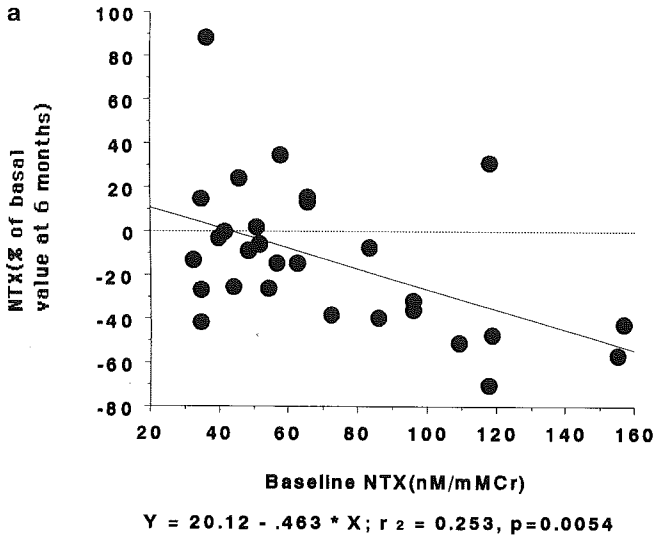

Fig. 3. Reduction of bone turnover by alfacalcidol treatment is dependent on the baseline bone turnover rate. **a** Correlation between baseline *N*-telopeptide (*NTX*) level and change in NTX after 6 months of treatment. **b** Correlation between baseline bone-derived alkaline phosphatase (*BAP*) level and change in BAP after 6 months treatment. Correlations between baseline levels of bone turnover markers and changes in these markers after 6 months of treatment with alfacalcidol were calculated. A highly significant negative correlation between the baseline level of NTX and the change in NTX after intervention was observed. ($Y = 20.12 - 0.463 * X$; $r_2 = 0.253$; $P = 0.0054$). The same trend was also found for BAP ($Y = 13.054 - 0.449 * X$; $r_2 = 0.253$; $P = 0.0054$). The results indicate that alfacalcidol reduces bone turnover in patients with osteoporosis with high bone turnover

incident fracture. Bisphosphonates are thought to be the most powerful agents for reducing bone resorption, and many recent reports have shown their beneficial effects on bone fracture prevention in osteoporosis [8–10]. However, bisphosphonate treatment induces at least transient secondary hyperparathyroidism, especially in elderly patients, because it inhibits bone resorption [3]. In this regard, alfacalcidol may still play an important role in the treatment of osteoporosis.

However, very little information has been available on changes in bone turnover rates after treatment with alfacalcidol [12,13]. The hormonal form of vitamin D_3 ($1,25[OH]_2$ vitamin D_3) is known to stimulate bone resorption in in vitro and animal studies [14], although previous clinical reports have shown no effect on bone resorption after active vitamin D_3 treatment at a clinical dosage [2,3]. We have reported that alfacalcidol 0.75 μg/day increased the urinary excretion of calcium without a concomitant increase in the urinary excretion of hydroxyproline [2]. These results indicates that alfacalcidol may not stimulate bone resorption as seen in in vitro studies, and thus the increase in urinary excretion of calcium probably resulted from enhanced intestinal calcium absorption [6].

In the present study, we attempted to investigate the effects of alfacalcidol on bone turnover in elderly patients with osteoporosis who were expected to have a calcium imbalance due to low intestinal calcium absorption or low intake of calcium [15]. The baseline characteristics in the present study revealed that the elderly patients with osteoporosis had markedly high levels of bone turnover markers and, thus, accelerated bone resorption rates.

Alfacalcidol effectively inhibited PTH secretion by about 30% compared with the baseline value, and it increased urinary calcium excretion by approximately 60%–100%. These results are in good agreement with those from previous studies [2,3]. Therefore, we can conclude that alfacalcidol is effective in improving the calcium imbalance in elderly women with osteoporosis.

Bone turnover marker levels decreased after alfacalcidol administration. However, the exact mechanism(s) by which alfacalcidol inhibits bone turnover in patients with osteoporotics remains unclear. The baseline level of I-PTH and the decrease in PTH after alfacalcidol treatment may account for the decrease in resorption, because PTH is known to facilitate resorption [16]. The baseline level of serum I-PTH was significantly correlated with the decrease in NTX and BAP levels (Table 4), suggesting that patients with secondary hyperparathyroidism respond well to alfacalcidol treatment in terms of reduction of bone turnover.

However, direct correlations between changes in bone resorption markers and serum I-PTH levels after treatment were not identified at any time point. The remote effects of the decrease in PTH on bone turnover were also not significant (Table 5). We have to consider the possibility that the present assay system for PTH

cross-reacts with both 1–84 PTH and N-truncated PTH fragments and is antagonistic to the whole PTH molecule [17]. Thus, whole PTH measurements are required before concluding whether the decreases in bone marker were a result of the reduced PTH secretion.

As shown in Table 1, the patients in the present study had very high levels of bone turnover markers. The same trend in the effect of alfacalcidol treatment on bone turnover in women immediately after menopause who had high bone turnover rates was reported [18], suggesting that alfacalcidol may depress bone resorption only in patients with accelerated bone turnover. No inhibitory effect of alfacalcidol on the urinary excretion of DPD was found in a phase III study of alendronate [3]. The mean baseline DPD excretion in group D in the present study was 10.3 nM/mM Cr, while it was 6.8 nM/mM Cr in the alendronate phase III study, even though osteoporotic patients were selected. To confirm the hypothesis that alfacalcidol reduces bone turnover only in osteoporotic patients with high bone turnover, we calculated the correlation coefficients between the baseline level of bone markers and their changes after 6 months of treatment. The results clearly demonstrate that the declines in bone resorption markers after the intervention are highly dependent on baseline values. Therefore, we may conclude that alfacalcidol inhibits bone resorption in patients with osteoporosis with high bone resorption rates.

BAP and OC levels were significantly decreased after 6 months of alfacalcidol treatment. The magnitude of the decrease in the serum level of BAP after alfacalcidol treatment also depended on its baseline level. However, there was no correlation between the baseline level of OC and the decrease in serum OC after 6 months of treatment. A possible explanation for this discrepancy between BAP and OC levels may be the time-course difference between them. That is, the time-course change in serum OC level after alfacalcidol administration tended to be delayed comparing with that in serum BAP. A longer observation period would be required to confirm this possibility. The effects of alfacalcidol on bone turnover were statistically significant but slight, and all changes were within the minimal significant change for each marker (22.5% for DPD, 24.7% for NTX, and 17.0% for BAP; from the study of the Committee of Guidelines for Bone Markers of the Japanese Society of Osteoporosis [16]). Therefore, changes in bone turnover after alfacalcidol treatment may be difficult to recognize in individual patients.

The effects of alfacalcidol on BMD in the present study are in good agreement with those found in previous studies. Alfacalcidol treatment increased LBMD by approximately 2%. This increase in LBMD was consistent in previous studies, regardless of bone turnover rates [1–3]. Because baseline BMD and bone turnover have been reported to be independent risk factors for future fracture [7,19,20], the slight inhibition of bone resorption together with the improvement of the calcium balance with alfacalcidol may explain the effects of alfacalcidol on BMD maintenance and fracture prevention.

In conclusion, alfacalcidol increased the urinary excretion of calcium and decreased the serum level of PTH, possibly through enhancement of intestinal calcium absorption. Alfacalcidol slightly decreased the accelerated bone turnover rate without a direct correlation to the effects on PTH metabolism. Thus, these metabolic effects of alfacalcidol may yield beneficial effects on BMD and on the prevention of osteoporotic fracture.

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Absolute height reduction and percent height ratio of the vertebral body in incident fracture in Japanese women

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Abstract Vertebral fractures are the most common complication associated with osteoporosis. The definition of prevalent or incident vertebral fractures is important in epidemiological evaluation and in clinical trials of osteoporosis treatment. There have been few reports regarding the morphometric cutoff criteria for incident fractures in Asians, and only a few reports of their occurrence in Caucasians. The aim of this study was to establish a definition for incident fractures in Japanese women. A total of 279 women, aged 64.8 ± 9.9 (mean ± SD) years, were recruited, and repeated thoracolumbar spinal radiographs were performed at a mean of 1.45 ± 0.44 years after the baseline film. Vertebral fractures were graded from 0 to 3 in both baseline and follow-up films using a semiquantitative assessment by the consensus of three readers. Incident fractures were defined as an increase of grade, except grade 3, and a vertebral body height of less than 12 mm on the baseline film. As morphometric criteria, both absolute height reduction (AHr) and the percent height ratio (PHr) were calculated. Prevalent fractures were observed in 207 vertebrae (6.2%) and 85 cases (30.5%), and incident fractures in 42 vertebrae (1.3%) and 29 cases (10.4%). As morphometric criteria for incident fracture, both a PHr ≥15% and an AHr ≥3 mm or ≥4 mm indicated sensitivity of 49.17% and 50.83% and specificity of 99.92% and 99.90%, respectively. In conclusion, the morphometric criteria of incident fracture combined with a semiquantitative assessment will provide useful information in the study of clinical osteoporosis, especially for international comparisons.

