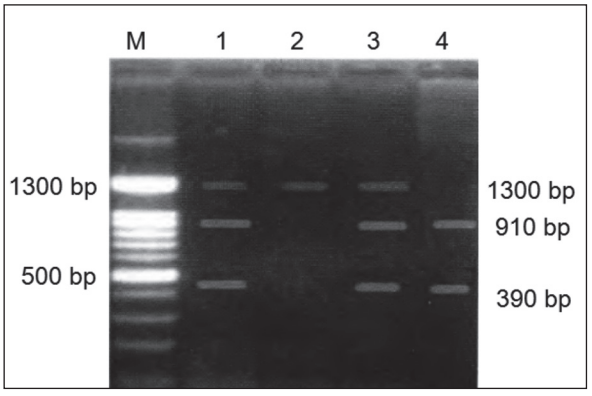

Figure 2. ERα Xba-I enzyme electrophoresis (M: DNA molecular weight markers, 1, 3: Xx genotype, 2: XX genotype, 4: xx genotype).

Table I. Comparison of genotype frequencies of ERα Pvu-II polymorphisms with allele frequencies in two groups [number of cases (%)].

Group	No. of cases	Genotype frequency			Allele frequency	
		PP	Pp	pp	P	p
Case group	122	25 (20.5)	50 (41)	56 (45.9)	90 (36.9)	154 (63.1)
Control group	106	2 (1.9)	48 (45.3)	54 (50.9)	44 (20.8)	168 (79.2)
X^2		16.789			14.224	
p		0.000			0.000	

Note: Genotype frequency (%) = number of the genotype frequency / number of samples × 100%, allele frequency (%) = number of the genotype frequency / (2 × number of samples) × 100%.

Table II. Comparison of genotype frequencies of ERαXba-I polymorphisms with allele frequencies in two groups [number of cases (%)].

Group	No. of cases	Genotype frequency			Allele frequency	
		XX	Xx	xx	X	x
Case group	122	8 (6.6)	56 (45.9)	52 (42.6)	74 (30.3)	170 (69.7)
Control group	106	7 (6.6)	41 (38.7)	51 (48.1)	60 (28.3)	152 (71.7)
X^2		1.058			0.224	
p		0.589			0.636	

Note: Genotype frequency (%) = number of the genotype frequency / number of samples × 100%, allele frequency (%) = number of the genotype frequency / (2 × number of samples) × 100%.

allele frequencies X and x, respectively, none of the differences were statistically significant ($p > 0.05$) (Table II).

Comparison of Gene Polymorphisms of ERĐ Alu-I and Rsa-I

ERβ gene was digested with restriction enzyme Alu-I, which could distinguish three genotypes: one band of aa type, with the length of 307 bp; three bands of Aa type, with the lengths of 307, 240, and 67 bp, and two bands of AA type, with the lengths of 240 and 67 bp (Figure 3). ERβ gene was digested with restriction enzyme Rsa-I, which could distinguish three genotypes: one band of rr type, with the length of 156 bp; three bands of Rr type, with the lengths of 156, 125 and 31 bp, and two bands of RR type, with the lengths of 125 and 31 bp (Figure 4). The genotype frequencies of ERβ Alu-I and Rsa-I polymorphisms in the case group and the control group were consistent with Hardy-Weinberg equilibrium law and featured with group representation. There were no significant differences ($p > 0.05$). By the comparison of genotype frequencies aa, Aa, and AA of ERβ Alu-I polymorphisms in the case group with those in the control group, the differences were statistically significant ($p < 0.05$). The frequency of P allele in the case group was significantly higher than that in the control group, and the difference was also statistically significant ($p < 0.05$) (Table III).

By the comparison of genotype frequencies rr, Rr, and RR of Rsa-I polymorphisms in the case group and in the control group with allele frequencies r and R, respectively, none of the differences were statistically significant ($p > 0.05$) (Table IV).

