Original Article
Estrogen receptor alpha gene polymorphisms contribute to fragility fracture susceptibility in elderly postmenopausal women with heart failure

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Abstract: Objective: The association between estrogen receptor alpha (ESR1) gene PvuII polymorphism and fracture risk is still ambiguous and inconclusive. Furthermore, this relationship in postmenopausal women with heart failure (HF) is rarely reported. The aim of the present study was to evaluate the effect of the PvuII polymorphism (rs2234693, C>T) of the ESR1 gene on fragility fractures in elderly postmenopausal women with HF. Methods: This was a hospital-based case-control study of postmenopausal women >60 years of age with HF, including 80 fracture patients and 80 controls between January 2009 and January 2014. The PvuII genotype was determined using a polymerase chain reaction-restriction fragment length polymorphism assay. Results: Fragility fracture was more common in smokers and those with a history of fragility fracture and less common in subjects who exercised daily. The PvuII T allele was present in 83.75% of patients with fractures vs. 62.50% of controls. Carriers of the variant rs2234693T allele had increased risk of fracture (P<0.05). Compared with the common genotype, the CT+TT rs2234693 genotype was associated with significantly increased risk of fracture (P = 0.036, adjusted odds ratio = 1.323, 95% confidence interval = 1.263-2.787). In stratified analyses, the association between the risk of fracture and the rs2234693 variant was more prominent in younger individuals (≤67 years) and non-smokers. Conclusion: The PvuII polymorphism (rs2234693, C>T) of the ESR1 gene may contribute to the development of fragility fractures in elderly postmenopausal women with HF.

Keywords: Estrogen receptor, postmenopausal women, heart failure, fragility fracture, gene polymorphisms

Introduction

Osteoporosis is a progressive systemic skeletal disease characterized by low bone mass and microarchitectural deterioration of bone tissue, with a consequent increase in bone fragility and susceptibility to fracture [1, 2]. The disease is highly prevalent in postmenopausal women [3]. Approximately one third of women older than 50 years will experience a fragility fracture, as will one in five men [4].

Fragility fracture affects quality of life in elderly postmenopausal women and contributes significantly to increased disability rates. Although the relationship between osteoporotic bones and the risk of non-union [5] and fixation loss [6] remains under debate, fast skillful surgery

with minimal tissue injury is desirable [5, 7], rather than prolonged bed rest and high-dose narcotics. Furthermore, the outcomes of conservative treatment are unsatisfactory [8] because a percentage of the comminuted, osteoporotic low energy fractures will be porous or unstable fractures that require operative stabilization [9]. Overall, the need for surgery to repair fragility fractures in the elderly is becoming more apparent as life expectancy and health requirement increase [9].

Heart failure (HF) is a complex clinical syndrome that results from any structural or functional impairment of ventricular filling or ejection of blood [10], and it has a high incidence in the elderly [11]. In patients with severe medical conditions, orthopedic surgery cannot be per-

formed because these patients cannot be anesthetized, as the perioperative cardiovascular risk in noncardiac surgical procedures is elevated [12], for example in patients with severe HF [13-15]. Studies show that pre-fracture cardiovascular disease is a strong predictor of post-operative cardiac failure [12] that may lead to increased mortality during hospitalization [16]. Considering the complications associated with fracture and subsequent operation, it is necessary and urgent to develop effective interventions to reduce the incidence of fragility fractures in high-risk populations, particularly in patients with HF.

Recently, numerous studies have attempted to explore the pathogenesis of this disease [17-20]. Bone mineral density (BMD) is an important clinical predictor of fracture risk. Age and gender are two factors that affect the fracture risk independently of BMD values [21, 22]. Significant progress has been made in recent years to identify genes and alleles affecting hip fracture risk, such as the vitamin D receptor (VDR), insulin-like growth factor I (IGF-I), collagen type I alpha 1 (COL1A1), and estrogen receptor α (ESR1) genes [23, 24].

