Polymorphism in vitamin D receptor is associated with bone mineral density in patients with adolescent idiopathic scoliosis

Kuen Tak Suh · Il-Soo Eun · Jung Sub Lee

Received: 13 October 2009 / Revised: 25 January 2010 / Accepted: 8 March 2010 / Published online: 2 April 2010
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Abstract Low bone mass and osteopenia have been reported in the axial and peripheral skeleton of adolescent idiopathic scoliosis (AIS) patients. Furthermore, several recent studies have shown that gene polymorphisms are related to osteoporosis. However, no study has yet linked polymorphisms in the vitamin D receptor (VDR) gene and bone mass in AIS. Accordingly, the authors examined the association between bone mass and VDR gene polymorphisms in 198 girls diagnosed with AIS. The VDR *BsmI* (rs1544410), *FokI* (rs2228670) and *Cdx2* (rs11568820) polymorphisms and bone mineral density at the lumbar spine (LSBMD) and femoral neck (FNBMD) were analyzed and compared to their levels in healthy controls. Mean LSBMD and FNBMD in AIS patients were lower than in age- and sex-matched healthy controls ($P = 0.0022$ and $P = 0.0013$, respectively). A comparison of genotype frequencies in AIS patients and controls revealed a significant difference for the *BsmI* polymorphism only ($P = 0.0054$). Furthermore, a significant association was found between the VDR *BsmI* polymorphism and LSBMD. In particular, LSBMD in AIS patients with the AA genotype was found to be significantly lower than in patients with the GA ($P < 0.05$) or GG ($P < 0.01$) genotypes. However, no significant association was found between LSBMD or FNBMD and the VDR *FokI* or *Cdx2* polymorphisms. These results suggest that the VDR *BsmI*

polymorphism is associated with LSBMD in girls with AIS.

Keywords Adolescent idiopathic scoliosis · Bone mineral density · Vitamin D receptor · Polymorphism

Introduction

The etiology and pathogenesis of adolescent idiopathic scoliosis (AIS) is unclear despite the large number of studies conducted. The cause of scoliosis is believed to be multifactorial due to the associations between the development of scoliosis and growth, hormonal secretion, gravity and others [1, 15, 16, 21, 26, 27, 33, 36, 43]. However, none of these parameters has been demonstrated to play a causative role in the development of AIS.

Burner et al. [5] first reported an association between osteopenia and idiopathic scoliosis using the Singh index. Generalized low bone mass and osteopenia in both the axial and peripheral skeletons have been reported in AIS [7, 8, 11, 13, 35, 38], and abnormal histomorphometric bone cell activity has been observed in AIS bone biopsies [9]. In addition, low bone mass is likely to persist through to adulthood in AIS patients [7], and there is increasing concern that adolescents with idiopathic scoliosis might have a lower peak bone mass, which would increase the risk of developing osteoporosis and related complications in later life [7, 8]. However, the precise mechanism and causes of bone loss in AIS have not been identified.

Osteoporosis is defined as a reduction in bone mass caused by a deterioration of the bone microarchitecture, which results in bone fragility and increases fracture risk. AIS is a complex disorder, which demonstrates interactions between environmental and genetic factors. In particular,

K. T. Suh · J. S. Lee (✉)
Department of Orthopaedic Surgery, Medical Research Institute,
Pusan National University School of Medicine, 1-10 Ami-Dong,
Seo-Gu, Busan 602-739, Korea
e-mail: jungsublee@pusan.ac.kr

I.-S. Eun
Department of Orthopaedic Surgery, Busan Medical Center,
Busan, Korea

genetic influences have been reported to account for 50–80% of inter-individual bone mineral density (BMD) variability. Furthermore, several genetic association studies have demonstrated relationships between polymorphisms of candidate genes and decreased BMD and fracture risk [14, 20, 30, 39, 40, 42].

1,25-Dihydroxyvitamin D₃ (1,25(OH)₂D₃) plays a central role in skeletal metabolism by binding to its nuclear steroid receptor, vitamin D receptor (VDR). 1,25(OH)₂D₃ can stimulate bone formation and resorption by acting on osteoblastic and osteoclastic lineage cells and thus regulates bone turnover [4]. Some have shown associations between serum 25(OH)D₃ status and BMD and osteoporotic fractures [10, 17], and that 25(OH)D₃ inadequacies are associated with elevated risks of osteoporosis and osteoporotic fractures [18, 23].

