Polymorphism in interleukin-6 gene is associated with bone mineral density in patients with adolescent idiopathic scoliosis

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Low bone mass and osteopenia have been described in the axial and peripheral skeleton of patients with adolescent idiopathic scoliosis (AIS). Recently, many studies have shown that gene polymorphism is related to osteoporosis. However, no studies have linked the association between IL6 gene polymorphism and bone mass in AIS. This study examined the association between bone mass and IL6 gene polymorphism in 198 girls with AIS. The polymorphisms of IL6-597 G→A, IL6-572 G→C and IL6-174 G→A and the bone mineral density in the lumbar spine and femoral neck were analysed and compared with their levels in healthy controls. The mean bone mineral density at both sites in patients with AIS was decreased compared with controls ($p = 0.0022$ and $p = 0.0013$, respectively). Comparison of genotype frequencies between AIS and healthy controls revealed a statistically significant difference in IL6-572 G→C polymorphism ($p = 0.0305$). There was a significant association between the IL6-572 G→C polymorphism and bone mineral density in the lumbar spine, with the CC genotype significantly higher with the GC ($p = 0.0124$) or GG ($p = 0.0066$) genotypes.

These results suggest that the IL6-572 G→C polymorphism is associated with bone mineral density in the lumbar spine in Korean girls with AIS.

The aetiology and pathogenesis of adolescent idiopathic scoliosis (AIS) remain unclear. The aetiology is believed to be multifactorial, including such factors as growth, hormonal secretion and gravity.¹⁻⁹ However, none of these parameters has been shown individually to play a causative role.

Turner, Badger and Sherman¹⁰ first noted an association between osteopenia and AIS using the Singh index.¹¹ Generalised low bone mass and osteopenia in the axial and the peripheral skeleton have been described in AIS¹²⁻¹⁶ along with abnormal histomorphometric bone cell activity in bone biopsies.¹⁷ As the low bone mass in AIS patients is likely to persist into adulthood,¹² there is increasing concern that adolescents with idiopathic scoliosis might have a lower peak bone mass, thereby increasing the risk of osteoporosis and related complications in later life.^{12,13} However, the precise mechanism and causes of bone loss in AIS have not been identified.

Osteoporosis is defined as a reduction in the microarchitecture of bone, resulting in an increase in fragility and the risk of fracture. It is a complex disorder, with interactions between environmental and genetic factors. The latter account for 50% to 80% of the

inter-individual variability in bone mineral density (BMD) and several studies have demonstrated a relationship between polymorphisms of candidate genes with a decrease in BMD and an increased risk of fracture.¹⁸⁻²³

Several studies have suggested that immunological factors such as interleukins and tumour necrosis factors might influence the development of osteoporosis. Interleukin-6 (IL-6) is a multi-functional cytokine essential in the differentiation and function of osteoclasts^{24,25} and IL-6 and its receptor are possible pathogenic factors in conditions associated with bone loss.^{24,25} Clinical studies have shown that IL-6 mRNA expression in bone is enhanced in 95% of patients with osteoporotic vertebral fracture but in only 50% of post-menopausal controls.²⁴ Therefore, the genes of any component of IL-6 might be candidates for osteoporosis. Some investigators have evaluated the association between IL-6 gene polymorphisms and BMD in post-menopausal women,²⁶⁻³¹ but there are no reports linking the association between bone mass in patients with AIS and IL-6 gene polymorphism. We examined the association between bone mass in girls with AIS and IL-6 gene polymorphism and compared these with their levels in healthy controls.

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Patients and Methods

We enrolled 198 girls with a mean age of 12.5 years (11.1 to 13.9), newly diagnosed with AIS and 120 healthy girls recruited from routine school screenings, with a mean age of 12.7 years (11.0 to 13.9). The diagnosis of AIS was confirmed through a detailed medical history, physical examination and standard radiographs. The year since menarche and the Risser sign were also evaluated. Those receiving any form of treatment for scoliosis were excluded. Girls with a history of congenital deformities, neuromuscular disease, endocrine disease, skeletal dysplasia, connective tissue abnormalities, mental retardation, inflammatory diseases and use of medication known to affect bone metabolism were also excluded. All subjects and their parents gave informed consent before participating in the study, which was approved by the Clinical Research Ethics Committee of the university and hospital.