Key words osteoporosis · incident fracture · cutoff value · vertebral height

Introduction

Osteoporosis is defined as a systemic skeletal disease characterized by low bone mass and microarchitectural deterioration of bone tissue, with a consequent increase in bone fragility and susceptibility to fractures [1]. Fractures in the spine, hip, and forearm are known as fragility fractures. Although vertebral fractures occur at a higher rate than other osteoporotic fractures, one-third of them are asymptomatic [2]. Therefore, accurate diagnosis of vertebral fractures depends on radiologic visual assessment or quantitative vertebral morphometry [3,4]. The definition of prevalent or incident vertebral fractures on radiograms is important not only in clinical trials of osteoporosis for drug therapy [5] but also in epidemiological studies of osteoporosis [6]. There are ethnic differences between Japanese and Caucasians in the number of vertebral fractures [7], incidence of hip fractures [8,9], and dosages of bisphosphonates, alendronate [10,11], and risedronate [12,13] for increased effects on lumbar bone mineral density (BMD) in osteoporotic patients. These ethnic differences are important in clarifying the pathoetiology of osteoporosis. In particular, it is necessary to employ the same criteria for vertebral fractures in comparative studies between different races. Many criteria, both visual semiquantitative and morphometric quantitative, for vertebral fractures have been reported [4,14–16].

In the present study, we determined the morphometric criteria for absolute height reduction (AHr) and the percent height ratio (PHr) of the vertebral body for incident fractures in Japanese women.

Materials and methods

Subjects

A total of 279 Japanese women, aged 64.8 ± 9.9 (mean ± SD) years, who visited the Research Institution and Practice for Involutional Diseases, Nagano, Japan, were enrolled in the study. Written informed consent was obtained from all subjects. Their mean lumbar BMD

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was −1.80 of the T score. The exclusion criteria were severe scoliosis, vertebral deformities other than nontraumatic fractures, and a history of disease known to cause secondary osteoporosis. All participants received no treatment or operation for osteoporosis.

Radiographs

Conventional lateral radiographs of the lumbar and thoracic vertebrae were taken at both baseline and follow-up time points, with a mean interval of 1.45 ± 0.44 years between them. The tube-to-film distance was 105 cm, and the X-ray beam was centered over T7 for the thoracic film and L3 for the lumbar film.

Vertebral fracture

Semiquantitative assessment

Both prevalent and incident vertebral fractures were initially evaluated using semiquantitative assessment (Fig. 1).

A visual semiquantitative grading of 0–3 of the vertebral body of T4 through L5 was done by three independent observers: grade 0 (normal); grade 1 (mildly deformed, approximately 20%–25% reduction in anterior, middle, and/or posterior height and a reduction of area of 10%–20%); grade 2 (moderately deformed, approximately 25%–40% reduction in any height and a reduction in area of 20%–40%); and grade 3 (severely deformed, approximately 40% reduction in any height and area) (Fig. 2) [3].

Three readers simultaneously viewed both the baseline and the follow-up films from T4 to L5 in chronological sequences, and graded vertebral fractures. After these independent readings, both films were reviewed by three observers. When inconsistency between the readings was recognized, the three readers negotiated among themselves to reach an agreement regarding the degree of vertebral fracture and made a consensus reading decision.

Morphometric assessment

The radiographs were digitized using a laser scanner (Hitachi Digitizer HDG-1717BL), and the digitized images were then displayed on a CRT monitor. Six points, superior and inferior points of anterior, middle, and posterior sites, were marked for each vertebral body by an independent trained assistant in a standard fashion (Fig. 3) [14]. With six pointization, the four

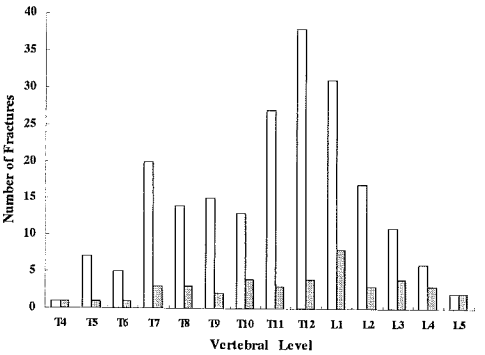

Fig. 1. Distribution of prevalent and incident fractures after consensus reading on semiquantitative assessment. □, Prevalent fracture; ▨, Incident fracture

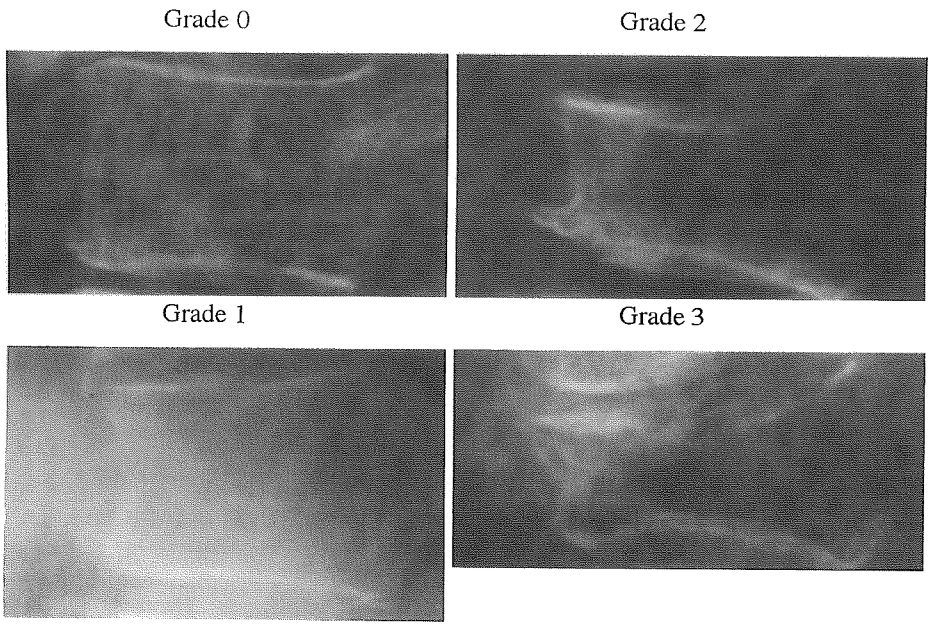

Fig. 2. Semiquantitative grading of vertebral deformity: examples of grades 0 to 3

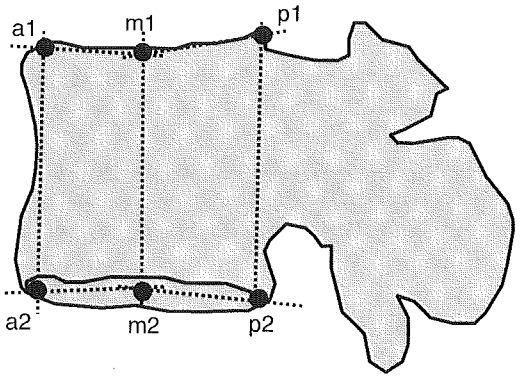

Fig. 3. Pointing method in morphometric assessment. *a*1, anterosuperior; *a*2, anteroinferior; *m*1, middle superior; *m*2, middle inferior; *p*1, posterosuperior; *p*2, posteroinferior; anterior vertebral height (Ha), a1 to a2; middle vertebral height (Hm), m1 to m2; posterior vertebral height (Hp), p1 to p2

corner points, anterosuperior (a1), anteroinferior (a2), posterosuperior (p1), and posteroinferior (p2), of the vertebral body were marked.

An additional point, middle-superior (m1) or middle-inferior (m2), was placed on the middle on a line bisecting the two projections of the upper and lower endplate: two lines, a1 to m1, and m1 to p1, were equal in length, and a2 to m2, and m2 to p2 were also equal. The anterior (Ha), middle (Hm), and posterior (Hp) heights and anteroposterior ratio (Ha/Hp), middle-posterior ratio (Hm/Hp), and middle-anterior ratio (Hm/Ha) were calculated.