The estrogen receptor α, a member of the nuclear receptor superfamily of ligand-activated transcription factors, is one of the key mediators of hormonal responses in estrogen-sensitive tissues [25, 26]. The estrogen-ESR1 complex is primarily responsible for regulating cellular signaling pathways *in vivo*, as well as bone mass in skeletal systems [27, 28]. Several genetic polymorphisms of the ESR1 gene, including ESR1 XbaI (rs9340799, A>G) and PvuII (rs2234693, C>T), have been investigated for their possible association with fracture risk [29-32]. However, two previous meta-analyses suggested that the *ESR1* gene *Pvu*II polymorphism is not associated with fracture risk in postmenopausal women [17, 33]. The specific association between PvuII polymorphism and fracture in postmenopausal women remains controversial [17, 29-36].

To the best of our knowledge, the relationship between *ESR1 Pvu*II (rs2234693, C>T) and fracture risk in postmenopausal women with HF has not been investigated to date. Furthermore, whether this polymorphism has functional consequences in postmenopausal women remains unclear. To resolve this issue,

we explored the association between the *ESR1* gene PvuII polymorphism in a hospital-based, case-control study of postmenopausal women >60 years of age and determined the susceptibility to fracture in this subpopulation.

Materials and methods

Ethics statement

This study was approved by the Ethics Committee of Jiangsu Province Geriatric Institute and written informed consent was obtained from all the subjects.

Subjects

This hospital-based, case-control study of elderly subjects with absence of menses for 2 years included 80 fracture patients and 80 controls with heart failure recruited from Suzhou Kowloon Hospital Affiliated to the Medical School of Shanghai Jiao Tong University between January 2009 and January 2014.

The Chinese participants were all from the same geographic region. Consecutive women admitted to the hospital because of fragility fractures were included. Control postmenopausal women were included if they did not meet the exclusion criteria. All eligible patients had heart failure with New York Heart Association (NYHA) class II-IV symptoms, an ejection fraction ≤40%, and a plasma B-type natriuretic peptide (BNP) concentration of ≥150 pg/ml (or an N-terminal pro-BNP [NT-proBNP] concentration of ≥600 pg/ml) or, if they had been hospitalized for heart failure within the previous 12 months, a BNP concentration of ≥100 pg/ml (or an NT-pro-BNP concentration of ≥400 pg/ml) [37-39]. Women were excluded if they had fractures of the toe, facial bone, and finger or fractures caused by excessive trauma (such as traffic accidents and falls from a height sufficient to cause fracture in a person without osteoporosis [40]), diseases causing secondary osteoporosis (cancer, rheumatoid arthritis, malabsorption, and thyroid gland dysfunction), or those taking drugs known to have a deleterious effect on bone metabolism (corticosteroids, anticonvulsants). Subjects with a history of alcohol abuse or severe renal and hepatic failure were also excluded from the study.

Table 1. Demographic information

Characteristics	Cases	Controls	P-value
Subjects (n)	80	80	
Age (years)	67.51 ± 5.92	67.59 ± 4.86	0.928
Menopause age (years)	48.83 ± 4.36	49.26 ± 3.74	0.514
BMI (kg/m^2)	23.47 ± 3.79	24.18 ± 3.64	0.230
SBP (mmHg)	135.18 ± 23.28	134.50 ± 17.81	0.838
DBP (mmHg)	83.43 ± 11.78	82.48±11.29	0.601
FPG (mmol/L)	5.92 ± 2.27	5.56 ± 1.56	0.231
TC (mmol/L)	5.04 ± 1.02	5.23 ± 0.99	0.229
TG (mmol/L)	2.03 ± 1.00	2.06 ± 1.14	0.862
HDL-C (mmol/L)	1.74 ± 0.55	1.74 ± 0.52	1.00
LDL-C (mmol/L)	3.15 ± 0.86	3.29 ± 0.98	0.340
TNF-α (pg/ml)	1.92 ± 0.85	2.14±1.65	0.298
IL-6 (pg/ml)	3.64 ± 2.28	3.92 ± 5.17	0.662
Smoking (n, %)	16 (20.00)	7 (8.75)	0.043
Daily exercise (n, %)	52 (65.00)	66 (82.50)	0.012
History of fragility fracture (n, %)	32 (40.00)	13 (16.25)	0.001
CHD (n, %)	10 (12.50)	11 (13.75)	0.815
Hypertension (n, %)	39 (48.75)	41 (51.25)	0.752
Dietary calcium supplement (n, %)	43 (53.75)	44 (55.00)	0.874

Clinical data collection

A structured questionnaire was used to retrieve information on the subjects, such as age, menopause age, hypertension, smoking or fragility fracture history, daily exercise, and calcium supplementation. The questionnaires were administered by trained interviewers who were not aware of the study hypothesis.

