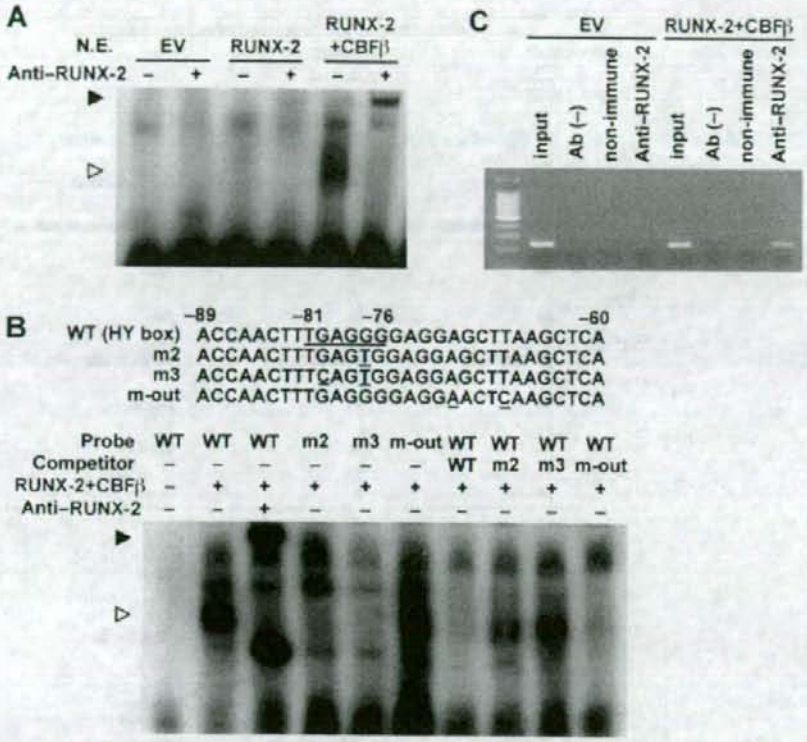

Figure 5. In vitro and in vivo binding of RUNX-2 and the hypertrophy box (HY box). **A**, Electrophoretic mobility shift assay (EMSA) for binding of the digoxigenin-labeled HY box oligonucleotide probe with nuclear extracts (N.E.) of COS-7 cells transfected with empty vector (control), RUNX-2 alone, or RUNX-2 and core-binding factor β (CBFβ). **B**, EMSA for binding of the wild-type (WT) and mutated HY box probes with nuclear extracts of COS-7 cells transfected with RUNX-2 and CBFβ. Mutations (underlined) were created inside (m2 and m3) and outside (m-out) the responsive region (from −81 bp to −76 bp; underlined). Cold competition with a 100-fold excess of unlabeled WT or mutated probes is shown. **C**, Chromatin immunoprecipitation assay for in vivo binding of RUNX-2 and the HY box. Cell lysates of HuH7 cells transfected with empty vector (control) or RUNX-2 and CBFβ were amplified with a primer set (from −113 bp to +119 bp) spanning the HY box before (input) and after immunoprecipitation with an antibody to RUNX-2 or the control nonimmune IgG or in the absence of antibody (Ab [−]). Open arrowheads indicate the shifted bands of the RUNX-2–DNA probe complex; solid arrowheads indicate the bands supershifted by an antibody to RUNX-2. See Figure 2 for other definitions.

mutations in the identified responsive region (mutation 1 at −80 bp, mutation 2 at −77 bp, and mutation 3 at both −80 bp and −77 bp) of the fragment between −339 bp and +39 bp (Figure 4A). Transactivation by RUNX-2 alone and in combination with CBFβ was suppressed by mutation 2 and mutation 3, but not by mutation 1, indicating that the −77 bp site is crucial for the transactivation of COL10A1 by RUNX-2. Luciferase assays by 1–15-bp deletions starting at the −81 bp site in the HY box confirmed that promoter activation by RUNX-2 was suppressed when the −77 bp site was included in the deletions (Figure 4A). Dose-response analysis of tandem repeats of the wild-type and the mutated (mutation 2) HY box clearly revealed that the wild-type HY box responded to RUNX-2 alone and in combination with CBFβ in a repeat number–dependent manner in all cells, while mutation 2 at the −77 bp site markedly suppressed the responses (Figure 4B).

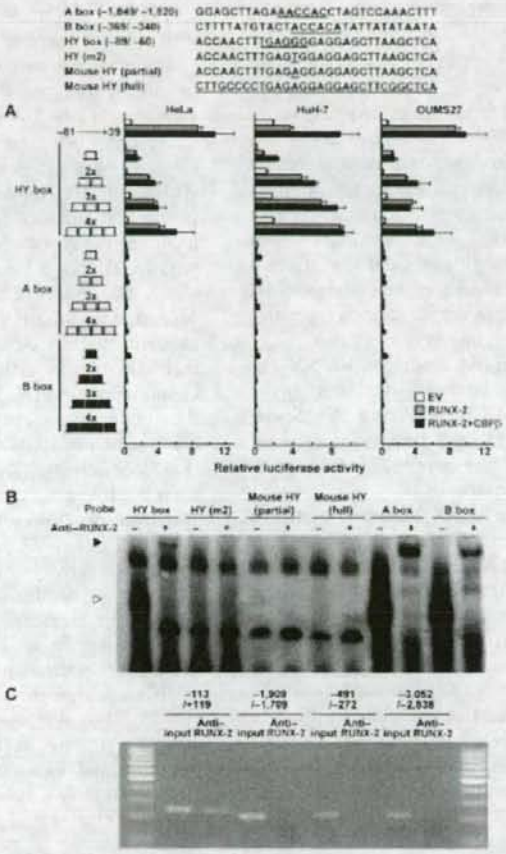

Figure 6. RUNX-2 transactivation of and binding to the A box (from –1849 bp to –1820 bp), B box (from –369 bp to –340 bp), and HY box (from –89 bp to –60 bp) in the human COL10A1 promoter. **A**, Response of luciferase activity to tandem repeats of the HY box, A box, and B box using human cells cotransfected with empty vector (control), RUNX-2 alone, or RUNX-2 and core-binding factor β (CBFβ). Bars show the mean and SD relative luciferase activity of 3 wells per group. **B**, Electrophoretic mobility shift assay (EMSA) for specific binding of digoxigenin-labeled oligonucleotide probes of the HY box, A box, and B box with nuclear extracts of COS-7 cells transfected with RUNX-2 and CBFβ. In addition to the 3 human elements, binding of 3 mutated HY box probes (m2) that mutated to the corresponding mouse sequence only at –77 bp in the core responsive region (mouse HY [partial]), and that fully mutated to the corresponding mouse sequence (mouse HY [full]) were examined. Open arrowheads indicate shifted bands of the RUNX-2–DNA complex; solid arrowheads indicate bands supershifted by an antibody to RUNX-2. **C**, Chromatin immunoprecipitation assay for in vivo binding of RUNX-2 with the human elements. Cell lysates of HuH7 cells transfected with RUNX-2 and CBFβ were amplified with a primer set spanning the HY box (from –113 bp to +119 bp), A box (from –1,909 bp to –1,709 bp), or B box (from –491 bp to –272 bp), or a primer set that did not include a RUNX-2 binding motif (from –3,052 bp to –2,838 bp) before (input) and after immunoprecipitation with an antibody to RUNX-2.

Specific binding of RUNX-2 to the HY box. We further examined in vitro and in vivo binding of RUNX-2 to the HY box by EMSA and ChIP assay, respectively. EMSA showed complex formation by the HY box oligonucleotide probe and nuclear extracts of COS-7 cells transfected with RUNX-2 and CBFβ (Figure 5A). The complex was barely detected with nuclear extracts of cells transfected with RUNX-2 alone, suggesting that CBFβ is necessary for binding. However, supershift of the complex by an antibody to RUNX-2

confirmed the binding of RUNX-2 to the HY box (Figure 5A).

Mutagenesis analyses in the HY box oligonucleotide probe showed that complex formation was abolished by the mutations inside the identified responsive region (from −81 bp to −76 bp) (mutations 2 and 3 in Figure 4), but not by mutations outside the region (Figure 5B). Cold competition with an excess amount of the unlabeled wild-type and outside mutation probes suppressed complex formation, while competition with the unlabeled inside mutation probes did not affect it (Figure 5B). These results demonstrate the specific binding between RUNX-2 and the identified responsive region (from −81 bp to −76 bp) of the HY box.

ChIP assay showed in vivo binding of RUNX-2 to the COL10A1 promoter regulatory region, including the HY box, in the presence of CBFβ (Figure 5C). Specificity was confirmed as RUNX-2 because it was not immunoprecipitated in the absence of the antibody or by the negative control nonimmune IgG.