The reproducibilities of morphometry were evaluated by measuring 20 randomly selected radiographs, 10 of grade 0 and 10 of grade ≥1, on semiquantitative assessment on 10 occasions in 2 weeks; the coefficients of variation (RMS) were 1.26%, 1.10%, and 1.31% for Ha, Hm, and Hp for grade 0, and 1.70%, 1.66%, and 1.65% for Ha, Hm, and Hp for grade ≥1, respectively.

Morphometric assessments, in addition to semiquantitative assessments, were done of all baseline and follow-up radiographs.

Definition of incident fractures

Incident fractures were defined as those in which vertebral bodies showed a distinct alteration in morphology resulting in a higher deformity grade on the follow-up radiograph, except for a vertebral deformity of grade 3 and a vertebral body height of less than 12mm on the baseline radiograph.

The results for which a consensus was obtained in the reading grade by semiquantitative assessment were considered as the "gold standard" for incident fractures. Chronological changes in absolute height reduction (AHr) and the percentage height ratio (PHr) were calculated in all cases with and without vertebral fracture by morphometry. In incident fractures as diagnosed by a consensus reading, the cutoff values for AHr and PHr were evaluated from sensitivity and specificity.

Results

Morphometry of vertebral heights and vertebral height ratios

The values of Ha, Hm, and Hp from T4 to L5 in grade 0 are shown in Table 1. The mean values of the vertebral heights (3348 vertebrae, 279 cases) were 25.3 ± 4.7mm, 25.2 ± 4.2mm, and 27.2 ± 4.2mm for Ha, Hm, and Hp, respectively.

The values of Ha/Hp, Hm/Hp, and Hm/Ha from T4 to L5 in grade 0 are shown in Table 2. The mean values of the vertebral height ratios (3348 vertebrae, 279 cases) were 0.93 ± 0.08, 0.93 ± 0.06, and 1.00 ± 0.05, respectively.

Prevalent fractures

Prevalent fractures as determined in 3338 vertebrae in 279 cases by consensus reading on semiquantitative assessment were observed in 207 vertebrae (6.2%) in 85 cases (30.5%). See Fig. 1 for the distribution of prevalent fractures. Prevalent fractures occurred more commonly in the thoracolumbar regions: 13.0%, 18.4%, and 15.0% in T11, T12, and L1, respectively.

Table 1. Values of Ha, Hm, and Hp from T4 to L5 in grade 0 at the baseline

Level	*n*	Ha (mm)	Hm (mm)	Hp (mm)
T4	215	19.9 ± 1.3	19.9 ± 1.2	21.7 ± 1.3
T5	240	20.5 ± 1.3	20.6 ± 1.2	22.5 ± 1.3
T6	255	20.5 ± 1.4	21.1 ± 1.2	23.2 ± 1.3
T7	246	21.0 ± 1.4	21.5 ± 1.2	23.8 ± 1.3
T8	255	21.8 ± 1.6	22.0 ± 1.4	24.1 ± 1.3
T9	253	23.0 ± 1.5	22.8 ± 1.3	24.6 ± 1.3
T10	250	24.0 ± 1.8	23.8 ± 1.4	25.9 ± 1.4
T11	231	24.7 ± 1.7	25.2 ± 1.5	28.2 ± 1.7
T12	219	26.8 ± 1.8	27.4 ± 1.6	30.8 ± 1.9
L1	242	28.7 ± 1.9	29.0 ± 1.7	32.5 ± 1.8
L2	256	30.4 ± 2.2	29.7 ± 1.9	32.6 ± 2.0
L3	260	31.3 ± 2.5	30.4 ± 2.0	32.1 ± 2.0
L4	259	30.8 ± 2.8	30.5 ± 2.3	30.3 ± 2.2
L5	167	31.2 ± 2.9	29.8 ± 2.3	27.9 ± 2.0
Totals	3348	25.3 ± 4.7	25.2 ± 4.2	27.2 ± 4.2

Ha, anterior vertebral height; Hm, middle vertebral height; Hp, posterior vertebral height
Values are mean ± SD

Incident fractures

Incident fractures were determined in 3231 vertebrae in 279 cases by consensus reading on semiquantitative assessment, and they were observed in 42 vertebrae (1.3%) in 29 cases (10.4%). The distribution of incident fractures is also shown in Fig. 1. L1 was the most frequent site (19.0%) of incident fractures. The changes in grade on the semiquantitative assessment are shown in Table 3. Thirty-five cases from grade 0, 5 from grade 1, and 2 from grade 2 were upgraded.

The cutoff values of PHr, AHr, and combined PHr and AHr, PAHr, determined employing sensitivity and specificity in incident fractures, are shown in Table 4. The cutoff values of 15% and 20% in PHr were 50.83% and 36.67% for sensitivity, and 99.90% and 99.93% for specificity, respectively. The cutoff values of 3mm and 4mm in AHr were 65.00% and 51.67% for sensitivity, and 99.77% and 99.92% for specificity, respectively. The cutoff values of 15% and 3mm and 15% and 4mm in PAHr, combined PHr and AHr, were 50.83% and 49.17% for sensitivity, and 99.90% and 99.92% for specificity, respectively. When the cutoff values for PAHr were 15% and 3mm, and 15% and 4mm, the frequencies of incident fractures, on a per case or per vertebra basis, were 27 (9.7%) per case and 43 (1.2%) per vertebra, and 26 (9.3%) per case and 41 (1.2%) per vertebra, respectively. These cutoff values of PAHr were better for sensitivity than those of 20% and 3mm (36.67%) and 20% and 4mm (36.67%), but these specificities were almost the same (99.93% and 99.94%).

Table 2. Values of Ha/Hp, Hm/Hp, and Hm/Ha ratios from T4 to L5 in grade 0 at the baseline

Level	*n*	Ha/Hp	Hm/Hp	Hm/Ha
T4	215	0.92 ± 0.05	0.92 ± 0.04	1.00 ± 0.04
T5	240	0.91 ± 0.05	0.92 ± 0.04	1.01 ± 0.04
T6	255	0.89 ± 0.05	0.91 ± 0.04	1.03 ± 0.05
T7	246	0.89 ± 0.05	0.91 ± 0.03	1.03 ± 0.05
T8	255	0.90 ± 0.06	0.91 ± 0.04	1.01 ± 0.05
T9	253	0.94 ± 0.05	0.93 ± 0.04	0.99 ± 0.04
T10	250	0.93 ± 0.06	0.92 ± 0.04	1.00 ± 0.05
T11	231	0.88 ± 0.06	0.89 ± 0.04	1.02 ± 0.05
T12	219	0.87 ± 0.05	0.89 ± 0.04	1.02 ± 0.04
L1	242	0.89 ± 0.06	0.89 ± 0.04	1.01 ± 0.05
L2	256	0.93 ± 0.06	0.91 ± 0.04	0.98 ± 0.05
L3	260	0.98 ± 0.07	0.95 ± 0.04	0.97 ± 0.05
L4	259	1.02 ± 0.09	1.01 ± 0.06	1.00 ± 0.06
L5	167	1.12 ± 0.09	1.07 ± 0.06	0.96 ± 0.06
Totals	3348	0.93 ± 0.08	0.93 ± 0.06	1.00 ± 0.05

Values are mean ± SD

Discussion

Morphological changes such as endplate deformity, buckling of the cortex, lack of parallelism of the endplate, and loss of vertebral continuity [17] are characteristics of vertebral fractures. These findings can be recognized easily by semiquantitative assessment, but they might be missed by other quantitative methods. However, associations with degenerative osteosclerotic changes and scoliosis are often difficult to determine in the diagnosis of incident fractures.

It is important to distinguish false positives based on radiographic problems, e.g., incorrect projection of the X-ray, from real incident vertebral fractures. When morphometric criteria are used to define incident fractures, it is essential to maintain good quality control and assurance in the radiographic procedure.

