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Association of the –381T/C promoter variation of the brain natriuretic peptide gene with low bone-mineral density and rapid postmenopausal bone loss

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Abstract Osteoporosis is believed to result from interplay among multiple environmental and genetic determinants, including factors that regulate bone-mineral density (BMD). Recent quantitative trait locus analysis in human suggested a possible involvement of chromosomal region 1p36.2–p36.3 for determination of BMD. The brain natriuretic peptide (*BNP*, also named *NPPB*) gene lies within this candidate region for BMD determination. Overexpression of the *BNP* resulted in skeletal overgrowth in transgenic mice. Association analysis between nucleotide variations of the *BNP* gene and radial BMD in 378 Japanese postmenopausal women revealed a significant association of the –381T/C variation of the *BNP* gene with radial BMD ($r = 0.17$, $P = 0.01$). Homozygous T-allele carriers had the lowest BMD values ($0.395 \pm 0.056\,g/cm^2$), homozygous C-allele carriers had the highest ($0.429 \pm 0.051\,g/cm^2$), and heterozygous individuals had intermediate radial BMD values ($0.405 \pm 0.048\,g/cm^2$), indicating a dosage effect. Accelerated bone loss also correlated with the –381 T allele in a 5-year follow-up study ($r = 0.21$, $P = 0.017$). These results suggest that variation of BNP may be an important determinant of postmenopausal osteoporosis, in part through the mechanism of accelerated postmenopausal bone loss.

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Introduction

Osteoporosis is characterized by low bone-mineral density (BMD) and by deterioration of the micro-architecture of bone tissue with a consequent increase in fragility and susceptibility to fracture. BMD, an important predictor of fracture, appears to be determined by genetic as well as environmental factors (Giguere and Rousseau 2000; Stewart and Ralston 2000).

Natriuretic peptides comprise a family of three structurally related molecules: atrial natriuretic peptide, brain natriuretic peptide (BNP; also known as B-type natriuretic peptide), and C-type natriuretic peptide (CNP) (LaPointe et al. 1996; Maack 1996). Accumulating evidence indicates that natriuretic peptides are important regulators of bone and cartilage differentiation and maintenance, whose biological actions are mediated through two types of guanylyl cyclase (GC)-coupled receptor subtypes (GC-A and GC-B) (Yamamoto et al. 1996; Suda et al. 1998; Chusho et al. 2001; Yasoda et al. 1998; Hasegawa et al. 1994). For instance, transgenic mice overexpressing BNP presented with skeletal overgrowth (Suda et al. 1998). The targeted disruption of a related gene, *CNP*, in mouse resulted in congenital abnormalities in skeletal development (Chusho et al. 2001).

A quantitative trait locus (QTL) analysis on BMD in human suggested a possible involvement of chromosomal region 1p36.2–p36.3, spanning approximately 7 Mb, for determination of BMD (Devoto et al. 2001). The responsible gene within the region has not been defined to date (Spotila et al. 2000; Albagha et al. 2002).

Because of the chromosomal location within the QTL candidate region, as well as skeletal abnormalities displayed in BNP-transgenic mice, we investigated the association between genetic variations in the *BNP* gene and radial

Table 1. Summary of analyzed polymorphisms on *BNP* gene

No.	Name	Location	JSNP ID[a]	NCBI dbSNP[b]	Allele frequency	% Heterozygosity
1	–2158A/G	Promoter	IMS-JST106520	—	0.88:0.12	19
2	–1563A/G	Promoter	—	rs1800773	Monomorphic	0
3	–1299G/T	Promoter	IMS-JST106522	—	0.86:0.14	21
4	–381T/C	Promoter	IMS-JST083611	—	0.84:0.16	24
5	R25L	Exon 1 (+)	—	rs5227	Monomorphic	0
6	R47H	Exon 2 (+)	—	rs5229	Monomorphic	0
7	M93L	Exon 2 (+)	—	rs5230	Monomorphic	0

SNP, Single-nucleotide polymorphism
[a] JSNP ID, Number from the Japanese SNP database (http://snp.ims.u-tokyo.ac.jp/index_ja.html)
[b] dbSNP ID, Number from the dbSNP database of NCBI (http://www.ncbi.nlm.nih.gov/SNP/)

BMD levels and rate of bone loss in a 5-year longitudinal follow-up study.

