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Laboratory Investigation

Association of Estrogen Receptor Gene Polymorphism in Patients with Degenerative Lumbar Spondylolisthesise

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Objective : The purpose of this study was to investigate the possible association of estrogen receptor alpha (ERα) gene polymorphisms in a cohort of degenerative spondylolisthesis (DS) patients.
Methods : Accordingly, the authors examined the association between DS and ERα gene polymorphisms in 174 patients diagnosed with DS. The *Pvu II* and *Xba I* polymorphisms, bone mineral density at the lumbar spine and femoral neck, and biochemical markers were analyzed and compared in the 174 patients with DS and 214 patients with spinal stenosis (SS).
Results : A comparison of genotype frequencies in DS and SS patients revealed a significant difference for the *Pvu II* polymorphism only (p=0.0452). No significant difference was found between these two groups with respect to the *Xba I* polymorphism, BMD or biochemical markers. No significant association was found between the *Pvu II* polymorphism of ERα and BMD, vertebral slip or biochemical markers in patients with DS.
Conclusion : These results suggest that the ERα gene polymorphism using *Pvu II* restriction enzyme influences the prevalence of DS.

Key Words : Degenerative spondylolisthesis · Estrogen receptor alpha · Polymorphism.

INTRODUCTION

Degenerative lumbar spondylolisthesis (DS) is a common and important condition of the aging spine, which frequently presents with symptoms of spinal canal stenosis. Several articles have addressed to identify the factors responsible for the development of DS[2-4,7,16,18,21,23,30], which is between four and five times more common in women than men[18]. The reason for this has not been fully explained although hormonal influences and pregnancy have been implicated[9,20].

Several investigators have suggested etiological factors including race, soft tissue abnormalities, the influence of lumbosacral angle, a lower intercristal line, lumbosacral bony anomalies, facet tropism and alignment[3,4,7,16,18,27]. Others have studied the orientations of the lumbar facet joints and the finding of an increased sagittal angle in patients with DS suggests the possibility of a developmental predisposition to this alignment[2,7].

It has been suggested that DS is part of generalized primary osteoarthritis which, is also more commonly found in women, but the exact pathogenesis of DS is not entirely clear. However, the prevalence of DS is significantly different between men and women, which suggests the potential involvement of sex hormones, especially estrogens in the pathogenesis of DS. Previous studies on estrogen-related arthritis and female predominance in DS have also raised the possibility that estrogen has a specific role in DS[9].

Cartilage is a sex-hormone-sensitive tissue[19]. Tsai and Liu[25] reported that estrogen is chondrodestructive via estrogen receptor-mediated mechanism. Furthermore, Ha et al.[8] reported that high levels of estrogen receptor expression aggravated degenerative changes in the articular cartilage of facet joints, and suggested that these changes contribute to the pathogenesis of DS in postmenopausal women. The degeneration of facet joints is similar to the degeneration of other diarthrodial joints. Thus, facet joints may be influenced by estrogen hormone in the same manner as other large joints.

Estrogen receptors are known to be associated with disease of bone and articular cartilage. In particular, the estrogen receptor alpha (ERα) gene is an important mediator of signal transduction, and ERα protein is expressed in cells of the musculoskeletal system including bone cells and chondrocytes[11], which suggests that the genes of any component of the estrogen endocrine pathway are candidate genes of facet joint degeneration. In addition,

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the ERα gene appears to play an important role in the genetic regulation of the arthritis of diarthrodial joints[10-12]. However, no study has been undertaken to address the effect of ERα polymorphisms in patients with DS. Therefore, we investigated the possible association of ERα *Pvu II* and *Xba I* polymorphisms in a cohort of DS patients.