Height and weight were measured in a standing position wearing indoor clothing without shoes. BMI was computed as weight in kilograms divided by height in meters squared (kg/m^2). Blood pressure was measured in all subjects. Subjects were divided into non-smokers and smokers. Individuals who formerly or currently smoked ≥10 cigarettes per day for at least 2 years were defined as smokers. Individuals who had persistent systolic blood pressure >140 mm Hg and diastolic blood pressure >90 mm Hg and/or were currently receiving anti-hypertensive treatment were defined as hypertensive. Cholecalciferol 400 IU/day or other related drugs taken prior to admission for at least 2 years was regarded as dietary calcium supplementation. A history of fragility fracture was considered as a fracture that was not caused by significant or slight external trauma 6 months before the individuals were enrol-

led in the study. Evidence included radiologic or surgical procedure reports or a copy of the radiograph. Daily exercise consisted of aerobic training for at least 30 minutes three times per week or physical exercises at a higher intensity level for at least 20 minutes two times per week or for 1 h three times per week [37, 41]. In addition, women were also considered to exercise if they walked more than 3 km daily or were engaged in other sports or activities [29].

Blood analyses

Each patient donated 20 ml venous blood after fasting for 10 hours and after providing written informed consent. The blood specimens were separated into serum and plasma and placed in the ultra-cold storage freezer at -80°C. Serum fasting plasma glucose (FPG), total cholesterol (TC), triglyceride (TG), high-density lipoprotein cholesterol (HDL-C), and low-density lipoprotein cholesterol (LDL-C) were measured using standard procedures by automatic biochemistry analyzers at Suzhou Kowloon Hospital Affiliated to the Medical School of Shanghai Jiao Tong University. Tumor necrosis factor α (TNF-α) and interleukin-6 (IL-6) were measured in plasma samples by ELISA (Mercodia, Shanghai Kexing Biological Technology, China).

Genotyping

Genotypes for the ESR1 PvuII gene polymorphism (rs2234693, C>T) were determined through polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis. Based on the GenBank reference sequence, the PCR primers were as follows: forward, 5'-CTGCCACCCTATCTGTATCTTTTCCTA-TTCACC-3' and reverse, 5'-TCTTTCTCTGCCACC-CTGGCGTCGATTATCTGA-3'. Genomic DNA was extracted from whole blood samples collected at baseline from each study subject using the QIAamp DNA Blood Mini Kit (QIAGEN 51106, Valencia, CA, USA). The reaction solution (20

Table 2. The logistic regression analysis of multiple factors for fragility fracture

Characteristics	Crude OR (95 % CI)	P value	Adjusted OR (95% CI)[a]	P value
Smoking	2.607 (1.009, 6.738)	0.043	1.145 (1.082, 1.479)	0.046
Daily exercise	0.394 (0.188, 0.823)	0.012	0.497 (0.425, 0.512)	0.031
History of fragility fracture	3.436 (1.633, 7.227)	0.001	1.897 (1.786, 2.001)	0.012

[a]Adjusted for age, menopause age, BMI, smoking status, daily exercise, history of fragility fracture, CHD, hypertension and dietary calcium supplement. BMI: body mass index; CHD: coronary heart disease.
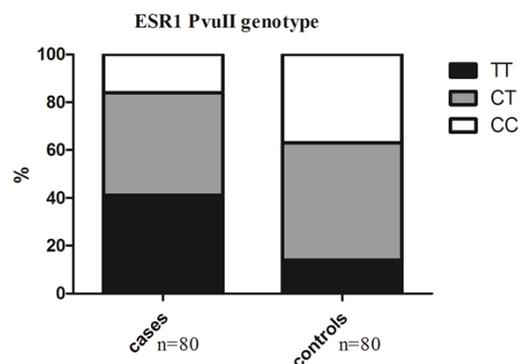

Figure 1. Percent distribution of patients with the ESR1 Pvull CC, CT and TT genotypes between cases and controls.