Involvement of other RUNX-2 binding motifs in the activation of the human COL10A1 promoter by RUNX-2. Next, we screened for other possible RUNX-2 binding motifs with the putative consensus sequences (20) in the 4.5-kb fragment of the 5′-end flanking region of the human COL10A1 promoter. Of the 6 identified regions, we selected the 2 most probable regions. The region from −1,839 bp to −1,834 bp was shown by comparative genomic analysis to correspond to the core responsive element of RUNX-2 in the mouse Col10a1 promoter identified in a previous study (20), and the region from −357 to −352 was the most proximal to the transcription start site of the 6 regions. We then prepared the 30-bp elements that included these 2 regions for further analyses, and called them A box (from −1,849 bp to −1,820 bp) and B box (from −369 bp to −340 bp) (Figure 6).

When we compared the dose-response effects of the tandem repeats on luciferase activity using the 3 human cell lines as described above, neither the A box nor the B box responded to RUNX-2 alone or to RUNX-2 in combination with CBFβ regardless of the number of repeats, in contrast to the HY box, which showed potent repeat number–dependent increases (Figure 6A). In addition to the 3 human elements, we examined binding of the partially or fully mutated HY box probe to the corresponding mouse sequence with RUNX-2 by EMSA. None of the mutated HY box probes formed a complex with the nuclear extract of RUNX-2 and CBFβ-transfected cells (Figure 6B), similar to mutation 2 in Figure 5B. However, both A box and B box oligonucleotide probes did form complexes with the nuclear extracts, which were supershifted by addition of an antibody to RUNX-2, indicating the specific binding of RUNX-2 to all human wild-type elements: HY box, A box, and B box (Figure 6B). More interestingly, when in vivo binding of RUNX-2 to the 3 elements was examined by ChIP assay using human HuH7 cells, RUNX-2 bound to the COL10A1 promoter region that included the HY box, but not to the region including the A box or B box or to the region without the putative RUNX-2 binding motif (Figure 6C).

The results of luciferase assay, EMSA, and ChIP suggested that the HY box was the principal responsive element of RUNX-2 under specific epigenetic environments in human cells. In fact, when we used mouse chondrogenic ATDC-5 cells instead of human cells for the luciferase assays, neither RUNX-2 alone nor a combination of RUNX-2 and CBFβ affected COL10A1 promoter activity. (Data are available online at http://www.h.u-tokyo.ac.jp/ortho/Supplemental_Figures.pdf.) Interestingly, however, there was a decrease in the region between −81 bp and −76 bp in not only RUNX-2–induced activity, but also basal promoter activity. Furthermore, luciferase assays of the tandem repeats of the human elements showed a moderate but repeat number–dependent increase in HY box activity in ATDC-5 cells whether or not they were transfected with RUNX-2, although neither the mutated HY box (mutation 2), A box, nor B box showed the response. (Data are available online at http://www.h.u-tokyo.ac.jp/ortho/Supplemental_Figures.pdf.) Hence, the HY box may also function as a basal responsive element for various transcriptional stimulations regardless of species.

DISCUSSION

The present study showed that human COL10A1 promoter activity was enhanced by RUNX-2 alone and was enhanced even more potently by RUNX-2 in combination with the coactivator CBFβ, and further identified the HY box as the principal and specific responsive element in the 3 human cell lines. Although this is the first study to identify the promoter element responsive to RUNX-2 in the human COL10A1 gene, RUNX-2 binding sites have previously been identified at more distal regions in the mouse Col10a1 promoter (20), which is not within the highly conserved region between mouse and human genes (Figure 3A). The human promoter region that corresponds to the mouse responsive element (A box) showed little response to RUNX-2 and did not show in vivo binding with RUNX-2 in

human cells (Figures 6A and C). The mouse promoter region that corresponds to the human HY box did not bind to RUNX-2 (Figure 6B), and the human HY box did not respond to RUNX-2 in mouse ATDC-5 cells. (Data are available online at http://www.h.u-tokyo.ac.jp/ortho/Supplemental_Figures.pdf.) These findings indicate that there are different mechanisms of type X collagen transactivation by RUNX-2 in humans and mice, and demonstrate that RUNX-2 binds to and activates the HY box in the specific microenvironment in human cells.

Nevertheless, the present study showed the colocalization of RUNX-2 and type X collagen in mouse specimens (Figure 1) and the induction of endogenous type X collagen expression by RUNX-2 in mouse cells (Figure 2). Our study had inevitable limitations, since neither human samples during endochondral ossification nor human chondrogenic cell lines that undergo hypertrophic differentiation are available. Indeed, the endogenous type X collagen induction in mouse cells by RUNX-2 shown in Figure 2 may not be due to transactivation of the mouse HY box, but to transactivation of the responsive element identified in a previous mouse study (20). However, mutations in the RUNX-2 gene cause cleidocranial dysplasia in both humans and mice (32,34,35). In addition, mutations in the COL10A1 gene cause skeletal abnormalities similar to Schmid-type metaphyseal chondrodysplasia in both species (9–12). We therefore believe that RUNX-2 and type X collagen play a role in endochondral ossification in humans as well as in mice.

The temporal and spatial specificity of type X collagen expression has been shown to be under the tight control of positive and negative regulators. In contrast to the positive regulator RUNX-2, parathyroid hormone (PTH) and PTH-related protein (PTHrP) are known to be crucial inhibitors of transcription (36–38). Previous searches in the human COL10A1 promoter have located a regulatory region between −2,410 bp and −1,875 bp upstream of the transcription start site, the activity of which was suppressed by PTH/PTHrP (38–42). In the central part of the region, a functional activator protein 1 (AP-1) site was identified between −2,144 bp and −2,135 bp as a responsive region of FosB and Fra-1 (42). Another enhancer region was identified between −2,273 bp and −2,244 bp, although the related transcription factor remains unknown (41). The deletion analysis of the luciferase assay that was performed in the present study, however, failed to detect a decrease in activity between −2,349 bp and −1,899 bp, which contains the 2 above-mentioned regions, with or without RUNX-2 transfection in any cell line (Figure 3B), indicating a different mechanism of RUNX-2 transcriptional regulation than of PTH/PTHrP and AP-1 transcriptional regulation in humans.

For the examination of RUNX-2 localization in the limb cartilage and the bone fracture callus, we used X-Gal staining in heterozygous *Runx2*-deficient mice with *lacZ* gene insertion at the *Runx2*-deletion site ($Runx2^{+/lacZ}$ mice) (32). This is because neither antibodies nor riboprobes worked appropriately in the localization of RUNX-2 by immunostaining or in situ hybridization, respectively, of the tissue of wild-type mice. Our preliminary studies confirmed that the $Runx2^{+/lacZ}$ mice showed normal skeletal phenotypes, except that they exhibited a dysplastic clavicle, which is typical of cleidocranial dysplasia, as previously reported (32) (Details are available online at http://www.h.u-tokyo.ac.jp/ortho/Supplemental_Figures.pdf.) Their growth plate phenotypes were also comparable with those of their wild-type littermates before and after birth. In addition, fracture callus formation and type X collagen expression in the callus were similar between the 2 genotypes.

These findings indicate that the RUNX-2 haploinsufficiency in $Runx2^{+/lacZ}$ mice did not cause abnormalities in skeletal growth or repair, confirming the adequacy of using $Runx2^{+/lacZ}$ mice instead of wild-type mice for the analysis. However, in a previous study in which we created an experimental OA model by induction of knee joint instability, $Runx2^{+/lacZ}$ mice exhibited suppression of type X collagen expression and degradation in joint cartilage (22). The fact that RUNX-2 haploinsufficiency prevented OA progression without affecting physiologic skeletal growth and repair suggests that RUNX-2–related signaling can be a therapeutic target of this disorder without severe skeletal side effects.

The HY box is indeed a core promoter element in the human COL10A1 gene responsive to RUNX-2 in human cells. However, basal luciferase activity without RUNX-2 transfection was also decreased in the region between −81 bp and −76 bp in the HY box not only in mouse ATDC-5 cells, but also in 3 human cell lines (Figure 3B), although not to as great an extent as RUNX-2–induced activity. Furthermore, the site-directed mutagenesis (Figure 4A) and tandem repeat experiments (Figure 4B) in the HY box showed a decrease and a repeat number–dependent increase, respectively, of basal luciferase activity in human cells without RUNX-2 transfection. These indicate that the HY box may also function as a potent universal enhancer in the human COL10A1 promoter, responding to

various transcriptional stimulations besides RUNX-2 in human cells. Considering that chondrocyte hypertrophy is a crucial step for skeletal growth, repair, and OA progression, the HY box will be useful in the comprehensive screening of transcription factors or cofactors for COL10A1 transactivation and chondrocyte hypertrophy, which might be therapeutic targets for skeletal growth retardation, fractures, and OA.

ACKNOWLEDGMENTS

We thank Dr. Michael Owen (GlaxoSmithKline, London, UK) for providing $Runx2^{+/tm2Z}$ mice. We also thank Reiko Yamaguchi and Hajime Kawahara for excellent technical assistance.

