ID4 Gene Polymorphism and Osteoporosis in Thai Menopausal Women

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Background: *The inhibitor of DNA binding 4 (ID4) protein regulates osteogenic and adipogenic cell fate and lack of ID4 gene expression decreased osteoblast differentiation. Variant in the ID4 gene polymorphism has not been reported with osteoporosis.*
Objective: *To identify whether ID4 can be a marker gene for osteoporosis in Thai menopausal women.*
Material and Method: *The 3'UTR of ID4 (rs3798339) single nucleotide polymorphism was examined by polymerase chain reaction-restriction fragment length polymorphism method (PCR-RFLP), together with lumbar spine bone mineral density (BMD) in 160 Thai menopausal women.*
Results: *Lumbar spine 3 (L3) had a significantly lower BMD score in women with the TT genotype, compared with the CT+CC genotypes (p = 0.037). This disappeared after the adjustment of various factors.*
Conclusion: *The polymorphism at 3'UTR of ID4 gene can alter ID4 mRNA stability, and may be linked to the function of proteins. However, this needs confirmation in larger populations. The present study is useful as an initial investigation into ID4 gene polymorphism in osteoporosis.*

Keywords: *ID4, Single nucleotide polymorphism, Menopausal women, Osteoporosis*

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Osteoporosis, a metabolic bone disease, has become a global public health problem. Primarily, the result of low bone density is one of the social and economic burdens. Rates of occurrence are increasing steadily due to ageing of the world's population[1]. Many risk factors, including genetic disturbances and environmental management, have been linked to decrease bone mineral density (BMD) and osteoporotic fractures[2].

Mesenchymal stem cells (MSC), a source of precursor cells, are linked to bone cell differentiation, and are connected to osteoporosis by rate of differentiation between osteoblasts and adipocytes[3]. Many basic helix-loop helix (bHLH) transcription factors are well recognized as regulators of differentiation in adipocytes and osteoblasts[4]. Interestingly, one bHLH member, inhibitor of DNA binding 4 or ID4 protein, has been reported to be a late marker of osteogenesis. In terms of the mesenchymal cell differentiation, it shows toward osteoblasts and adipocytes[5,6].

ID4 protein is encoded by the ID4 gene located in chromosome 6p22.3, and may be targeted for predicting and preventing the onset of age-related osteoporosis. ID4 regulates osteogenic and adipogenic cell fate[5] and lack of ID4 gene expression enormously decreases osteoblast differentiation, while increasing adipocyte levels[6].

At present, little is known regarding the effects of ID4 gene polymorphism on osteoporosis. Therefore, the present study was aimed at investigating links between single nucleotide gene polymorphism (SNP) in the ID4 gene that might affect ID4 protein function and BMD in Thai women.

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Material and Method

Subjects

One hundred sixty Thai females (age range 40 to 70 years) who attended the Menopause Clinic,

Department of Obstetrics and Gynecology at Ramathibodi Hospital, Bangkok for a physical check-up were recruited. All subjects were apparently in good health, and signed an informed consent form to participate in the study. The study protocol was approved by the Ethics Committees of the Faculty of Tropical Medicine and the Faculty of Medicine (Ramathibodi Hospital), Mahidol University, Bangkok, Thailand.

BMD measurement

Bone mineral density (BMD, g/cm^2) was assessed at the lumbar spine (L1, L2, L3, L4), using dual-energy X-ray absorptiometry (DEXA) (Lunar Prodigy, Lunar, USA).

Genotyping of the ID4 SNP at position rs3798339 T/C in 3'untranslated region (3'UTR) (database from NCBI)

Genomic DNA was extracted from frozen blood samples (stored at -80°C) using a Flexi Gene DNA Kit (Qiagen, Hilden, Germany). Genotyping of the genetic variants of ID4 was amplified by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP), with the forward and reverse primers being designed as 5'CAA ACA GAC CAC GTT ATA CAC ACA3' and 5'CGC TAA GCT ACT GTC CAA TCT C3', respectively. The amplification products yielded a 350-bp fragment. Each variant was digested by *SspI* restriction endonuclease in a 37°C incubator for three hours, before being visualized under UV detection using 4% (w/v) agarose gel electrophoresis containing 0.5 ug/mL ethidium bromide.

Statistical analysis

Statistical analysis was performed using the software program SPSS 11.5 for Windows (SPSS Inc., Chicago, Illinois, USA). Links between genotypes, BMD, and biochemical parameters were examined by Mann-Whitney U test and Kruskal-Wallis test. A *p*-value less than 0.05 was considered statistically significant.

