Table 3 Baseline data in the patients with or without incident fractures during amino-bisphosphonates (amino-BP) treatment

Items	Incident fracture (−)	Incident fracture (+)	*P*
Number of cases	160	71	–
Age (years)	69.0 ± 0.7	74.3 ± 1.1	<0.0001
BMI (kg/m^2)	21.8 ± 0.2	22.3 ± 0.4	0.272
Serum Ca (mg/dl)	9.23 ± 0.03	9.14 ± 0.05	0.060
Serum P (mg/dl)	3.53 ± 0.04	3.41 ± 0.05	0.083
NTX (nM/mM Cr)	54.1 ± 2.5	57.2 ± 3.7	0.448
Initial BMD (g/cm^2)	0.788 ± 0.010	0.743 ± 0.015	0.0157
Number of prevalent vertebral fractures	1.09 ± 0.16	2.58 ± 0.25	<0.0001

The baseline data were compared between the patients with or without incident fractures, retrospectively. A total of 71 patients had new fractures during amino-BP treatment. The patients with incident fractures were of higher age, with a greater number of prevalent vertebral fractures and lower lumbar bone mineral density (LBMD) at baseline. Data are expressed as mean ± SE. Statistical analysis was made by analysis of variance (ANOVA)

Table 4 Bone-related outcomes at the end of observation in the patients with or without incident fractures

Outcomes	Incident fracture (−)	Incident fracture (+)	*P*
LBMD (g/cm^2)	0.854 ± 0.011	0.823 ± 0.017	0.131
Ca (mg/dl)	9.26 ± 0.03	9.16 ± 0.05	0.091
P (mg/dl)	3.54 ± 0.04	3.43 ± 0.06	0.140
NTX (nM/mM Cr)	28.6 ± 1.5	33.2 ± 2.3	0.104
ucOC (ng/ml)	2.28 ± 0.13	2.75 ± 0.19	0.038

Data listed in Table 4 were obtained at the end of the observations. In a case having incident fracture, serum and urinary samples were taken 1 or more years after the occurrence of incident fracture or 1 or more years after the recognition of morphometric fracture (2.6 ± 0.6 years after the recognition of incident fracture). Data are expressed as mean ± SE. ucOC, undercarboxylated osteocalcin

Table 5 Stepwise regression analyses of the risks for fracture susceptibility in amino-BP users

Risk	χ^2	*P*	R^2
Number of prevalent vertebral fracture	21.999	0.0000	0.0772
Age (years)	9.1164	0.0025	0.1092
ucOC (ng/ml)	4.4385	0.0351	0.1247
Baseline LBMD (g/cm^2)	3.8923	0.0485	0.1384

Stepwise regression analysis was performed among the four risks, which may relate to having incident fractures during amino-BP treatment. All four risks were significantly associated with incident fractures

and 9 patients with incident fractures, respectively. The Kaplan–Meier plot indicated a significantly higher susceptibility of incident fractures in accordance with an increasing number of risks (see Fig. 1). Furthermore, the number of patients with high ucOC (>2.6 ng/ml) in each category is shown in Table 6. The percentages for the patients with high ucOC were significantly increased in

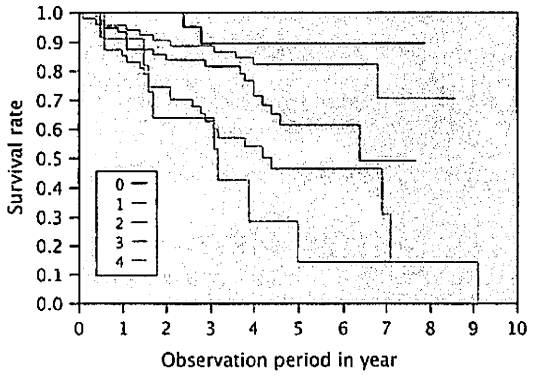

Fig. 1 Kaplan–Meier plot of the categorized patients under amino-bisphosphonate (amino-BP) treatment in accordance with the number of fracture risks (0–4). The lower survival rate from incident fractures was observed in accordance with the number of fracture risk in patients with amino-BP-treated osteoporosis ($P < 0.001$ in log-rank and Wilcoxon test)

accordance with increase in number of risks ($P < 0.0001$), providing further proof that high ucOC was an independent risk factor for incident fractures in osteoporosis during bisphosphonates treatment.

Discussion

In this study, traditional risk factors, such as older age, low BMD, and the presence of preexisting fractures [18], for incident fractures in osteoporosis were also recognized as risks for incident fractures in bisphosphonate users. However, no significant contribution of incident fractures to changes in LBMD and bone resorption markers was seen, although such changes considerably favored fracture prevention; that is, LBMD increased by about 11% and 8% for patients with and without incident fractures, respectively, and urinary excretion of NTX decreased by about 60% and

Table 6 Rate of patients with high ucOC in five risk categories

Risk category	ucOC < 2.6 ng/ml	ucOC ≥ 2.6 ng/ml	Percent (%)
0	31	0	0
1	48	19	28.4
2	46	29	38.7
3	19	28	59.6
4	0	11	100

The trend for the rate of patients with high ucOC was apparently increased in accordance with increase in number of risks. $\chi^2 = 49.1$ and $P < 0.0001$ by Peason's Chi-square test

50% for patients with and without incident fractures, respectively. These changes in LBMD and urinary NTX were not significantly different between patients with and without incident fractures. These changes in parameters were in good agreement with previous reports obtained from Japanese postmenopausal osteoporosis [19]. Therefore, the biological effects of bisphosphonates on bone density and resorption were identical regardless of the occurrence of new fractures during treatment. Previous reports have indicated that increase in BMD and decrease in bone markers were not strong predictors for future fractures in bisphosphonates users [20–24].

On the other hand, the serum level of ucOC of fractured patients was significantly higher than that of patients without any new fractures. Secondary analysis using stepwise regression analysis revealed that the level of ucOC in amino-BP users was a significant independent risk for incident fractures (Table 5). Because ucOC was measured after an incident fracture occurred, it was thought that the difference in ucOC levels between patients with and without incident fracture may have reflected the occurrence of a fracture but not a vitamin K deficiency in bone. However, this possibility is unlikely because urinary excretion of NTX did not differ between these two groups, indicating that there was no significant difference in bone resorption. It has been recently reported that the biological action of amino-BP and activation of vitamin K have a point of contact, that is, the geranylgeranylation of molecules. Amino-BP inhibits this process [1, 2] and, in contrast, vitamin K activation requires geranylgeranylation of the side chain [13]. Therefore, it is possible that bisphosphonates may interfere with vitamin K activation in bone cells, especially in patients with preexisting vitamin K deficiency. This hypothesis prompted the authors to measure ucOC in patients treated with amino-BP. Results indicate that patients with incident fractures during amino-BP treatment may be lacking vitamin K in bone cell levels consequent to a higher level of ucOC. However, Hirano et al. [24] reported that amino-BP treatment caused a decrease in ucOC level but resulted in no change in carboxylated osteocalcin (cOC), suggesting that amino-BP did not decrease the carboxylation of osteocalcin. So, further clarification is required whether amino-BP treatment affects carboxylation on osteocalcin in both patients with or without vitamin K deficiency in a prospective study design. This is the first report that suggests the usefulness of measuring ucOC in amino-BP users in terms of fracture prediction. Because ucOC decreased after vitamin K_2 treatment [11], concurrent use of vitamin K_2 with amino-BP may be effective in preventing the occurrence of new fractures in patients with higher ucOC levels during treatment with only amino-BP.

The limitations of this study were as follows. First, the present results must be evaluated as a prospective study design because the present study was a retrospective study. Second, a larger number of participants is necessary to increase statistical power. Third, direct evidence that shows that bisphosphonates inhibit vitamin K activation in bone cells is required in vitro, and we have to measure serum carboxylated osteocalcin level together with ucOC to assess gamma-carboxylation during amino-BP treatment in vivo. Last, because other risk factors for fracture such as the physical activity of the participants was not evaluated in the present study, those factors related to increased susceptibility to fractures should be incorporated into the model in future.

In conclusion, it is useful to evaluate the serum level of ucOC in patients with amino-BP to predict future fracture occurrence.

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REVIEW ARTICLE

Genetic aspects of osteoporosis

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Abstract The multiple factors contributing to the pathogenesis of osteoporosis include genetic and environmental factors. Because decrease in bone mineral density (BMD) is the major clinical indicator and a useful quantitative trait, many association and linkage studies of BMD have been conducted. Although the series of studies showed apparently significant associations, the genes have not been found that can be utilized in clinical practice. Several genes identified in robust genome-wide association studies will be the new cutting edge in genetic studies of osteoporosis. Our recent reports of functional single nucleotide polymorphism in the tissue-nonspecific alkaline phosphatase gene and gamma-carboxylase gene are presented in this review to discuss the future prospects in the genetic research of osteoporosis from the point of view of genome–nutrition interaction.