AUTHOR CONTRIBUTIONS

Dr. Kawaguchi had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study design. Higashikawa, Saito, Ohba, Takeshita, Nakamura, Chung, Kawaguchi.

Acquisition of data. Higashikawa, Saito, Kamekura, Kan, Oshima.

Analysis and interpretation of data. Higashikawa, Ikeda, Ogata, Takeshita, Chung, Kawaguchi.

Manuscript preparation. Higashikawa, Takeshita, Kawaguchi.

Statistical analysis. Higashikawa, Saito, Kawamura, Kawaguchi.

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DOI 10.1002/art.24376

Errata

In the articles by Valdes et al in the January 2007 and February 2006 issues of *Arthritis & Rheumatism* (pages 137–146 and 533–539, respectively), some information on sample collection and a funding source was omitted. Collection of some of the samples used for genetic association was carried out by Andrew Carr, MD (Nuffield Orthopaedic Centre, Botnar Research Centre, Oxford University, Oxford, UK), and funding was provided by the Norman Collisson Foundation, the Botnar Foundation, and the Lord Nuffield Orthopaedic Trust).

We regret the errors.

Osteoporos Int (2008) 19:429–435
DOI 10.1007/s00198-007-0544-4

SPECIAL POSITION PAPER

Development and application of a Japanese model of the WHO fracture risk assessment tool (FRAX™)

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Received: 5 November 2007 / Accepted: 10 December 2007 / Published online: 22 February 2008

Abstract

Summary The present study estimated the 10-year probability using the Japanese version of WHO fracture risk assessment tool (FRAX™) in order to determine fracture probabilities that correspond to intervention thresholds currently used in Japan and to resolve some issues for its use in Japan.

Introduction The objective of the present study was to evaluate a Japanese version of the WHO fracture risk assessment (FRAX™) tool to compute 10-year probabilities of osteoporotic fracture in Japanese men and women. Since lumbar spine bone mineral density (BMD) is used preferentially as a site for assessment, and densitometers use Japanese reference data, a second aim was to investigate the suitability and impact of this practice in Japan.

Methods Fracture probabilities were computed from published data on the fracture and death hazards in Japan. Probabilities took account of age, sex, the presence of clinical risk factors and femoral neck BMD. Fracture probabilities were determined that were equivalent to intervention thresholds currently used in Japan. The difference between T-scores derived from international reference data and that using Japanese-specific normal ranges was estimated from published sources. The gradient of risk of BMD for fracture in Japan was compared to that for BMD at the lumbar spine in the Hiroshima cohort.

Results The 10-year probabilities of a major osteoporosis-related fracture that corresponded to current intervention thresholds ranged from approximately 5% at the age of 50 years to more than 20% at the age of 80 years. The use of femoral neck BMD predicts fracture as well as or better than BMD tests at the lumbar spine. There were small differences in T-scores between those used for the model and those derived from a Japanese reference population.

Conclusions The FRAX™ tool has been used to determine possible thresholds for therapeutic intervention, based on equivalence of risk with current guidelines. The approach will need to be supported by appropriate health economic analyses. Femoral neck BMD is suitable for the prediction of fracture risk among Japanese. However, when applying the FRAX™ model to Japan, T-scores and Z-scores should be converted to those derived from the international reference.

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Keywords Bone mineral density · Fracture · Fracture probability · Fracture risk assessment tool · Intervention thresholds · Japan

Introduction

Fractures related to osteoporosis have become a major health and economic burden in Asian countries just as they have in North America and Europe. An estimated 117,900 cases of hip fracture occurred in 2002 [1], and the incidence in Japan has increased in the past 10 years [1, 2]. Asia will be expected to have the highest absolute increase in fracture number because it has the largest population. Early detection of individuals with high fracture risk using clinical risk factors would have a substantial impact on reducing the burden of fractures in Asia.

A series of meta-analyses on prospective population-based cohorts has identified a number of clinical risk factors that contribute to fracture risk independently of BMD at the femoral neck [3]. The integration of these risks would, therefore, enhance the predictive value of BMD [4]. The risk factors comprise age, sex, bone mineral density, body mass index (BMI), long-term use of glucocorticoids, parental history of hip fracture, history of fragility fracture, smoking, alcohol consumption (3 or more units/day), and secondary osteoporosis such as rheumatoid arthritis. A WHO scientific group has proposed that the 10-year probability for fracture is used to express fracture risk for clinical assessment [5] and to determine intervention thresholds [3] The aim of this study was to create a fracture probability model based on the methodology of the WHO risk assessment tool (FRAX™) [6] calibrated to the epidemiology of Japan.

In addition, several problems need to be resolved before the FRAX™ model is applied to Japan. First, the FRAX™ tool inputs femoral neck BMD and the Z-score or T-score is based on the NHANES III reference data base. In Japan, BMD at the lumbar spine is widely used clinically because the physical size of Japanese people is smaller than that of Western people, giving rise to a view based on little evidence that the reproducibility of measurements at the femoral neck BMD would be poorer than that at the lumbar spine. Furthermore, data on the young adult mean (YAM) and the mean at each age are installed in the DXA systems in Japan, and programmed to calculate T- and Z-scores from Japanese reference data. In addition, the Japanese Society for Bone and Mineral Research [7, 8] provide recommendations for the diagnosis of osteoporosis and intervention based on YAM, and these are widely used in clinical practice.

Against this background, additional aims of the present study were to provide fracture probabilities based on the FRAX™ tool that were equivalent to currently accepted intervention thresholds, explore the impact of using Japanese-specific normative data for femoral neck BMD, and reassess the respective performance characteristics of BMD at the femoral neck and lumbar spine.

Methods

Models were constructed to compute the 10-year probability of hip fracture and a major osteoporosis-related fracture in Japan. A major osteoporosis-related fracture was defined as a clinical spine, hip, proximal humeral and forearm fracture. Poisson modelling was used to calculate the hazard functions. The relationship between probability and hazard functions were used to calculate the 10-year probability or fracture for a combination of the risk factors. The mortality estimates for Japan were those published by the World Health Organization for 1999, which accord with estimates from Japan [9]. The incidence of hip fractures was taken from previously published sources [1] as was the incidence of fractures at the proximal humerus and distal forearm [10]. Since the incidence of a clinical vertebral fracture was not known in Japan, we assumed that the ratio of clinical vertebral fracture incidence to that of a vertebral fracture diagnosed by radiographic surveys [11] would be the same in the Japan as it was for Sweden [12].

The relationship of clinical risk factors to fracture outcomes was assumed to be the same as that determined in a large meta-analysis of risk factors of 190,000 patient years from nine prospectively studied population-based cohorts from Europe, Australia, North America and Asia [3]. The relationship has been validated in a further 11 cohorts of population-based samples with 1.2 million patient years of observation from the same regions [4]. The independent contribution of each risk factor was used to compute probabilities of fracture in the absence of clinical risk factors or in the presence of any combination [13, 14].

In Japan, the criteria for the diagnosis of osteoporosis prepared by the Japanese Society for Bone and Mineral Research [7] are based on BMD measurements expressed as a percentages of the young adult mean (YAM) for women. In patients with no prior fragility fracture a diagnosis of osteoporosis is made where the BMD is less than 70% of YAM. In patients with a previous fracture, osteoporosis is diagnosed where the BMD is less than 80% of YAM. These diagnostic thresholds, derived by maximising sensitivity and specificity for fracture detection, are also used as intervention thresholds. In order to compare intervention thresholds using YAM with probabilities derived from the FRAX™ algorithm, T-score equivalents were used. The T-score equivalent to 70% and 80% of

YAM for Japanese people is −2.7 SD and −1.8 SD, respectively, using the NHANES III reference for BMD at the femoral neck in Caucasian women aged 20–29 years [15].

The relative performance characteristics of BMD at the lumbar spine and femoral neck were examined in a population-based prospective study in Hiroshima. The Hiroshima cohort comprised 2,596 men and women (69% female, 9,803 person years, mean age 65.1 years). Details of the cohort have been previously published [11]. In brief, the participants received measurement of lumbar spine and femoral neck BMD using dual X-ray absorptiometry (DXA, QDR-2000, Hologic) during the period from 1994 to 1995 and were followed for a mean period of 4 years. Information about hip fracture, fracture of the distal radius, proximal humeral fracture and clinical spinal fracture was collected at interview by trained nurses and physicians during the biennial health examinations. One hundred eighty-six fractures were detected during the follow-up period, of which 89 were categorized as osteoporotic fractures and 31 were hip fractures. The gradient of fracture risk (increase in fracture risk per SD change in Z-score for BMD and increase in fracture risk per 0.1 g/cm^2 change in BMD) at the two sites was determined by the use of Poisson models [16]. The fracture outcomes were calculated for hip fracture, a major osteoporosis-related fracture (femoral neck, distal radius, proximal humerus and clinical spine fracture) and all fractures.