Table 3. Changes of grade in incident fracture on semiquantitative assessment

Incident fracture	Grade			Number	
	Baseline		Follow-up	Vertebrae	Cases
(−)	0	→	0	3384	
	1	→	1	122	
	2	→	2	61	
	3	→	3	17	
Totals				3584	250
(+)	0	→	1	23	
	0	→	2	7	
	0	→	3	5	
	1	→	2	4	
	1	→	3	1	
	2	→	3	2	
				42	29
Totals				3626	279

Table 4. Cutoff values of percent height ratio (PHr), absolute height reduction (AHr), and combined PHr and AHr (PAHr), and sensitivity and specificity for incident fracture, and frequencies of incident fracture on per case basis and per vertebra basis

Value	Cutoff	Sensitivity (%)	Specificity (%)	Frequency of incident fractures			
				Per case basis		Per vertebra basis	
				n	%	*n*	%
PHr	≧15%	50.83	99.90	27	9.7	43	1.2
	≧20%	36.67	99.93	22	7.9	35	1.0
AHr	≧3mm	65.00	99.77	35	12.5	54	1.5
	≧4mm	51.67	99.92	28	10.0	43	1.2
PAHr	≧15%, 3mm	50.83	99.90	27	9.7	43	1.2
	≧15%, 4mm	49.17	99.92	26	9.3	41	1.2
	≧20%, 3mm	36.67	99.93	22	7.9	35	1.0
	≧20%, 4mm	36.67	99.94	22	7.9	34	1.0

It is important to define incident fractures, especially in the evaluation of drug therapy and international comparisons of the epidemiology of osteoporosis. In defining incident fractures, many methods, including visual semiquantitative assessment and quantitative morphometry, have been proposed.

In alendronate clinical trials, an incident fracture was defined as a decrease of ≥20% and ≥4mm in the height of any vertebrae, relative to the baseline [2,15]. The effect of risedronate on vertebral incident fractures was evaluated by both quantitative and semiquantitative assessments [12,18]. An incident fracture was defined as ≥15% reduction at anterior, middle, or posterior heights of the vertebral body for quantitative assessment [19], and an increase of one or more grade for semiquantitative assessment [3], compared with baseline thoracic and lumbar radiographs.

In raloxifen clinical trials, the criterion for diagnosis of an incident vertebral fracture was based on a reduction in the anterior, middle, and/or posterior vertebral height of ≥20% and at least 4mm, compared with the baseline radiograph [20,21], as well as a grade change of at least one for semiquantitative assessment [21]. In a parathyroid hormone [(1–34)PTH] clinical trial, an incident vertebral fracture was assessed by the grade of deformity, a decrease in height of approximately more than 20%, whereas worsening of the preexisting deformity was not analyzed [22].

Although vertebral fractures associated with osteoporosis are frequently observed, the criteria for incident vertebral fractures have not yet been defined in Japan. In the present study, the cutoff values of vertebral height reduction and ratio in Japanese women were evaluated using morphometry, and semiquantitative assessment as the gold standard.

The morphometry of vertebral heights at the anterior, middle, and posterior of vertebral bodies from T4 to L4 showed excellent reproducibilities (RMS = 1.10%–1.70%). However, in the diagnosis of incident vertebral deformities, semiquantitative assessments by trained radiologists might be better than morphometric techniques [23]. In addition, a comparative study of semiquantitative and morphometric methods has shown similar results for the assessment of vertebral fractures in osteoporosis [24]. For these reasons, we adopted a semiquantitative assessment as the gold standard.

Several methods for defining incident vertebral fractures, including the spinal deformity index and point prevalence [25,26], have been reported [27]. The percentage change in anterior, middle, and posterior vertebral heights, and/or absolute vertebral height reductions, are used as criteria [28]. Recently, among these methods the relative vertebral height ratio and/or absolute height reduction have been mainly used. Regarding the height ratio, 15% and 20% reductions have been often used to define deformity, but there is no information on which criterion is better. In the present study, in Japanese women, a cutoff value of 15% reduction of vertebral height was better than that of 20%, because the 15% reduction had better sensitivity (50.83% vs 36.67%), and almost the same specificity (99.90% vs 99.93%).

In clinical trials for osteoporosis treatment, it is necessary to consider two points in the assessment of incident vertebral fractures; incident fractures are rare events, but stringent morphometric criteria can also lead to failure to detect incident fractures observed on radiograms. Therefore, to reduce false positives or false negatives, it is essential to establish cutoff levels for the relative ratio and absolute reduction of vertebral heights that can satisfy the requirements for both good sensitivity and specificity.

In the present study, we selected, as relative ratios and absolute reduction of vertebral heights, 15% and 3mm, and 15% and 4mm, respectively. The sensitivity

and specificity of these criteria were 50.83% and 49.17%, and 99.90% and 99.92%, respectively.

Using quantitative morphometry and the semiquantitative approach with 350 Caucasian and Asian postmenopausal women with one to seven prevalent vertebral fractures and a T score of no more than 2 SD of lumbar BMD, the cutoff values of PAHr were ≥15% and 3mm, and 20% and 4mm for the relative ratio and absolute vertebral height reduction. The sensitivity and specificity rates for incident fractures were 75.44% and 47.37%, and 98.75% and 99.84%, respectively [17]. Compared with the results of our present study, the sensitivity rate for incident fractures differed, whereas the specificity rate was almost the same. The difference in the sensitivity rate might be related to differences in the subjects or different criteria for participation in the study.

We adopted as defining criteria for incident fractures both an absolute vertebral height reduction ≥3mm or ≥4mm and a relative height ratio ≥15%. The adaptation of these two cutoff criteria can decrease the possibility of artifacts that might occur in baseline and follow-up radiography.

Vertebral heights and ratios differ between races, e.g., Caucasian and Japanese women. The mean Japanese vertebral heights were 1–2mm shorter than those for Caucasians [29], reflecting the possibility that the Japanese are of a shorter stature. However, it has been reported that the prevalence of vertebral fractures was similar among Hong Kong Chinese and American Caucasians when population-specific means and SD were used for defining vertebral fractures [30].

In conclusion, the morphometric criteria, both a relative height ratio ≥15% and an absolute height reduction ≥3mm or ≥4mm of incident fracture combined with a semiquantitative assessment were determined, and these measures will provide useful information in the study of clinical osteoporosis, especially for international comparisons.

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Alendronate reduced vertebral fracture risk in postmenopausal Japanese women with osteoporosis: a 3-year follow-up study

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Abstract The risk-reducing effect of alendronate on vertebral fractures has been consistently reported. In a 2-year, randomized, double-blind, active drug-controlled (1 μg alfacalcidol) double-dummy study, we also reported that alendronate (5.0 mg) had a fracture-reducing effect in Japanese patients with preexisting vertebral fractures. The present report describes the risk-reducing effect of alendronate (5.0 mg) for 3 years in postmenopausal osteoporotic patients. The 3-year treatment period consisted of the original 2-year double-blind study followed by a 1-year extension. A total of 170 postmenopausal female patients were involved in the third year; 90 received alendronate and 80 received alfacalcidol. Both efficacy and safety were analyzed in these 170 patients. Vertebral fracture was determined by quantitative morphometry, and vertebral bone mineral density (BMD) was measured by the DXA method (dual-energy X-ray absorptiometry). The primary efficacy endpoint was the incidence of vertebral fracture, excluding fracture cases that occurred in the first 6 months after treatment initiation. The cumulative incidence of vertebral fracture at 3 years was 7.8% (7/90) in the alendronate group and 18.8% (15/80) in the alfacalcidol group, indicating a significantly reduced risk of fractures in the alendronate group (relative risk = 0.41, 95% CI = 0.18–0.97). Lumbar spine BMD increased by 9.2% in the alendronate group (n = 26) and by 1.4% in the alfacalcidol group (n = 22) at 3 years. The safety profile of alendronate during 3 years of treatment was similar to that of alfacalcidol. The present study thus demonstrated that treatment with alendronate 5.0 mg for 3 years increased vertebral BMD and reduced the risk of vertebral fractures in Japanese, postmenopausal women with osteoporosis.

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Key words alendronate · osteoporosis · vertebral fracture · alfacalcidol

Introduction

Osteoporosis is a common disorder in the elderly population and is associated with increased risk of bone fractures and spinal deformity. The prevention and treatment of osteoporosis have been challenging issues for the medical community in Japan, which is a rapidly aging society. In 1997, approximately 92 000 cases of hip fracture occurred in Japan, and about 80% of these patients were women [1]. The lifetime risk of vertebral fracture was estimated to be 37% or greater in women over 50 years old [2]. Low bone mineral density (BMD) is associated with increased fracture risk, and pharmacological therapy is directed at reducing fracture risk by increasing BMD.