Subjects and methods

Subjects

DNA samples were obtained from the peripheral blood of 378 postmenopausal Japanese women. Mean age and body mass index (BMI) with standard deviation (SD) was 58.4 ± 8.6 (range 32–69) years and 23.7 ± 3.61 (range 14.7–38.5) kg/m^2, respectively. The BMD of radial bone (expressed in g/cm^2) of each participant was measured by dual energy X-ray absorptiometry using a DTX-200 (Osteometer Meditech, Hawthorne, CA, USA). To calculate adjusted BMD, we normalized the measured BMD for differences in age and BMI by multiple regression analysis (Kleinbaum et al. 1998; Tsukamoto et al. 2000), using the Instat 3 software package (GraphPad Software, San Diego, CA, USA). The adjustment equation for the study samples was as follows: [adjusted BMD (g/cm^2)] = [measured BMD (g/cm^2)] − 0.006375 × {58.39 − [age (years)]} + 0.008961 × {23.65 − [BMI (kg/cm^2)]}. BMD in the distal radius was measured according to the Guidelines for Osteoporosis Screening in a health check-up program in Japan (Orimo et al. 2001). All subjects were nonrelated volunteers who gave their informed consent prior to the study. No participant had medical complications or was undergoing treatment for conditions known to affect bone metabolism, such as pituitary diseases, hyperthyroidism, primary hyperparathyroidism, renal failure, adrenal diseases, or rheumatic diseases, and none was receiving estrogen replacement therapy.

Genotyping for molecular variants in the *BNP* gene

We examined in our test population seven polymorphisms (SNPs) archived in the National Center for Biotechnology Information database for SNPs (NCBI dbSNP; http://www.ncbi.nlm.nih.gov/SNP/) and the Japanese SNP (JSNP) database (http://snp.ims.u-tokyo.ac.jp/index.htmlamino); three SNPs from JSNP were confirmed to be polymorphic in our test population, whereas four other SNPs archived in the NCBI dbSNP turned out to be monomorphic in our test population, as described in Results (Table 1).

Genotypes of SNPs were determined using the SNP dependent PCR (Sd-PCR) method, a refined allele-specific PCR to discriminate polymorphic sequences, as described previously (Iwasaki et al. 2002). In brief, the Sd-PCR transforms nucleotide differences (G, A, T, or C) between two alleles at a single site into size differences between the respective alleles. The procedure incorporates double-nucleotide mismatches at the 3′ end of polymorphic (forward) primers representing each allele, one mismatch corresponding to the natural SNP to be tested and the other designed to allow distinct allelic discrimination through almost exclusive amplification of one allele over the other.

Two allele-specific primers (AS primers) and one reverse primer were prepared per SNP. AS primers (long and short) have a five-base difference between them, and each has a polymorphic nucleotide of the SNP sequence at the 3′ ends and an additional artificial mismatch introduced near the 3′ end. These primer sets allowed distinct discrimination of alleles. Each genomic DNA sample (10 ng) was amplified with 250 nM of each primer (two polymorphic forward, and a reverse) in a 10-μl reaction mixture containing 10 mM deoxyribonucleoside triphosphates, 10 mM Tris-HCl, 1.5 mM $MgCl_2$, 50 mM KCl, 1 U Taq DNA polymerase, and 0.5 mM fluorescence-labeled deoxycytidine triphosphate (ROX-dCTP; Perkin-Elmer, Norwalk, CT, USA). The Sd-PCR reaction was carried out on a thermal cycler (Geneamp system 9600, Perkin-Elmer) with initial denaturation at 94°C for 4 min, followed by five cycles of stringent amplification (94°C for 20 s, 64°C for 20 s, 72°C for 20 s) and then 25 cycles of 94°C for 20 s, 62°C for 20 s, and 72°C for 20 s, terminating with a 2-min extension at 72°C. Allele discrimination was carried out by electrophoresis and laser scanning of the DNA fragments on an ABI Prism 377 DNA system using GeneScan Analysis Software ver2.1 (Applied Biosystems, Foster City, CA, USA). To confirm the accuracy of the Sd-PCR method, we carried out direct resequencing using the ABI Prism BigDye Terminator system (Applied Biosystems).

Statistical analysis

BMD data of each subject were normalized with age and BMI, using Instat 3 software package (GraphPad Software) via multiple regression analysis (Iwasaki et al. 2002). Quantitative association between genotypes and adjusted BMD values (g/cm^2) was analyzed via one-way analysis of variance (ANOVA) with regression analysis as a post hoc test. Three genotypic categories of each SNP were converted into incremental values, 0, 1, and 2, corresponding to the number of chromosomes possessing a minor allele nucleotide. Statistical significance was determined by ANOVA, *F*-test. To ascertain the Hardy-Weinberg equilibrium among genotypes of the subjects, a chi-square test was used. Prediction of binding sites for transcription factors was performed using MatInspector V2.2 (http://transfac.gbf.de/cgi-bin/matSearch/matsearch.pl) based on the TRANSFAC4.0 database (http://transfac.gbf.de/TRANSFAC/index.html).