Adolescence is a critical time for the acquisition of bone mass, around 40% of skeletal mass is acquired during pubertal maturation [32], and vitamin D is known to play an important role in this bone gain during adolescence. Moreover, it has been reported that pubertal girls with hypovitaminosis D are at risk of failing to achieve maximum peak bone mass [24], which suggests that the genes of any component of the vitamin D endocrine pathway are candidate loci of the osteoporosis gene. In addition, the VDR gene appears to play an important role in the genetic regulation of bone mass and in the pathogenesis of fractures.

Several investigators have evaluated the association between VDR gene polymorphisms and BMD in postmenopausal women, and a smaller number of studies have been conducted in children [2, 22, 25, 34, 37, 44]. However, no report has yet linked bone mass in AIS and a VDR gene polymorphism. Accordingly, we undertook to examine association between bone mass and VDR gene polymorphisms in girls with AIS.

Materials and methods

A total of 198 Korean girls (11–13 years of age), newly diagnosed with AIS at the authors' institution, and 120 age-matched healthy girls were enrolled after applying the exclusion criteria detailed below. The following candidates were excluded: those who had received any type of treatment for scoliosis, those with a history of a congenital deformity, a neuromuscular disease, an endocrine disease, skeletal dysplasia or a connective tissue abnormality, those with mental retardation or psychiatric disease, and those who had taken medication known to affect bone metabolism. All subjects and parents provided informed consent before the study commencement. This study was approved by the clinical research ethics committee at our institute.

Evaluation of scoliosis severity

Normal standing whole spine antero-posterior radiographs were obtained for each AIS patient at the first presentation. A standard technique was used to measure Cobb's angle, and when more than one curve was found, the most severe was selected for measurements. Curves of <10° were excluded.

Anthropometric measurements

Anthropometric measurements included body height and body weight. Corrected heights for patients were calculated using Bjure's formula ($\text{Log } y = 0.011x - 0.177$, where y is the loss of trunk height (cm) due to a deformed spine and x is the greatest Cobb angle of the primary curve) [8]. The body mass indices (BMIs) were calculated by dividing weight (kg) by uncorrected height squared (m²).

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 (DEXA) (XR-36; Norland Corp., Fort Atkinson, WI, USA). LSBMDs were measured at L1–L4 in anterior–posterior view. The scoliotic curvature can make it difficult to measure spinal BMD reliably. To minimize this problem, the amount of spinal rotation in patients was determined by pre-scanning the spine and measuring LSBMD in the neutral position [35].

Biochemical markers of bone turnover

Blood samples were collected between 8:00 and 10 a.m. 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 (ALP) by RIA (Tandem-R Ostase, Beckman Coulter, Fullerton, CA, USA) and serum 25(OH)D₃ and 1,25(OH)₂D₃ levels were measured by RIA using an IDS (Immunodiagnostic System Limited, UK). The intra-assay and inter-assay variabilities for 25(OH)D₃ and 1,25(OH)₂D₃ were both below 10%.

Genotyping

Genomic DNA was extracted from peripheral blood leukocytes using a QIAamp DNA blood kit (Qiagen GmbH, Hilden, Germany). Polymerase chain reaction (PCR)

primers were designed for the *BsmI* (rs1544410), *FokI* (rs2228670) and *Cdx2* (rs11568820) SNPs of the *VDR* gene. Polymorphic regions of the *VDR* gene were amplified by PCR using the specific forward primers (GGCAACCTGAAGGGAGACGTA for rs1544410, AGCTGGCCCTGGCACTGACTCTGCTCT for rs2228670 and ACTGCAGCCTTGACCTCCTA for rs11568820) and the specific reverse primers (CTCTTTGGACCTCATCACCGAC for rs1544410, ATGGAAACACCTTGCTTCTTCTCCCTC for rs2228670 and AAAGCAAACCAAGGGGTCTT for rs11568820). PCR products were digested with *BsmI*, *FokI* or *HypCHIII* restriction enzyme, respectively, and then electrophoresed through 1.5% agarose gel to confirm the reaction. Products were purified directly from PCR 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 mean $\pm$ standard deviations. The Hardy–Weinberg equilibrium was tested for each SNP in the patient and control groups using the chi-square test. Frequency distributions of genotypes in patients and controls 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

The genotype frequencies of all studied SNPs were determined by screening DNA samples from the 318 subjects. The genotype frequencies of the two study groups are summarized in Table 1. The genotype frequency distributions of all three polymorphic SNPs were in Hardy–Weinberg equilibrium. Comparisons of genotype frequencies of the three polymorphisms studied in AIS patients and healthy controls revealed a significant difference for only the *BsmI* polymorphism ($P = 0.0054$).