Keywords Osteoporosis · Single nucleotide polymorphism · Bone mineral density · Genetics

Introduction

Osteoporosis brings about deterioration in activities of daily living (ADL) and quality of life (QOL) of the affected patients. Although fragility fractures resulting from osteoporosis continue to increase in the current aging society, it is assumed that this disease is still undertreated [1]. In the 1990s, osteoporosis was defined as a disease characterized by low bone mass and microarchitectural deterioration of bone tissue, leading to enhanced bone fragility and a consequent increase in fracture risk [2]. This definition reflects the importance of bone mass and microarchitecture in determining bone strength. Because there have not been practical measures of microarchitecture, bone mass or bone mineral density (BMD) has been used as a quantitative trait in searching the genes for osteoporosis. It seems reasonable that a vast series of association and linkage studies have been conducted with BMD, but one should keep in mind that BMD is one of the complex traits of osteoporosis and one of the surrogate markers for bone fragility.

Recently, osteoporosis was redefined as a skeletal disorder characterized by compromised bone strength predisposing a person to an increased risk of fracture [3]. In the previous definition of osteoporosis, low BMD was not considered as a sole factor of osteoporosis, but the new definition declares more clearly that bone strength is determined not only by BMD but also by factors other than BMD [3]. According to the new definition, the genes of osteoporosis should be a group of genes contributing to the multiple aspects of pathogenesis. Although case–control studies by defining the case with the diagnostic criteria of this disease are suggested, the diagnosis of osteoporosis might not be suitable as a "phenotype" in genetic studies because the diagnosis contains biologically heterogeneous components. In this review, genetic aspects of osteoporosis are discussed mainly using BMD as one of the measurable phenotypes of osteoporosis.

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Genetic aspects of bone phenotypes

Predisposing factors of osteoporosis include both lifestyle factors and genetic factors. The first step in preventing osteoporosis should be the reduction of lifestyle-related risk factors. On the other hand, the genetic factors cannot be removed even when these are identified. However, it will be useful if one can learn that she or he has the genetically predisposing factor(s) and is thus motivated to avoid certain lifestyle risk factors.

Family history of fractures is included among the established risk factors for osteoporotic fractures [4], indicating the importance of the genetic background of osteoporosis. Twin studies also supported the heritability of BMD [5], which is the most valuable indicator of bone strength. On the other hand, it was reported that the possibility that the genetic determinants of BMD and those of fractures might be different [6]. Factors contributing to the variation in bone quality will be studied further from genetic aspects. For example, femoral neck cross-sectional geometry was successfully used as a clinical measure in quantitative trait locus analyses [7].

Approaches for the pathogenesis of diseases can be classified as deductive or inductive. Analyses about the roles of known substances or genes would be classified into deductive approaches and belong to the genetic approach. On the other hand, the recent availability of whole genome information has made the inductive approach possible, which is named a genome-wide association study (GWAS). In this mini-review, the recent genetic and genomic approaches for osteoporosis are reviewed, and our studies on the functional single-nucleotide polymorphisms (SNPs) related to osteoporosis are introduced.

Candidate gene approaches to the determinants of bone mineral density

Until now, BMD has been utilized most widely as a quantitative measure in genetic and genomic studies for osteoporosis. Quite a few association studies with BMD have been done with so-called candidate gene approaches [8]. Candidate genes have been chosen based on basic bone cell biology and clinical observations. In addition, the genome-wide linkage and association studies will show novel series of candidate genes that should be investigated further.

Association studies with the polymorphisms of these genes were done using genetic polymorphisms. Among the polymorphisms, SNPs were most commonly utilized. SNPs in the regulatory region (rSNPs) and those in coding regions (cSNPs) could be related to quantitative or qualitative variations of the gene expressions. In addition, other SNPs, for example, those in introns, could affect the gene expression or could be markers for genomic study. Microsatellite polymorphisms (e.g., dinucleotide repeat or triple repeat) are other kinds of polymorphisms that have also been utilized in osteoporosis research.

Association studies with candidate gene polymorphisms have been published by many groups including ours [9–40]. If you search the database using the key words "gene polymorphisms and bone mineral density," 1,000 and more articles will be hit. The genes analyzed are classified into nuclear receptors and related molecules, collagen and other matrix proteins, receptor activator of nuclear factor-kappa B ligand (RANKL)/RANK system, cytokines and related molecules, hormones and related molecules, enzymes, cell cycle-related molecules, lipoprotein receptor-related peptides (LRPs) and Wnt signals, cell-surface molecules, transcription factors, and others (Table 1). However, the contribution of most genes to determining BMD is small and the result is not always reproducible [41, 42]. Lifestyle-related factors as confounding factors against genetic factors should be managed in the association studies. In addition, ethnic factors have to be considered appropriately [43].

The vitamin D receptor gene has been studied most extensively, but the implications of vitamin D receptor gene polymorphisms have not been established [44]. Recent extensive meta-analyses [45] showed that the effects of the vitamin D receptor gene polymorphisms seem modest, although the significant effects of the polymorphisms on BMD and osteoporotic fractures were proved.

Searches for functional SNPs affecting variation in bone metabolism

When polymorphisms of genes were significantly and reproducibly associated with bone phenotypes, biological relevancy should be confirmed, and the methods of clinical application should be considered following that process. In other words, it would be a rational method in the genetic approach for osteoporosis to examine the association of functional polymorphisms with bone phenotypes. Although the contribution of each polymorphism to BMD would be small, the significant effects of each polymorphism supported by functional studies will be a clue suggesting that the gene should play important roles in the pathogenesis of osteoporosis.

We reported two functional SNPs in two genes that are related to the variation in BMD of the elderly. The first one was an SNP in the tissue-nonspecific alkaline phosphatase (TNSALP) gene [46]. TNSALP resides in the plasma membrane of osteoblasts and supplies phosphate to the

Table 1 Genes studied in candidate gene approaches for bone mineral density (BMD)

Nuclear receptors and related molecules
Vitamin D receptor
Estrogen receptor-α
Estrogen receptor-β
Androgen receptor
Glucocorticoid receptor
Peroxisome activator receptor-γ
Nuclear receptor co-activator-3
Erα co-factor retinoblastoma-interacting zinc finger protein
Collagen and other matrix protein
Type I collagen-α1
Type I collagen-α2
Osteocalcin
Matrix gla protein
Alpha 2-HS glycoprotein
RANKL/RANK system
RANKL
RANK
Osteonectin/SPARC
Cytokines and related molecules
Transforming growth factor-β1
Insulin-like growth factor-1
Tissue necrosis factor-α
TNFRSF1B
TNFRSF11B
TNF receptor-associated factor-6
Bone morphogenetic protein-2
Bone morphogenetic protein-4
LTBP3
Interleukin-6
Interleukin-1
Interleukin-1 receptor antagonist
Interleukin-1β
Interleukin-10
Tissue necrosis factor
Tissue necrosis factor receptor
Smad 6
TGF-β receptor-3
Adiponectin
Myostatin
Hormones and related molecules
Calcitonin
Calcitonin receptor
Thyroid hormone receptor
TSH receptor
Calcium-sensing receptor
PTH
PTH/PTHRP receptor
Dopamine receptor D4
Prepro-NPY
Growth hormone
Growth hormone receptor
POMC
Cannabinoid receptor type 2
Leptin receptor
β3-adrenergic receptor
Vitamin D-binding protein
SHBG
Enzymes
Adenyl cyclase
Methylene tetrahydrofolate reductase
Methionine synthase
Catalase
Farnesyl diphosphate synthase
Farnesyl pyrophosphate synthase
FMS-related tyrosine kinase
Aromatase
p450
Carbon anhydrase
Angiotensin-converting enzyme
CYP1A1
CYP1B1
CYP3A7
CYP3A4*18
CYP17
CYT19
COMT
eNOS
GGCX
Urokinase
PAI-1
ALDH2
Pituitary glutamyl cyclase
Phosphodiesterase 40
Tissue-nonspecific alkaline phosphatase
CYP1A1
ALOX15
ALOX12
Lactase
Paraoxonase
Procollagen-lysine, 2-oxoglutarate 5-dioxygenase
Rho GTPase-Rho REF
WRN
Matrix metalloproteinase-1
Cathepsin K
Mature metalloproteinase-9
Delta-aminolevulinic dehydrogenase
Uridine diphosphate glucuronyl transferase 2B7

Table 1 continued

Cell cycle-related molecules
p57
Cdx-2
Cyclin D1
CD38
LRPs and Wnt signals
LRP5
LRP6
WISP 1
FZD 1
"multiple Wnt pathway genes"
SOST
Cell-surface molecules
Duffy antigen receptor
Toll-like receptor 4
GALR3 receptor
CC domain receptor 2
CD38
CD40
CLCN7 (chloride channel)
Osteoclast-associated receptor
Vascular proton pomp
Purinergic P2RX7 receptor
Semaphorin 7
GALR3 receptor
Transcription factors
RUNX2/CBRA1
Microphthalmia-associated transcription factor
Forkhead box C2
Others
Perilipin

calcification site. We searched for nonsynonymous and functional SNPs in the exons of this gene. As a result, an SNP in exon 7 (787C > T), which replaces tyrosine at codon 246 to histidine, gives the biochemical differences between the products of each genotype. The K_m value of 787 His was smaller than that of 778 Tyr, which means that persons with 787 His may supply phosphate to the calcification site more efficiently. Elderly Japanese women with 787 His had higher radial BMD than those with other genotypes. This study demonstrated the importance of phosphate metabolism in bone metabolism in the elderly. Additional in vitro experiments supported the biochemical variations resulting from this polymorphism [47, 48]. Further studies are underway to examine the clinical meaning of this variation, for example, the effects of this genotype on the relationship between phosphate intake and hormones in calcium metabolism and aging.