The mean height and body weight for Japanese women in the Hiroshima cohort was 150 cm and 52.6 kg, respectively, giving a BMI of 23.4 kg/m^2. The calculation of fracture probability was made at this BMI, but differences in BMI have little effect on predictive value for fracture risk assessment in the presence of BMD [17].

Japanese-specific T- and Z-scores and those derived from NHANES III were compared using the database of the Japanese Society for Bone and Mineral Research [7]. Data using both methods of calculation were entered into the FRAX™ tool.

Results

Ten-year probability of fracture

The 10-year probability of a major osteoporotic fracture for individuals without clinical risk factors is shown in Fig. 1 grouped by sex, age and T-score. The 10-year probability increased with age and with decreasing T-score. At younger ages, the fracture probability was similar in men and women. With advancing age, probabilities rose as expected, but the increase was greater in women than in men. In men aged 80 years, the 10-year probability for osteoporosis-related fracture exceeded 10% at a T-score of −3 SD, whereas in women of the same age, fracture probabilities exceeded 10% with T-score of −1 SD.

The contribution of clinical risk factors to fracture probability is shown in Fig. 2 for women aged 65 years with a BMI of 23.4 kg/m^2. In women without clinical risk factors, the 10-year probability for an osteoporosis-related fracture was 7.5%. The 10-year probability was higher in the presence of clinical risk factors. Smoking and alcohol were relatively weak risk factors, the use of long-term glucocorticoids of intermediate weight, and a parental history of hip fracture or a prior fragility fracture were associated with the highest risks. For example, the 10-year probability was 8.1% for smokers and 14.5% for individuals with a prior fracture. The 10-year probability for hip fracture was 1.1% in women without a clinical risk factor, 1.6% in smokers and 2.7% in women with a previous fracture (see Fig. 2).

Fracture probabilities were computed in women at the diagnostic threshold recommended in Japan. Thus, the cut-off level of BMD was set at 70% of YAM in women without a previous fracture and at 80% of YAM in those with a previous fragility fracture. In women aged 50, 60, 70 and 80 years without clinical risk factors and with BMD equivalent to 70% of YAM, the 10-year probability was 5.4%, 8.7%, 13.8% and 23%, respectively. In women having BMD equivalent to 80% of YAM and existing fracture but no other clinical risk factors, the 10-year probability was 7.1%, 10.5%, 14.7% and 23.4% at the same ages, respectively. Thus, at each age, the fracture probability was similar using the two diagnostic criteria. In contrast, the fracture probability equivalent to the diagnostic threshold in Japan rose with age, and at the age of 80 years was about four times higher than that at age 50 years (Fig. 3). Similar findings were apparent for hip fracture probability in that probabilities equivalent to the diagnostic threshold in Japan rose with age. The increase with age was more marked than for all major fractures and at the age of 80 years was about 6–40 times higher than that at age 50 years depending on the threshold used (see Fig. 3).

Comparison of lumbar spine and femoral neck BMD

The gradient of fracture risk for spine BMD and femoral neck BMD in the Hiroshima cohort indicated that lumbar spine measurements predicted all fractures, osteoporosis-related fracture and hip fracture with approximately equal gradients of risk that ranged from 1.25/SD for all fractures to 1.17/SD for hip fractures. There was no difference in the gradient of risk between men and women. In the case of hip fracture risk, the gradient of risk in men and women combined was not statistically significant with BMD measured at the lumbar spine. BMD at femoral neck had

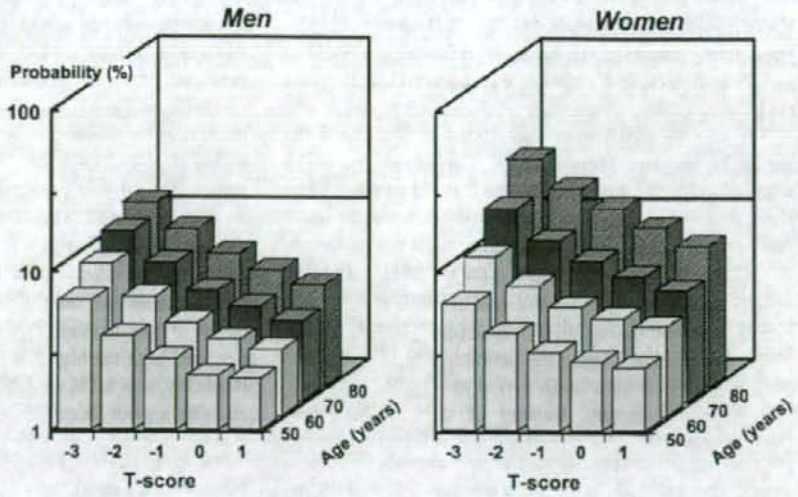

Fig. 1 Ten-year probability (%) of osteoporotic fracture (hip, clinical spine, humerus, forearm) in Japanese men and women without clinical risk factors according to age and T-score for BMD at the femoral neck

a similar or slightly higher gradient of risk for fractures compared with spine BMD, particularly in the case of hip fracture (Table 1). There was no significant difference in gradient of risk between lumbar spine BMD and femoral neck BMD with the exception of that for hip fracture where the gradient of risk was significantly higher for measurements made at the femoral neck. When gradient of risk was standardized to a constant denominator (i.e., RR/0.1 g/cm^2) the findings remained unchanged (see Table 1).

Japanese reference values

The reference mean in women aged 20–29 years at the femoral neck was 0.858 g/cm^2 (SD=0.120 g/cm^2) using the NHANES III data. When young normal values were computed from the Japanese population the mean BMD was 0.786 g/cm^2 (SD=0.107 g/cm^2). Thus the threshold for osteoporosis using the NHANES III data was 0.558 g/cm^2 and that derived from the Japanese data was 0.519 g/cm^2. The thresholds for osteopenia (WHO definition) were 0.738 g/cm^2 and 0.679 g/cm^2, respectively. Thus there were systematic differences in the T-score derived from the two data sets. A comparison of fracture probabilities computed from the z-scores using the two approaches is shown in Fig. 4 for different combinations of risk factors. The differences in probabilities were relatively modest, but as expected, the use of Japanese reference values overestimated fracture probabilities.

Discussion

This paper describes the development of the WHO fracture risk assessment tool calibrated to the epidemiology of

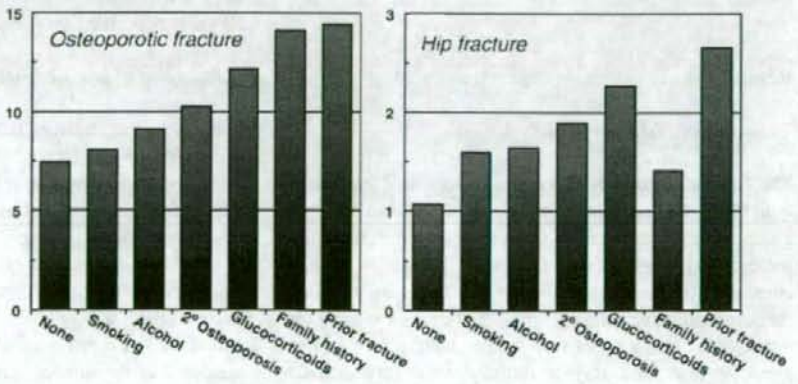

Fig. 2 Ten-year probability for osteoporotic (hip, clinical spine, humerus, forearm) and hip fracture (%) according to the presence of a clinical risk factor, in women at the age of 65 years and with a BMI of 23.4 kg/m^2

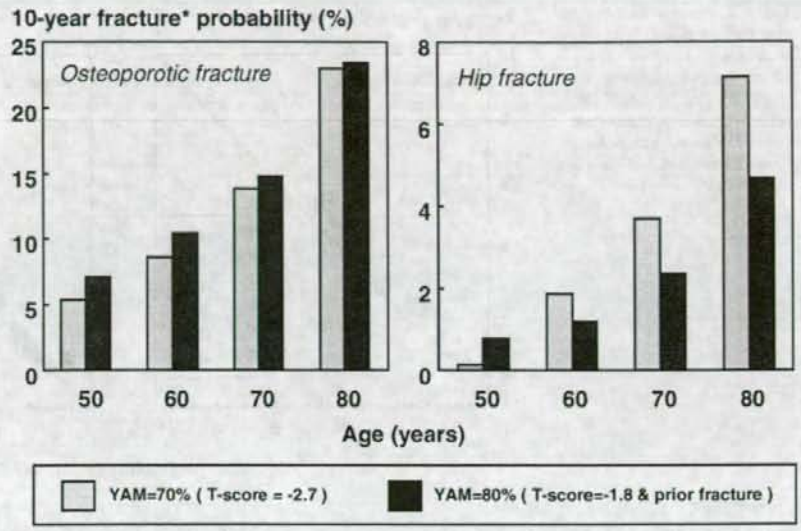

Fig. 3 Ten-year probability of osteoporotic (hip, clinical spine, humerus, forearm) and hip fracture based on women at the threshold for the diagnosis of osteoporosis using the criteria of the Japanese Bone Mineral Metabolism Association

Japan. The incidence of hip fracture, distal radius fracture and proximal humeral fracture in Japan is lower than that in North America or Northern Europe [1, 10]. However, the prevalence and incidence of spinal fracture are higher in Japan [11, 18]. A minority of all cases of morphological spinal fracture are assumed to be clinical spine fractures in the Japanese FRAX™ models. The multiplier is age and sex specific. For men the multiplier goes from approximately 33% at age 50 to 48% at age 85. For women the corresponding figures are 19% and 24%. These estimates, derived from the epidemiology of fracture in Sweden [12], have been shown to hold true for Japan [19].