Subject characteristics are presented in Table 2. For each genotype, the difference in age, BMI, cBMI and biochemical markers were compared in the two study groups. No statistically significant differences were identified. Mean LSBMD and FNBMD in patients were

Table 1 Genotype frequency distributions in AIS patients and healthy controls

	AIS (<i>n</i> = 198)	Control (<i>n</i> = 120)	<i>P</i> value
<i>BsmI</i>			0.0054
GG	50	46	
AG	102	61	
AA	46	13	
<i>FokI</i>			0.7665
CC	64	42	
CT	104	63	
TT	30	15	
<i>Cdx2</i>			0.7254
GG	136	84	
GA	49	26	
AA	13	10	

Table 2 Genotype frequency distributions in AIS patients and healthy controls

	AIS (<i>n</i> = 198)	Control (<i>n</i> = 120)	<i>P</i> value
Age (years)	12.5 $\pm$ 0.8	12.7 $\pm$ 1.0	0.1766
BMI	18.0 $\pm$ 1.6	18.2 $\pm$ 2.3	0.5051
cBMI	17.7 $\pm$ 1.6	18.2 $\pm$ 2.3	0.0582
25(OH)D ₃ (ng/mL)	14.3 $\pm$ 9.4	13.4 $\pm$ 6.7	0.5700
1,25(OH) ₂ D ₃ (pg/mL)	79.4 $\pm$ 28.9	81.0 $\pm$ 19.5	0.3833
Osteocalcin (μ g/L)	25.6 $\pm$ 7.5	21.8 $\pm$ 10.2	0.3301
Alkaline phosphatase (μ g/L)	12.7 $\pm$ 5.9	11.4 $\pm$ 7.8	0.3725
LSBMD (g/cm ²)	0.717 $\pm$ 0.050	0.737 $\pm$ 0.068	0.0022
FNBMD (g/cm ²)	0.705 $\pm$ 0.044	0.725 $\pm$ 0.064	0.0013
cBMI Corrected BMI			

decreased to be lower than in controls ($P = 0.0022$ and $P = 0.0013$, respectively).

The *BsmI* polymorphism was found to be significantly associated with LSBMD, but not with FNBMD (Table 3). In particular, AIS patients with the AA genotype had a significantly lower LSBMD than patients with the GA ($P < 0.05$) or GG ($P < 0.01$) genotype. However, no significant association was found between either LSBMD or FNBMD and the *FokI* or *Cdx2* polymorphisms.

Discussion

Adolescent idiopathic scoliosis patients have been reported to have a low bone mass and osteopenia in both the axial and peripheral skeletons [6, 8, 11, 13, 35, 38]. However, the precise mechanism of bone loss in AIS patients is

Table 3 Bone mineral densities in AIS patients with different genotypes

	GG	GA	AA	<i>P</i> value
<i>BsmI</i>				
LSBMD	0.728 ± 0.054	0.720 ± 0.048	0.696 ± 0.048	0.0046
FNBMD	0.707 ± 0.047	0.701 ± 0.039	0.712 ± 0.052	0.3298
	CC	CT	TT	<i>P</i> value
<i>FokI</i>				
LSBMD	0.719 ± 0.054	0.718 ± 0.048	0.707 ± 0.050	0.4863
FNBMD	0.705 ± 0.047	0.707 ± 0.042	0.699 ± 0.046	0.7019
	GG	GA	AA	<i>P</i> value
<i>Cdx2</i>				
LSBMD	0.715 ± 0.050	0.718 ± 0.051	0.730 ± 0.057	0.7190
FNBMD	0.705 ± 0.046	0.701 ± 0.040	0.722 ± 0.038	0.2475

unclear. As osteoporosis is a disease with a strong genetic component, much effort is focused on the identification of SNPs associated with the osteoporotic phenotype [31]. Morrison et al. [29] were the first to report that the *BsmI* polymorphism of the VDR gene is responsible for part of the genetic effect on peak bone mass. Subsequent association studies between VDR gene polymorphism and BMD have been conducted [2, 22, 25, 34, 37, 44]. However, no report has yet linked bone mass in AIS and a VDR gene polymorphism.

