Table 1. Linkage Analyses of BMD or Osteoporosis.

Chromosomal locus	Marker	Candidate gene	Phenotype	Reference
1p36, 2p24-23, 4qter	*D1S450, D2S149, D4S1539*		Low BMD (spine, hip)	Devoto et al. 1998
1p36.3-36.2	*D1S214*		Low BMD (femoral neck)	Devoto et al. 2001
1q25-31	*D1S3737*	Osteocalcin	BMD	Raymond et al. 1999
3p22-21.2		PTH receptor type 1	BMD	Duncan et al. 1999
7p21		Interleukin-6	osteopenia	Ota et al. 1999
6p21.3		Tumor necrosis factor–α	osteoporosis, osteopenia	Ota et al. 2000
11q12-13	*D11S987*		High BMD (spine)	Johnson et al. 1997

PTH, parathyroid hormone

located on chromosome 1; interleukin (IL)–1β and IL-1 receptor antagonist on chromosome 2; CC chemokine receptor 2 (CCR2), peroxisome proliferator–activated receptor–γ, and the calcium sensing receptor on chromosome 3; runt-related gene 2 and estrogen receptor α (ERα) on chromosome 6; IL-6 and the calcitonin receptor on chromosome 7; osteoprotegerin on chromosome 8; calcitonin, parathyroid hormone, p57 (Kip2), and matrix metalloproteinase–1 (MMP-1) on chromosome 11; the vitamin D receptor (VDR) and insulin-like growth factor–I on chromosome 12; aromatase on chromosome 15; collagen type Iα1 on chromosome 17; and transforming growth factor–β1 (TGF-β1) and apolipoprotein E on chromosome 19. These genes are candidate loci for determination of BMD or susceptibility to osteoporosis or osteoporotic fracture. However, it is also possible that polymorphisms in these genes are in linkage disequilibrium with other polymorphisms in nearby genes that are determinants of these conditions. In this review, I will discuss several candidate genes that are of particular interest.

OSTEOCALCIN GENE (*BGLAP*)

Osteocalcin is an extracellular matrix protein that is abundant in bone and is a marker of bone turnover in both normal and diseased states. Calcitriol acting through the VDR and a specific vitamin D–responsive element in the *BGLAP* promoter induce its synthesis [Morrison *et al.* 1989]. Characterization of osteocalcin-deficient mice demonstrated that this protein functions as a negative regulator of bone formation [Ducy *et al.* 1996]. A C→T single nucleotide polymorphism (SNP) at nucleotide 298 in the promoter region of *BGLAP* was identified, and was suggested to be associated with BMD [Dohi *et al.* 1998]. BMD for the lumbar spine increased according to the rank order of genotypes *CC* < *CT* < *TT* for this 298C→T polymorphism in 160 postmenopausal Japanese women, although the observed differences were not statistically significant [Dohi *et al.* 1998]. Association of this SNP with BMD for other sites was not examined. The molecular mechanism that might be responsible for an association of the 298C→T SNP of *BGLAP* with BMD remains unknown.

CC CHEMOKINE RECEPTOR 2 GENE (*CCR2*)

CCR2 is a receptor for monocyte chemoattractant protein (MCP)–1 and for closely related proteins including MCP-2, -3, -4, and -5 [Luster 1998]. MCP-1 is chemotactic for monocytes and other leukocyte subsets. It is expressed in osteoblastic cells and is induced during both bone inflammation [Rahimi *et al.*, 1995] and developmentally regulated bone remodeling [Volejnikova *et al.*, 1997]. The recruitment of monocytes to bone induced by this chemokine has been associated with an increase in the number of osteoblasts lining the bone surface. The fact that monocytes produce factors that regulate bone formation or resorption, including platelet-derived growth factor, IL-1, and TNF-α, suggests that the recruitment of these cells by MCP-1 is important in the regulation of bone remodeling.