The FRAX™ algorithm is suitable for assessment in men and women from the age of 40 years and calculates the 10-year probability for both hip fracture and a major osteoporosis-related fracture. One of its strengths is that it can capture the independent contribution of several clinical risk factors to fracture risk and can be used with or without information on femoral neck BMD. A more detailed account of the properties of the FRAX™ models is provided elsewhere [13]. In this paper, we focused on its application to decision-making in clinical practice with the estimation of intervention threshold i.e., the fracture probability at which intervention is currently considered to be worthwhile. The approach should be distinguished from intervention thresholds that are based on health economic analysis.

Table 1 Predictive ability of spine and femoral neck BMD for any, osteoporotic and hip fracture in men and women from Japan

	RR/SD		RR/0.1 g/cm^2	
	RR	95% CI	RR	95% CI
a. Any fracture				
Femoral neck	1.45	1.23–1.70	1.43	1.22–1.68
Lumbar spine	1.25	1.13–1.39	1.38	1.19–1.61
b. Osteoporosis-related fractures				
Femoral neck	1.40	1.09–1.78	1.38	1.09–1.74
Lumbar spine	1.20	1.04–1.40	1.30	1.05–1.61
c. Hip fracture				
Femoral neck	2.08[b]	1.34–3.22	2.11	1.38–3.23
Lumbar spine	1.17	0.91–1.50	1.25	0.87–1.80

[a] Hip, clinical spine, forearm and proximal humerus
[b] Significantly higher than lumbar spine (P=0.049)

The WHO makes no specific recommendation concerning intervention thresholds, since these depend on many local factors [6]. Rather, it is suggested they should be determined by each country, based on the local healthcare situation and cost-effectiveness of the treatment of osteoporosis. Intervention thresholds, based on cost-effectiveness have been formulated in the UK, the USA and in Sweden [20–22]. In Japan, diagnostic thresholds are used as intervention thresholds. When the probabilities of osteoporosis-related fracture were determined at these thresholds, they varied with age (see Fig. 3), ranging from approximately 5% at the age of 50 years to more than 20% at the age of 80 years. Against this background, a 10-year probability of 10% for osteoporosis-related fracture may be an acceptable intervention threshold for Japan, though an optimization should take account of health economic consequences for individuals and for the health care budget.

The FRAX™ tools are designed to be extensively used in the world as a means of identifying individuals with elevated risk for fracture and aid in the determination of the threshold for therapeutic intervention, but there will be hurdles to be faced in the ease of its acceptance. Such

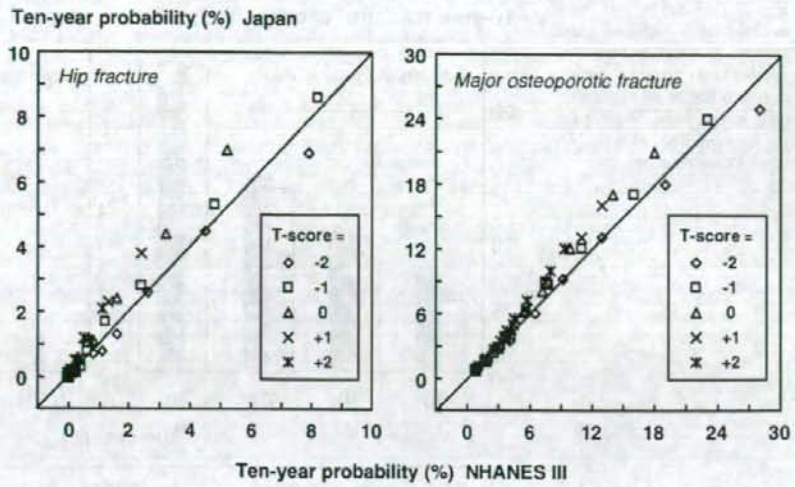

Fig. 4 Correlation between 10-year fracture probabilities (%) in women without clinical risk factors computed from normative data using NHANES III reference values and Japanese-derived reference values for femoral neck BMD. BMI is set at 23.4 kg/m^2

hurdles are likely to differ from country to country. In Japan, the choice of clinical risk factors is not at issue since the risk factors adopted in the FRAX™ algorithms included the data from the Hiroshima cohort, Japan [11, 23, 24], and the validation included the Japanese Miyama cohort [13]. More problematic is the inclusion of femoral neck BMD, since the lumbar spine measurement is the most widely used in Japan. Asian physiques are smaller than those of Caucasians, and the geometric characteristics of the femoral neck in Japanese differ from those in American women, in that the femoral neck length is shorter than in the Japanese [25, 26]. Because of uncertainty regarding measurement of femoral neck BMD, measurement of lumbar BMD is widely used in Japan. The present study indicates that concerns over the use of femoral neck BMD are unfounded.

Femoral neck BMD was superior in its ability to predict hip fractures compared with spine BMD, and BMD at the spine and femoral neck had similar predictive value for fractures other than hip fracture. Fujiwara et al. [11] have also shown that the ability to predict the risk for morphological spinal fracture was similar between femoral neck BMD and lumbar spine BMD. These data on gradients of fracture risk, derived in Japan, did not differ from those reported in Western countries. Indeed, the meta-analysis used to inform the FRAX™ tool showed no evidence for heterogeneity in gradients of risk between cohorts [16]. Thus the evidence suggests that fracture risk assessment is not disadvantaged by the use of femoral neck BMD. Indeed, the converse may be true.

A further hurdle, unique to Japan, is that diagnostic thresholds differ from the WHO description of osteoporosis which defines osteoporosis on the basis of a fixed T-score threshold (≤ −2.5 SD) using an international reference standard for young (aged 20–29 years) Caucasian women [15]. In Japan, diagnostic thresholds are also derived by reference to a young population, but differ from the WHO in that a local (i.e., Japanese) standard is used and that the criteria differ in patients with or without previous fracture. In view of the widespread use of data derived from the Japanese population, the question arises whether T-scores or Z-scores derived from Japanese databases could be used in the FRAX™ tool, rather than those derived from the international reference base. In the present study, mean BMD at the femoral neck was lower in Japanese women than in the NHANES III sample from the USA, as previously shown [27]. The difference was not, however, large (approximately a half a SD). There were also differences in the SD which was smaller in Japanese women than in the NHANES III sample (0.107 and 0.120 g/cm2, respectively). Not surprisingly, the DXA-based T-score obtained from Japanese and USA populations differed as did the computed probabilities. Although the differences were small at low T-scores, when applying the FRAX™ model to Japan, it is preferable to program the system so that the Japanese T- and Z-scores are converted into the appropriate T- and Z-scores based on NHANES III.

In conclusion, a FRAX™ tool has been developed to compute fracture probabilities calibrated to the epidemiology of Japan. The tool has been used to determine possible thresholds for therapeutic intervention, based on equivalence of risk with current guidelines. The approach will need to be supported by appropriate health economic analyses. The present study indicates that the femoral neck BMD is suitable for prediction of the risk for fracture among Japanese people. However, when applying the FRAX™ model to Japan, T-scores and Z-scores should be converted into those derived from the international reference.

Conflicts of interest None.

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Hiroshima J. Med. Sci.
Vol. 57, No. 1, 17–25, March, 2008
HIJM 57–3

Effect of Angiotensin Converting Enzyme Inhibitor and Benzodiazepine Intake on Bone Loss in Older Japanese

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ABSTRACT

We investigated the effects of several frequently described medication regimens on annual percentage change in bone mineral density (BMD). A longitudinal cohort study (a retrospective analysis) was conducted. Subjects in the Adult Health Study (a prospective cohort study begun in 1958) have been followed through biennial medical examinations in Hiroshima, Japan. Participants were 2,111 subjects (67% women; aged 47-95 years) who were undergoing biennial health examinations from 1994 to 2000. The subjects were examined for the effect of certain drugs on bone mineral change during baseline and one follow-up (4 year later) measurements. Mean annual percentage change in BMD at the femoral neck was −0.38% for men, and −1.14% for women. After adjustment for sex, age, change of weight, alcohol consumption, and smoking status, annual percentage change in BMD decreased by 0.61% among individuals taking angiotensin converting enzyme (ACE) inhibitors continuously in comparison with individuals who had not taken them (p=0.002): also decreased 0.40% among individuals taking benzodiazepines (BZDs) continuously (p=0.034). Our results suggest that careful consideration should be given to the use of ACE inhibitors and BZDs in a cohort of Japanese elderly.