Results

The *BNP* gene was examined for association with BMD as one of the likely candidates for osteoporosis susceptibility genes. We first examined the polymorphic nature of seven archived SNPs in 32 chromosomes of the subjects from our test population; three SNPs were archived in the JSNP database, and four SNPs were archived in the NCBI-dbSNP database, as shown in Table 1. Three SNPs from the JSNP database were moderately polymorphic, whereas the other four SNPs from the NCBI-dbSNP database turned out to be monomorphic in the test population.

Among the three polymorphic SNPs as defined earlier, promoter SNP –381T/C, localized at 283 bp upstream of the transcription initiation site (referenced contig; NT_004488.10 from GenBank), revealed a significant correlation with variation in radial BMD ($r = 0.13$, $P = 0.01$). Statistical analyses of two other SNPs did not reach statistical significance (–2158A/G; $r = 0.06$, $P = 0.23$, –1299G/T; $r = 0.03$, $P = 0.58$). As to the –381T/C SNP, homozygous T-allele carriers had the lowest adjusted BMD (0.395 ± 0.056 g/cm^2), heterozygous individuals had an intermediate adjusted BMD (0.405 ± 0.048 g/cm^2), and homozygous C-allele carriers had the highest adjusted BMD (0.429 ± 0.051 g/cm^2), implying an allelic dose effect of this variation on influences to BMD (Fig. 1).

Accelerated bone loss in T-allele carriers in a 5-year follow-up study. To test whether bone loss of postmenopausal women is affected by the variation of the *BNP* gene, we examined the 5-year bone loss of the 126 subjects who had been followed up longitudinally for over 5 years. Five-year bone loss (g/cm^2) was calculated for each individual by subtracting the adjusted BMD value obtained in the BMD measurement carried out 5 years previously from the BMD value measured in the recent analysis (Fig. 2). A significant difference in 5-year bone loss was identified between women with the T-allele homozygotes for the –381T/C SNP

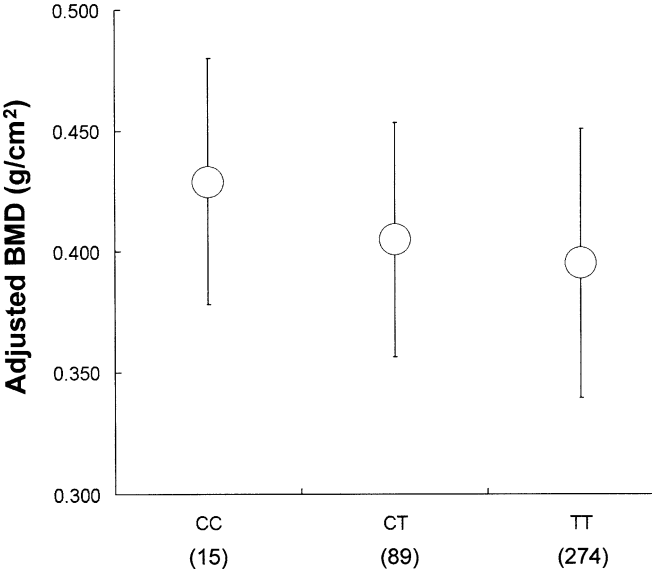

Fig. 1. Association of –381T/C variations with adjusted-radial bone mineral density (BMD). Adjusted BMD of three genotypically classified subgroups among 378 subjects were plotted. *Open circles* indicate mean values and *error bars* indicate standard deviations. The correlation between the number of minor alleles possessed and the adjusted BMD was tested by linear regression analysis ($P = 0.01$)

(0.048 ± 0.030 g/cm^2, $n = 90$) and other women (0.036 ± 0.023 g/cm^2, $n = 36$) ($P = 0.017$). The results suggest that genetic variation of BNP contributes to the development of osteoporosis, in part through the mechanism of accelerated postmenopausal bone loss. We hypothesized that T-allele homozygotes for the BNP –381 SNP is an important risk factor for decreased BMD in postmenopausal women.