Another gene is vitamin K-dependent gamma-glutamyl carboxylase (GGCX) [49]. GGCX carboxylates vitamin K-dependent proteins including bone Gla protein (osteocalcin) and matrix Gla protein. Functional polymorphisms in the GGCX gene, if any, might explain the variation in bone metabolism and BMD. Also in this case, polymorphisms in the exons were screened in Japanese elderly women and a nonsynonymous SNPs was found: about 8762 G > A (Arg325Gln). When the kinetic parameters of GGCX325-Gln and GGCX325-Arg were compared in vitro, V_{max}/K_m was significantly higher for GGCX325-Gln than for GGCX325-Arg. Association study of this polymorphism with radial BMD of Japanese postmenopausal women showed that the body mass index (BMI)-adjusted Z score in the subpopulation older than 75 years was higher in those with 325 Gln than those with 325 Arg/Gln or 325 Arg. In this study, we first reported the different activities of GGCX between the common genotypes and their association with BMD. Vitamin K deficiency is known as a nutritional risk factor for osteoporotic fractures, and a regimen of vitamin K_2 is utilized for osteoporosis treatment. The common allelic variation in the GGCX gene may explain the individual variation in the response to nutritional and/or pharmacological intervention with vitamin K. It would be rational to utilize the allele information in finding the level of vitamin K intake at which the effects of the genotype with lower enzymatic activity can be avoided. We have already reported that this GGCX gene polymorphism affects the correlation between the vitamin K status and gamma-carboxylation of osteocalcin in young males [50], and this kind of study is awaited in the group of elderly.

Implication from monogenic bone diseases

There are rare diseases involving bone that are caused by mutations of single genes and considered to be monogenic diseases. The causative genes of these diseases were identified by linkage analyses of the affected families. These genes would have important implications for the variations of bone phenotype also in the general population. A distinguished example is the gene for osteoporosis-pseudoglioma syndrome (OPPG) [51]. Positional cloning with the affected pedigrees showed that rare mutations in lipoprotein receptor-related peptide 5 (LRP5) gene cause the disease. In addition, another mutation in the same gene was demonstrated to cause a syndrome with high BMD [52]. It is also interesting that the LRP5 gene resides in the locus that has been among the loci related to BMD in the linkage studies [53]. Several groups including ours examined the relationship between the polymorphisms of LRP5 gene and BMD, and the results were reproducible [54–56].

Table 2 Candidate genes suggested by genome-wide association studies

Genes	Chromosome location
ADAMTS18 (ADAM metallopeptidase with thrombospondin type 1 motif, 18)	16q23
TGFBR3 (transforming growth factor-beta receptor III)	1p33–32
TNFRSF11B (tumor necrosis factor receptor superfamily, member 11b, osteoprotegerin)	8q24
LRP5 (lipoprotein receptor-related protein 5)	11q13.4
Receptor activator of nuclear factor-kappa B ligand (RANKL)	13q14
Osteoprotegerin (OPG)	8q24
Estrogen receptor-1 gene (ESR1)	6q25.1
Zinc finger and BTB domain containing 40 genes (ZBTB40)	1q36
Major histocompatibility complex region	6p21

In addition, the LRP5 gene was screened out by the recent GWAS, as mentioned below [57]. These results strongly suggest that variations in this gene would contribute to the variation of BMD in the general population.

Genes suggested by genome-wide association studies

Systematic search for the genes for osteoporosis has been done by genome-wide linkage studies with pedigrees, which have shown some hotspots linked to BMD, for example, those on chromosome 11 [53]. Further fine mappings were required to specify the genes contributing to the pathophysiology of osteoporosis and consequent analyses of their functions in bone biology. Recent advances in analyzing SNPs distributing to the whole genome area made it possible to conduct a GWAS (Table 2). One of the GWAS studies identified two SNPs, rs4355801 on chromosome 8 and rs3736228 on chromosome 11 [57]. The former is close to the osteoprotegerin gene and the latter nonsynonymous SNP is in the LRP5 gene, both of which are major components in bone biology. In the series of candidate gene approach, the significant correlation between the polymorphisms in LRP5 gene has been reproducible. The identification of the LRP5 gene in the GWAS study further strengthens the importance of this gene in the pathogenesis of osteoporosis. Osteoprotegerin was also identified to be correlated with BMD in another GWAS study [58].

Other examples of genes identified in GWAS studies are RANKL [58], estrogen receptor 1 (ESR1) [58], ADAM metallopeptidase with thrombospondin type 1 motif, 18 (ADAMTS18) [59], and transforming growth factor-beta receptor III (TGFBR3) [59].

Prevention of osteoporotic fractures is the major clinical goal of osteoporosis therapy, and the incidence of osteoporotic fractures should be an ideal phenotype used in the genetic studies searching the genes for osteoporosis. Recently, Kung et al. [60] reported the association of the JAG1 gene with osteoporotic fractures as well as BMD with GWAS study. They also demonstrated the possible molecular mechanism with which the genetic variation of this gene affects bone metabolism [60].

Discussion

Selection of candidate genes for polymorphism studies of osteoporosis is rather arbitrary. This situation cannot be avoided because we do not know how many genes are involved in the pathogenesis of osteoporosis or in the determination of BMD. Recent genome-wide studies with a large population size are successfully overcoming this issue, and several genes were identified for osteoporosis. These genes include the novel series of candidate genes whose implications should be studies. So far the new list of genes contains "previous" candidate genes that are well known in the field of bone biology.

BMD is a surrogate marker for bone fragility, and one should not consider the genes for low BMD as immediately being those for osteoporosis. Although BMD is still a useful quantitative measure in genetic studies for osteoporosis, other phenotypes, particularly the incidence of fractures, should be kept in mind. Further studies are required to utilize the products of genetic studies for the advancement of osteoporosis practice.

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The Fracture and Immobilization Score (FRISC) for risk assessment of osteoporotic fracture and immobilization in postmenopausal women—A joint analysis of the Nagano, Miyama, and Taiji Cohorts

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ABSTRACT

Introduction: We aimed to (i) explore risk factors for major osteoporotic fracture or immobilization; (ii) develop a prediction model that can be used to assess the risk of fracture and immobilization; and (iii) assess external validity of the final model.

Methods: A total of 1787 postmenopausal Japanese women were followed in a hospital-based cohort study. Endpoints included the annual incidence of major osteoporotic fracture and immobilization. For each endpoint, multivariate Poisson regression models were fitted separately and risk factors were screened through backward variable selection. The predictive accuracy of the final model (FRISC) was evaluated in two independent community-based cohorts.

Results: Over a median follow-up of 5.3 years, a total of 383 major osteoporotic fractures (279 clinical vertebral, 44 hip, 60 distal forearm) and 83 immobilizations occurred in the developmental dataset. Backward variable selection confirmed that the following are risk factors for major osteoporotic fracture: age, weight, prior fracture, back pain, and lumbar bone mineral density (BMD). Age, prior fracture and dementia were significant risk factors for immobilization. Hosmer–Lemeshow tests did not indicate any significant deviation between the observed fracture frequency and prediction from the FRISC in the independent validation dataset. The C statistic for the FRISC was 0.727 (95% confidence interval: 0.660 to 0.794) and was higher than that for BMD alone significantly ($p = 0.03$).

Conclusions: We developed a novel prediction model for fracture and immobilization, FRISC, and the clinical risk factors in the FRISC allows better identification of populations at high risk of fracture than BMD alone. A web application is available at http://www.biostatistics.jp/prediction/frisc.

Introduction

Fracture due to osteoporosis results in increased mortality, morbidity and medical expense in the US and Japan [1–3]. As an adjunct to the development of effective treatments, early identification of populations at high risk of fracture is regarded as an effective strategy for decreasing both the burden of illness and the associated cost in countries with aging populations [4]. The US and Japanese guidelines both recommend that bone mineral density (BMD) should be used to determine when to intervene in patients with osteoporosis [5,6]. However, epidemiological studies have demonstrated that many major osteoporotic fractures occur among individuals with a BMD T score value above the intervention threshold value, although the incidence of fracture certainly increases with decreasing BMD [7,8]. As a solution to this problem, a WHO scientific group proposed the use of 10-year probabilities of osteoporotic or hip fracture, calculated using multiple risk factors. The result was the WHO fracture risk assessment tool (FRAX) [9]. Several other prediction models/risk assessment tools were also developed based on data from cohort studies and clinical trials [10–13].

Abbreviations: BMD, bone mineral density; FRAX, fracture risk assessment tool; FRISC, Fracture and Immobilization Score; CHD, coronary heart disease; CVD, cerebrovascular disease; ROC, receiver operating characteristic; CI, confidence interval.

