Association of bone mineral density with vitamin D and estrogen receptor gene polymorphisms during GnRH agonist treatment

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Abstract

Aims: This study examined whether or not a decrease in bone mineral density (BMD) induced by the use of gonadotropin-releasing hormone agonist (GnRHa) during sexual maturation is affected by vitamin D receptor and/or estrogen receptor gene polymorphisms, like the phenomenon observed during the postmenopausal period.

Methods: In 43 patients who received GnRHa therapy for 6 months to treat uterine myoma or endometriosis at our department and who were confirmed to have pituitary down-regulation, we measured bone density before and after GnRHa treatment using DXA and analyzed the bone metabolism turnover using bone metabolic markers. Polymorphisms were analyzed by RFLP using FokI and TaqI for the vitamin D receptor gene and PvuII and XbaI for the estrogen receptor gene. The then determined gene polymorphism was analyzed in relation to the percentage decreases in BMD following GnRHa treatment.

Results: The patients were divided by \( f, t \) into two groups: \((f, t) < 2 \) (Group V-I) and \((f, t) \geq 2 \) (Group V-II). They were also divided by \( P, x \) into two groups \((P, x) < 3 \) (Group E-I) and \((P, x) \geq 3 \) (Group E-II). The BMD change was significantly higher in Group V-II than in Group V-I. Group E-II tended to have a higher BMD change than Group E-I, although this difference was not statistically significant.

Conclusions: Patients who often have \( f \) and \( t \) polymorphism are more likely to show BMD reduction following GnRHa therapy, like the phenomenon seen during the postmenopausal period, than patients with other gene polymorphisms. Measures to avoid BMD reduction are required when using GnRHa in such patients.

Key words: BMD, estrogen receptor, GnRH agonist, osteoporosis, vitamin D receptor.

Introduction

As the average life expectancy of the population has been prolonged, close attention has begun to be paid to osteoporosis, which can affect the quality of life of elderly people. Bone mass is determined by environmental factors (nutrition, exercise etc.) and genetic factors. Regarding genetic factors, the gene encoding vitamin D receptors (VDR gene) has been reported to show polymorphisms, and bone mass varies depending on the polymorphisms of this gene. It has also been reported that estrogen is involved in the rapid reduction of bone mass after menopause. Women can undergo a sharp decrease in bone mass following menopause. Following recent changes in the lifestyle of women, the prevalence of estrogen-dependent diseases such as endometriosis and uterine myoma has been increasing. The frequency of using gonadotropin-
releasing hormone agonist (GnRHa) as drug therapy for these estrogen-dependent diseases has also been increasing. GnRHa induces estrogen deficiency and reportedly reduces bone mass by about 2-5% when used for 6 months. GnRHa is used after peak bone mass is reached. A decrease in bone mass during this period can serve as a significant risk factor for postmenopausal osteoporosis.

The present study examined whether or not the degree of decrease in bone mass and changes in bone metabolic markers following GnRHa therapy were associated with the polymorphism of genes encoding VDR and estrogen.

Materials and Methods
Materials
The subjects of this study were 76 patients, 20-35 years old, who visited our department between April 1996 and June 2002, and received GnRHa for the treatment of endometriosis or uterine myoma. This study was approved by the Juntendo Ethical Committee, and all blood specimens were obtained with written informed consent. The GnRHa preparations used were buserelin acetate (Suprecur Mochida Pharmaceutical Co. Ltd, Tokyo) 900 µg/day, nafarelin acetate (Nasanyl Yamanouchi Pharmaceutical Co. Ltd, Tokyo) 200 µg/day and leuprorelin acetate (Leuplin Takeda Chemical Industries Ltd, Tokyo) 1.88 or 3.75 mg/month. Of these 76 patients, 43 who satisfied all of the following requirements were included in the analysis: (i) no history of disease or drug therapy affecting bone mass; (ii) received treatment with GnRHa for 6 months; (iii) pre-dosing serum FSH level below 20 mIU/mL; (iv) serum estradiol (E₂) level below 30 pg/mL after 2 and 6 months of treatment; (v) complete data were available; and (vi) DNA analysis was possible. Thirty-three patients dropped out of the study prematurely, including 19 cases where E₂ was not lower than 30 pg at one or both of the measurements made 2 or 6 months after the start of dosing, five cases where data were incomplete, two cases where DNA analysis was not possible, and seven cases where GnRHa was discontinued within 6 months. Before the start and 6 months after the start of dosing, serum bone alkaline phosphatase (B-ALP) and osteocalcin (OC) levels were measured as markers of ossification. At the same time, measurements of urinary deoxypyridinoline (D-PYR), an indicator of bone resorption, were taken.