We showed that a 190G→A SNP of *CCR2*, which results in substitution of Ile for Val at amino acid 64 in the first transmembrane domain of the encoded protein, was associated with BMD at various sites in community-dwelling Japanese middle-aged men and elderly women who participated in a population-based study of aging and age-related diseases, with the *AA* genotype representing a contributing factor to increased bone mass [Yamada *et al.*, 2002a]. The molecular mechanism by which the *A* allele of this SNP protects against age- related bone loss remains unclear. Other ligands of CCR2, including MCP-2, -3, -4, and -5, might help to explain the *in vivo* effects of the 190G→A SNP. Alternatively, these effects may be mediated through intracellular interactions of variant CCR2 proteins with other chemokine receptors, such as CCR5 and CXC chemokine receptor 4 [Lee *et al.* 1998]. However, our results suggest that *CCR2* is a candidate locus for the determination of BMD, especially in middle-aged men and elderly women.

ESTROGEN RECEPTOR α GENE (*ESR1*)

The importance of ERα in the regulation of bone mass was indicated by the occurrence of osteoporosis in a man with a nonsense mutation in *ESR1* [Smith *et al.*, 1994] and by the observation that the BMD of mice lacking a functional *ESR1* is 20 to 25% less than that of wild-type

Table 2. Candidate gene association studies of BMD or osteoporosis

Candidate gene	Chromosomal locus	Phenotype	Reference
Tumor necrosis factor receptor 2	1p36.3-36.2	Low BMD (spine)	Spotila et al. 2000
Methylenetetrahydrofolate reductase	1p36.3	BMD	Miyao et al. 2000b
Osteocalcin	1q25-31	BMD, osteopenia	Dohi et al. 1998
Interleukin-1β	2q14	BMD	Nemetz et al. 2001
Interleukin-1 receptor antagonist	2q14.2	Osteoporotic fracture Bone loss (spine)	Langdahl et al. 2000a Keen et al. 1998
CC chemokine receptor 2	3p21	BMD	Yamada et al. 2002a
Peroxisome proliferator-activated receptor-γ	3p25	BMD	Ogawa et al. 1999
Calcium sensing receptor	3q13.3-21	BMD	Lorentzon et al. 2001
Runt-related gene 2	6p21	BMD, osteoporotic fracture	Vaughan et al. 2002
Estrogen receptor α	6q25.1	BMD	Kobayashi et al. 1996
Interleukin-6	7p21	BMD BMD BMD	Lorentzon et al. 2000 Ota et al. 2001 Murray et al. 1997
Calcitonin receptor	7q21.3	BMD, osteoporotic fracture	Taboulet et al. 1998
Osteoprotegerin	8q24	Vertebral fracture BMD	Langdahl et al. 2002 Ohmori et al. 2002
Calcitonin	11p15.2-15.1	BMD	Miyao et al. 2000a
Parathyroid hormone	11p15.3-15.1	BMD	Hosoi et al. 1999
p57 (Kip2)	11p15.5	BMD	Urano et al. 2000
Matrix metalloproteinase-1	11q22-23	BMD	Yamada et al. 2002c
Vitamin D receptor	12q12-14	BMD BMD BMD	Morrison et al. 1994 Gross et al. 1996 Arai et al. 2001
Insulin-like growth factor-I	12q22-24.1	BMD, osteoporosis	Kim et al. 2002
Aromatase	15q21.2	BMD, osteoporosis, spinal fracture	Masi et al. 2001
Collagen type Iα1	17q21.31-22	Osteoporosis Osteoporotic fracture BMD	Grant et al. 1996 Uitterlinden et al. 1998 Garcia-Giralt et al. 2002
Transforming growth factor-β1	19q13.1	BMD BMD, osteoporosis BMD, osteoporosis Osteoporosis (femoral neck)	Langdahl et al. 1997 Yamada et al. 1998 Yamada et al. 2001b Keen et al. 2001
Apolipoprotein E	19q13.2	BMD	Shiraki et al. 1997

mice [Korach, 1994]. Two SNPs have been identified in the first intron of *ESR1*: a T→C SNP that is recognized by the restriction endonuclease *Pvu* II [*T* and *C* alleles correspond to the presence (*p* allele) and absence (*P* allele) of the restriction site, respectively], and an A→G SNP that is recognized by *Xba* I [*A* and *G* alleles correspond to the presence (*x* allele) and absence (*X* allele) of the restriction site, respectively]. These SNPs, alone or in combination, have been associated with BMD in postmenopausal women [Kobayashi *et al.*, 1996; Albagha *et al.*, 2001] or pre-menopausal women [Willing *et al.*, 1998; Patel *et al.*, 2000], or with the response to hormone replacement therapy (HRT) [Salmén *et al.*, 2000]. However, other studies did not confirm these observations [Han *et al.*, 1997; Gennari *et al.*, 1998; Vandevyver *et al.*, 1999; Becherini *et al.*, 2000; Langdahl *et al.*, 2000b; Brown *et al.*, 2001]. In addition, a microsatellite (TA repeat) polymorphism of *ESR1*, but not the T→C and A→G SNPs in the first intron, was associated with BMD and with the prevalence of fractures [Becherini *et al.*, 2000; Albagha *et al.*, 2001; Langdahl *et al.*, 2000b].