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Although case-finding strategies optimized by risk assessment tool for fracture appear to be promising, there are several problems which must be addressed before clinical application. First, whether combination of BMD and clinical risk factors allows better discrimination of fracture than BMD alone is still controversial; the discriminatory power of the FRAX was excellent in eleven population-based cohorts [14] but was similar to BMD alone in a cohort study [15]. Second, since the Japanese version of FRAX was validated only in the community dwelling cohorts in Japan [9], there has been no available data whether the FRAX probability performs well in hospital-based population. As Justice et al. pointed out, the effectiveness of prediction models in clinical practice depends on the extent to which they can be generalized to the population in question [16]. In general practice, the FRAX was often adapted to assess fracture risk in a patient who has a more complicated risk for fracture, such as atherosclerosis, diabetes or other potential risks to deteriorate bone strength. In this regard, it is necessary to evaluate prediction models for fracture not only in community but also hospital-based populations. Third, outcomes other than fracture, such as immobilization, appear to be also important for optimizing osteoporotic treatment from the health economic perspective. In fact, we have found that osteoporotic fracture was an independent risk for immobilization and this morbid state would require a large amount of resources of care [17].

The present study was therefore performed with three main aims. The first was to explore risk factors for the incidence of major osteoporotic fracture or immobilization. This was done using data from the Nagano Cohort, a hospital-based cohort study of postmenopausal Japanese women. The second aim of this study was to develop a prediction model named as the Fracture and Immobilization Score (FRISC), to assess the risk of major osteoporotic fracture and immobilization, based on the risk factors confirmed in the first part of the study. Finally, we assess the external validity of the FRISC and investigate whether the predictive accuracy was improved from BMD alone using pooled data from the Miyama and Taiji Cohorts, which followed 400 Japanese women from communities over a 10-year period.

Methods

Development and validation datasets

We used two independent datasets in the current analysis; a developmental dataset from the Nagano Cohort and a validation dataset from the Miyama and Taiji Cohorts. Profiles of these three cohorts have been detailed previously [17–28]. The Nagano Cohort recruited and followed up postmenopausal women who were receiving medical care as outpatients or visitors at a medical institute in Nagano Prefecture, Japan since April 1993 [16–20]. A total of 1787 participants were included in the developmental dataset; exclusion criteria were (i) metabolic bone disease and (ii) secondary osteoporosis (e.g. hyperparathyroidism, hyperthyroidism other than patients on T4 replacement and with euthyroid for more than one year, chronic renal failure or osteomalacia). We excluded those who met the exclusion criteria regardless of BMD.

However, steroid users were enrolled to the present study because the history of steroid use was required in the FRAX. The protocol was approved by the ethics committee at the Research Institute and Practice for Involutional Diseases and we obtained written informed consent from all participants. The Miyama Cohort was set up in 1988 as subsets of nationwide community-based cohort studies sponsored by the Ministry of Education or Ministry of Health and Welfare [21–23]. A total of 1453 inhabitants aged 40–79 years in Miyama Village were listed from the resident registration in December 1988. Then, 200 men and 200 women were recruited and followed up between 1990 and 2000. The Taiji Cohort is a community-based cohort study in Taiji Town, Wakayama Prefecture, Japan [25–27]. From a list of 2261 inhabitants aged 40–79 years obtained from the resident registration in June 1992, 50 men and 50 women in each decade age group between 40 and 79 years (a total of 400 participants) were recruited randomly and followed up between 1993 and 2003. All the sampled participants were contracted and agreed to participate. The validation dataset included all the women in the Miyama and Taiji Cohorts.

Data collection in the Nagano Cohort

At baseline, anthropometric indices, including body weight and body height, were obtained for all patients. Subjects were also interviewed to obtain data about age at menopause, smoking habit, alcohol consumption, past and present occupation, presence of pain, medical history (including rheumatoid arthritis, diabetes mellitus, hypertension, dyslipidemia, cancer, dementia, coronary heart disease [CHD] and cerebrovascular disease [CVD]). Pain was defined as any symptom of pain in the back, hip muscles, ribs, legs, knees, neck, shoulders, wrists, or other joints. Back pain was defined as any symptom of pain in the back trunk area, regardless of the degree or consistency of the pain [18]. Rheumatoid arthritis was diagnosed according to the diagnostic criteria proposed by the American Rheumatism Association [28]. The Japanese version of the Mini-mental State Examination (MMSE) was performed in the subjects who were suspicious dementia and a subject with the total score of MMSE less than 20 points was considered to have obvious cognitive dysfunction [29]. A history of CHD was defined as any previous acute coronary symptoms or events requiring coronary intervention, and was confirmed by coronary angiography, echocardiogram, or MD-CT imaging study. A history of CVD was diagnosed based on evidence of apparent brain attack or an existing brain lesion as observed by magnetic resonance imaging or computer-assisted X-ray tomography. Self-reports of a history of malignancy were confirmed by referring to the patient's medical records [19]. The BMD of the lumbar spine was measured at baseline using dual-energy X-ray absorptiometry (Lunar DPX-L or DPX-IQ; Lunar Corporation, Madison, WI) and a quality assurance test was carried out for every measurement to detect machine drift. The inter-assay variance of the lumbar BMD measurements in our laboratory was $0.5 \pm 0.5\%$ (coefficient of variation ± standard deviation) [20]. T score was calculated by using Japanese standard values [30].

Data collection in the Miyama and Taiji Cohorts

A self-administered questionnaire was used for baseline data collection in the Miyama Cohort, while both self-administered and interviewer-administered questionnaires were used in the Taiji Cohort [22–27]. The items in these questionnaires included birth date, body weight, body height, current smoking status, current alcohol intake, presence of back pain, use of steroids, and medical history such as rheumatoid arthritis. Parental history of fracture was asked only in the Taiji Cohort. The BMD of L2–4 and BMD at femoral neck, Ward's triangle and the trochanteric region were measured by dual-energy X-ray absorptiometry (Lunar DPX; Lunar Corporation, Madison, WI in the Miyama Cohort, Hologic QDR-1000; Hologic Inc., Crosby Drive Bedford, MA in the Taiji Cohort) and treated as T scores. The incidence of clinical fracture was evaluated in both the cohorts. However, radiographs for morphometrical vertebral fracture were available only in the Miyama Cohort. Consequently, parental history or morphometrical vertebral fracture was missing data in either cohort systematically and thus we assumed that participants with these missing data did not have parental history or prior fracture. We calculated the 10-year probability of major osteoporotic fracture by entering the following data into online version of the FRAX; age, sex, weight, height, previous fracture, parental history of hip fracture, current smoking status, glucocorticoid use, rheumatoid arthritis, alcohol intake and femoral neck BMD.

Endpoints

Endpoints included the annual incidence of major osteoporotic fracture and immobilization. Major osteoporotic fracture was defined

as first occurrence of any clinical fracture (hip fracture, surgical neck fracture of the humerus, distal forearm fracture, or clinical vertebral fracture). We also evaluated a radiographical vertebral fracture by the semi-quantitative visual method [31], but major osteoporotic fracture does not include morphometrical fracture by definition. A validation analysis of our semi-quantitative method for analyzing incident vertebral fracture has been reported elsewhere [32]. Prior vertebral fractures were defined as those fractures for which the ratio of the height of the central or anterior vertebral body to that of the posterior vertebral body was less than 0.8, or when any of these three vertebral body heights was less than 80% of the height of the adjacent vertebral body [6]. Immobility was defined in accordance with the subject's locomotive ability. Subjects bed-bound at home (lying in bed almost all day) for more than 6 months or institutionalized in nursing homes (lying in bed or using a wheelchair for locomotion), were defined as immobile [17]. Some immobile subjects could be sitting on a bed and could be going to a portable toilet which located besides the bed. Participants who died from any cause, moved to the home of a relative because they were not able to perform the activities of daily living independently, were lost to follow-up, or were followed up until June 1, 2009 were treated as censored. For each endpoint, the accumulation of person-years at risk started from registration of each patient.

Statistical considerations

For each endpoint, we fitted multivariate Poisson regression models separately and rate ratios for risk factors estimated by the Poisson regression models were reported with 95% confidence intervals (CI) and p values. The following variables were initially identified from the literature as the traditional risk factors for osteoporotic fracture: covariates included in the FRAX other than femoral neck BMD (age, height, weight, prior fracture, parental history of fracture, current smoking status, use of steroids, rheumatoid arthritis, alcohol intake), lumbar BMD, presence of back pain, presence of any pain, and drug treatment for osteoporosis [9,18,22]. These covariates were screened via backward variable selection with a significance level of $p=0.2$. We constructed a prediction model for immobilization using the same procedure, except that three covariates (dementia, history of CVD, and history of malignancy) were used in addition to those used in the osteoporotic fracture analysis. Finally, the FRISC was developed using the following formula:

$$\mathrm{Prob}(t) = \int_0^t \lambda \exp(\mathbf{X}\beta) \exp\left[-\int_0^u \{\lambda \exp(\mathbf{X}\beta) + m(v)\} dv\right] du$$

Here, t is a time point for prediction (i.e. the formula calculates 10-year probability if $t=10$), β is a vector of log-rate ratios for covariates $\mathbf{X}$, λ denotes baseline incidence rate, and $m(v)$ is mortality at time v obtained from sex- and age-specific mortality in Vital Statistics of Japan in 2008 [33].

We assessed the predictive accuracy of the FRISC in terms of calibration and discrimination [34] using occurrence of major osteoporotic fracture within a 10-year period, which was treated as a binary event, in the validation dataset. Calibration, namely how closely the prediction reflects actual events, was assessed using ratio of observed and predicted events and the Hosmer–Lemehshow test. Discrimination, the ability to distinguish between those who experience the event and those who do not, was evaluated using receiver operating characteristic (ROC) curves and Harrell's C statistic. Improvement in the C statistics of the two models from BMD alone was assessed by using contrast tests.