Furthermore, bone mineral density (BMD) was measured by dual energy X-ray absorptiometry (DXA) before and 6 months after the start of GnRHa therapy. Serum LH, FSH and E₂ levels were measured by enzyme immunoassay (EIA) before and 2 and 6 months after the start of dosing.

Methods
(1) RFLP analysis of VDR and estrogen receptor genes
Heparinated whole blood samples were used for the extraction of genomic DNA. Whole blood (10 mL) was combined with 30 mL of 1 x Lysis buffer. The mixture was agitated with the container placed upside down. The specimen was then placed in an ice bath for 30 min. After 5-min centrifugation at 2000 r.p.m., the supernatant was discarded and the remainder was suspended again in 30 mL of 1 x Lysis buffer. After further centrifugation under the same conditions, the supernatant was discarded. The remainder was combined with 1 mg of proteinase K (Wako Pure Chemical Industries, Ltd, Osaka), 4 mL of HMW buffer and 1 mL of 10% SDS. The mixture was agitated overnight at room temperature. The specimen was then combined with 5 mL of TE-saturated phenol (Nippon Gene Co., Ltd, Tokyo) and agitated aggressively for 10 min. This was followed by 30 min centrifugation at 3000 r.p.m. The upper layer was transferred into a 15-mL tube and combined with 5 mL of TE-saturated phenol/CHCL3 (Nippon Gene Co., Ltd, Tokyo). The mixture was agitated aggressively for 20 min and then centrifuged at 3000 r.p.m. for 30 min. The supernatant was combined with cold 100% ethanol (10 mL) and centrifuged at 3000 r.p.m. for 30 min. The supernatant was discarded, and the remainder was slightly rinsed with cold 80% ethanol and then with cold 100% ethanol. The specimen was then air-dried. The dried specimen was combined with 300 µL of distilled water and left to stand at 4°C for 3 days to dissolve the DNA. The then obtained DNA solution was diluted to a concentration of 100 µg/mL for use in PCR.

To check each sample for restriction fragment length polymorphism (RFLP), the following sites of VDR and estrogen receptor (ER) genes were amplified by PCR: the site of the EDR gene containing exon 2 (the site recognized by restriction enzyme FokI) using 2 primers (primer #1: 5-AGCTGGCCCTGGCACTGACTCTGCTCT-3, and primer #2: 5-ATGGAAACACCTGCTTCTTCTCCCT, and primer #2: 5-ATGGAAACACCTTGTCTTCTCCCT, and the site of the VDR gene containing exon 9 (recognized by TaqI) using two primers (primer #3: 5-CAGACGATGGACGGAGCAAG-3, and primer #4: 5-GCAACTCCTCATTGCTGAGTCTC), and the portion of the ER gene containing intron 1
(recognized by restriction enzyme PvuII and XbaI) using two primers (primer #5: CTGCCACCTATCTGT TACTTTTCTATTCTCC-3, and primer #6: 5-TCTTTCTGCACCTGCGTGATTATCTGA).

Each PCR product was subjected to RFLP polymorphism analysis using FokI (Roche, USA) and TaqI (Roche, USA) for the VDR gene and PvuII (Roche, USA) and XbaI (Roche, USA) for the ER gene. The allele of the gene digested with restriction enzymes was called f, t in the case of VDR and p, x in the case of ER. The alleles that were not digested were called F, T, P and X. Genotypes of the VDR gene were expressed as FF, Ff, ff, TT, Tt and tt, and those of the ER gene as PP, Pp, pp, XX, Xx and xx.

(2) Bone density measurement
Bone density at the anterior and posterior planes of the second through fourth lumbar vertebrae was measured by DXA (QDR-200, Hologic) before and 6 months after the start of GnRHa therapy. The percentage decrease in bone density was calculated by dividing the difference between pre- and post-dosing density by the predosing density.

(3) Markers of bone metabolism
As markers of ossification, blood bone type ALP (EIA) and OC (IRMA) were quantified, and as a marker of bone resorption, urinary D-PRY (EIA) was quantified. All of these parameters were measured during the hospital visit.

(4) Statistical analysis
A Mann–Whitney test was used for statistical analysis of the data. \( P < 0.05 \) was regarded as significant.

Results

Haplotyping of VDR and ER
Shiraki reported that the effects of individual genes have a limit and that individual conditions can be evaluated more accurately by using combinations of related genes.\(^9\) Based on this report, we classified cases according to combinations of VDR and ER gene polymorphism. Since cases where VDR genes have f or t tend to show lower bone density,\(^{10–18}\) we divided the cases into two groups: Group V-I where \((f, t) < 2\) and Group V-II where \((f, t) \geq 2\). Since cases where ER genes have P or x tend to show lower BMD,\(^{19–21}\) the cases were divided into two groups: Group E-I where \((P, x) < 3\) and Group E-II where \((P, x) \geq 3\). There was no significant difference in background of age and body mass index (BMI) between Groups V-I and V-II or between Groups E-I and E-II.