MATERIALS AND METHODS

Three-hundred-eighty-eight women with symptomatic degenerative lumbar stenosis treated from March 2006 to December 2010, were enrolled. Diagnoses were DS in 174 patients and spinal stenosis (SS) without anterior displacement in 214 patients. Average ages were 58.2±6.9 in the DS group 58.7±6.9 in the SS group, which were not significantly different (p>0.05). The level of both vertebral slippage and stenosis was L4-5 in all patients. We selected the patients with a facet angle of <45° on computed tomographic scans to minimize the effects of the morphologic abnormalities of facet joints. Average facet angles were 37.7°±4.3° in the DS and 37.5°±4.4° in the SS. The difference of facet angle was not statistically significant between groups (p>0.05). DS was defined as a vertebral slip of >5% using the Morgan and King[17] method on lateral radiographs. Mean slip in the DS group was 13.2%±3.0%. All subjects provided informed consent before study commencement and this study was approved by the Clinical Research Ethics Committee at our institute.

Anthropometric measurements

Anthropometric measurements included body height and body weight. The body mass indices (BMIs) were calculated by dividing weight (kg) by height squared (m^2).

Dual energy X-ray absorptiometry

Lumbar spinal bone mineral density (LSBMD) and femoral neck BMD (FNBMD) of the non-dominant proximal femur were measured by dual-energy X-ray absorptiometry (XR-36; Norland Corp., Fort Atkinson, WI, USA). LSBMDs were measured at L1 to L4 in anterior-posterior view.

Biochemical markers of bone turnover

Blood samples were collected between 8 am and 10 am after an overnight fast. Plasma and serum samples were analyzed in a routine laboratory using standard procedures. Osteocalcin in heparinized plasma was measured using a solid-phase, two-site chemiluminescent enzyme-labeled immunometric assay (Immulite Osteocalcin, Diagnostic Product Corporation, Los Angeles, CA, USA); serum alkaline phosphatase by RIA (Tandem-R Ostase, Beckman Coulter, Fullerton, CA, USA); and serum $25(OH)D_3$ and $1,25(OH)_2D_3$ levels were measured by RIA using an IDS (Immunodiagnostic Systems Limited, UK). The intra-assay and inter-assay variabilities for $25(OH)D_3$ and $1,25(OH)_2D_3$ were both below 10%.

Genotyping

Genomic DNA was extracted from peripheral blood leukocytes using QIAamp DNA blood kit (Qiagen GmbH, Hilden, Germany). A 346 base-pair polymerase chain reaction (PCR) fragment of ERα was generated using a primer pair (forward, GATATCCAGGGTTATGTGGCA, and reverse, AGGTGTT GCCTATTATATTAACCTTGA) in a 20 μL reaction mixture containing 20 ng of genomic DNA. PCR products were digested with *Pvu II* or *Xba I* restriction enzyme, and then electrophoresed through 1.5% agarose gel to confirm the reactions. PCR products were purified directly using a PCR cleaning kit (Qiagen GmbH). Sequences were determined by cycle sequencing using an ABI PRISM Bigdye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems, Foster City, CA, USA) on an automated DNA sequencer (ABI PRISM 310, Perkin Elmer Applied Biosystems, Foster City, CA, USA).

Statistical analysis

Statistical analysis was performed using SPSS 11.5 software for Windows (SPSS, Chicago, IL, USA). Results are presented as means± standard deviations. The Hardy-Weinberg equilibrium was tested for each SNP in the DS and SS groups using the chi-square test. Frequency distributions of genotypes in the two groups were compared using the chi-square test for each SNP studied. Inter-group comparisons were made using the t-test, ANOVA, and the non-parametric Kruskal-Wallis test. Statistical significance was accepted for p values of <0.05.

RESULTS

Table 1. Genotype frequency distributions in DS and SS (control) patients

	DS (n=174)	SS (n=214)	p value
Pvu II			0.0452
TT	73	64	
TC	83	106	
CC	18	44	
Xba I			0.4376
AA	111	120	
AG	53	83	
GG	10	11	

DS : degenerative spondylolisthesis, SS : spinal stenosis

The genotype frequencies of all studied SNPs were determined by screening DNA samples from the 388 subjects. The genotype frequencies of the DS and SS groups are summarized in Table 1. The genotype frequency distributions of both polymorphic SNPs were in Hardy-Weinberg equilibrium. Comparisons of genotype frequencies of the *Pvu II* or *Xba I* polymorphisms in the two groups revealed a significant difference for only

the *Pvu II* polymorphism (p=0.0452). The prevalences of the three *Pvu II* genotypes in DS/SS patients were TT 42.0%/29.9%, TC 47.7%/49.5%, and CC 10.3%/20.6%, respectively.