All reported p values for statistical tests are two-tailed, and $p<0.05$ was taken to indicate statistical significance. All statistical analyses were performed using SAS version 9.2 (SAS Institute, Cary, NC).

Results

Characteristics of participants and follow-up

Baseline characteristics of participants in the Nagano, Miyama and Taiji Cohorts are summarized in Table 1. The Nagano Cohort included older participants and mean lumbar BMD in this cohort was lower than the other cohorts. Prior fracture, back pain and parental history were observed more frequently in the Miyama and Taiji Cohorts. In the Nagano Cohort, 37.4% of participants were being treated with bone resorption inhibitors (bisphosphonates or a selective estrogen receptor modulator), and 16.7% were receiving 1-alpha-OH vitamin D_3 or vitamin K_2 at baseline. Table 2 describes the incidence of fractures and immobilization. In the Nagano Cohort, over a median follow-up time of 5.3 years (range, 0.03–16.5 years), a total of 383 major osteoporotic fractures occurred (279 clinical vertebral fractures, 44 hip fractures, 60 distal forearm fracture, Table 2). In the Miyama and Taiji Cohorts, 337 of 400 participants completed the planned follow-up of the 10-year period (84%) and a total of 60 major osteoporotic fractures occurred (44 clinical vertebral fractures, 8 hip fractures, 8 distal forearm fracture, Table 2). Incidence rates of fractures in the two cohorts were much lower than the Nagano Cohort possibly due to the difference in average age and lumbar BMD at baseline (Tables 1 and 2). Immobilization occurred in 83 participants in the Nagano Cohort.

Risk factors for fracture and immobilization

We fitted multivariate Poisson regression models to the validation dataset of 1787 participants. Backward variable selection identified the following six risk factors for major osteoporotic fracture: age, weight, lumbar BMD, prior fracture and presence of back pain (Table 3). That is, parental history of fracture, smoking status, alcohol consumption, rheumatoid arthritis, and use of steroids, which are all

Table 1
Characteristics of participants in the three cohorts.

	The Nagano Cohort (N = 1787)			The Miyama and Taiji Cohorts (N = 400)		
	Mean	SD	5–95 percentile	Mean	SD	5–95 percentile
Age (years)	63.4	11.1	45–81	59.5	11.3	41–77
Height (cm)	150.9	6.6	140–161	150.2	6.2	140–159
Weight (kg)	51.1	8.5	38–65	51.2	9.3	37–66.5
Lumbar BMD (T score)	−1.55	1.22	−3.5–0.5	−1.36	1.19	−3.85–1.57
Femoral neck BMD (T score)	*			−1.61	1.84	−3.29–0.53
		Frequency	%	Frequency	%	
Prior fracture		403	22.6	49†	25.0	
Presence of pain	Back	572	32.0	251	63.0	
	Other sites	449	25.1	‡‡		
Parental history		22	1.2	20‡	10.0	
Current smoker		38	2.1	16	4.0	
Current alcohol drinker		137	7.7	46	11.5	
Medication	Bone resorption inhibitors	369	37.4	‡‡		
	Active vitamin D_3 or vitamin K_2	299	16.7	‡‡		
	Steroids	27	1.5	0‡	0.0	
Rheumatoid arthritis		224	12.5	0	0.0	
Dementia		97	5.4	‡‡		

SD: standard deviation; BMD: bone mineral density.
*Not measured in the Nagano Cohort. †Not measured in the Miyama Cohort (N = 200).
‡Not measured in the Taiji Cohort (N = 200).

Table 2
Frequencies and incidence rates of fracture and immobilization in participants in the three cohorts.

	The Nagano Cohort (N = 1787)				The Miyama and Taiji Cohorts (N = 400)			
	Frequency	IR	95% CI		Frequency	IR	95% CI	
Major osteoporotic fracture	383	34.1	30.9	37.7	60	16.1	12.5	20.7
Clinical vertebral fracture	279	24.9	22.1	28.0	44	11.8	08.8	15.9
Hip fracture	44	3.9	2.9	5.3	8	2.2	1.1	4.3
Immobilization	83	7.4	6.0	9.2	-			

IR, incidence rate per 1,000 person-years; CI, confidence interval.

included in the FRAX, were excluded based on having p values less than 0.2. Importantly, incidence rate of major osteoporotic fracture increased as weight elevated and this direction is opposite to the FRAX and this trend remains significant even when all the other risk factors listed initially in the variable selection procedure are adjusted for (rate ratio for 10 kg increase in weight: 1.22, 95% CI: 1.07 to 1.40, $p<0.01$). Multivariate analysis for immobilization, using the same variable selection procedure, showed that age, prior fracture and dementia were associated with the incidence of immobilization (Table 3).

Input and output of the FRISC

All the risk factors that were retained through the variable selection procedure were incorporated into the final prediction model named as the FRISC. Interface of web application of the FRISC is displayed in Fig. 1. The input comprises the sex risk factors and, menopausal status and secondary osteoporosis which were used only for assessment of the applicability. The output comprises the 1, 3, 5 and 10-year probabilities of major osteoporotic fracture and those of immobilization and is calculated by using the algorithm described in Supplementary Data.

External validation of the FRISC

Fig. 2 displays histograms of the calculated 10-year probabilities of major osteoporotic fracture for the 400 participants in the validation dataset (upper: the FRISC, lower: the FRAX). An apparent difference was observed in the left tail of the two histograms; in the upper figure participants with fracture probability less than 0.05 were very few, while the FRAX gave the fracture probability less than 0.05 to a substantial portion of the participants. As a result, the fracture probabilities from the FRISC were much higher on average. Table 4 compares the predictive accuracy of the two prediction models and prediction from BMD alone. Over the 10-year follow-up, major osteoporotic fracture developed in 60 of 400 participants in the validation dataset. The predicted event frequency calculated from the FRISC was slightly higher than the observation (observed/predicted ratio: 0.74), while the FRAX tended to underestimate (observed/predicted ratio: 1.59). The Hosmer-Lemeshow test did not indicate any significant deviation between the observed event frequency and prediction from the FRISC. The C statistics for the FRISC was 0.727, indicating that the discriminatory power of the FRISC is moderate, while that for prediction from BMD alone was 0.651. That is, the discriminatory power of the FRISC, which combines BMD and additional clinical risk factors, was better than BMD alone significantly even in independent community-based cohort studies ($p=0.03$, Table 4). Fig. 3 shows ROC curves for major osteoporotic fracture probability from the FRISC (solid curve), the FRAX (dashed curve) and BMD alone (dotted curve). Both the ROC curves of the prediction models increased almost identically at first, but the curve for the FRISC was slightly above the curve for the FRAX where sensitivity is higher than 0.7 and where lower probability is used as a cutoff point (i.e. 16% or lower in the FRISC, 14% or lower in the FRAX), indicating that the FRISC is advantageous over the FRAX for screening of low-risk osteoporotic patients.

Table 3
Multivariate Poisson regression analysis of risk factors for major osteoporotic fracture and immobilization in the development dataset of 1,787 participants.

	Major osteoporotic fracture				Immobilization			
	Rate ratio	95% CI		p	Rate ratio	95% CI		p
Age, + 10 years	1.62	1.43	1.83	<0.01	2.80	2.09	3.73	<0.01
Weight, + 10 kg	1.25	1.10	1.42	<0.01	-	-	-	-
Lumbar BMD, + 1 T score point	0.85	0.76	0.94	<0.01	-	-	-	-
Prior fracture, yes/no	2.00	1.57	2.54	<0.01	2.04	1.21	3.44	0.01
Back pain, yes/no	1.58	1.27	1.96	<0.01	-	-	-	-
Dementia, yes/no	-	-	-	-	2.09	1.32	3.29	<0.01

BMD: bone mineral density; CI: confidence interval.

Discussion

In the current study, we explored clinical risk factors for major osteoporotic fracture and immobilization and developed a novel prediction model, the FRISC. Importantly, the assessment of external validity showed that the FRISC allows accurate prediction of major osteoporotic fracture even in the community-based setting and after a long-term follow-up of ten years, although it was developed in a hospital-based cohort study (i.e. for outpatients and visitors to a clinic). Therefore, the FRISC is useful both not only for patients who have a more complicated risk for fracture, such as atherosclerosis, diabetes or other potential risks to deteriorate bone strength, but also general postmenopausal women. Further the discriminatory power of the FRISC was shown to be better than BMD alone. We have previously noted that there is a close relationship between bone fractures and subsequent immobilization in postmenopausal Japanese women, and that these two conditions are morbid states that require a large amount of health resources [17]. Therefore, an accurate measure to predict these two conditions is particularly valuable in the context of an aging society. A web application of the FRISC is available at http://www.biostatistics.jp/prediction/frisc (Fig. 1).