We showed that the A→G (*Xba* I) SNP in the first intron of *ESR1*, alone or in combination with the T→C (*Pvu* II) SNP, was associated with BMD for the femoral neck in community-dwelling elderly women recruited to our large-scale population-based study. The combined *CC/GG* genotype was a genetic risk factor for predisposition to reduced BMD whereas the *TT/AA* genotype was a contributing factor to increased BMD, although the contribution of these SNPs to BMD appeared relatively small [Yamada *et al.*, 2002b]. For the T→C (*Pvu* II) SNP of *ESR1*, BMD tended to be reduced in elderly women with the *CC* genotype compared with those with the *TT* or *TC* genotypes, consistent with a previous observation in postmenopausal Japanese women [Kobayashi *et al.*, 1996]. For the A→G (*Xba* I) SNP of *ESR1*, however, BMD tended to be lower in elderly women with the *GG* genotype than in those with the *AA* or *AG* genotypes. This finding differs from the previous observation for postmenopausal Japanese women that individuals with the *GG* genotype showed the highest BMD for the lumbar spine or total body and those with the *AA* genotype showed the lowest BMD, although statistical significance for these differences was not achieved [Kobayashi *et al.*, 1996].

For women aged <60 years, we did not detect any association between the T→C (*Pvu* II) or A→G (*Xba* I) SNPs and BMD at various sites. In contrast, ERα genotype was previously shown both to be associated with BMD for the femoral neck or lumbar spine in women aged 24 to 44 years [Willing *et al.*, 1998] and to be an independent predictor of heel stiffness index determined by quantitative bone ultrasound in women aged 18 to 35 years [Patel *et al.*, 2000]. However, the association of *ESR1* genotype with BMD differed between these two previous studies: individuals with the *TT* genotype or the *AA* genotype showed the lowest BMD in the former study, whereas individuals with these genotypes showed the highest heel stiffness index in the latter study. Our data are consistent with the results of the latter study [Patel *et al.*, 2000]. Although Albagha *et al.* (2001) found no association between BMD and either of these two *ESR1* SNPs alone in women; these researchers did detect an association of BMD for the lumbar spine or femoral neck with the haplotype of these SNPs. No association was detected between estrogen responsiveness of BMD and ERα genotype in postmenopausal Korean women who had undergone HRT [Han *et al.*, 1997]. In contrast, women with the *TT* genotype (*Pvu* II SNP) have been suggested to be relatively estrogen insensitive; those with the *C* allele appeared to benefit more from the protective effect of HRT on fracture risk than did women with the *TT* genotype [Salmén *et al.*, 2000]. The molecular mechanisms that underlie the association of the T→C (*Pvu* II) and A→G (*Xba* I) SNPs of *ESR1* with BMD or genetic susceptibility to osteoporosis remain unclear. However, *ESR1* may be a determinant of bone mass or of genetic predisposition to postmenopausal osteoporosis.

INTERLEUKIN-6 GENE (*IL6*)

IL-6 is a multifunctional cytokine that is important in the development of postmenopausal osteoporosis [Manolagas and Jilka, 1995]. A VNTR (variable number of tandem repeats) polymorphism in the 3' flanking region of *IL6* has been associated with BMD in postmenopausal white women [Murray *et al.*, 1997], and sibling-pair analysis has provided evidence of linkage between the *IL6* locus and reduced BMD in postmenopausal Japanese women [Ota *et al.*, 1999]. A −174G→C SNP in the *IL6* promoter has been shown to affect both promoter activity and the plasma concentration of IL-6 [Fishman *et al.*, 1998]. This polymorphism was also associated with peak bone mass in healthy white men [Lorentzon *et al.*, 2000], however, we have not detected this SNP in the Japanese population [Y. Yamada, unpublished data]. Three polymorphisms of *IL6* have been identified in Japanese, among which a −634C→G SNP in the promoter region has been associated with BMD for the radius [Ota *et al.*, 2001]. For this SNP, BMD decreased according to the rank order of genotypes *CC* > *CG* > *GG* in postmenopausal Japanese women [Ota *et al.*, 2001]; BMD for other sites was not measured. The molecular mechanism responsible for the association of the −634C→G SNP of *IL6* with BMD has not been determined.