For the evaluation of scoliosis, normal standing whole-spine anteroposterior radiographs were taken for each patient at their first presentation, using a standard technique to measure the Cobb angle. If more than one curve was found, the most severe was selected for the measurement. Curves $< 10^\circ$ were excluded.

Anthropometric measurements included height and weight. For the patients with AIS, the corrected height was derived from Bjure's formula ($\text{Log } y = 0.011x + 0.177$, where y is the loss of trunk height (cm) due to the deformed spine and x is the greatest Cobb angle of the primary curve).¹³ The body mass index (BMI) was determined by dividing the weight (kg) by the square of the uncorrected height (m^2).

The BMD of the lumbar spine and that of the neck of the non-dominant proximal femur were measured by dual-energy X-ray absorptiometry (DEXA, XR-36; Norland Corp., Fort Atkinson, Wisconsin). The lumbar BMD was measured in L1-4 in the anteroposterior view. The curvature might present difficulties in measuring the spinal BMD reliably and in order to minimise this problem, the level of rotation in patients with AIS was determined by pre-scanning the spine and measuring the lumbar spine BMD in the neutral position.^{15,32}

For the measurement of biochemical markers of bone turnover, blood samples were collected between 8:00 am and 10:00 am after an overnight fast. The samples of plasma and serum were analysed in a routine laboratory using standard procedures according to the specifications of the manufacturers. Osteocalcin in heparinised plasma was measured by a solid-phase two-site chemiluminescent enzyme-labelled immunometric assay (Immulate Osteocalcin, Diagnostic Product Corporation, Los Angeles, California). Serum alkaline phosphatase was measured by radioimmuno assay (Tandem-R Ostase, Beckman Coulter, Fullerton, California). Serum $25(\text{OH})\text{D}_3$ and $1,25(\text{OH})_2\text{D}_3$ levels were measured by radio immuno-assay (RIA) using the IDS (Immunodiagnostic System Limited, Boldon, United Kingdom). The intra- and inter-assay variabilities for $25(\text{OH})\text{D}_3$ and $1,25(\text{OH})_2\text{D}_3$ are $< 10\%$.

For genotyping the genomic DNA was extracted from the peripheral blood leucocytes using a QIAamp DNA blood kit (Qiagen GmbH, Hilden, Germany). The polymorphic regions of the IL-6 gene were amplified by polymerase chain reaction (PCR) with the specific forward primers (GCAAAGTCCTCACTGGGAGGA for -597 G $\rightarrow$ A, GCAAAGTCCTCACTGGGAGGA for -572 G $\rightarrow$ C and AATGACGACCTAAGCTGCAC for -174 G $\rightarrow$ C), and with the specific reverse primers (GGGCTGCGATGGAGTCAGA for -597 G $\rightarrow$ A, GGGCTGCGATGGAGTCAGA for -572 G $\rightarrow$ C and TTGATAAATCTTTGTTGGAGGGTG for -174 G $\rightarrow$ C). The PCR was carried out in a mixture of 1.25 pmol of each primer, 50 ng genomic DNA, 250 M dNTPs and 0.15 U TaqMan nuclease method (Applied Biosystems, Foster City, California) provided by the manufacturer. Amplification was carried out in a GeneAmp PCR system 9700 thermal cycler (Applied Biosystems). Their sequences were determined by cycle sequencing using an ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems) on an automated DNA sequencer (ABI PRISM 310, Perkin Elmer Applied Biosystems, Foster City, California).

Statistical analysis. This was performed using SPSS 11.5 software for Windows (SPSS Inc., Chicago, Illinois). The data are expressed as the mean (SD). The Hardy-Weinberg equilibrium was tested for each single nucleotide polymorphism (SNP) and group of participants using the chi-squared test. The frequency distributions of genotypes in the AIS and healthy controls were compared for each SNP studied using the chi-squared test. The groups were compared using a *t*-test, ANOVA and non-parametric Kruskal-Wallis test, where appropriate. A *p*-value < 0.05 was considered significant.