The major finding of the current study is that inclusion of the four clinical risk factors, namely age, weight, prior fracture and back pain, in addition to BMD significantly improved the accuracy of the prediction model for major osteoporotic fracture. In contrast, parental history of fracture, smoking status, alcohol consumption, rheumatoid arthritis and use of steroids, which are all included in the FRAX, were not associated with incidence of fracture in the present analysis. The reason for this observation does not appear to be a lack of power given the number of observed events in the Nagano Cohort. Diet and other lifestyle factors, which were Westernized among smokers in this cohort, may have contributed to this unexpected result. One implication of these findings is that the association between lifestyle factors and fracture risk is possibly biased due to confounding factors, and it is necessary for prediction models to reflect the multidimensional nature of lifestyles. Although there were smokers and drinkers in the present population, the extent of their smoking and drinking was very mild, and smaller percentages of patients had these habits than in comparable Caucasian populations. In the practical point of view, a more parsimonious model is desirable and the FRISC would therefore provide a simple but sufficiently accurate measure for prediction of major osteoporotic fracture.

The present results indicated that incidence of fracture increases with heavier body weight, although low BMI has been considered as a significant risk factor of fracture as proposed in the FRAX. This trend remained even after the adjustment for the other risk factors

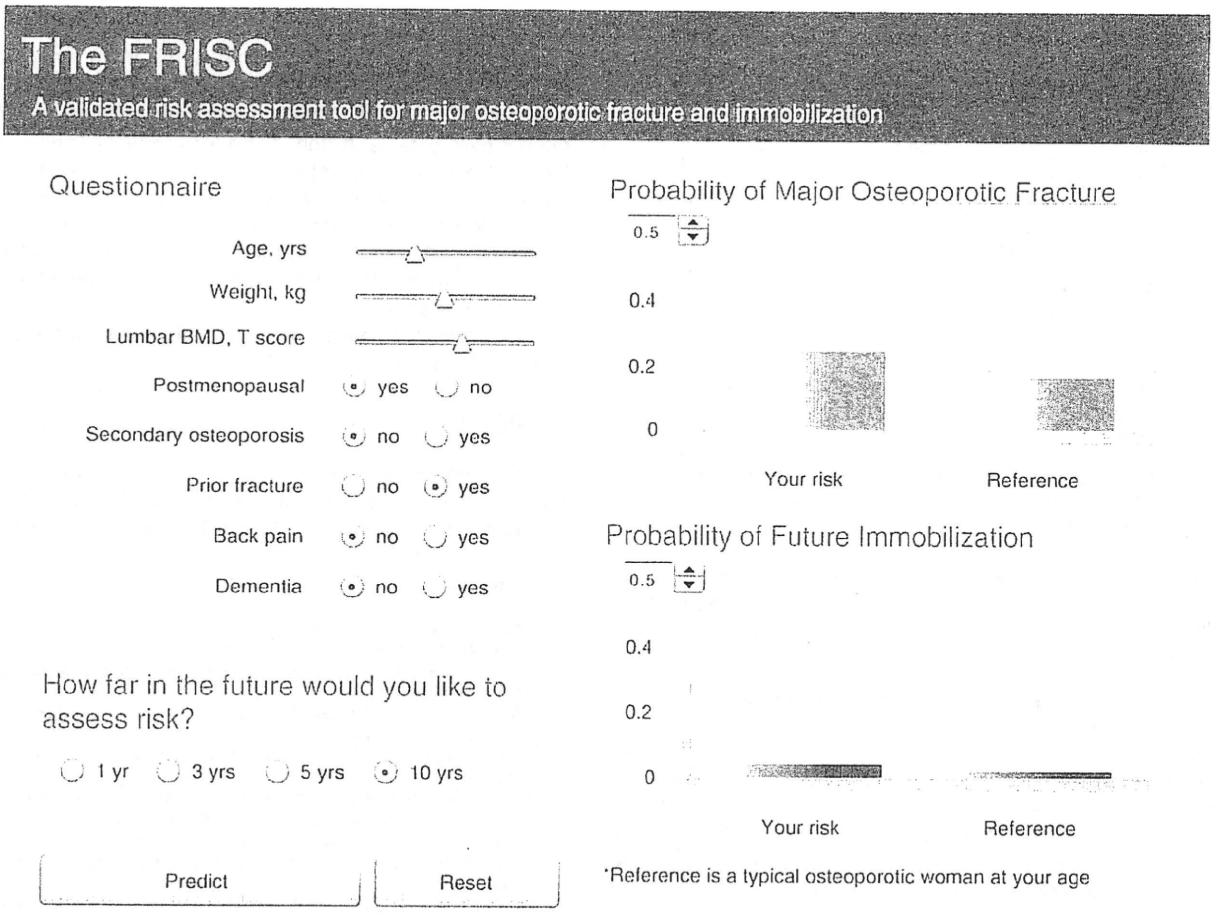

Fig. 1. Input and output of the web application of the FRISC.

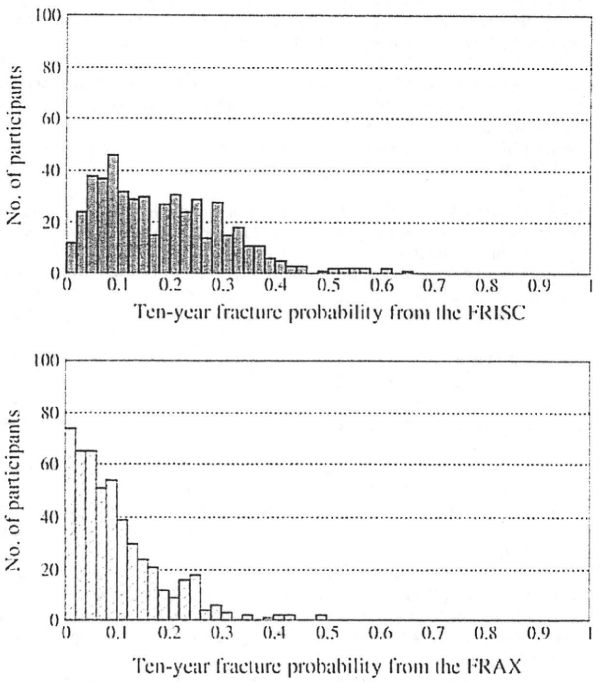

Fig. 2. Histogram of 10-year probabilities of major osteoporotic fracture from the FRISC (upper) and the FRAX (lower) in the Miyama and Taiji Cohorts.

($p<0.01$) and therefore seemed to be attributable to confounders. This may be one of the causes of discrepancy in 10-year probability between the FRAX and the actual fracture rate in the three cohorts. Recent report indicated that morbid obesity had a higher susceptibility of fractures comparing to the postmenopausal women with normal weight although the BMD of the obesity was higher than the controls [35]. As it is well known that obesity will connect to have diabetes mellitus or at least to have glucose intolerance and diabetes may deteriorate bone quality due to an increase in non-enzymatic glycation induced cross-links of collagen, which increased collagen

Table 4
Predictive accuracy of major osteoporotic fracture probability from the FRISC compared the FRAX evaluated in the validation dataset from general population.

	Calibration			Discrimination			
	Predicted no. of cases	Observed/predicted ratio	p^*	C statistics[†]	95% CI		$p^‡$
BMD alone	–	–	–	0.651	0.575	0.728	–
The FRAX	37.8	1.59	<0.01	0.699	0.629	0.768	0.23
The FRISC	81.2	0.74	0.17	0.727	0.660	0.794	0.03

CI: confidence interval.

* Hosmer–Lemeshow test, p value less than 0.05 indicates a significant deviation between the observed and predicted event frequencies. Number of strata and degree of freedom are 10 and 8, respectively.

† The proportion of all patient pairs in which prediction and observed occurrence of event are concordant.

‡ Contrast test comparing C statistics of the FRAX and FRISC from that of BMD alone, p value less than 0.05 indicates a significant improvement from BMD alone.

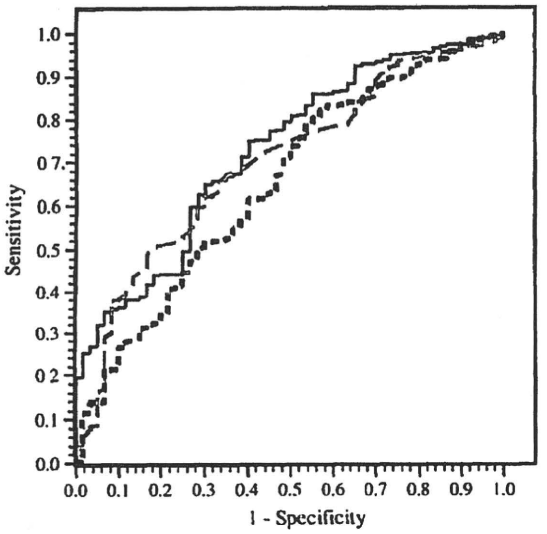

Fig. 3. Receiver operating characteristic curve for major osteoporotic fracture probability from the FRISC (solid curve), the FRAX (dashed curve) and BMD alone (dotted curve) in the Miyama and Taiji Cohorts.

brittleness [36,37]. Lifestyle factors such as diet and exercise may also be other explanations for this observation.

Although the intrinsic properties of the bone are important components of fracture risk, assessment of these factors alone does not adequately reflect the full range of factors associated with the occurrence of fracture [38]. Loss of bone mass and impaired bone quality are commonly held to be the two major causes of increased bone fragility in osteoporosis [2]; however, existing prediction models do not directly take bone quality into consideration. Despite recent progress in understanding the composition and structure of the bone, there are currently no standard assessments of bone quality. Novel bone quality-related markers such as homocysteine [39,40] and pentosidine [41] appear to improve predictive accuracy, but further research is required to determine whether they will be useful in the context of predicting osteoporotic fracture.