Markers of bone metabolism
All cases showed higher bone metabolic turnovers during GnRHa treatment, irrespective of VDR or ER gene polymorphisms. In Groups V-I and V-II, all of OC, B-ALP and D-PRY increased significantly after GnRHa treatment, as compared to their pretreatment levels. In Groups E-I and E-II, all markers (except for D-PRY in Group E-II) increased significantly after treatment.

BMD
Pre-treatment BMD did not differ significantly between any two groups. The percentage decrease in BMD following GnRHa treatment ranged from 0.9 to 7.2% in individual groups. The decrease in BMD was significantly greater in Group V-II than in Group V-I \((P < 0.05)\). The decrease in BMD was greater in Group E-II than in Group E-I, although the difference was not significant.

Discussion
GnRHa decreased the production of estrogen in the ovaries by suppressing the pituitary function. This drug has been used for hormone-dependent gynecologic tumors such as uterine myoma and endometriosis.

Estrogen deficiency induced by GnRHa shortens the nucleus of uterine myoma and alleviates the intrapelvic inflammation associated with endometriosis. This is why GnRHa has been used for the treatment of these conditions.

Table 1 Patients characteristics for the vitamin D receptor and estrogen receptor polymorphism

<table>
<thead>
<tr>
<th></th>
<th>Group V-I</th>
<th>Group V-II</th>
<th>Group E-I</th>
<th>Group E-II</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number</td>
<td>31</td>
<td>12</td>
<td>33</td>
<td>10</td>
</tr>
<tr>
<td>Age (mean ± SD)</td>
<td>31.3 ± 3.4</td>
<td>29.5 ± 2.6</td>
<td>31.1 ± 2.7</td>
<td>30.6 ± 3.2</td>
</tr>
<tr>
<td>Body mass index (mean ± SD)</td>
<td>20.0 ± 1.3</td>
<td>20.4 ± 1.7</td>
<td>19.7 ± 1.0</td>
<td>20.3 ± 1.7</td>
</tr>
</tbody>
</table>

Note: \( P \) values are not significant.
In recent years, endoscopic surgery using a laparoscope or hysteroscope has advanced remarkably in the field of gynecology. At present, most cases of uterine myoma or endometriosis are indicated for endoscopic surgery. To allow safe endoscopic operation, which involves many more limitations than open surgery, preoperative GnRHa tends to be used frequently. Since GnRHa induces temporary menopause in women during sexual maturation, various adverse reactions to this drug, resembling climacteric disturbances, have been reported. Most of these reactions are transient in nature and often mild. A decrease in the bone mineral level can be viewed as the most serious adverse reaction to this drug.

Although a number of reports on GnRHa-reduced bone mass have been published, few of them have dealt with genetic factors responsible for the reduction in BMD.

In Japan, a variety of GnRHa preparations have been used, including nasal sprays and depots, and the effect in suppressing the pituitary varies among different preparations. The subjects of the present study were confined to those patients in whom complete suppression of the pituitary gland was confirmed, irrespective of the type of GnRHa preparation used.

Bone mass density is reportedly reduced by 2–5% following 6 months of GnRHa therapy. In the present study, bone mass decreased by 3.7% on aver-

**Table 2** Bone Metabolic Markers during GnRHa treatment in vitamin D receptor polymorphism

<table>
<thead>
<tr>
<th></th>
<th>Group V-I (n = 31)</th>
<th></th>
<th></th>
<th>Group V-II (n = 12)</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pre-treatment</td>
<td>post-treatment</td>
<td>P-value</td>
<td>pre-treatment</td>
<td>post-treatment</td>
<td>P-value</td>
</tr>
<tr>
<td>Serum osteocalcin</td>
<td>7.1 ± 2.1</td>
<td>10.2 ± 3.4</td>
<td>0.01</td>
<td>5.3 ± 1.2</td>
<td>7.3 ± 1.9</td>
<td>0.01</td>
</tr>
<tr>
<td>B-ALP</td>
<td>57.6 ± 38.4</td>
<td>71.6 ± 24.2</td>
<td>0.01</td>
<td>45.5 ± 14.0</td>
<td>57.4 ± 18.7</td>
<td>0.01</td>
</tr>
<tr>
<td>Urinary deoxypiridinoline</td>
<td>5.3 ± 1.3</td>
<td>6.4 ± 1.7</td>
<td>0.01</td>
<td>4.6 ± 1.0</td>
<td>5.9 ± 1.7</td>
<td>0.01</td>
</tr>
</tbody>
</table>

All data are presented as the mean ± SD. B-ALP, serum bone alkaline phosphatase.