Subject characteristics are presented in Table 2. The two groups were compared for each genotype, the difference in age, BMI, biochemical markers, and facet angle. No statistically significant differences were identified.

The *Pvu II* and *Xba I* polymorphisms were not found to be significantly associated with vertebral slip in DS patients (Table 3).

Combined polymorphisms

Three different haplotype alleles (TA, CA, CG) were identified by haplotype analysis of the *Pvu II* and *Xba I* polymorphisms of ERα in combination in DS patients. Six different genotypes, TATA (40.5%), TACA (24.1%), TACG (23.3%), CACA (4.3%), CACG (5.2%) and CGCG (2.6%), were identified when these haplotypes were combined. However, these haplotypes and genotypes were not found to be associated with vertebral slip.

DISCUSSION

DS is a disorder of the adult spine with a distinct female predominance[18,20]. It is approximately four times more common in woman than in man. Although the existence of this gender difference has long been known, no study has been performed to elucidate the possible explanations for it. Rosenberg[18] considered osteoporosis and hormonal factors, but found no evidence for either. Ha et al.[8] reported a significant increase in estrogen receptor in the facet articular cartilage of DS patients, and postulated that this elevated expression may be a the causative factors of DS, particularly in postmenopausal women. However, the effect of estrogen on the development of osteoarthritis remains controversial[5,22,24,26]. In degenerative lumbar spinal stenosis, degeneration of the facet joints resembles the degeneration that occurs in other diarthrodial joints and if the estrogen hormone affects the facet joints in the same way as other large joints, severe facet arthritis caused by estrogen is likely to progress into DS.

Sagittal plane facet has been believed to play a major role in facet joint slippage[2,4,7], but this does not always induce DS. Love et al.[15] concluded that an increased angle of the facet joint is the result of arthritic remodeling and that it is not the primary cause of DS. Imada et al.[9] investigated the influence of oophorectomy on the development of DS and found that patients had threefold greater incidence of DS after this procedure. Sanderson and Fraser[20] reported a twofold incidence of DS among parous as compared with nulliparous women. There is no clear explanation for this finding, although it has been speculated that the effect of childbearing on the abdominal musculature and hormonal changes in pregnancy, particularly the production of relaxin, could be contributing factors. Furthermore, the distinct gender difference observed in DS indicates that DS is related in some way to sex hormones. ERα is a steroid hormone nuclear receptors that transactivates many down-stream target genes, including estrogen responsive element. Estrogen receptors are classified based on modes of alternate gene splicing as ERα or ERβ. Both receptor types are known to be important regulators of skeletal growth and maturation[13,31]. Although the issue remains controversial[6,14], the relationship between ERα *Pvu II* or *Xba I* polymorphisms and osteoarthritis has been observed on a number of occasions[1,10,28,29]. This suggests that the gene of any component of the estrogen endocrine pathway is a candidate locus for the pathogenesis of DS. However, no previous attempts have been made to consider the effects of the ERα *Pvu II* or *Xba I* polymorphisms in DS.

In the present study, we examined the *Pvu II* and *Xba I* SNPs of the ERα gene to determine whether they are associated with vertebral slip in Korean patients with DS. In addition, we com-