The incidence of clinical vertebral fracture in the Japanese population is substantially high (Table 2). As a result, the 10-year fracture probabilities generated using the FRISC is much higher than the FRAX (Fig. 2). The major underlying cause of the discrepancy in the 10-year probabilities is likely to be the difference in population. The FRISC was developed in a cohort study conducted at one medical institute and included subjects who were receiving treatment for osteoporosis, whereas the FRAX was developed using data from a community-based population. Although the effectiveness of bisphosphonate and selective estrogen receptor modulators in reducing fracture risk has been demonstrated, in the current analysis, drug treatment for osteoporosis was not a significant factor at the site of major osteoporotic fracture after adjustment for other risk factors, suggesting that its influence on risk is smaller than that of the risk factors. People who visit a hospital or clinic possibly have a higher prevalence of co-morbid conditions than people in the general population, yielding an increased incidence of fracture because of deterioration in both bone quality and quantity.

Given the large difference in incidence rates of fracture between the Nagano Cohort and the Miyama and Taiji Cohorts (Table 2), it may not seem to be sensible to choose the Miyama and Taiji Cohorts as validation cohorts since a good fit is unexpected. However, as shown in Table 1, the Nagano cohort included older participants and the mean lumbar BMD in this cohort was lower than the other cohorts. Therefore the difference in participants' characteristics may be attributable to the higher incidence rate in the Nagano cohort relative to the other cohorts. Further, the Miyama and Taiji Cohorts followed participants over a 10-year period and are more suitable for the validation analysis. A limitation of our validation analysis was that parental history or morphometrical vertebral fracture was missing data in either of the validation cohorts systematically. We assumed that participants with these missing data did not have parental history or prior fracture, yielding a somewhat lower 10-year probability of major osteoporotic fracture. Given that we did not find any evidence of deviation between the observed fracture frequency and prediction from the FRISC even in independent community-based cohort studies, the FRISC appears to allow accurate prediction of major osteoporotic fracture both in community-based and hospital-based settings.

Supplementary materials related to this article can be found online at doi:10.1016/j.bone.2010.08.019.

Conclusion

We developed a novel prediction model for fracture and immobilization, FRISC, and the clinical risk factors in the FRISC allows better identification of populations at high risk of fracture than BMD alone.

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Effects of long-term vitamin K_1 (phylloquinone) or vitamin K_2 (menaquinone-4) supplementation on body composition and serum parameters in rats

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ABSTRACT

Vitamin K is a cofactor for γ-glutamyl carboxylase, which is an essential enzyme for the γ-carboxylation of vitamin K-dependent proteins such as osteocalcin and matrix Gla protein. Although it has been suggested that vitamin K plays an important role in the improvement of bone metabolism, the relationship between dietary vitamin K intake and bone metabolism has not been thoroughly investigated. Moreover, vitamin K is thought to have other actions beyond influencing the γ-carboxylation status. In the present study, we examined the effects of the long-term addition of phylloquinone (PK) or menaquinone-4 (MK-4) to a control diet on bone mineral density, bone strength, body composition, and serum parameters in rats. A total of 23 female Sprague–Dawley strain rats (6 weeks old) were divided into three groups: basic control diet group, PK diet (PK: 600 mg/kg diet) group, and MK diet (MK-4: 600 mg/kg diet) group. Three months after starting the experimental diet, the addition of PK to the basic control diet significantly increased the bone mineral density (BMD) of the femur ($p<0.05$). In the MK group, there was no significant difference in the BMD of the femur. However, two types of bone strength parameter: the minimum cross-sectional moment of inertia and the polar moment of inertia, were significantly higher in the MK group than in the control ($p<0.05$, respectively). Furthermore, the femoral bone parameters (the width, dry weight and ash weight, and cortical, cancellous, trabecular, and total bone mineral contents) in the MK group were increased significantly compared with the control. Interestingly, the addition of PK or MK-4 significantly decreased the total fat accumulation ($p<0.01$ and $p<0.05$, respectively), and serum triglycerides were reduced by 48% in the PK group and 29% in the MK group compared with the control. There were no significant differences in the levels of serum calcium, phosphorus, alkaline phosphatase, growth hormone, insulin-like growth hormone-1, insulin-like growth hormone binding protein-3, and cross-linked N-teleopeptide of type I collagen among the three groups. This is the first study to demonstrate the effect of the long-term addition of PK or MK-4 to the control diet on body composition and serum parameters in an *in vivo* system using rats. Further studies on the mechanism of vitamin K supplementation in the regulation of bone metabolism would provide valuable data on the prevention of lifestyle-related disorders, including osteoporosis.

Introduction

Vitamin K was originally recognized as a factor involved in blood clotting, and is known to be important in bone metabolism. In nature, vitamin K exists in two forms: vitamin K_1 (phylloquinone: PK), and vitamin K_2 (menaquinone: MK-n), which comprises a family of naphthoquinones with differing numbers of isoprenoid residues (1 to 14) at the 3-position of naphthoquinone. PK is found in leafy, green vegetables, and menaquinone-4 (MK-4) is present in meat, eggs, and dairy products. Japanese fermented beans (referred to as natto) *Bacillus natto*, contain large amounts of menaquinone-7 (MK-7) synthesized by the bacteria.

Several epidemiologic studies have shown the association between biological makers of bone metabolism and vitamin K intake. A low dietary phylloquinone intake was associated with an increased risk of hip fracture in the elderly [1]. Kaneki et al. reported a significant inverse correlation between the incidence of hip fracture in women and the consumption of natto, one of the major sources of vitamin K_2 [2].

Vitamin K is a cofactor for vitamin K-dependent carboxylase, known as γ-glutamyl carboxylase (GGCX), which facilitates the post-translational modification of glutamic acid (Glu) to γ-carboxyglutamic acid (Gla) residues in selected proteins [3–7]. Three vitamin K-dependent proteins, osteocalcin (OC), matrix Gla protein (MGP), and protein S, are found in

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bone; OC is the most abundant [8–10]. OC is produced in osteoblasts, and fully carboxylated OC binds the calcium ions of hydroxyapatite [11].

Recently, Hosoi et al. clarified a significantly higher association between the single nucleotide polymorphism (SNP) of GGCX (R325Q, 974G>A) (rs699664) and bone mineral density (BMD) among postmenopausal women [12]. Moreover, we suggested that the requirement of vitamin K for γ-carboxylation may be different depending on the GGCX genotypes in healthy young males [13].

Vitamin K is thought to have other actions beyond the function of a coagulation factor associated with the γ-carboxylation status.

In this study, we investigated the effect of long-term PK or MK-4 supplementation on the BMD, bone strength, fat accumulation, serum parameters and bone metabolism markers in an *in vivo* system using rats.

Materials and methods

Experimental animals

The care and use of rats in the present study followed the guidelines of governmental legislation in Japan on the proper use of laboratory animals. Six-week-old female Sprague Dawley rats were used, and all rats were initially fed the control diet (AIN-93 diet) [14] for eight days. Then, they were divided into three groups, and were each fed experimental diets for eighty-five days: control (Cont.) diet, vitamin K_1 (phylloquinone: PK) diet, or vitamin K_2 (menaquinone-4: MK-4) diet. The vitamin K diets were modified from AIN-93 and contained PK or MK-4 at 600 mg/kg, respectively [15]. PK and MK-4 were kindly supplied by Eisai Co., Ltd. (Tokyo, Japan). Ca, P, protein, and lipid contents were identical in these diets. The animals were housed individually in wire cages with free access to ion-exchanged distilled water. Twelve-hour light/dark cycles, a constant temperature (23 ± 1 °C), and constant humidity (50 ± 5%) were maintained. All rats were observed each day. Their food intake was monitored, and body weight measurements were obtained every second day.

X-ray computed tomography (CT) scanning

Eighty-two days after starting the experimental diet, the body composition (fat and muscle amounts) and bone mineral contents, bone volume, and bone mineral density (BMD) were measured using an X-ray CT system for small experimental animals with a rat mode (LaTheta LCT-100, Aloka, INC., Tokyo, Japan) [16]. The visceral and subcutaneous fat volumes computed automatically were compared with those after the radiologist's adjustments. Ratios of volumetric visceral fat-to-total fat and visceral fat-to-subcutaneous fat were compared on average and with single-slice measurements obtained at L4 and L5 vertebral body levels. The visceral and subcutaneous fat volumes were computed on the tomographic scanning images at 1.5-mm intervals in the measurement area presented with 2 yellow lines in Fig. 1A.

Bone parameters (bone mineral contents, bone volume, and BMD) were computed on the tomographic scanning images at 1.0-mm intervals in the measurement area between the proximal and distal epiphyses of the right femur [17]. The minimum cross-sectional moment of inertia and polar moment of inertia that represent the flexural rigidity and torsional rigidity, respectively, were also calculated automatically employing the software provided with the device. According to the manufacturer, the precision error (as % CV) was within 2% range for all measurements.

The length of the femur and the width of the femur were measured using a dial caliper, and the fresh weight of femur was measured. Then, the femurs were dried at 95 °C for 24 h to measure the dry weight of the femur. All the femurs were burnt to ash at 550–600 °C for 24 h, and the ash weight was measured. The ashed bone was dissolved in 1 M nitric acid to determine the calcium and phosphorus content in the femur.