Key words: *Bone loss, Angiotensin converting enzyme inhibitor, Benzodiazepine, Pharmacoepidemiology*

Change in bone mineral density (BMD) is known to be a useful measure for evaluating the risk of bone fractures, and there have been many reports studying various factors that might be associated with change of BMD. While early reports indicate that intake of glucocorticoids[3] and thyroid hormones[29] tends to reduce BMD and that intake of estrogens[9] and thiazide[6] tends to increase it, recent studies have reported that other medications that are not intended for bone therapies could affect bone loss. For example, statins[7], beta-adrenergic blockers (β -blockers)[24], and cyclooxygenase-2-selective non-steroidal anti-inflammatory drugs (COX-2-selective NSAIDs)[5] reportedly tend to increase BMD. Another study reports that low serum vitamin B12 levels may be associated with increased rates of hip bone loss[31]. On the other hand, there are also reports with contrary results[25,30], so there is not yet a unified consensus. In addition, most of these reports are studies based on populations of Caucasians in Western countries. Medications among Caucasians in Western countries are expected to be very different from those among other populations, including Japanese; e.g., the doses and types of medication administered in Japan differ from those in Western countries. Therefore, it is important to find evidence of the effects on BMD, positive or negative, of a variety of drugs and supplements, including those not targeting osteoporosis, such as antihypertensive drugs. The main purpose of this study is to examine the association between BMD change and drug use in a 2,111 cohort of elderly Japanese men and women. This study was a retrospective analysis, and we had no intervention for the effects of medications.

MATERIALS AND METHODS

Study Population

In 1958, the Atomic Bomb Casualty Commission (predecessor to the Radiation Effects Research Foundation) initiated the so-called Adult Health Study (AHS), a cohort follow-up study which relies upon data obtained in the course of biennial health examinations of participants in a fixed cohort to shed light on the many changes in physiological and biochemical functions that might be attributable to atomic bomb radiation exposure[17,36]. The initial AHS cohort consisted of ~15,000 atomic bomb survivors and ~5,000 controls, all of whom were selected from the residents of Hiroshima and Nagasaki on the basis of a supplementary questionnaire that was included in the 1950 national census and a survey of atomic bomb survivors. Participation rates in the study were ~80% throughout the follow-up period. Further information about the expansion of the cohort and details of the health examinations are available elsewhere[1,11].

A total of 2,613 AHS subjects aged between 47 and 95 years underwent physical examination in Hiroshima in the 1994-1995 examination cycle. We then selected 2,111 of these subjects (703 men and 1,408 women) for our analysis. The remaining 73 subjects were excluded either because of a diagnosis suggesting impairment of bone metabolism (such as hyperparathyroidism, renal osteodystrophy, or bilateral oophorectomy) or because they were only available for one examination during the 1994-2000 period. The other remaining 429 subjects were excluded because they were premenopausal or they took drugs that interfered with bone metabolism, including estrogens, glucocorticoids, calcitonins, ipriflavones, bisphosphonates, and thyroid hormones, and the sample sizes of them were too small to attain statistical significance.

Bone Mineral Density

BMD was measured at the femoral neck at each biennial health examination using a dual x-ray absorptiometer (DXA, QDR-2000; Hologic Inc, Waltham, MA, USA). An anthropomorphic spine phantom was scanned daily to calibrate the DXA. There was no significant fluctuation in machine performance during the study period. The precision of the DXA was also carefully monitored over the study period using the anthropomorphic phantom, and was found to be less than 1%.

In our study, annual percentage change in BMD was defined as follows:

$$\text{Annual Percentage Change in BMD (\%)} = \frac{\text{BMD at second exam} - \text{BMD at baseline exam}}{\text{BMD at baseline exam} \times \text{follow-up period (yrs.)}} \times 100$$

Other Measurements

Subjects were asked to bring every over-the-counter or prescription medication they were taking, and in interviews by trained nurses were asked how often and how much of these medications they were taking. Subjects known to be taking at least one medication were then categorized into 4 groups for each medication according to "on" vs. "off" status of the use of each drug in the baseline (1994-1995)/second follow-up (1996-2000) surveys: "New" if the status in the baseline/follow-up was "off"/"on", "Continuing" if "on"/"on", "Stop" if "on"/"off", and, "No" if "off/ "off".

Height, body weight[8], and blood pressure measurements[4] were recorded during every survey. Subjects were interviewed by nurses to obtain disease histories, years since menopause, and lifestyle information including smoking status and drinking habits[14,22,23,32]. After examination and interview, every subject consulted a doctor, who ascertained the diagnoses. Diagnosis of hypertension was based on a systolic blood pressure $\geq$ 140 mm Hg, a diastolic blood pressure $\geq$ 90 mm Hg, or current treatment with antihypertensive drugs.

Because we did not have data about subjects' physical activity at baseline, we used the data about status of going out in 1996-1997 as a substitute for activity of daily living at the baseline. We asked subjects whether they could go out alone or with a helper, or if it was impossible to go out in 1996-1997.

Since we had previously reported that there was no evidence of an association between atomic bomb radiation dose and BMD in our study cohort[10], we did not include radiation dose as a possible factor.

All subjects gave written informed consent for BMD measurements and all other examination items.

Statistical analyses

Linear regression models were used to examine the relationship of drug use to annual percentage change in BMD at the femoral neck. For the response variable of annual percentage change in BMD at the femoral neck, a preliminary univariate linear regression model was fitted using each drug use as an indicator covariate. The effect of drug use was adjusted by including in each model factors that expected to affect BMD change at the femoral neck, such as sex, age (years), change of weight (kg), diastolic blood pressure (mm Hg), smoking status (non-smoker, ex-smoker, or current smoker), and alcohol consumption (non-drinker, ex-drinker, or current drinker). For adjustment, we examined height, body mass index, systolic blood pressure, baseline disease such as hypertension, diabetes mellitus, cardiovascular disease, coronary heart disease and stroke. Hypertension and cardiovascular disease had associations with

annual percentage change in BMD at the femoral neck in univariate regression models (regression coefficient yes/no (p value); -0.256 (p = 0.01), -0.178 (p = 0.04), respectively), but did not in multivariate models (regression coefficient (p value); -0.146 (p = 0.16), -0.126 (p = 0.20), respectively). Other factors did not have associations.

The medications that appeared to have strong associations (p<0.10) with the response variable were calcium channel blockades (CCBs), benzodiazepines (BZDs), and angiotensin converting enzyme (ACE) inhibitors (regression coefficient of continuing/no (p value): -0.244 (p = 0.043), -0.435 (p = 0.020), -0.632 (p = 0.002), respectively). Each of them was negatively associated with annual percentage change in BMD at the femoral neck.

Next, a preliminary multivariate linear regression model was fitted including those medications that exhibited significant associations with the response variable in the univariate linear regressions. Again, the effects of the drugs were modified by sex, age, change of weight, diastolic blood pressure, smoking status, and alcohol consumption. We came to a definite final model with non-significant terms, i.e. CCB use and diastolic blood pressure, removed.

In addition, we fit each model separately for the two sex groups, because annual percentage changes in BMD at the femoral neck level generally differ between men and women.

Statistical analysis software SAS 8.2 (SAS software version 8.2 for regression analysis, SAS Institute, Cary, North Carolina, U.S.) was used for all statistical analyses. Two-sided p values were calculated, with p <0.05 as the cut off point for statistical significance.

RESULTS

The mean interval between the baseline and second survey, i.e. the average observation period, was 4.0 years. Characteristics of the subjects in our cohort are summarized in Table 1. Mean (standard deviation (SD)) age at the time of baseline survey was 62.8 (9.9) years for men and 67.0 (9.0) years for women. Mean (SD) annual percentage change in BMD measured at the femoral neck was −0.38% (1.49) for men and −1.14% (1.95) for women. The numbers of subjects who were taking main medications are shown by sex and groups (No, Stop, New, and Continuing) in Table 2. In our cohort, 35% of all subjects used one or more antihypertensive drugs.

Table 3 contains the estimated regression coefficients of the final full model with non-significant terms removed. In this model, after the adjustment for sex, age, change of weight, smoking status, and alcohol consumption, we found that the BMD at the femoral neck annually decreased by 0.61% of the baseline on average among the continuing users of ACE inhibitors compared with the non-users (p = 0.002), and by 0.40% among the continuing users of BZDs compared with the non-users (p = 0.034). It should be noted that diastolic blood pressure and CCB intake did not show a significant association with annual percentage change in femoral neck BMD (p = 0.113, p = 0.199, respectively) in the multivariate regression model. There were few differences in sex, age, BMD, blood pressure, and other measurements between CCB users and ACE inhibitor users. It should be noted that 44% of ACE inhibitor users also used CCB. On the other hand, 18% of CCB users concurrently used ACE inhibitor.