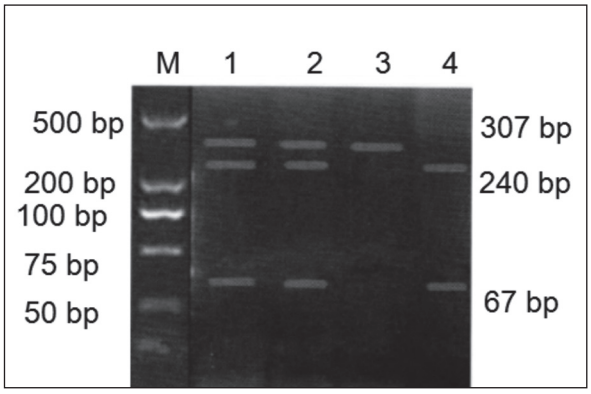

Figure 3. ERβ A1u-I enzyme electrophoresis (M: DNA molecular weight markers, 1, 2: Aa genotype, 2: aa genotype, 4: AA genotype).

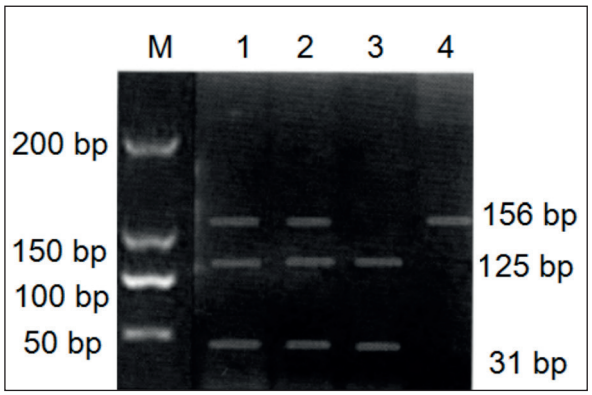

Figure 4. ERβ Rsa-I enzyme electrophoresis (M: DNA molecular weight markers, 1, 2: Rr genotype, 2: RR genotype, 4: rr genotype).

Discussion

Osteoporosis is a complex disease in which genetic and environmental factors work together. Female postmenopausal osteoporosis is a progressive bone loss occurred after menstrual cessation due to ovarian function decline caused by natural or surgical and other causes. It is mainly due to the lack of estrogen, but the detailed pathogenesis is still not very clear[11]. Female postmenopausal osteoporosis is a serious metabolic bone disease that seriously affects the health of the elderly. The hindrance and mortality of the hip fracture are extremely high, resulting in a great social burden and greatly shortening the life expectancy of the patients. At present, people have been exploring osteoporosis a lot, and also have accumulated a lot of valuable experience. However, the previous study was more concentrated in bone mineral density and bone morphology, and there was less research on the genetic types of osteoporosis[12]. ER gene is a gene that is found earlier in relation to osteoporosis. The ER gene may be different with countries and races. Relevant data[13] show that, the incidence of osteoporosis and breast cancer and other female diseases in postmenopausal women, may be related to ER gene polymorphisms. Therefore, the study on the relationship between genetic polymorphisms and osteoporosis and other diseases, helps from the genetic level to elucidate the pathogenesis of the above diseases. At present, the study on ER Pvu-II and Xba-I polymorphisms is the most extensive in the world. The study on the polymorphisms of the introns of ERα gene was mainly focused on the cleavage sites of the first intron Pvu-II

Table III. Comparison of genotype frequencies of ERβ Alu-I polymorphisms with allele frequencies in two groups [number of cases (%)].

Group	No. of cases	Genotype frequency			Allele frequency	
		aa	Aa	AA	A	a
Case group	122	48 (39.3)	48 (39.3)	11 (9)	72 (29.5)	172 (70.5)
Control group	106	70 (66)	29 (27.4)	2 (1.9)	38 (17.9)	174 (82.1)
X^2		14.860			8.316	
p		0.001			0.004	

Note: Genotype frequency (%) = number of the genotype frequency / number of samples × 100%, allele frequency (%) = number of the genotype frequency / (2 × number of samples) × 100%.