Alendronate (ALN) exerts a potent inhibitory effect on bone resorption [3,4]. In European and American studies, ALN at a dose of 10 mg/day has consistently been shown to increase BMD at the spine, hip, and other skeletal sites and to reduce the risk of all types of fractures [5–12]. In long-term extensions of the original phase III studies, BMD continued to increase at the spine during ALN treatment for at least 7 years; ALN was generally well tolerated and had an adverse experience profile similar to that of placebo [13,14].

In Japan, we conducted a 2-year, double-blind comparative study and found that ALN 5 mg significantly lowered the incidence of one or more vertebral fractures to a greater extent than did alfacalcidol after 6

months or more of treatment [15]. In the same study, ALN also significantly reduced the incidence of multiple vertebral fractures compared with alfacalcidol during 2 years of treatment [15].

Bisphosphonates with increase in lumbar spine BMD in Japanese patient with osteoporosis have been widely studied, but preparations with a risk-reducing effect on vertebral fracture in Japanese patients with osteoporosis for 3 years have apparently not been reported. We herein describe the safety and efficacy of ALN in reducing the incidence of vertebral fractures during a total study period of 3 years, which consisted of the original 2-year double-blind study plus a 1-year extension.

Patients and methods

Study design

The study was first conducted as a 2-year, double-blind, comparative trial and then was extended for another year. The study was performed at 57 departments of 55 institutional centers in Japan nationwide. Patients were randomized to receive either ALN (5 mg; Merck, Whitehouse Station, NJ, USA, and Banyu Pharmaceutical, Tokyo, Japan) or alfacalcidol (1 μg; Teijin, Tokyo, Japan) once daily in a double-blind fashion.

The study extension was approved in advance by the institutional review boards (IRB) of the individual participating institutional sites. The subjects enrolled in the preceding 2-year double-blind study who completed the study and were judged eligible for the extended doses were advised of the objectives and procedures of the extension study. Informed written consent was then reobtained from these patients. The present study, including the 1-year extension, was conducted from September 1998 through November 2001 in accordance with the spirit of the Declaration of Helsinki and the Guideline for Good Clinical Practice (Ministry of Health, Labour and Welfare of Japan, Notification No. Yakuhatsu 874, dated October 2, 1989).

Every patient enrolled in the 1-year extension study was given the same study drugs as administered in the preceding 2-year, double-blind, comparative study. The following study drugs were administered: one tablet of ALN 5 mg plus one tablet of alfacalcidol placebo in the ALN group and one tablet of alfacalcidol 1 μg plus one tablet of ALN placebo in the alfacalcidol group. All study drugs were taken once daily on arising with approximately 180 ml water. The patients were instructed to remain upright and refrain from any food, beverage, or other drug intake for at least 30 min after drug ingestion. Calcium lactate powder (1.5 g) was taken once daily after the evening meal.

Subject population

Of 242 patients who completed the preceding 2-year double-blind study, 179 participated in this 1-year extension. Nine patients (n = 3 in the ALN group and n = 6 in the alfacalcidol group) were excluded from the current analysis, leaving 170 postmenopausal female patients (n = 90 in the ALN group and n = 80 in the alfacalcidol group) for analysis. As defined in the patient criteria, the female patients were 65 years old or older, ambulatory, and had one to four preexisting vertebral fractures associated with osteoporosis at the start of the preceding double-blind study.

Patients were excluded if they had ever been treated with a bisphosphonate or had been treated with any of the following within 8 weeks of the start of the study: pharmacologically active vitamin D preparations (including alfacalcidol), anabolic steroid, calcitonin, ipriflavone, vitamin K, male sex hormone (androgen), female sex hormone (estrogen), antiestrogen, or calcium preparations. Other exclusion criteria included metabolic bone diseases (e.g., hyperthyroidism, osteomalacia, renal osteodystrophy), diabetes, history of peptic ulcer, reflux esophagitis, rheumatoid arthritis, history of malignancy, serious liver or heart disease, renal dysfunction, or serum creatinine concentration ≥1.5 mg/dl at the start of the preceding double-blind study. Table 1 presents the baseline characteristics at the start of the original 2-year double-blind study for the female patients enrolled in this 1-year extension.

Evaluation of vertebral fractures

Vertebral fractures were evaluated using radiographs of thoracic (T8 centered) and lumbar vertebrae (L3 centered) that were taken from the anterior and lateral sides at baseline and every 6 months during treatment. New vertebral fractures were identified on radiographs by experienced researchers and were confirmed using quantitative morphometry. Three vertebral heights, Ha, Hc, and Hp, were measured, and a new vertebral fracture was defined as a decrease of 20% or more in any of these heights, relative to baseline.

The primary efficacy endpoint was the proportion of patients with a new vertebral fracture more than 6 months after initiating treatment (vertebral fractures that occurred within 6 months after the start of treatment were not considered). The secondary endpoints were (1) the 3-year cumulative incidence of patients who experienced vertebral fractures (excluding those in the first 6 months) and (2) the percentage of patients who developed multiple (more than one) new vertebral fractures.

Table 1. Demographics and baseline characteristics of female patients (n = 170) enrolled in the full 3-year study[a]

Characteristics	Alendronate (n = 90)	Alfacalcidol (n = 80)
Age, years	71.2 (5.3)	72.6 (5.7)
Height, cm	146.5 (6.0)	145.0 (5.9)
Body weight, kg	49.5 (8.5)	48.9 (7.7)
Years since menopause	22.0 (7.2)	22.4 (7.6)
No. of vertebral fractures at baseline[a]		
1	46	35
2	26	26
3	9	10
4	9	9
ALP (IU/l)	211 (82, 93–482)[b]	224 (90, 52–535)[b]

[a] Baseline refers to the beginning of the original 2-year double-blind study
[b] Values are presented as mean (SD), except for ALP, which is given as mean (SD, Min.–Max.)

Measurement of vertebral BMD

BMD of the spine (L2–L4) was measured by dual-energy X-ray absorptiometry (DXA) at baseline and at 12, 24, and 36 months of treatment in a subset of patients (at centers with bone densitometry equipment).

Safety evaluation

The safety of the study drugs was assessed at each examination by evaluating adverse experiences (AEs) and abnormal changes in clinical laboratory test values. The following laboratory tests were performed: hematology: red blood cell count, white blood cell count, differential white blood cells (basophils, eosinophils, neutrophils, lymphocytes, monocytes), hemoglobin, hematocrit, and platelet count; blood chemistry: AST (GOT), ALT (GPT), γ-GTP, alkaline phosphatase (ALP), LDH, CPK, BUN, creatinine, albumin, total bilirubin, total cholesterol, Na, K, Cl, Ca, and P; and urinalysis: protein and sugar.

Statistical analyses

Efficacy was analyzed by intention to treat (ITT) using all randomized patients with at least 12 months of follow-up. The primary efficacy endpoint was the proportion of patients with a new vertebral fracture more than 6 months after initiating treatment (vertebral fractures that occurred within 6 months after the start of treatment were not considered). It was decided before unblinding that the primary analyses would exclude fractures occurring during the first 6 months, because this is the minimum time that is required to refill existing resorption sites and begin to restore bone strength, as predicted by bone remodeling theory. A 95% confidence interval for the mean difference in fracture incidence between the treatment groups was calculated. The time to the first new vertebral fracture was profiled in a survival analysis (estimation of survival function and log-rank test) using the life table method.

BMD was assessed as the percentage change from baseline in mean density (g/cm^2) of the lumbar vertebrae L2–L4 at 12, 24, and 36 months of treatment and was compared between the treatment groups using the two-sample t test procedure after adjusting for multiplicity of data points.

Safety was analyzed using the primary endpoint of clinical symptoms and laboratory abnormalities. The incidences of adverse events and drug-related adverse events were compared between the treatment groups using Fisher's exact test.

The level of significance was 0.05 (two-tailed) for the efficacy analysis (primary and secondary endpoints) and the safety analysis (primary endpoint).

Results

Frequency of vertebral fractures

Baseline characteristics (before initiating treatment) were similar for women who entered the third year in both treatment groups; there were no significant differences (see Table 1). The 3-year cumulative increase in new vertebral fractures was significantly less in the ALN group than in the alfacalcidol group (log-rank test, P = 0.0341; Fig. 1). Overall, 7/90 (7.8%) and 15/80 (18.8%) in the ALN and alfacalcidol groups, respectively, experienced new vertebral fractures (relative risk = 0.42, 95% CI = 0.18–0.97; Fig. 2). The between-treatment difference in the percentage of patients with vertebral fractures was 11% (95%CI = 0.8%–21.2%). Thus, ALN reduced vertebral fracture risk relative to alfacalcidol ($P < 0.05$).