Table 2. Characteristics in DS and SS (control) patients

	DS (n=174)	Control (n=214)	p value
Age (years)	58.2±6.9	58.7±6.9	0.5898
Height (cm)	159.0±3.4	158.6±3.3	0.4275
Weight (kg)	57.3±4.8	57.1±4.3	0.7907
BMI (kg/m^2)	22.7±2.0	22.7±1.8	0.9118
Menopause, n (%)	151 (86.8)	186 (86.9)	0.8687
Age at menopause (years)	51.7±3.1	51.3±2.8	0.3158
Smoking, n (%)	5 (2.9)	4 (1.9)	1.0000
Combined disease, n (%)	35 (20.1)	55 (25.7)	0.1966
DM/HT	22 (12.6)/21 (12.1)	30 (14.0)/28 (13.1)	
25(OH)D$_3$ (ng/mL)	10.3±5.4	11.4±6.7	0.4836
1,25(OH)$_2$D$_3$ (pg/mL)	59.3±25.9	61.0±20.4	0.3779
Osteocalcin (μg/L)	28.6±7.7	25.8±10.3	0.4001
Alkaline phosphatase (μg/L)	12.7±5.7	11.4±7.8	0.3852
Facet angle (°)	32.7±4.3	32.5±4.4	0.7040
Vertebral slip (%)	13.2±3.0		
LSBMD (g/cm^2)	0.806±0.071	0.792±0.062	0.1039
FNBMD (g/cm^2)	0.797±0.066	0.787±0.060	0.2201

DS : degenerative spondylolisthesis, SS : spinal stenosis, DM : Diabetes, HT : Hypertension, BMI : body mass indices, LSBMD : lumbar spinal bone mineral density, FNBMD : femoral neck bone mineral density.

Table 3. Vertebral slip in DS patients with different genotypes

Pvu II	TT	TC	CC	p value
Vertebral slip	13.3±3.1	13.4±2.7	11.7±3.2	0.1503
Xba I	AA	AG	GG	p value
Vertebral slip	13.7±3.1	12.5±2.6	11.9±2.1	0.0789

DS : degenerative spondylolisthesis

pared genotype frequencies in DS and SS patients. These comparisons revealed a significant difference in the genotype frequencies of the *Pvu II* polymorphism in the two study groups. However, the *Pvu II* polymorphism was not found to be associated with vertebral slip in these patients. In addition, no significant difference was observed between DS patients and SS controls in term of the genotype frequencies of the *Xba I* polymorphisms, and the genotypes of this polymorphism were not found to influence vertebral slip in DS patients either.

Haplotype analysis is believed to be an effective tool for determining the genetic contributions of common diseases. However, haplotype analysis of the *Pvu II* and *Xba I* polymorphisms of ERα in combination showed no significant association with the amount of vertebral slip or the prevalence of DS. This indicates that a gene-to-gene interaction between the polymorphisms in the ERα genes does not significantly modulate the vertebral slip in DS patients.

There have been reports that in the polymorphisms of the ERα are associated with differences in BMD. Bergink et al.[1)] found a relationship between osteophytes and increased risk of knee osteoarthritis for the *Pvu II* and *Xba I* polymorphisms of ERα. They further hypothesized that variations in the ERα may be associated with changes in juxtaarticular bone rather than articular cartilage and this might lead to more severe osteoarthritis later in life. Lian et al.[12)] found that hip BMD was associated with radiographic hip osteoarthritis in their cohort study. Therefore, we also investigated associations between the ERα polymorphisms and LSBMD or FNBMD in both DS and SS groups (data not shown). However, the ERα genotypes were not found to influence on the LSBMD or FNBMD in either group.

A number of limitations of the present study require consideration. First, the number of samples tested was relatively small, and the normal population was not included in this study. Thus, further studies in a larger patient population are required. Second, we did not evaluate interactions with other genes associated with normal development and maintenance of cartilage, such as, the type IX collagen gene. Third, although the occurrence of degenerative disease can be associated with environmental and occupational conditions, we did not evaluate interactions with these factors. Furthermore, associations with other factors, such as, the radiological and histological findings of facet joints in patients with DS, should also be evaluated.

CONCLUSION

In this study, associations between the ERα *Pvu II* or *Xba I* gene polymorphisms and DS patients were studied. We found ERα gene polymorphism using *Pvu II* restriction enzyme influences the prevalence of DS. Therefore, early diagnosis of the disease using noted genetic markers will provide beneficial information regarding individual susceptibility to DS. However, further studies on a larger number of subjects are required to determine the mechanism responsible for DS.

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