OSTEOPROTEGERIN GENE (*OPG*)

Osteoprotegerin is a soluble member of the TNF receptor superfamily of proteins. *In vitro* studies suggest that osteoprotegerin inhibits osteoclastogenesis by interrupting the intercellular signaling between osteoblastic stromal cells and osteoclast progenitors [Simonet *et al.*, 1997]. Osteoprotegerin-deficient mice develop severe osteoporosis [Bucay *et al.*, 1998], whereas the systemic administration of recombinant osteoprotegerin results in a marked increase in BMD in normal rats as well as in the prevention of bone loss in ovariectomized rats [Simonet *et al.*, 1997; Yasuda *et al.*, 1998]. Both 209G→A and 245T→G SNPs in the *OPG* promoter have been associated with BMD in postmenopausal women [Arko *et al.*, 2002], and 163A→G and 245T→G SNPs were associated with vertebral fracture [Langdahl *et al.*, 2002]. A −223T→C SNP was also associated with BMD in postmenopausal Japanese women [Ohmori *et al.*, 2002]. Given that osteoprotegerin plays important roles in bone remodeling; *OPG* may be a determinant of BMD or predisposition to osteoporosis.

MATRIX METALLOPROTEINASE-1 GENE (*MMP1*)

MMP-1 is an interstitial collagenase that is expressed widely among tissues, and therefore plays a prominent role in collagen degradation. Given that collagens are the most abundant proteins in bone; MMP-1 may contribute to the modeling and remodeling of the bone matrix. A single base insertion (G→GG) at nucleotide position −1607 in the promoter region of *MMP1* results in the creation of a binding site for the Ets family of transcription factors adjacent to an AP-1 (activator protein-1) site as well as in increased transcription of *MMP1* and increased enzyme activity [Rutter *et al.*, 1998].

We showed that the −1607G→GG polymorphism of *MMP1* was associated with BMD for the distal radius in community-dwelling postmenopausal Japanese women, and that the *GG/GG* genotype represents a risk factor for genetic susceptibility to reduced bone mass at the distal radius

[Yamada *et al.*, 2002c]. Three polymorphisms in the 5' region (nucleotides –524 to +52) of *MMP1* were shown not to be associated with osteoporosis [Thiry-Blaise *et al.*, 1995]. These polymorphisms have also not been shown to affect transcriptional activity or other gene function. Our results, together with the fact that the –1607G→GG polymorphism affects the transcriptional activity of the gene, suggest that *MMP1* may be a susceptibility locus for reduced BMD for the distal radius in postmenopausal women.

VITAMIN D RECEPTOR GENE (*VDR*)

Vitamin D is a potent regulator of bone and calcium homeostasis as well as of cellular differentiation and replication in many tissues. Its active form, 1,25-dihydroxyvitamin D_3 (calcitriol), interacts with the highly specific VDR, which mediates the effects of calcitriol on the expression of target genes. A *Bsm* I restriction fragment length polymorphism of *VDR* was associated with BMD in Australian women [Morrison *et al.*, 1994]. Of the many studies performed since this first report, some [Tokita *et al.*, 1996; Uitterlinden *et al.*, 1996; Sainz *et al.*, 1997] have supported this association whereas others [Hustmyer *et al.*, 1994; Melhus *et al.*, 1994; Garnero *et al.*, 1996] have not. These apparently contradictory results are possibly attributable to differences in several factors, including sample size, as well as ethnic background, age, and calcium intake of the subjects, among the various studies. In addition, the effects of *VDR* genotype on BMD appear to be relatively small. A meta-analysis of 16 studies concluded that BMD was 2.5% lower for the spine and 2.4% lower for the femoral neck in individuals with the *BB* genotype (*B* allele, absence of the *Bsm* I restriction site) than in those with the *bb* genotype (*b* allele, presence of the restriction site) [Cooper and Umbach, 1996]. Another meta-analysis of 75 studies, including those of polymorphisms (*Apa* I, *Taq* I, and *Fok* I) other than the *Bsm* I polymorphism, confirmed the association of *VDR* polymorphisms with BMD [Gong *et al.*, 1999]. However, no consensus regarding the impact of the *Bsm* I polymorphism of *VDR* on bone mass or bone loss has yet been established.