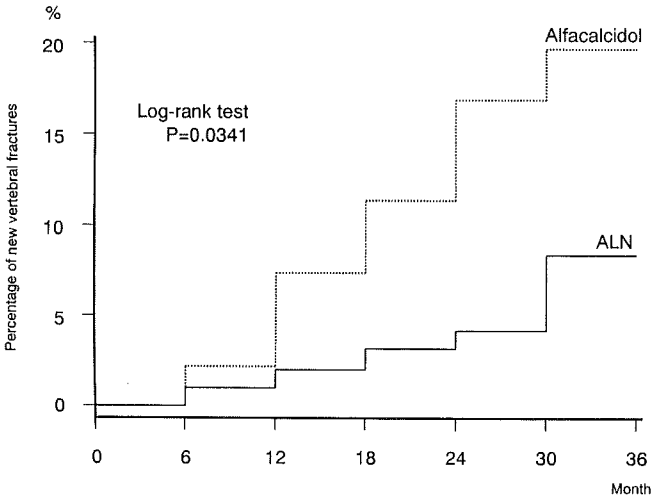

Fig. 1. Cumulative distribution of patients with new vertebral fractures. *ALN*, alendronate

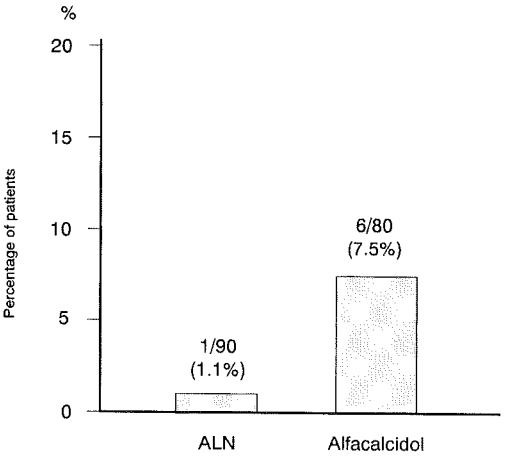

Fig. 3. Percentage of patients with multiple new vertebral fractures: the difference in incidence between groups was 6.4% (95% CI, 0.2%–12.6%)

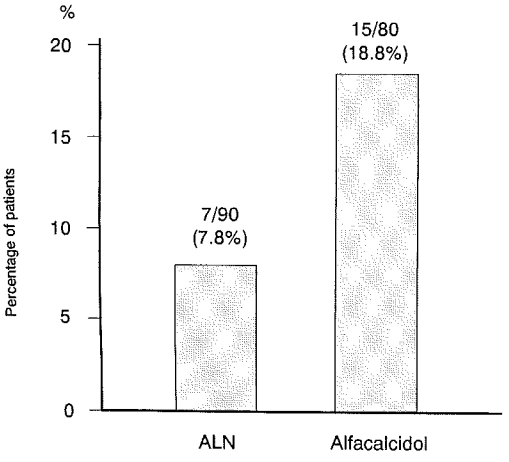

Fig. 2. Percentage of patients with new vertebral fractures: the difference in incidence between groups was 11.0% (95% CI, 0.8%–21.2%)

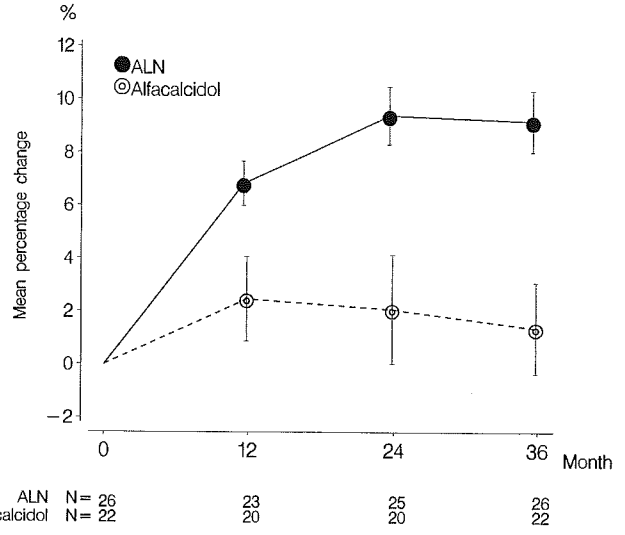

Fig. 4. Percent change profile of L2–L4 BMD (mean ± SE)

Multiple vertebral fractures

The number and percentage of patients who experienced multiple new vertebral fractures were 1/90 (1.1%) and 6/80 (7.5%) in the ALN and alfacalcidol groups, respectively (Fig. 3). The between-treatment difference in the percentage of patients with multiple new vertebral fractures was 6.4% (95% CI = 0.2–12.6, Fig. 3). Thus, ALN significantly reduced the risk of multiple vertebral fractures compared with alfacalcidol ($P < 0.05$).

Lumbar spine BMD

Percent changes from baseline in L2–L4 BMD were calculated at 12, 24, and 36 months in patients who had not developed lumbar spine fractures at any measurement. As shown in Fig. 4, there was a greater increase in lumbar spine BMD in the ALN group than in the alfacalcidol group (9.2% vs 1.4% increase at 3 years of treatment). The increases in spine BMD were significantly greater for ALN compared to alfacalcidol at these three time points (two-sample t test using Bonferroni's multiple comparison method).

Laboratory data

Serum levels of ALP, Ca, and phosphorus were considered parameters of bone metabolism. Serum ALP levels decreased significantly during the first 6 months of ALN treatment and remained consistently decreased for 3

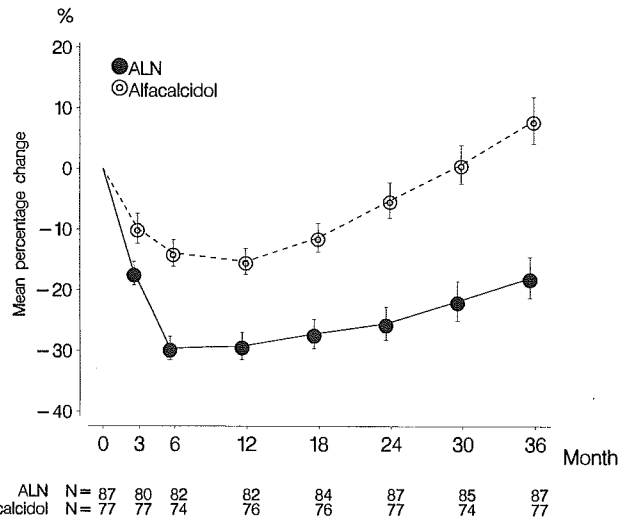

Fig. 5. Percent change profile of serum ALP (mean ± SE)

years. In contrast, alfacalcidol produced only a small decrease in ALP at 6 to 12 months, which gradually returned to baseline levels by month 24. Although serum Ca slightly decreased with ALN and increased with alfacalcidol, the between-group difference was not statistically significant. Serum phosphorus also tended to decrease in the ALN group, but no statistically significant difference was observed between the treatment groups.

Safety

No statistically significant difference in the incidence of drug-related AEs was observed between the ALN and alfacalcidol groups (22.2% vs 16.3% for subjective symptoms/objective symptoms and 12.2% vs 15.0% for laboratory abnormal findings). Similarly, the incidence of gastrointestinal AEs did not differ significantly between the treatment groups (ALN 14.4% vs alfacalcidol 13.8%). The drug-related gastrointestinal AEs occurring in 2% or more of patients (i.e., two events or more in either treatment group) were constipation (3.3%, three events), stomach heaviness (2.2%, two events), stomach discomfort (2.2%, two events), and gastritis (5.6%, five events) in the ALN group, and constipation (3.8%, three events), abdominal pain (2.5%, two events), stomach discomfort (2.5%, two events), and gastritis (3.8%, three events) in the alfacalcidol group.

Discussion

In this study, postmenopausal Japanese women with osteoporosis manifested as preexisting vertebral fractures were treated with ALN (5 mg/day) or alfacalcidol (1 μg/day) for 3 years. The results demonstrated that the incidence of vertebral fractures after 6 months was significantly lowered by ALN compared with alfacalcidol. The increases in spine BMD were significantly greater for ALN compared to alfacalcidol ($P < 0.001$), and the BMD-elevating effect of ALN lasted continuously throughout the 3-year period. Furthermore, serum ALP levels for ALN treatment group showed significant decrease compared to alfacalcidol group beyond month 6 ($P < 0.001$), and this decrease was rather persistent for 3 years, suggesting a long-lasting inhibitory action of the drug on the bone metabolism.