Results

In the present study, links between ID4 gene polymorphism at rs3798339 and lumbar spine bone mineral density in 160 Thai women were found. Baselines of age, weight, height, body mass index (BMI), nutritional parameters, and lumbar spine BMD were presented in Table 1. ID4 polymorphism was identified by PCR-RFLP and visualized on gel

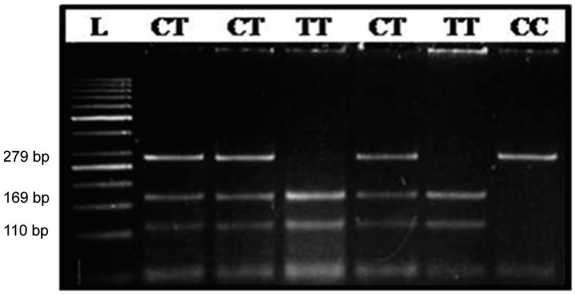

Fig. 1 Fragment patterns of ID4 SNP at rs3798339.

electrophoresis; the fragment patterns are shown in Fig. 1. Fragments with 169 and 110 bp for wild-type (TT), 279, 169, and 110 bp for heterozygous (CT), as well as 279 bp for homozygous (CC), were observed. No statistical differences among genotypes, weight, height, BMI, and serum calcium, total protein, globulin, hemoglobin, and hematocrit were detected (Table 2).

When classifying ID4 polymorphism into wild-type (TT) and variant type (CT+CC) groups, the genotypes of the former group suggested a trend of reduced lumbar spine BMD (L1, L2, L3, and L4) in comparison with the variant type of CT+CC (Fig. 2). However, only lumbar spine 3 exhibited a significantly lower BMD in relation to the TT genotype ($p = 0.037$). Combinations of the effects on different genotypes, and factors related to osteoporosis risk, were analyzed by logistic regression model (Table 3). After

Table 1. Demographic characteristic of women (n = 160)

Characteristic	Median (min-max)
Age (years)	56 (40-70)
Height (cm)	155 (142-173)
Weight (kg)	56.6 (38.1-75.0)
Body mass index (BMI) (kg/m^2)	24.09 (15.46-39.54)
Calcium (mmol/L)	9.3 (7.8-10.8)
Total protein (g/L)	75.9 (66.8-85.3)
Albumin (g/L)	45 (34.7-51.7)
Globulin (g/L)	31.0 (22.0-43.0)
Hemoglobin (g/dL)	12.9 (10.5-15.4)
Hematocrit (%)	38.6 (32.1-45.0)
Lumbar spine1 (g/cm^2)	0.925 (0.610-1.363)
Lumbar spine 2 (g/cm^2)	0.968 (0.638-1.485)
Lumbar spine 3 (g/cm^2)	1.028 (0.710-1.628)
Lumbar spine 4 (g/cm^2)	1.046 (0.710-1.628)

classification of the lumbar spine 3 BMD cut-off point T-score for osteoporosis[1], older aged (>60 years) individuals were found to possess a higher osteoporosis/osteopenia cut-off in terms of risk (odds ratio for age = 3.30); BMI showed a positive effect, however, with an odds ratio = 0.19. Albumin level was included in this calculation because it showed a significant difference among genotypes. It had no significant effect on the BMD cut-off in this equation as ID4 polymorphism. Therefore, age and BMI might be employed as the adjustment factors when analyzing the effect of genotype on osteoporosis risk.

Discussion

The objective of the present study was to investigate determinants of a new osteoporosis marker gene, involving inhibitors of DNA binding 4; ID4 with lumbar spines (L1, L2, L3, and L4) BMD

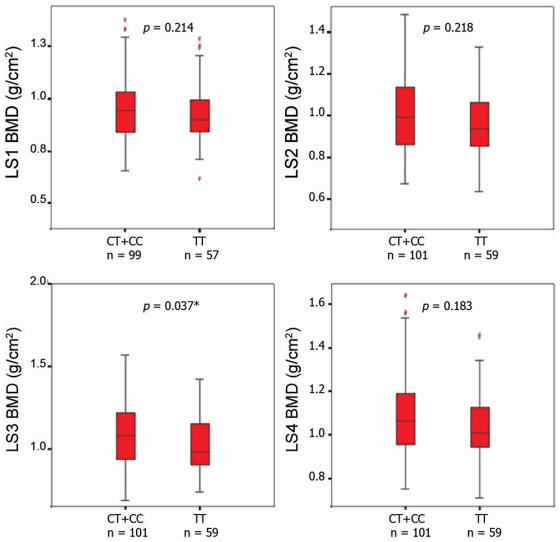

Fig. 2 Association of genotypes and Lumbar spine BMDs.