Discussion

In the work reported here, we showed an association of the –381T/C SNP variation in the promoter region of the *BNP* gene with radial BMD in a population of postmenopausal Japanese women. Adjusted BMD was lowest in T/T homozygotes, intermediate among heterozygotes, and highest among C/C homozygotes in the test population. The data implied that variation in the promoter region of the *BNP* gene might have affected bone metabolism in these women, eventually introducing variation in BMD. Lowered BMD in postmenopausal women could be a result of accelerated bone loss and/or lesser acquisition of bone mass before maturation. A correlation between rate of bone loss and variation of the *BNP* gene was indicated by analysis of bone loss over 5 years in a longitudinal follow-up study. The correlation may suggest that the main contribution of the BNP variation is to increase bone turnover and bone loss.

The –381 variation may be important on theoretical grounds because it is located just upstream of the gene transcription initiation site. Predictive analysis of binding motifs for transcription factors using the MatInspector pro-

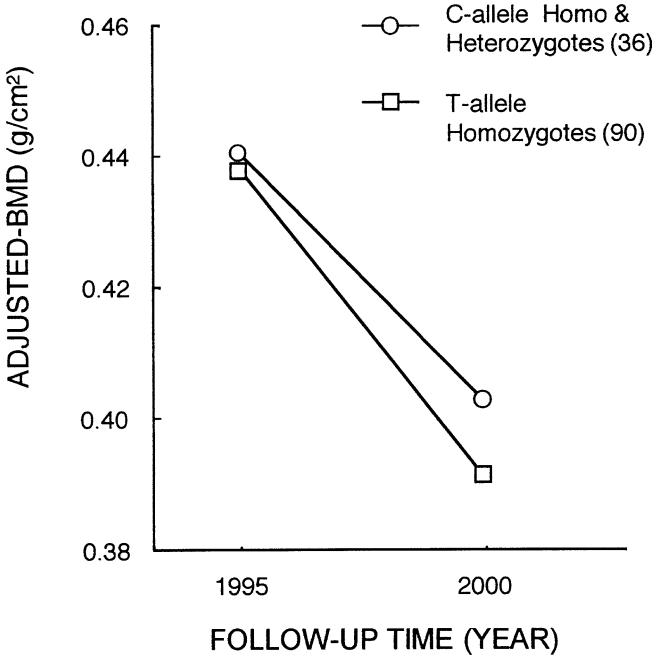

Fig. 2. Five-year longitudinal bone loss between subjects harboring the T allele of the –381T/C single-nucleotide polymorphism and other subjects in 126 postmenopausal women. Mean values of adjusted BMD are plotted on the diagram from data at 5 years previously (1995) to those at a recent measurement (2000). *Open circles* indicate values among C homozygous and heterozygous individuals. *Open squares* are for individuals with T homozygotes. *Lines* are synonymous to regression lines drawn for each genotypically classified subject group. A significant difference was indicated for the inclination of two lines ($P = 0.017$)

gram revealed that the –381T/C variation is located within the consensus binding sequence of δ-crystallin/E2-box factor 1 (δ-EF1; GGACACCTGGA) and upstream stimulatory factor (ACACCTGG), both of which contain an E-box binding element (CAnnTG) inside them (Sekido et al. 1994; Dillner and Sanders 2002; Viollet et al. 1996). Because δ-EF1 is an important transcription factor that distributes in many organs including connective tissues of the skeletal system (Davies et al. 2002; Funahashi et al. 1993; Terraz et al. 2001; Sooy and Demay 2002), it regulates not only tissue-specific expression of the crystalline gene in the lens, but also many genes including collagens and osteocalcin genes important for bone and cartilage development and maintenance (Funahashi et al. 1993; Terraz et al. 2001; Sooy and Demay 2002). Altered promoter function could account for the different clinical features of bone mass in individuals, which should be examined in the future by means of a binding assay for each transcription factor, or by a promoter activity test using reporter constructs, for example.

In summary, we showed a significant association of the –381T/C variation in the promoter region of the *BNP* gene with radial BMD of postmenopausal Japanese women. Structural inspection suggests a possible contribution of a transcription factor delta-EF1 binding to the SNP site. The possibility cannot be ruled out, however, that this SNP marker may itself be in linkage disequilibrium with other unmeasured and functional variants at or near *BNP* that are the true mechanistic basis for the associations. Functional studies will be required to rule out these possibilities. Nevertheless, our data is in accord with the previous data from human QTL linkage analysis in search of an osteoporosis susceptibility gene at 1p36.2–p36.3.

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