Upon absorption, bisphosphonates are rapidly localized to active sites of bone remodeling, where they exhibit their pharmacological actions on osteoclasts. Although the precise pharmacological mechanism of action of ALN remains elusive, it is known that nitrogen-containing bisphosphonates, including ALN, block the mevalonic acid pathway, thereby inhibiting the prenylation of G protein(s), which play a key role in intracellular signal transduction. As a result, the bone-resorbing activity of osteoclasts is suppressed [16–18]. It is believed that the four-carbon amino side chain of ALN enables the drug to suppress osteoclastic bone resorption without interfering with bone calcification [19], thus normalizing bone metabolism [20]. This conclusion is supported by data from animal studies showing that ALN increases bone strength along with BMD [21,22]. Moreover, histological investigation of iliac bone biopsies revealed normal bone calcification in patients treated with ALN over 3 years [23]. Furthermore, bone specimens from postmenopausal women with osteoporosis treated with ALN for 2–3 years showed that the mean degree of mineralization was restored to normal levels [24,25].

In clinical studies conducted in Western countries, ALN (5–10 mg/day) significantly reduced the incidence of both vertebral and nonvertebral fractures by about half, which was associated with increased BMD and decreased bone resorption markers [5–13].

Clinical studies have also been conducted in Japan to assess the efficacy and safety of alendronate. Of relevance to the current study, a double-blind, alfacalcidol-controlled study showed that ALN 5 mg resulted in an increase of 6% or more in lumbar spine BMD at 48 weeks compared with an increase of approximately 1% with alfacalcidol [26–29]. In an earlier report from the current alfacalcidol-controlled study, treatment with ALN 5 mg/day for 2 years significantly reduced vertebral fracture risk in Japanese osteoporotic patients; this risk reduction was similar to that observed in studies conducted outside Japan with ALN 5–10 mg/day [15]. In addition, ALN was generally well tolerated

during 2 years of treatment, and the safety profile of ALN was similar to that of alfacalcidol [15].

The safety analysis in the present study revealed no statistically significant differences in the incidence of drug-related AEs between the ALN and alfacalcidol groups. The incidences of drug-related AEs observed in this study were similar to those reported in the preceding 2-year comparative study [15]. Moreover, the incidences of gastrointestinal AEs did not differ significantly between the treatment groups.

Esophageal ulcer resulting from mucosal irritation has occasionally been described with the use of bisphosphonates [30–32]. In the present study, however, no severe cases of esophageal ulcer were observed during the 1-year extension or the preceding 2-year double-blind study. Previous placebo-controlled studies have demonstrated that ALN was well tolerated, with an incidence of AEs similar to that of placebo [13,32]. In the Fracture Intervention Trial (FIT), the incidences of gastroduodenal perforation, ulcer, and/or bleeding were similar in the ALN and placebo groups [10]. Other studies have demonstrated that the occurrence of gastrointestinal disorders, including esophagitis, may be markedly reduced when the recommended dosing instructions are adhered to [32–34].

In conclusion, treatment with ALN 5 mg/day for 3 years significantly reduced the risk of vertebral fractures and was generally well tolerated in postmenopausal Japanese women with osteoporosis.

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Association of a single-nucleotide polymorphism in low-density lipoprotein receptor-related protein 5 gene with bone mineral density

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Abstract Low-density lipoprotein receptor-related protein 5 (LRP5) is an important regulator of osteoblast growth and differentiation, affecting peak bone mass in vertebrates. Here, we analyzed whether the *LRP5* gene was involved in the etiology of postmenopausal osteoporosis, using association analysis between bone mineral density (BMD) and an *LRP5* gene single-nucleotide polymorphism (SNP). Association of an SNP in the *LRP5* gene at IVS17-1677C > A (intron 17) with BMD was examined in 308 postmenopausal Japanese women (65.2 ± 9.6 years; mean ± SD). The subjects bearing at least one variant A allele (CA + AA; $n = 142$) had significantly lower Z scores for total body and lumbar BMD than the subjects with no A allele (CC; $n = 166$) (total body, 0.08 ± 1.09 versus 0.50 ± 1.03; $P = 0.0022$; lumbar spine, −0.42 ± 1.43 versus −0.02 ± 1.42; $P = 0.013$). These findings suggest that the *LRP5* gene is a candidate for the genetic determinants of BMD in postmenopausal women, and this SNP could be useful as a genetic marker for predicting the risk of osteoporosis.

Key words wnt · LRP5 · osteoporosis · bone mineral density · polymorphism

Introduction

Osteoporotic fracture is a serious event in an increasingly aging population. Low bone mass is one of the most significant risk factors. Twin and sibling studies have revealed that the proportion of variance of bone mineral density (BMD) accounted for by genetic factors is around 50%–90% [1–6]. These studies have suggested that the variation in BMD among individuals is largely caused by genetic factors. Therefore, genetic markers that are correlated with BMD would be useful for predicting future bone loss and for clarifying the mechanism of bone loss in osteoporosis. After an association of BMD with vitamin D receptor (VDR) genotypes was reported [7], polymorphisms in several other genes were investigated [8]. These genes included those implicated in bone formation by the regulation of osteoblast growth and differentiation, such as transforming growth factor beta 1 (TGFβ1) [9], collagen type Ia1 (COLIA1) [10], parathyroid hormone (PTH) [11], and p57Kip2 (CDKN1C) [12]. Considering the polygenetic nature of BMD distribution and the multiplicity of endocrine factors known to regulate bone mass and bone turnover, it is important that the panel of candidate genes could be expanded to elucidate the whole genetic background of osteoporosis.

The Wnt signaling pathway plays a pivotal role in embryonic development and oncogenesis [13,14]. Studies using *Drosophila*, *Xenopus*, and mammalian cells have established a canonical signaling pathway [15–17]. Both genetic and biochemical results have provided solid evidence indicating that FZ proteins function as Wnt receptors. Wnt proteins bind Frizzled (FZ) and prevent glycogen synthase kinase 3 (GSK3)-dependent phosphonylation of β-catenin, leading to the stabilization of β-catenin. Meanwhile, the low-density lipoprotein receptor-related proteins 5 and 6 (LRP5 and LRP6) were found to be also required for the Wnt signaling pathway as Wnt co-receptors [18,19]. Recent reports have demonstrated that the Wnt-β-catenin signaling pathway regulates bone density through LRP5 [20–23]. Inactivating mutations in LRP5 decrease bone mass and cause the autosomal-recessive disorder osteoporosis-pseudoglioma syndrome in humans [20] and mice [21]. Conversely, activating mutations in LRP5 are linked to autosomal-dominant high-bone mass traits [22,23]. These data suggest that LRP5, which modulates

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Wnt signaling, controls bone metabolism in vivo in mammals. To examine the possible contribution of the *LRP5* gene to the etiology of involutional osteoporosis, we investigated an association between polymorphism in this gene and BMD in Japanese women.

Subjects and methods

Subjects

Genotypes were analyzed in DNA samples obtained from 308 healthy postmenopausal Japanese women (mean age ± SD; 65.2 ± 9.6 years) living in Nagano prefecture, Japan. Exclusion criteria included endocrine disorders such as hyperthyroidism, hyperparathyroidism, diabetes mellitus, liver disease, renal disease, use of medications known to affect bone metabolism (e.g., corticosteroids, anticonvulsants, heparin), or unusual gynecologic history. All women were non-related volunteers and provided informed consent before this study.

Measurement of BMD and biochemical markers

The lumbar spine BMD and total body BMD (in g/cm^2) of each participant were measured by dual-energy X-ray absorptiometry, using fast-scan mode (DPX-L; Lunar, Madison, WI, USA). We measured serum concentrations of calcium (Ca), phosphate (P), alkaline phosphatase (ALP), intact osteocalcin (I-OC; enzyme-linked immunosorbent assay [ELISA]; Teijin, Tokyo, Japan), intact parathyroid hormone (PTH), calcitonin, 1, 25$(OH)_2D_3$, total cholesterol (TC), and triglyceride (TG). We also measured urinary pyridinoline (PD; HPLC method) and deoxypyridinoline (DPD; HPLC method). The BMD data were recorded as "Z scores"; that is, deviation from the weight-adjusted average BMD for each age. The Z scores were calculated using installed software (Lunar DPX-L) on the basis of data from 20000 Japanese women.