Results

The mean Cobb angle for patients with scoliosis was 24.8° (16° to 69°). A total of 16.7% ($n = 33$) of girls with AIS were premenarchal when their curvature was detected and 165 were post menarchal, with a mean of 1.4 years (0.2 to 4.0) since menarche. In the AIS group, 76.8% ($n = 152$) were of Risser grades 0 ($n = 42$), 1 ($n = 59$), and 2 ($n = 51$). There were 131 thoracic, 47 double, ten thoracolumbar and ten lumbar curves.

The genotype frequencies of all SNPs studied were determined by screening DNA samples from 318 subjects (AIS group = 198, control = 120). The genotype frequencies of the subgroups are summarised in Table I. The genotype frequency distributions of all three polymorphic SNPs were in Hardy-Weinberg equilibrium. Comparison of genotype frequencies between patients with AIS and controls revealed statistically significant differences in IL6-572 G $\rightarrow$ C polymorphism ($p = 0.0305$). IL6-597 G $\rightarrow$ A and IL6-174 G $\rightarrow$ C were totally linked together and showed very rare allele frequencies in both groups.

The investigations for the two groups are presented in Table II. For each genotype, the difference in age, BMI,

Table I. Genotype frequency distributions in patients with adolescent idiopathic scoliosis (AIS) and healthy controls

Genotype	AIS (n = 198)	Controls (n = 120)	p-value
-597 G→A			
GG	197	119	
GA	1	1	
AA	0	0	
-572 G→C			0.0305
GG	130	67	
GC	64	44	
CC	4	9	
-174 G→C			
GG	197	119	
GA	1	1	
AA	0	0	

Table II. Genotype frequency distributions in adolescent idiopathic scoliosis (AIS) and healthy controls

	AIS (n = 198)	Controls (n = 120)	p-value
Age (yrs)	12.5 (11.1 to 13.9)	12.7 (11.0 to 13.9)	0.1766
BMI*	18.0 (15.0 to 23.9)	18.2 (14.7 to 29.3)	0.5051
cBMI†	17.7 (14.8 to 23.6)	18.2 (14.7 to 29.3)	0.0582
25(OH)D ₃ (ng/ml)	14.3 (5.2 to 26.7)	13.4 (6.6 to 23.4)	0.5700
1,25(OH) ₂ D ₃ (ng/ml)	79.4 (29.4 to 127.5)	81.0 (32.0 to 132.3)	0.3833
Osteocalcin (mg/L)	25.6 (7.3 to 43.0)	21.8 (6.0 to 42.1)	0.3301
Alkaline phosphatase (mg/L)	12.7 (5.9 to 21.1)	11.4 (5.0 to 21.7)	0.3725
Lumbar spine BMD‡ (g/cm ²)	0.717 (0.619 to 0.879)	0.737 (0.619 to 0.897)	0.0022
Femoral neck BMD (g/cm ²)	0.705 (0.615 to 0.835)	0.725 (0.613 to 0.908)	0.0013

* BMI, body mass index

† cBMI, corrected body mass index

‡ BMD, bone mineral density

Table III. Bone mineral density in different genotype subgroups of adolescent idiopathic scoliosis

-572 G→C	GG	GC	CC	p-value
BMD*				
Lumbar spine	0.713 (0.619 to 0.854)	0.720 (0.631 to 0.879)	0.804 (0.731 to 0.861)	0.0159
Femoral neck	0.706 (0.629 to 0.835)	0.704 (0.615 to 0.807)	0.708 (0.690 to 0.732)	0.8865

* BMD, bone mineral density

cBMI and biochemical markers between genotype subgroups were compared in AIS and the healthy controls. No statistically significant differences were identified. The mean lumbar spine and femoral neck BMD in patients with AIS were decreased compared with those controls ($p = 0.0022$ and $p = 0.0013$, respectively).