Table 2. Variant analysis of genotypes

	TT (n = 59)	CT (n = 88)	CC (n = 13)	*p*-value
Weight (kg)	56.6 (38.1-75.0)	55 (39.0-74.3)	55.3 (41.6-65.0)	0.874
Height (cm)	155 (145-173)	155 (142-167)	154 (148-161)	0.947
BMI (kg/m^2)	23.44 (15.46-39.54)	22.77 (16.23-31.53)	22.15 (18.74-28.13)	0.830
Calcium (mmol/L)	9.3 (8.7-10.7)	9.4 (7.8-10.8)	9.2 (8.6-10.0)	0.478
Total protein (g/L)	75.4 (68.0-83.2)	76.6 (66.8-85.3)	75.6 (71.2-79.8)	0.493
Albumin (g/L)	43.9 (34.7-51.7)	45.4 (36.7-51.1)	45.5 (40.2-50.3)	0.031
Globulin (g/L)	32.0 (22.0-41.9)	31.0 (23.1-43.0)	30.5 (22.9-37.5)	0.552
Hemoglobin (g/dL)	13.3 (10.5-15.4)	12.8 (10.6-14.6)	11.4 (10.9-12.7)	0.152
Hematocrit (%)	39.2 (32.1-45.0)	38.6 (32.8-43.0)	35.0 (33.9-36.8)	0.087
Lumbar spine1 (g/cm^2)	0.900 (0.610-1.276)	0.944 (0.657-1.363)	0.940 (0.681-1.198)	0.394
Lumbar spine 2 (g/cm^2)	0.938 (0.638-1.331)	0.986 (0.675-1.485)	1.029 (0.796-1.286)	0.452
Lumbar spine 3 (g/cm^2)	0.986 (0.741-1.425)	1.087 (0.690-1.569)	1.066 (0.866-1.403)	0.113
Lumbar spine 4 (g/cm^2)	1.008 (0.710-1.448)	1.081 (0.690-1.569)	1.022 (0.802-1.407)	0.293

Kruskal-wallis H test, $p<0.05$ was considered statistically significant
Values are expressed as median (min-max)

Table 3. Univariate and multivariate logistic regression analysis of risk factors (age, BMI, serum albumin and ID4 genotypes) for osteoporosis/osteopenia by using lumbar spine 3 BMD cut-off point T-score for osteoporosis

Factors	Crude OR	(95% CI)	*p*-value	Adjusted OR	(95% CI)	*p*-value
Age (>60 year)	3.23	1.63-6.43	0.001	3.30	1.74-6.25	<0.001
BMI (≥25 kg/m^2)	0.24	0.10-0.58	0.001	0.19	0.08-0.42	<0.001
Albumin (>43.1 g/L)	0.71	0.35-1.44	0.366	0.76	0.44-1.31	0.763
ID4 (CT+CC, TT)	1.46	0.74-2.87	0.299	1.56	0.74-3.27	0.239

BMD = bone mineral density; OR = odds ratio; 95% CI = 95% confidence interval
$p<0.05$ was considered statistically significant
A multivariate logistic regression analysis using a backward Wald method

by PCR-RFLP method. The genotype of ID4 was associated with the BMD of L3, possibly led to the discovery of a relationship in terms of protein function.

The ID4 protein is a member of the basic helix-loop helix (bHLH) transcription factor superfamily, which has been reported in osteogenesis regulations[7-9] by proliferation and differentiation of mesenchymal stem cells (MSCs) and by controlling osteoblasts or adipocytes[10]. Unbalanced differentiation of MSCs into bone marrow adipocytes or osteoblasts can cause an excessive accumulation of bone marrow adipocytes, as observed in senile osteoporosis[11]. Furthermore, a reduction of osteoblast differentiation in an ID4 gene-deficient mice study was observed with adipocytes instead of osteoblasts[6].

The authors hypothesize that polymorphism at 3'untranslated region (3'UTR) of the ID4 gene in the present study (rs3798339) is associated with BMD by regulation of transcription. It is possible 3'UTR of this gene alters its mRNA stability, which relates to the appearance and function of proteins[12] in mesenchymal stem cell differentiation in osteoblasts or adipocytes. These cells ultimately affect the occurrence of osteoporosis[5].