μL) was placed into each well of a 96-well reaction plate (the remainder of the reaction solution was used to prevent experimental errors), and 5 μL of DNA sample or water control was added to each tube. PCR amplification for each polymorphism was performed using a MJ Mini thermal cycler (Bio-Rad, Hercules, CA, USA) as described previously [42].

Statistical analysis

All statistical analyses were performed using SPSS 20.0 (SPSS Inc., Chicago, IL, USA). All tests were two sided and the criterion of statistical significance was set at $P<0.05$. Differences in demographic characteristics and genotype frequencies of ESR1 Pvull between cases and controls were calculated using Student's t-tests (for continuous variables) and χ^2 tests (for categorical variables). Stepwise logistic regression was performed to determine the significant independent predictors of fragility fracture.

The Hardy-Weinberg equilibrium was assessed for controls using the goodness-of-χ^2 test. Associations between the genotypes and alleles and risk of GC were estimated by odds ratios (OR) and 95% confidence intervals (CIs).

The crude OR was calculated using the Woolf approximation method and the adjusted OR was evaluated by the unconditional logistic regression method, with adjustments for age, menopause age, BMI, smoking status, daily exercise, history of fragility fracture, CHD, hypertension, and dietary calcium supplementation.

Results

Demographic information

The demographic, clinical, and biochemical characteristics of all 160 patients and those of cases and controls after stratification are shown in **Table 1**. There were significant differences in smoking status, daily exercise, and history of fragility fracture between cases and controls. No differences in other characteristics, such as age, menopause age, BMI, SBP, DBP, the levels of FPG, TC, TG, HDL-C, LDL-C, TNF-α, and IL-6, CHD or dietary calcium supplement were observed between the groups.

Logistic regression analysis of multiple risk factors for fragility fracture

The baseline characteristics affecting the likelihood of having a fragility fracture were determined by stepwise logistic regression. Variables considered included age, menopause age, BMI, smoking status, daily exercise, history of fragility fracture, CHD, hypertension, and dietary calcium supplementation. The results of this analysis (**Table 2**) showed that fragility fracture was more common in smokers and those with a history of fragility fracture and less common in those who exercised daily.

Percent distribution of patients with the ESR1 Pvull CC, CT, and TT genotypes between cases and controls

Among cases, 16.25% of subjects had the CC genotype, 42.50% had the CT genotype, and

Table 3. Genotype and allelotype of ESR1 PvuII rs2234693 (C > T) polymorphism specific risks

Genotype	Crude OR (95% CI)	P value	Adjusted OR (95% CI)ᵃ	P value
CC	1.00		1.00	
CT	2.012 (0.907, 4.464)	0.083	1.014 (0.900, 2.034)	0.103
TT	6.923 (2.696, 17.776)	**0.000**	2.491 (2.132, 4.216)	**0.028**
CT+TT	3.092 (1.465, 6.525)	**0.002**	1.323 (1.263, 2.787)	**0.036**
CC+CT	1.00			
TT	4.404 (2.026, 9.575)	**0.000**	1.884 (1.589, 2.458)	**0.031**
C	1.00			
T	2.705 (1.721, 4.250)	**0.000**		

The bold in the table indicates statistically significant data. ᵃAdjusted for age, menopause age, BMI, smoking status, daily exercise, history of fragility fracture, CHD, hypertension and dietary calcium supplement. BMI: body mass index; CHD: coronary heart disease.

41.25% had the TT genotype. Among controls, 37.50% of subjects had the CC genotype, 48.75% had the CT genotype, and 13.75% had the TT genotype (**Figure 1**).