**Table 3** Bone metabolic markers during GnRHa treatment in estrogen receptor polymorphism

<table>
<thead>
<tr>
<th></th>
<th>Group E-I (n = 33)</th>
<th></th>
<th></th>
<th>Group E-II (n = 10)</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pre-treatment</td>
<td>post-treatment</td>
<td>P-value</td>
<td>pre-treatment</td>
<td>post-treatment</td>
<td>P-value</td>
</tr>
<tr>
<td>Serum osteocalcin</td>
<td>5.3 ± 1.3</td>
<td>8 ± 2.7</td>
<td>0.01</td>
<td>6.3 ± 2.0</td>
<td>8.2 ± 2.4</td>
<td>0.01</td>
</tr>
<tr>
<td>B-ALP</td>
<td>46.4 ± 26.5</td>
<td>58.5 ± 20.8</td>
<td>0.01</td>
<td>52.4 ± 9.0</td>
<td>67.6 ± 20.0</td>
<td>0.01</td>
</tr>
<tr>
<td>Urinary deoxypiridinoline</td>
<td>4.7 ± 1.2</td>
<td>5.9 ± 1.6</td>
<td>0.01</td>
<td>4.9 ± 1.1</td>
<td>6.1 ± 1.9</td>
<td>NS</td>
</tr>
</tbody>
</table>

All data are presented as the mean ± SD. B-ALP, serum bone alkaline phosphatase.

**Figure 1** Percentage decrease in lumbal spine mineral density (LS-BMD) during GnRHa treatment: (a) vitamin D receptor polymorphism; (b) estrogen receptor polymorphism.
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age (range: 0.9–7.2%) following 6 months of GnRHa therapy, consistent with previous reports.

In the present study, OC and B-ALP were used as the markers of ossification, and urinary D-PY was examined as a marker of bone resorption. All of these markers increased after treatment, indicating that the decrease in BMD during GnRHa therapy involves the enhanced metabolic turnover of bone. Thus, the present study revealed that treatment with GnRHa increases the metabolic turnover of bone, and that even in women during sexual maturation, the GnRHa-induced reduction of estrogen can lead to a state of bone metabolism resembling that which prevails during the postmenopausal period.

Women assigned to Group V-II have a risk factor for genetic bone mass reduction. Ferrari et al. reported that the BMD in this group of preadolescent girls was lower than that in the control group. Other investigators reported that BMD did not differ between women assigned to Group V-II and the other women. In the present study, the predosing bone density did not differ significantly between Groups V-I and V-II. The absence of a significant difference in this parameter may be associated with the fact that the number of cases studied was relatively small.

The percentage change in BMD was significantly higher in Group V-II than in Group V-I. In view of the report that women who have f and t tend to have lower bone mass even when they receive no GnRHa treatment, it is likely that BMD is further reduced by GnRHa treatment in these women. This finding of the GnRHa-induced enhancement of bone metabolism suggests that women belonging to Group E-II, whose bone metabolism is more dependent on estrogen, are likely to undergo a decrease in BMD. Kobayashi et al. examined the bone mass of postmenopausal women and reported that a significant decrease in BMD was noted in the group possessing many (P, x). In this study, BMD did not differ significantly between Groups V-I and V-II. However, the BMD in Group E-II tended to be low, as compared to the control group.

It has been reported that f and t of the VDR gene and P and x of the ER gene are associated with the low bone density of sexually mature women and the percentage decrease in bone density during the peri-menopausal period. The present study showed that even in cases of estrogen deficiency, artificially induced by GnRHa during sexual maturation, the f and t of VDR and the P and x of ER are involved in the decrease of BMD.

Because GnRHa is used during the peak bone mass maintenance stage, the decrease in BMD induced by its use may serve as a risk factor for the future onset of osteoporosis. The women belonging to Groups V-II and E-II have gene polymorphisms that can reduce the bone density of sexually mature women and cause a more rapid decrease in bone density during the postmenopausal period. The results of the present study indicate that treatment of these high-risk groups with GnRHa can cause a higher percentage of decrease in bone density as compared to the other groups of women.

The decrease in BMD induced by pseudomenopausal therapy using GnRHa was of a type similar to that seen in postmenopausal women. These patterns of decrease in BMD are of a high metabolic turnover type. The number of patients with endometriosis or uterine myoma has been increasing recently. Gynecologists often encounter cases where GnRHa needs to be administered repeatedly.

This study suggests that f and t serve as risk factors for bone mass reduction associated with GnRHa therapy. When using GnRH agonists, it is advisable to check the BMD and bone metabolic marker of individual patients, bearing in mind the possibility of VDR gene polymorphisms f and t, and to take active measures for the prevention of BMD that may be induced by the drug.

References

Association of BMD with polymorphism