Nevertheless, the significance of this association was not found when calculating combinations of these factors with logistic regression. Age and BMI may be the most important points for consideration in this genetic polymorphism study, as well as the impact of the small sample size[13] used in the present pilot.

Conclusion

The polymorphism at 3'UTR of ID4 can alter ID4 mRNA stability, and may be linked to the function of proteins. However, this needs confirmation in larger populations. The present study is useful as an initial investigation into ID4 gene polymorphism in osteoporosis. For further analysis, experiments should be conducted in larger populations to confirm the impact of ID4 as a marker gene for osteoporosis. However, the present study is useful as an initial investigation into ID4 gene polymorphism in osteoporosis.

What is already known on this topic?

Osteoporosis risk factors are genetic disturbances and environmental management. Genetic polymorphism has been linked to bone mineral density (BMD) and osteoporotic fractures.

What this study adds?

The 3'UTR of ID4 (rs3798339) single nucleotide polymorphism was examined by polymerase chain reaction-restriction fragment length polymorphism method (PCR-RFLP), together with lumbar spine bone mineral density (BMD) in 160 Thai menopause women. Lumbar spine 3 (L3) had a significantly lower BMD score in women with the TT genotype, compared with the CT+CC genotypes (p = 0.037). This disappeared after the adjustment of various factors. However, this study is useful as an initial investigation into ID4 gene polymorphism in osteoporosis.

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Potential conflicts of interest

None.

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ความแปรผันทางพันธุกรรมของโปรตีนยับยั้งการจับของดีเอ็นเอ 4 (ID4) ต่อความหนาแน่นมวลกระดูกสันหลังที่ลดลงในผู้หญิงไทยวัยหมดประจำเดือน

เชาวนี ชูพีรัชน์, สิริกุล กุลานุวัฒน์, วนิดา ชื่นตา, ศิวพร วรรณะเอี่ยมพิกุล, วาเนสซ่า แอนเน่ ชูช์, แสงชัย พฤทธิพันธุ์, รังสรรค์ ตั้งตรงจิตร

ภูมิหลัง: *โปรตีนยับยั้งการจับของดีเอ็นเอ 4 (ID4) ควบคุมการสร้างเซลล์กระดูกและเซลล์ไขมัน การไม่ทำงานของจีน ID4 ส่งผลให้การเปลี่ยนไปทำหน้าที่ของเซลล์ออสติโอบลาสต์ลดลง ความแปรผันทางพันธุกรรมของจีน ID4 ยังไม่เคยมีรายงานในโรคกระดูกพรุนมาก่อน*

วัตถุประสงค์: *การศึกษานี้จึงมีวัตถุประสงค์เพื่อระบุว่า ID4 สามารถเป็นจีนตัวติดตามสำหรับโรคกระดูกพรุนในผู้หญิงไทยได้*

วัสดุและวิธีการ: *การตรวจความสัมพันธ์ระหว่างความแปรผันทางพันธุกรรมของจีน ID4 กับความหนาแน่นมวลกระดูกในครั้งนี้ศึกษาตำแหน่งด้าน 3' UTR (rs3798339) ของจีน ID4 ด้วยวิธีการเพิ่มปริมาณสารพันธุกรรม และใช้เอนไซม์ตัดจำเพาะในผู้หญิงไทยวัยหมดประจำเดือน จำนวน 160 คน*

ผลการศึกษา: *พบว่าจีโนไทป์ TT สัมพันธ์กับค่าความหนาแน่นมวลกระดูกตำแหน่งกระดูกสันหลัง L3 ที่ลดลงเมื่อเปรียบเทียบกับจีโนไทป์ CT รวมกับ CC อย่างมีนัยสำคัญทางสถิติ ($p = 0.037$) แต่เมื่อมีการปรับปัจจัยต่าง ๆ ในการคำนวณพบว่า ความสัมพันธ์ระหว่างจีโนไทป์กับมวลกระดูกนั้นไม่มีนัยสำคัญทางสถิติ*

สรุป: *ความแปรผันของจีนตำแหน่ง 3' UTR สามารถเปลี่ยนแปลงความเสถียรของ mRNA และอาจจะเชื่อมโยงกับหน้าที่ของโปรตีน ID4 ได้ อย่างไรก็ตามต้องมีการศึกษาเพิ่มเติมในประชากรจำนวนเพิ่มขึ้น ประโยชน์ในการศึกษาในครั้งนี้สามารถเป็นจุดเริ่มต้นของการตรวจสอบความแปรผันทางพันธุกรรมของจีน ID4 ในโรคกระดูกพรุน*