Genotype and allelotype of ESR1 PvuII rs2234693 (C>T) polymorphism specific risks

The relationship between the genotype and allele of ESR1 PvuII and fragility fracture is summarized in **Table 3**. The TT genotype and T allele of ESR1 PvuII rs2234693 were associated with a significantly increased risk of fragility fracture compared with the CC genotype and C allele (TT vs. CC: P = 0.028, adjusted OR = 2.491, 95% CI = 2.132-4.216; T vs. C: P = 0.000, adjusted OR = 2.705, 95% CI = 1.721-4.250). Compared with individuals with the wild-type CC genotype, subjects with the variant genotypes (CT+TT) had a significantly increased risk of fragility fracture (P = 0.036, adjusted OR = 1.323, 95% CI = 1.263-2.787).

Stratified analyses for ESR1 PvuII rs2234693 genotypes in cases and controls

Stratified analyses were performed to evaluate the effects of the variant genotypes on the risk of fragility fracture in elderly postmenopausal women with heart failure according to age (67 years), smoking status, daily exercise and history of fracture (**Table 4**). For the ESR1 PvuII polymorphism rs2234693, an elevated risk of fragility fractures associated with the variant genotypes was evident in younger subjects (age ≤67 years) (P = 0.038, adjusted OR = 3.344, 95% CI = 1.921-4.128), but not in older subjects (P = 0.624). Stratification by smoking status revealed a significant association of rs2234693 with fragility fracture risk among non-smokers (P = 0.022, adjusted OR = 2.987, 95% CI = 1.856-4.643), but not among smokers (P = 0.847) (**Table 4**). There was no significant association between polymorphisms and susceptibility to fragility fractures according to daily exercise or history of fracture.

Discussion

The present study investigated the association between the ESR polymorphism rs2234693 and fragility fracture susceptibility in a postmenopausal population with heart failure. The variant rs2234693T (CT/TT) was associated with a significantly increased risk of fragility fracture. Our findings are consistent with those of previous reports [28, 32, 34, 43]. Recently, a Genome Wide Association Study further confirmed the association between the ESR1 gene and osteoporotic fractures [44].

The most extensively studied variants of the *ESR1* gene are the PvuII and XbaI polymorphisms, which have been linked to a lower sensitivity to estrogen [45, 46]. Because of the important effects of estrogens on bone mass and bone remodeling, numerous studies have evaluated the role of *ESR1* PvuII polymorphisms in the genetic regulation of fractures. However, the association between *ESR1* gene polymorphisms and fracture risk remains controversial and ambiguous. Our data also showed that the increased risk of fragility fracture associated with the variant genotypes of rs2234693 was more pronounced in younger subjects (<67 years) than in older subjects. This may be related to accumulated exposure to environmental risk factors in older individuals [21, 22, 47, 48]. Similarly, analyses stratified by smoking status identified a significant association between polymorphism status in nonsmokers, but not in smokers. Tobacco smoking is an accepted independent risk factor for fragility fracture [47, 48]. However, further studies are needed to verify these results.

The polymorphism is situated in a non-coding region. Therefore, its association with osteoporosis should be attributed to differences in

Table 4. Stratified analyses for ESR1 PvuII rs2234693 genotypes in cases and controls

Variables	(CT+TT)/CC for rs2234693		Allelic odds ratios and 95% confidence intervals for rs2234693	
	Cases, n (%)	Controls, n (%)	Adjusted OR (95% CI)[a]	P value
Age (y), median				
≤67	38 (47.50)/5 (6.25)	23 (28.75)/20 (25.00)	3.344 (1.921, 4.128)	**0.038**
>67	29 (36.25)/8 (10.00)	27 (33.75)/10 (12.50)	1.134 (0.311, 2.234)	0.624
Smoking				
Yes	10 (12.50)/6 (7.50)	4 (5.00)/3 (3.75)	1.198 (0.199, 4.618)	0.847
NO	57 (71.25)/7 (8.75)	46 (57.5)/27 (33.75)	2.987 (1.856, 4.643)	**0.022**
Daily exercise				
Yes	43 (53.75)/9 (11.25)	48 (60.00)/18 (22.50)	1.458 (0.647, 3.201)	0.248
NO	24 (30.00)/4 (5.00)	7 (15.00)/7 (15.00)	1.225 (0.818, 7.784)	0.107
History of fracture				
Yes	26 (32.50)/6 (7.5)	8 (10.00)/5 (6.25)	1.887 (0.632, 8.748)	0.229
NO	41 (51.25)/7 (8.75)	42 (52.5)/25 (31.25)	1.312 (0.568, 4.447)	0.141