Since BMD generally changes differently between men and women, it seems more appropriate to fit the models separately for the two sex groups. The results tell us that, while no significant association was found between use of any drug and BMD change for men, there was a significant association for women (Table 4). The average BMD at the femoral neck annually decreased by 0.74% of the baseline among the continuing female users of ACE inhibitors compared with those women who never used them (p=0.002), after the adjustment for age, change of weight, smoking status, and alcohol consumption. The result also suggested that, among the continuing female users of BZDs, BMD decreased by 0.45% of the baseline per year on average compared with non-users (p=0.058).

To validate the effect of ACE inhibitors, an antihypertensive drug, on BMD change, we need to analyse the data for female subjects diagnosed with hypertension. In this cohort, the number of females diagnosed with hypertension was 628 with the mean (SD) age 69.6 (8.6) years, among which the users of ACE inhibiters were 133 with mean age 70.4 (8.2) and the non-users were 495 with mean age 69.3 (8.7). Since the age distributions of these two groups show no major discrepancy, age is unlikely to be a confounder. In this linear regression analysis, BMD at the femoral neck appeared to decrease on average by 0.86% of the baseline per year among the continuing female users of ACE inhibitors in comparison with those who never took them, after adjustments were made for age and change of weight (Table 5). Interestingly, both systolic blood pressure and diastolic blood pressure did not exhibit a significant association with BMD change among hypertensive women.

It was suspected, in the case of users of BZDs and ACE inhibitors, that physical activities would be low, and there was an observable association between drug use and bone loss. Although we did not have data about physical activities in subjects at baseline, we did have data for the next examination cycle (1996-1997), when we interviewed subjects about whether they could go out alone,

Table 1. Characteristics of Study Subjects

Sex	Men	Women
Subjects, number	703	1408
At the Time of Baseline Survey:		
Age, mean (SD), years	62.8 (9.9)	67.0 (9.0)
40-49 years, %	14.8	3.5
50-59 years, %	17.5	14.2
60-69 years, %	48.7	44.9
70-79 years, %	12.2	28.2
80 years or over, %	6.8	9.2
Femoral neck BMD, mean (SD), g/cm^2	0.736 (0.115)	0.610 (0.100)
Height, mean (SD), cm	163.4 (6.2)	149.9 (5.8)
Body weight, mean (SD), kg	60.8 (9.0)	52.1 (8.8)
Body mass index, mean (SD), kg/m^2	22.7 (2.9)	23.2 (3.6)
Systolic blood pressure, mean (SD), mm Hg	132.5 (20.9)	132.8 (11.1)
Diastolic blood pressure, mean (SD), mm Hg	80.0 (12.2)	77.2 (11.8)
Non drinker, %	19.3	71.3
Current drinker, %	73.4	27.3
Exit drinker, %	7.3	1.4
Non smoker, %	17.0	86.9
Current smoker, %	50.2	9.7
Exit smoker, %	32.8	3.4
Hypertension, %	46.9	44.6
Diabetes mellitus, %	16.2	11.0
Cardiovascular disease, %	31.3	31.9
Coronary heart disease, %	5.3	6.9
Stroke, %	4.4	3.4
Cancer, %	4.6	6.0
Years after menopause, mean (SD), years	-	18.3 (9.9)
At the Time of Second Survey:		
Femoral neck BMD, mean (SD), g/cm^2	0.725 (0.123)	0.583 (0.107)
Body weight, mean (SD), kg	60.4 (9.1)	51.7 (9.1)
Annual percentage change in femoral neck BMD, mean (SD), %	-0.38 (1.49)	-1.14 (1.95)
Change of weight, mean (SD), kg	-0.27 (2.6)	-0.36 (2.7)

No, number; SD, standard deviation; BMD, bone mineral density.

Table 2. Number of Subjects on Main Medications

Sex	Men				Women			
Used drug	No	Stop	New	Continuing	No	Stop	New	Continuing
Calcium channel blockade	515	19	88	81	998	23	203	184
Vitamin D_3	678	4	17	4	1112	39	165	92
Benzodiazepine	619	7	48	29	1178	31	129	70
Angiotensin converting enzyme inhibitor	630	24	30	19	1218	16	107	67
Calcium	670	12	12	9	1231	40	103	34
Vitamin B_{12}	651	7	31	14	1246	28	103	31
COX-2 selective NSAID*	676	6	18	3	1283	19	86	20
Statin	679	2	14	8	1297	19	68	24
Beta-adrenergic antagonist	663	7	19	14	1326	14	41	27
Thiazide diuretics agent	690	3	4	6	1348	13	23	24
Vitamin K_2	699	0	4	0	1357	2	47	2

*COX-2 selective NSAID: cyclooxygenase-2-selective non-steroidal anti-inflammatory drugs (etodolac, piroxicam, sulindac, and zaltoprofen were categorized in this group).

Table 3. Multivariate Linear Regression with Adjustment for Sex, Age, Change of Weight, Smoking Status, and Alcohol Consumption

Annual Percentage Change in Femoral Neck BMD (R^2=0.1039)	Regression Coefficient	Standard Error	p value
Sex*	-0.682	0.122	<.001
Age (years)	0.120	0.044	.006
Age-square	-0.001	0.0003	.001
Change of weight (kg)	0.092	0.015	<.001
Alcohol consumption (current-/non-drinker)	0.278	0.092	.003
Alcohol consumption (ex-/non-drinker)	0.335	0.118	.005
Smoking status (current-/non-smoker)	-0.368	0.121	.002
Smoking status (ex-/non-smoker)	-0.045	0.073	.535
Angiotensin converting enzyme inhibitor use			
Stop/No	-0.583	0.280	.037
New/No	-0.143	0.160	.374
Continuing/No	-0.607	0.200	.002
Benzodiazepine use			
Stop/No	0.536	0.302	.076
New/No	0.057	0.143	.692
Continuing/No	-0.395	0.186	.034

*: Men=0 and women=1.

Table 4. Multivariate Linear Regression with Adjustment for Age, Change of Weight, Smoking Status, and Alcohol Consumption (Women)

Annual Percentage Change in Femoral Neck BMD (R^2=0.0756)	Regression Coefficient	Standard Error	p value
Age (years)	0.222	0.063	<.001
Age-square	-0.002	0.0005	<.001
Change of weight (kg)	0.094	0.019	<.001
Alcohol consumption (current-/non-drinker)	0.298	0.118	.012
Alcohol consumption (ex-/non-drinker)	0.468	0.230	.042
Smoking status (current-/non-smoker)	-0.343	0.178	.054
Smoking status (ex-/non-smoker)	-0.239	0.142	.092
Angiotensin converting enzyme inhibitor use			
Stop/No	-0.443	0.472	.348
New/No	-0.136	0.191	.482
Continuing/No	-0.743	0.243	.002
Benzodiazepine use			
Stop/No	0.303	0.365	.406
New/No	0.065	0.179	.716
Continuing/No	-0.449	0.236	.058

Table 5. Multivariate Linear Regression with Adjustment for Age, and Change of Weight (Hypertensive Women)

Annual Percentage Change in Femoral Neck BMD (R^2=0.1048)	Regression Coefficient	Standard Error	p value
Age (years)	0.235	0.104	.024
Age-square	-0.002	0.0007	.010
Change of weight (kg)	0.166	0.030	<.001
Angiotensin converting enzyme inhibitor use			
Stop/No	-0.517	0.538	.337
New/No	0.074	0.256	.772
Continuing/No	-0.855	0.300	.005

Table 6. Multivariate Linear Regression with Adjustment for Sex, Age, Change of Weight, Smoking Status, and Alcohol Consumption (Ambulatory subjects: N = 1932)

Annual Percentage Change in Femoral Neck BMD (R^2=0.0978)	Regression Coefficient	Standard Error	p value
Sex*	-0.716	0.121	<.001
Age (years)	0.159	0.045	.001
Age-square	-0.001	0.0003	<.001
Change of weight (kg)	0.073	0.015	<.001
Alcohol consumption (current-/non-drinker)	0.200	0.092	.030
Alcohol consumption (ex-/non-drinker)	0.270	0.120	.025
Smoking status (current-/non-smoker)	-0.357	0.120	.003
Smoking status (ex-/non-smoker)	-0.023	0.073	.754
Angiotensin converting enzyme inhibitor use			
Stop/No	-0.653	0.287	.023
New/No	-0.071	0.161	.660
Continuing/No	-0.554	0.202	.006
Benzodiazepine use			
Stop/No	0.349	0.308	.257
New/No	0.149	0.143	.300
Continuing/No	-0.271	0.193	.160

*: Men=0 and women=1

or with a helper, or if it was impossible to go out. Assuming that these data at 1996-1997 were so similar that they could be substituted for physical activities at 1994-1995, we examined the relation between these data, drug use, and bone loss.