SNP Selection

A polymorphic variation of the *LRP5* gene was extracted from the JSNP-database (http://snp.ims.u-tokyo.ac.jp/index.html), and was denoted as IVS17-1677C > A according to its localization on the gene.

Genotyping procedure

Genotypes of IVS17-1677C > A were determined using the SNP-dependent (Sd)-polymerase chain reaction (PCR) method, a modified allele-specific PCR of polymorphic sequence as previously described [24,25]. Two allele-specific primers (AS-primers) and one reverse primer were prepared per single-nucleotide polymorphism (SNP). The AS-primers (long and short) have a five-base difference between them; each has a polymorphic nucleotide of the SNP sequence at the 3′ ends, and an additional artificial mismatch introduced near the 3′ end. Primer sequences used were as follows: IVS17-1677C > A FL-primer: 5′-TTTTTGGGCGGTAAAT ACACGTCTCTCGAG-3′; IVS17-1677C > A FS-primer: 5′-CCGCGGTAAATACACGTCTCTCGAT-3′; and IVS17-1677C > A reverse-primer: 5′-GTTTCC GTCAGAAC GCTGCACTA-3′.

This primer set allowed distinct discrimination of alleles. For the assay, a genomic DNA sample (10 ng) was amplified with 250 nM of each primer (two polymorphic forward, and a reverse) in a 10-μl reaction mixture containing 10 mM dNTPs, 10 mM Tris-HCl, 1.5 mM $MgCl_2$, 50 mM KCl, 1 U Taq DNA polymerase, and 0.5 mM fluorescence-labeled dCTP (ROX-dCTP; Perkin-Elmer, Norwalk, CT, USA). The Sd-PCR reaction was carried out in a thermal cycler (Gene-amp system 9600; Perkin-Elmer) with initial denaturalization at 94°C for 4 min, followed by 5 cycles of stringent amplification (94°C for 20 s, 64°C for 20 s, 72°C for 20 s) and then 25 cycles at 94°C for 20 s, 62°C for 20 s, 72°C for 20 s), terminating with a 2-min extension at 72°C. Allele discrimination was carried out by electrophoresis and laser scanning of the DNA fragments on an ABI Prism 377 DNA system, using GeneScan Analysis Software ver2.1 (Applied Biosystems, Foster City, CA, USA). To confirm the accuracy of the Sd-PCR method, direct re-sequencing was carried out using the ABI Prism BigDye Terminator system (Applied Biosystems).

Statistical analysis

Comparisons of Z scores and biochemical markers between the group of individuals possessing one or two chromosomes of the minor A-allele and the group with only the major C-allele encoded at that locus were subjected to analysis. Coefficients of skewness and kurtosis were calculated to test deviation from a normal distribution. Because the clinical and biochemical traits in each genotypic group were normally distributed, we applied Student's *t*-test, using StatView-J4.5 software (SAS Institute, Cary, NC, USA). A P value of less than 0.05 was considered statistically significant.

Results

Association of LRP5 *gene polymorphism in intron 17 with BMD*

We analyzed the genotypes for the *LRP5* IVS17-1677C > A polymorphism (rs3781586 in the National Center for Biotechnology Information [NCBI] dbSNP data-

Table 1. Comparison of background and biochemical data between subjects bearing at least one A allele (AA + CA) and subjects with no A allele (CC) at IVS17-1677 (intron 17)

Items	Genotype (mean ± SD)		*P* value
	CC	CA + AA	
No. of subjects	166	142	
Age (years)	65.1 ± 9.6	65.4 ± 9.9	NS
Height (kg)	151.0 ± 6.2	150.3 ± 6.4	NS
Body weight (kg)	50.7 ± 8.4	50.3 ± 8.1	NS
Lumbar spine BMD (g/cm^2)	0.92 ± 0.20	0.87 ± 0.19	0.025
Lumbar spine BMD (Z score)	−0.02 ± 1.42	−0.42 ± 1.43	0.013
Total body BMD (g/cm^2)	1.00 ± 0.11	0.96 ± 0.12	0.015
Total body BMD (Z score)	0.50 ± 1.03	0.08 ± 1.09	0.0022
Ca (mg/dl)	9.2 ± 0.43	9.2 ± 0.45	NS
P (mg/dl)	3.4 ± 0.46	3.4 ± 0.48	NS
ALP (IU/l)	183.7 ± 62.6	195.4 ± 71.0	NS
I-OC (ng/ml)	7.6 ± 4.2	8.3 ± 3.7	NS
PD (pmol/μmol of Cr)	36.1 ± 24.7	34.8 ± 12.0	NS
DPD (pmol/μmol of Cr)	7.6 ± 5.2	7.4 ± 2.4	NS
Intact PTH (pg/ml)	35.1 ± 16.4	35.8 ± 16.6	NS
Calcitonin (pg/ml)	22.8 ± 11.1	23.4 ± 11.7	NS
1,25 $(OH)_2D_3$ (pg/ml)	37.5 ± 12.6	34.3 ± 10.4	NS
TC (mg/dl)	198.7 ± 37.5	195.7 ± 39.2	NS
TG (mg/dl)	141.5 ± 81.4	136.8 ± 71.4	NS
Percent fat	32.1 ± 7.9	31.6 ± 7.4	NS
BMI	22.2 ± 3.2	22.2 ± 2.9	NS

Statistical analysis was performed according to the method described in the text
BMD, bone mineral density; Ca, calcium; P, phosphate; ALP, alkaline phosphatase; I-OC, intact-osteocalcin; PD, pyridinoline; DPD, deoxypyridinoline; PTH, parathyroid hormone; TC, total cholesterol; TG, triglyceride; BMI, body mass index; NS, not significant

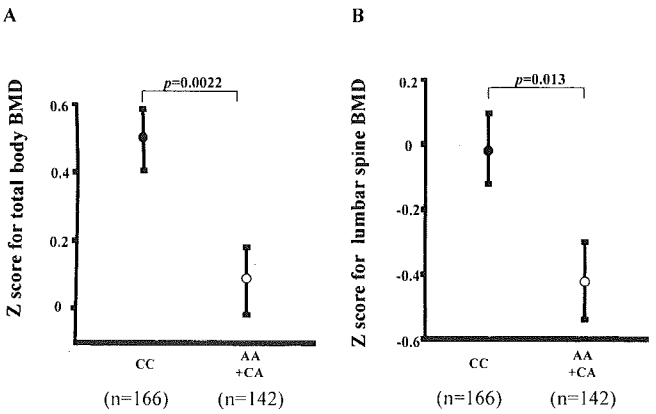

Fig. 1. Z Score values for total body and lumbar bone mineral density (*BMD*) in the groups with each genotype of the *LRP5* gene in intron 17 (IVS17-1677C > A). **A** Z Score values for total body BMD are shown as the *solid circle* for genotype CC at IVS17-1677 and as the *open circle* for genotype AA + CA at IVS17-1677. Values are expressed as means ± SE. Numbers of subjects are shown *in parentheses*. **B** Z Score values for lumbar spine BMD are shown in the same manner as in **A**

base) in 308 subjects, using Sd-PCR methods [25]. Among the 308 postmenopausal volunteers, 24 were AA homozygotes, 118 were CA heterozygotes, and 166 were CC homozygotes. Allelic frequencies were 0.731 for the C allele and 0.269 for the A allele in this population.

We compared Z scores for BMD of total body and lumbar spine between subjects bearing at least one chromosome with the A allele (genotype AA + CA; $n = 142$) and subjects with no A allele (CC; $n = 166$). The former subjects had significantly lower Z scores for total body BMD (0.08 ± 1.09 versus 0.50 ± 1.03; $P = 0.0022$, Fig. 1A) and lumbar BMD (−0.42 ± 1.43 versus −0.02 ± 1.42; $P = 0.013$; Fig. 1B). As shown in Table 1, the background data were not significantly different between these groups.

Discussion

We investigated the influence of a genetic variation of the *LRP5* gene on bone mineral properties. The allelic frequencies of an SNP in intron 17 (0.731 for IVS17-1677C and 0.269 for IVS17-1677A) in Japanese postmenopausal women were in Hardy-Weinberg equilibrium. The allelic frequencies of this SNP in the general Japanese population were reported in the JSNP database (IMS-JST137897). The database reported that the allelic frequencies were 0.726 for IVS17-1677C and 0.274 for IVS17-1677A, indicating that the allelic frequencies in the present study were in line with the JSNP database.