The bold in the table indicates statistically significant data. [a]Adjusted for age, menopause age, BMI, smoking status, daily exercise, history of fragility fracture, CHD, hypertension and dietary calcium supplement. BMI: body mass index; CHD: coronary heart disease.

gene expression, rather than to changes in protein sequence [29]. The rs2234693 SNP in intron1 of the estrogen receptor has been suggested to modulate gene transcription. The C allele, but not the T allele, contains a functional Myb binding site, and transfection experiments with luciferase reporters showed a four-fold higher transcription rate in cells incorporating constructs with the C allele than in those transfected with constructs bearing the T allele [49]. These results would suggest that cells with T alleles express fewer estrogen receptor molecules, which could render them more sensitive to a reduced supply of estrogen [29].

On the other hand, *ESR1* gene variants (*Pvu*II) are associated with increased susceptibility to cardiovascular disease in both sexes [50, 51]. A large number of studies have examined the association between *ESR1* gene polymorphisms and bone mineral density. However, several studies in women have reported inconsistent associations between polymorphism of the *ESR1* gene and bone mineral density [52-55]. The existence of ethnic differences between populations, the case-control designs, and a health-based selection bias in several studies could explain the findings [36, 54, 56].

A number of clinical risk factors have been identified that provide information on fracture risk. These include age and aging, gender, a prior fragility fracture or a parental history of

fracture, use of systemic corticosteroids, low body mass index, falls, diet disorders, smoking, excess alcohol intake, and certain diseases. The independent contribution of these risk factors can be integrated by the calculation of fracture probability with or without the use of bone mineral density [47, 48]. It is not possible to modify some risk factors such as age and gender [21, 22]. Although it is possible in theory to modify other risk factors such as calcium or vitamin D deficiency, the resulting benefits are often small. In the present study, more than half of the subjects performed daily exercise, that may be attribute to the deepened cognition of exercise training to improve the clinical symptoms of HF [37]. Additionally, exercise improves agility, strength of bone and muscle, posture, and balance. Consequently, it contributes to the decreased risk of falls and fractures [57].

The occurrence of HF increases with age [11] and the lifetime risk of developing HF is 20% for Americans 40 years of age [58]. In the present study, certain patients with fracture were not eligible for surgery mostly because of intolerance caused by heart failure. This can lead to system-wide complications associated with prolonged bed rest. The ability to reduce the incidence of fracture for this subpopulation is important to ensure quality of life and improve life expectancy.

The present study had several limitations. First, because only symptomatic fractures were considered, selection bias could not be avoided. Second, demographic and personal information, such as smoking history or daily exercise, was collected by questionnaire, which may have introduced bias and may in turn have led to insufficient statistical power in our stratified analysis. Third, the sample size was relatively small, and the statistical power of our study was limited. Fourth, although BMD is an independent risk factor for fragility fracture, we did not examine this variable because it was unethical to perform a dual-energy X-ray absorptiometry test in every subject, especially in controls. Finally, further analyses were prevented by missing clinical information, such as data on alcohol consumption.

In conclusion, this hospital-based, case-control study showed that the *ESR1* PvuII rs2234693 (C>T) polymorphism was significantly associated with increased risk of fragility fracture in postmenopausal women older than 60 years. We found that the inheritance risk for fracture is common, particularly in younger patients and non-smokers. In addition, previous fractures are associated with an increased risk for future osteoporotic fractures, whereas, daily exercise can reduce their incidence. Our findings highlight the need to perform large studies to achieve sufficient statistical power to further elucidate the complex, multigenic character of fragility fracture.

Disclosure of conflict of interest

None.

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