Table IV. Comparison of genotype frequencies of ERβ Rsa-I polymorphisms with allele frequencies in two groups [number of cases (%)].

Group	No. of cases	Genotype frequency			Allele frequency	
		rr	Rr	RR	R	r
Case group	122	25 (20.5)	50 (41)	37 (30.3)	126 (51.6)	108 (48.4)
Control group	106	22 (20.7)	62 (58.5)	30 (28.3)	110 (51.9)	102 (48.1)
X^2		2.191			0.171	
p		0.334			0.679	

Note: Genotype frequency (%) = number of the genotype frequency / number of samples × 100%, allele frequency (%) = number of the genotype frequency / (2 × number of samples) × 100%.

and Xba-I of ERα gene[14]. Among them, Pvu-II polymorphisms are caused by the occurrence of T-C point mutation in the first intron at about 0.4 kb from the upstream of the second exon, and the Xba-I polymorphisms are caused by G to A replacement at 50 bp from the downstream of Pvu-II, leading to the polymorphisms of ERα Pvu-II and Xba-I. The results of this study showed that by the comparison of genotype frequencies pp, Pp and PP in the ERα Pvu-II polymorphisms in the case group with those in the control group, the differences were statistically significant ($p < 0.05$). The frequency of P allele in the case group was significantly higher than that in the control group, and the difference was statistically significant ($p < 0.05$). By the comparison of genotype frequencies xx, Xx and XX in the ERα Xba-I polymorphisms in the case group and the control group with allele frequencies X and x, respectively, none of the differences were statistically significant ($p < 0.05$). It is suggested that the polymorphisms of ERα Pvu-II may be related to the susceptibility of osteoporosis in Han women in the region, and the genotype containing P allele may increase the risk of osteoporosis, while there was no correlation between the polymorphisms of ERα Xba-I and the risk of osteoporosis in Han women. The study of ERβ gene polymorphisms is mainly concentrated in the exon. GA point mutation can occur in the ligand binding region of the fifth exon (No.1082 nucleotide), and AG point mutation can occur in the 3 non-coding region (NCR) of the eighth exon (No. 1730 nucleotide); after these two point mutations occur, the recognition sites of restriction enzymes RsA-I and Alu-I are shown[15]. Thus, the ERβ gene fragment digested by restriction enzymes RsA-I and Alu-I can differentiate the different genotypes of the ERβ gene. ERβ gene polymorphisms may affect the expression levels and functions of ERβ, and thus affect the biological effects of estrogen. The results of this study showed that by the comparison of the genotype frequencies of ERβ Alu-I and Rsa-I polymorphisms in the case group and the control group, and by the comparison of genotype frequencies aa, Aa, and AA of ERβ Alu-I polymorphisms in the two groups, the differences were statistically significant ($p<0.05$). The frequency of allele A in the case group was significantly higher than that in the control group ($p < 0.05$). By the comparison of genotype frequencies rr, Rr, and RR of Rsa-I polymorphisms in the case group and the control group with allele frequencies r and R, there were no significant differences ($p > 0.05$). It is suggested that the Alu-I polymorphisms of ERβ gene may be related to the susceptibility of osteoporosis in Han women in this region, and the genotype containing allele A may increase the risk of osteoporosis, and there was no correlation between the Rsa-I polymorphisms of ERβ gene and the risk of osteoporosis in Han women. The limitations of this study are that the number of subjects is limited, and only limited to a certain region and a country. In the future, the sample size can be increased to carry out cross-regional, multi-national, multi-ethnic research to further explore the specific role of the ER gene in the pathogenesis of osteoporosis.

Conclusions

We observed that in Han Chinese women, susceptibility to osteoporosis may be affected by ERα Pvu-II polymorphisms and ERβ Alu-I polymorphisms; those carrying genotypes containing A and P alleles may have a higher risk in osteoporosis.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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