The IL6-572 G→C polymorphism was significantly associated with lumbar spine BMD but not with femoral neck BMD (Table III). The former in AIS patients with the CC genotype was significantly higher than in the AIS patients with the GC ($p = 0.0124$) or GG ($p = 0.0066$) genotypes. However, the loci IL6-597 G→A and IL6-174 G→A were not analysed statistically because the rare allele frequencies in the AIS group were too low ($p = 0.002$).

Discussion

Generalised low bone mass and osteopenia in the axial and peripheral skeleton have been described in patients with AIS.¹³⁻¹⁶ The precise mechanism of bone loss in these patients is unclear. Recently, many studies have reported that gene polymorphism was related to osteoporosis, but few have linked gene polymorphism and bone mass in AIS.³³

The IL-6 receptor gene is one of the candidate genes for osteopenia and osteoporosis. Several genetic association studies with IL-6 receptor polymorphism have yielded different genetic backgrounds.^{26-28,32-36} The allele frequencies of IL6-174 and IL6-597 in Caucasians are much higher than those in Koreans and Chinese,^{26-28,34-36} but the frequency of IL6-572 in Caucasians is much lower than that in Koreans and Japanese.^{26,27} Many studies have

been conducted regarding IL6-174 in Caucasians; C homozygotes of IL6-174 are associated with lower bone resorption and a smaller decrease in bone mass in older post-menopausal women.³¹ This SNP is also associated with systemic juvenile arthritis³⁷ and increased bone resorption.³¹ In pubertal girls, decreased IL-6 may be partly responsible for the development of maximal peak vertebral bone mass.³⁸ It was difficult to study the genetic effects of the IL6-174 polymorphism because of its very low frequency in the Korean population. However, the IL6-572 G→C polymorphism is relatively common in Koreans and Japanese and IL6-572 might therefore be a useful marker for association studies in Asian rather than Caucasian populations, because highly frequent population alleles are particularly useful in mapping those genes responsible for disease susceptibility and other traits.

A study using haplotype analysis is believed to be as effective in determining the genetic contributions of common diseases. The SNPs in the IL-6 promoter region are in complete and/or absolute linkage disequilibrium, so that only three haplotypes (ACC, GCG and GGG) of the possible eight are observed. Generally, haplotypes are more informative than single SNPs, but, the case haplotypes in the IL-6 promoter region, are not informative in association studies of complex trait diseases such as osteoporosis because of the very low frequencies of IL6-597 G→A and IL6-174 G→C in Asians.^{26,27} Our results also revealed the very low frequencies of IL6-597 G→A and IL6-174 G→C.

This study examined the IL6-597 G→A, IL6-572 G→C and IL6-174 G→C polymorphisms in the IL-6 gene promoter region in order to identify the genes involved in the regulation of bone mass in Korean patients with AIS, who represent an ethnically homogeneous population. We compared the frequencies of genotype in AIS with those in healthy controls and observed a significant difference in the genotype frequencies for IL6-572 G→C polymorphism, which was associated with lumbar spine BMD in patients with AIS. The prevalence of the three IL6-572 G→C genotypes in these subjects were GG 62.6%, GC 32.3% and CC 2.0%. Patients with AIS who had the C allele had a significantly higher lumbar spine BMD but not femoral neck BMD. Our findings are similar to those reported in other studies.^{26,27}

There were some limitations to this study. The number of samples tested was relatively small, which diminishes its statistical power and the possibility of detecting correlations. Only some patients had a lumbar curve of > 30°, which can diminish reliability in the measurement of BMD of the lumbar spine. Further studies with a larger and more homogeneous group of patients are recommended. We did not evaluate interactions with other genes, such as the oestrogen receptor gene, or other relevant gene polymorphisms, such as the calcium-sensing receptor gene. The association with other factors, such as the markers of bone metabolism, bone quality and other candidate genes, should also be tested.

We examined the association between BMD and IL6-572 G→C gene polymorphisms in Korean girls diagnosed with AIS. The IL6-572 G→C polymorphism was found to influence lumbar spine BMD, but the definite mechanism for the low bone mass in AIS is unknown.

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