Biochemical analysis of serum

Eighty-five days after starting the experimental diet, the animals were fasted overnight and sacrificed by bleeding from the abdominal aorta under anesthesia. Blood was collected and centrifuged at 2500 rpm for 15 min to extract the serum. Sera were collected and stored at −80 °C until being thawed for analyses.

Calcium was measured employing the o-cresol-phthalein complexion color development method [18], and inorganic phosphorus was determined using the method of p-methylaminophenol reduction [19]. Total amounts of protein were measured applying the Biuret method [20]. Alkaline phosphatase was determined employing the method of Bessey et al. [21]. Cross-linked N-teleopeptide of type I collagen (NTx) was measured using an enzyme-linked immunosorbent assay (Mochida Seiyaku Co., Ltd., Tokyo, Japan). Serum glucose, triglycerides, and total cholesterol were assayed using the glucose-enzyme [22], GK-GPO (glycerokinase-glycerol-3-phosphate oxidase) [23], and enzymatic determination [24] methods, respectively.

Serum growth hormone (GH) was measured employing the immuno-radiometric assay (TFB Inc., Tokyo, Japan). Insulin-Like Growth Factor-1 (IGF-1) was measured using the immuno-radiometric assay (Mitsubishi Kagaku Yatoron Inc., Tokyo, Japan). Insulin-Like Growth Factor Binding Protein 3 (IGFBP-3) was measured by radioimmunoassay (Bioclone Australia Pty Ltd., Sydney, Australia).

Statistical analysis

Values are shown as the mean ± standard error (S.E.). Comparisons between treatments (Cont. vs. PK, Cont. vs. MK) were conducted using the unpaired Student's t-test. Differences were considered significant at $p<0.05$. Analysis was conducted using SPSS17.0J (SPSS Inc., USA).

Results

Animals and diets

There were no significant differences in the final body weight at 85 days among the Cont. (mean ± S.E., 287.3 ± 7.0 g), PK (272.0 ± 3.7 g), and MK (281.5 ± 3.7 g) groups. In addition, there were no significant differences in the food intake (g/day) among the Cont. (mean ± S.E., 15.6 ± 0.3 g/day), PK (15.2 ± 0.4 g/day), and MK (16.2 ± 0.2 g/day) groups. No significant differences among the three groups (Cont., PK and MK) in the body weight gain (g/day) or food efficiency (body weight gain/food intake) were noted (data not shown). The vitamin K diets contained 0.06% PK or MK-4, respectively. Therefore, the amount of vitamin K intake from the experiment diets was calculated approximately 9–10 mg/day (30–35 mg/kg body weight).

Bone mass and bone mineral density of femur

As shown in Table 1, the width of the femur in the MK group was significantly higher than in the Cont. group ($p<0.05$). The dry and ash weights of the femur in the MK group were also significantly higher than those of the Cont. group ($p<0.05$, respectively). There was no significant difference in the width, dry weight, and ash weight of the femur between the Cont. and PK groups.

Moreover, the addition of MK-4 led to a significant increase in the total, cortical, cancellous, and trabecular volumes of the femur in the MK group compared with the control group ($p<0.01$, $p<0.05$, $p<0.01$, and $p<0.05$, respectively, Table 2). There was no significant difference in the total, cortical, cancellous, and trabecular volumes of the femur between the Cont. and PK groups.

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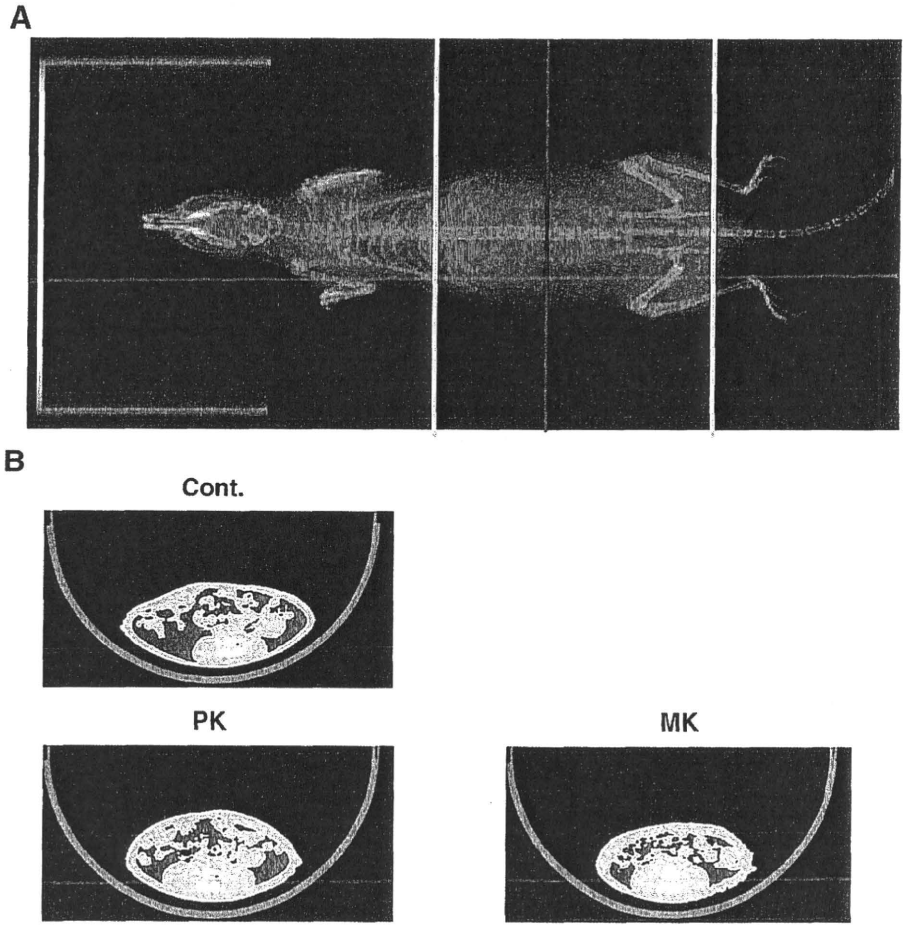

Fig. 1. X-ray computed tomography (CT) scanning after 82 days on the control (Cont.), PK (PK), or MK (MK) diet. (A) Representative images on X-ray CT scanning of the whole bodies of rats. For body composition measurements, tomographic images were acquired at 1.5-mm intervals in the measurement area presented as 2 yellow lines. (B) Cross-sectional appearance of rats in the Cont., PK, and MK groups. Tomographic X-ray CT images of the same 4th lumbar vertebral regions shown with a red line in panel A. The areas indicated in pink, yellow, and light-blue are visceral fat, subcutaneous fat, and muscle, respectively.

As shown in Table 3, the total bone mineral content (BMC) of the femur was higher in the PK and MK groups than that in the Cont. group ($p<0.05$ and $p<0.01$, respectively). The cortical, cancellous, and trabecular BMC in the MK group were significantly higher than those in the Cont. group ($p<0.05$, respectively, Table 3).

The BMD of the femur is shown in Fig. 2A. The BMD of the PK group was significantly higher than that of the Cont. group ($p<0.05$).

Bone strength

Two types of bone strength parameters: the minimum moment of inertia and polar moment of inertia of cross-sectional areas of the femur, are shown in Figs. 2B and C, respectively. The former parameter represents the flexural rigidity, and the latter torsional rigidity. Both of the minimum moment of inertia and polar moment of inertia of cross-sectional areas of the femur were significantly higher in the MK than in the Cont. group ($p<0.05$, respectively, Figs. 2B and C).

X-ray CT scanning of the fat area

Fig. 3 shows the results for the fat pad (g) after 82 days in the Cont., PK, and MK groups using an X-ray CT system for laboratory animals. The total fat weights were significantly lower in the PK and MK groups than in the Cont. group ($p<0.05$) (Fig. 3A). In the PK group, the weight of visceral fat was significantly lower than in the Cont. group ($p<0.01$, Fig. 3B). In the MK group, both the weights of visceral and subcutaneous fat were significantly lower than in the Cont. group ($p<0.05$, respectively, Figs. 3B and C). There was no significant difference in the amount of muscle among the Cont. (mean ± S.E., 97.9 ± 2.1 g), PK (101.4 ± 2.5 g), and MK (104.4 ± 2.2 g) groups.

Table 1
Length and weight of femur.

Groups	Length			Weight		
	Length (cm)	Width (cm)	Thickness (cm)	Fresh weight (g)	Dry weight (g)	Ash weight (g)
Cont.	3.48 ± 0.02	0.35 ± 0.01	0.28 ± 0.01	0.810 ± 0.018	0.534 ± 0.010	0.357 ± 0.008
PK	3.53 ± 0.03	0.35 ± 0.00 #	0.28 ± 0.01	0.830 ± 0.013	0.554 ± 0.010 #	0.374 ± 0.006 #
MK	3.51 ± 0.02	0.39 ± 0.01	0.29 ± 0.00	0.863 ± 0.019	0.576 ± 0.010	0.384 ± 0.007

Each value represents mean ± S.E.
#: Significant difference between the value of the control group and the MK group (#: $p<0.05$).

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