In this cohort, 1932 subjects answered that they could go out alone, 56 subjects needed helpers, 16 subjects found it impossible to go out, and 107 subjects did not answer. We did not find the relation between bone loss and these categories. We then analysed the procedure described previously concerning subjects who could go out alone (Table 6). It should be noted that BZD intake did not show a significant association with annual percentage change in femoral neck BMD (regression coefficient of continuing/no (p value); -0.271 ($p = 0.160$)) in the multivariate regression model.

DISCUSSION

The main purpose of the present study was to investigate the associations between BMD change and the use of several drugs in a cohort of 2,111 Japanese subjects who underwent one baseline measurement and one follow-up measurement (4 year later) of BMD. This study was motivated by the fact that, while most earlier studies investigating factors associated with BMD change have been for populations in the United States and Europe, few were found for other populations including the Japanese. Although this is a retrospective study, the result suggests that loss of BMD at the femoral neck might be attributed to ACE inhibitor and BZD intake among older Japanese. Other factors that appeared to be associated with BMD change were sex, age, change of weight, alcohol consumption, and smoking status.

Among those medications that might have an effect on BMD, some drugs such as glucocorticoids, thyroid hormones, estrogens, thiazide diuretics, and vitamin K_2 were not commonly prescribed in Japan at the time of survey, and thus we were unable to examine their associations with BMD change due to the small sample sizes. Although intake of statins, β-blockers, COX-2-selective NSAIDs, or vitamin B_{12} was occasionally observed in our cohort, no significant association was found for any of these drugs with BMD change. Vitamin D_3 and calcium supplements were frequently used in our cohort but did not show significant associations with BMD change. This is in agreement with some earlier studies that report that these drugs are effective not to increase but rather to maintain BMD[2,18].

There are some papers indicating that women diagnosed with hypertension express low BMD compared with normotensive women[4,35]. In preclinical studies, there is some discussion about a possibility of the rennin-angiotensin system (RAS) being associated with regulation of bone resorption. Hagiwara et al. reported that angiotensin II controlled differentiation of bone blast cells, and consequently bone formation was contained[13]. Hatton et al demonstrated that angiotensin II might be a stimulator of osteoclastic bone resorption[15].

The synthetic pathway of angiotensin II, which was well known in terms of high blood pressure, is called RAS. Angiotensinogen, which is secreted from the liver and used for the formation of angiotensin II, is converted to angiotensin I by renin secreted from the kidney. Angiotensin I is then converted to angiotensin II by angiotensin-converting enzyme (ACE). Angiotensin II is the major bioactive product of RAS. Angiotensin II has a variety of effects on the body. As with most other capillary beds in the body, constriction of efferent arterioles increases arterioler resistance, raising systemic arterial blood pressure and decreasing blood flow. Angiotensin II promotes secretion of aldosterone, which causes blood vessel contraction through specific receptors of the organism, and promotes maintenance of Na-level and discharge of K.

ACE acts not only on RAS but also simultaneously on the bradykinin system (BKS), and stimulates degradation of bradykinin (BK), converting it into an inactive substance. Therefore, ACE inhibitors also work on BKS, and maintain BK as high concentrations; at the same time, they work on RAS and suppress generation of angiotensin II. As mentioned above, although angiotensin II controls differentiation of bone blast cells, and bone formation is controlled as a result, some papers have reported that BK also stimulated bone resorption and reduced BMD[12,19,20]. From such reports, we consider that use of ACE inhibitors may increase BMD through reduced concentration of angiotensin II. On the other hand, BMD may be decreased through increased BK concentration.

Recently, in an experiment using rats, one group reported that angiotensin receptor blocker (ARB) restrained bone resorption[28], although ARB was designed as an antihypertensive drug without BK effect. The above results, however, seem to be consistent with the interpretation that use of ACE inhibitors may increase BMD through reduced concentration of angiotensin II, and may decrease BMD because of increased BK concentration on the other hand.

Another paper reported that ACE inhibitors might have possible benefits in treating not only hypertension but also osteoporosis among older Chinese[21]. A cross-sectional study of 3,887 Chinese men (n = 1958) and women (n = 1929), aged 65 years and over, was used to explore the association between ACE inhibitor use and BMD. The study consisted of a comparison of BMDs between users and nonusers of ACE inhibitors. However, mean weight in users was heavier than

that in nonusers. Also the rates of concomitant administration of ACE inhibitor, thiazide and statin were higher than those in nonusers. Although these effective factors were adjusted in multiple linear regression analysis, weight, thiazide and statin use have strong effects on BMD[6-8]. The existence of bias might be suspected. Furthermore, the use of antihypertensive medications in China was different from that in Japan. Women and men were more frequently prescribed beta-blockers and calcium supplements in China than patients in Japan. But women were less frequently prescribed ACE inhibitors and CCB in China than women in Japan. We would like to know the duration of antihypertensive treatment use there.

Additionally, one paper demonstrated that treatment with beta-blockers, ACE inhibitors, and CCBs was associated with reduced fracture risk[26]. That research represented the results of a large, nationwide, population-based pharmaco-epidemiological case-control study. The study subjects consisted of 124,655 cases that had experienced a fracture and 373,962 age- and gender-matched controls. The study found that treatment with ACE inhibitors was associated with reduced fracture risk. But the category of ACE inhibitors in the study also included ARBs. The mean age of cases was 43 years. The cases included hip fractures, forearm fractures, and spinal fractures. The cases tended to have a higher frequency of comorbidity. As no information on patients' compliance was available in the study, a prescription was used as a proxy for actual use of a drug. In addition, adjustment for differences in body weight was not possible.

Other than these papers, few have reported on the relationship between ACE inhibitor use and osteoporosis incidence. The actual mechanism behind ACE-inhibitor effects on BMD is not entirely understood, but we assume that use of ACE inhibitors decreases angiotensin II, resulting in increased BMD, and increases BK, resulting in decreased BMD. In our human observation study, however, we were only able to observe decreased BMD. This suggests that the effect of BK is greater than that of angiotensin II on BMD at the femoral neck in Japanese elderly. By contrast, angiotensin II may have a greater effect on BMD than BK in Chinese elderly. We do not know, however, whether the different results are due to race, differences in medication, differences of acting sites such as osteoblasts or osteoclasts, or other factors.

These considerations do not contradict the report that ARB increased BMD in Japanese. In the future, we would like to investigate the use of ARBs and consider the association of such use with BMD in our cohort.

ACE inhibitors and CCBs were the two most frequently used antihypertensive drugs in our cohort. Each of them appeared to be significantly associated with bone loss in the univariate linear regression adjusted for sex, age, change of weight, smoking status, and alcohol consumption. This observation made us suspect that antihypertensive drug rather than hypertension[4,33] might affects on BMD. The direct effect of hypertension on BMD, adjusted for possible effects of antihypertensive drugs, could be examined by comparing two groups of hypertensive and non-hypertensive subjects with equal drug use histories. However, this was impossible in this study because those antihypertensive drugs were used only by hypertensive subjects. We, therefore, examined the effect of ACE inhibitors on BMD among hypertensive subjects. In this analysis, a significant association was still found to exist between ACE inhibitor intake and loss of BMD, which indicates that use of ACE inhibitors might tend to accelerate bone loss among hypertensive women.

ARBs are now the first choice among medicines for about half of hypertension patients in Japan[34]. This is because ARBs have the same antihypertensive effects as ACE inhibitors and fewer side effects. However, the effects of ARBs on myocardial infarction are considered to be less serious than those of ACE inhibitors[16]. ACE inhibitors are still making clinical contributions. Other than the above reports, few papers have been published about the association between human BMD and ACE inhibitors. We therefore eagerly await elucidation of the specific roles that angiotensins and ACE inhibitors play in bone metabolism.

The effect of BZD intake, which appeared to be associated with BMD change in this study, may not be direct, since BZD users tend to have lower physical activities[27], which likely leads to larger decreases in BMD through the confounding effect of the physical activity. It would be possible to assess more accurately the direct effect of BZDs on BMD if the physical activity data were available for each individual. As we did not have the data for physical activity at baseline, we had no alternative but to assess that for 1996-1997. It should be noted that BZD intake did not show a significant association with annual percentage change in femoral neck with the adjustment for physical activity in 1996-1997. On the other hand, after the adjustment for physical activity in 1996-1997, there were still negative associations between ACE inhibitor use and BMD change. Our cohort consisted of outpatients, and therefore patients with severe cardiovascular diseases were excluded.

Our study had some obvious limitations. First, we could examine only a few frequently prescribed drugs in our cohort, and thus could not certify the effects of other medications, such as estrogen and glucocorticoid, which are more frequently prescribed and known to have associations with