The VDR gene is one of the most widely studied osteoporosis-related genes. However, the numerous genetic association studies undertaken on the topic have yielded conflicting results [2, 22, 25, 34, 37, 44]. Arabi et al. [3] concluded that VDR gene polymorphisms, identified using *BsmI* restriction enzyme, are associated with the bone mineral contents of the lumbar spine and femoral neck in healthy girls, whereas Lorentzon et al. [25] reported that the VDR gene polymorphism defined by *ApaI* and *BsmI*, is related to LSBMD in healthy adolescent girls. However, meta-analyses of the *BsmI* polymorphism have produced contradictory results for an association with FNBMD [12, 19]. Furthermore, in the GENOMOS study, no association was found between the *BsmI-ApaI-TaqI* (VDR) haplotype and any osteoporotic phenotype, whereas *Cdx2* polymorphism was found to be associated with LSBMD, FNBMD and the risk of vertebral fracture [28, 41]. Laaksonen et al. [22] concluded that the VDR *FokI* polymorphism is not associated with BMD or quantitative ultrasound parameters in girls, whereas Ames et al. [2] found that the *FokI* polymorphism at the VDR translation initiation site was associated with BMD and calcium deposition in children. On the other hand, Strandberg et al. [34] concluded that *FokI* genotypes are independently related to LSBMD, but not to FNBMD.

Since the first report was issued on the relationship between VDR genotypes and BMD in 1994 [29], a large number of studies have been conducted. However, the majority of these studies involved adults rather than children [2, 22, 25, 34]. Nevertheless, published results are conflicting, regardless of age, and in children appear to differ by gender, pubertal status and the restriction enzyme used [2, 22, 25, 34]. Thus, in the present study, we enrolled an age- and sex-matched healthy control group to minimize these effects.

We examined the *BsmI* (rs1544410), *FokI* (rs2228670) and *Cdx2* (rs11568820) SNPs of the VDR gene to identify those genes involved in the regulation of bone mass in Korean AIS patients, who incidentally represent a near ethnically homogenous population. In addition, we compared genotype frequencies in AIS patients and healthy controls. These comparisons revealed a significant difference in the genotype frequencies of the *BsmI* polymorphism in the two study groups. In addition, *BsmI* polymorphism was associated with LSBMD in AIS patients. The prevalences of the three *BsmI* genotypes in patients were GG 25.3%, GA 51.5% and AA 23.2%. In particular, AIS patients with the A allele had a significantly lower LSBMD, but this was not observed for FNBMD. However, no significant differences were observed between patients and controls in terms of the genotype frequencies of the *FokI* and *Cdx2* polymorphisms, and the genotypes of these two polymorphisms were not found to influence LSBMD or FNBMD in patients. Our findings concur with those of some previous studies, but not all of these studies assessed relationships in adolescent girls [2, 22, 25, 28, 34].

A number of limitations of the present study require consideration. First, the number of samples tested was relatively small and thus further studies in a larger patient population are required. Second, we did not evaluate interactions with other genes, such as the estrogen receptor gene, or with other relevant gene polymorphisms, such as those of the calcium sensing receptor gene. Furthermore, associations with other factors, such as the markers of bone metabolism, bone quality, and other candidate genes, should also be evaluated.

Summarizing, we examined the association between VDR gene polymorphisms and BMD in Korean girls with AIS. We found VDR gene polymorphism using *BsmI* restriction enzyme influences on LSBMD. Therefore, early diagnosis of the disease using well-known genetic markers will provide beneficial information regarding individual susceptibility to low bone mass and may help high-risk individuals to take precautions against further reduction of bone mass. Further studies on a larger number of subjects are required to determine the mechanism responsible for low bone mass in AIS.

Acknowledgments The authors thank the study participants and their parents for making this study possible and Frederick N. Dyer, Ph.D., for his suggestions.

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