The nucleotide sequence of the human *VDR* [Baker *et al.*, 1988] revealed two potential translation initiation sites, the most 5' of which is affected by a T→C SNP (ATG→ACG). Individuals with the *T* allele of this SNP thus have two start sites and may initiate translation from the first ATG codon, whereas those with the *C* allele initiate translation at the second ATG. Initiation at the first ATG lengthens the encoded VDR protein by three amino acids. The T→C SNP of *VDR* has been associated with BMD in postmenopausal Mexican-American women [Gross *et al.*, 1996], pre-menopausal American white women [Harris *et al.*, 1997], and Japanese women [Arai *et al.*, 1997], with the *TT* genotype implicated as a risk factor for reduced BMD. This SNP was also associated both with BMD in small populations of Caucasian men, with the *T* allele being a predisposing factor to reduced bone mass [Ferrari *et al.*, 1999], and with calcium absorption and BMD in girls and boys of various ethnic ancestries [Ames *et al.*, 1999]. However, no association of this SNP with BMD was detected in pre-menopausal American black [Harris *et al.*, 1997] or French [Eccleshall *et al.*, 1998] women. The T→C SNP of *VDR* was shown to affect both the molecular mass of the encoded protein (*T* allele, 50 kDa; *C* allele, 49.5 kDa) and transcriptional activation of the gene by vitamin D (*T* allele < *C* allele) [Arai *et al.*, 1997]. These observations were not independently confirmed, however [Gross *et al.*, 1998]. The functional impact of this SNP thus remains to be determined. A –3731A→G SNP that affects the binding site of the caudal-related homeodomain protein Cdx-2 in the *VDR* promoter was also recently associated both with transcriptional activity of the promoter and with BMD for the lumbar spine in Japanese women, with the *G* allele representing reduced transcriptional activity and low BMD [Arai *et al.*, 2001]. These various observations thus suggest that *VDR* is a susceptibility locus for reduced BMD or predisposition to osteoporosis.

COLLAGEN TYPE Iα1 GENE (*COL1A1*)

Type I collagen is the most abundant protein of bone matrix. Mutations in the coding regions of the genes for the two type I collagen chains (*COL1A1* and *COL1A2*) result in a severe autosomal dominant pediatric condition known as osteogenesis imperfecta [Sykes, 1990]. A G→T SNP at the first base of a consensus binding site for the transcription factor Sp1 in the first intron of *COL1A1* was associated not only with BMD in white women [Grant *et al.*, 1996] but also with osteoporotic fracture in postmenopausal women [Langdahl *et al.*, 1998; Uitterlinden *et al.*, 1998]. Other studies, however, showed only a weak association of this SNP with BMD or osteoporotic fracture in pre-menopausal French women [Garnero *et al.*, 1998], or a lack of association in postmenopausal women in Sweden [Liden *et al.*, 1998], in American women [Hustmyer *et al.*, 1999], or in postmenopausal Danish women [Heegaard *et al.*, 2000]. The *T* allele of the Sp1 binding site polymorphism affects collagen gene regulation in such a manner that it increases the production of the α1(I) collagen chain relative to that of the α2(I) chain and leads to reduced bone strength by a mechanism that is partly independent of bone mass [Mann *et al.*, 2001]. A –1997G→T SNP in the *COL1A1* promoter was also recently associated with BMD for the lumbar spine [Garcia-Giralt *et al.*, 2002]. The –1997G→T SNP, and the G→T SNP of the Sp1 binding site were shown to be in linkage disequilibrium [Garcia-Giralt *et al.*, 2002]. These observations thus suggest that genetic variants that affect type I collagen metabolism are important determinants of the development of osteoporosis or osteoporotic fracture.

TRANSFORMING GROWTH FACTOR-β1 GENE (*TGFB1*)

TGF-β1, which is produced by osteoblasts and is stored in substantial amounts in the bone matrix, is released during bone resorption and subsequently activated by the acidic microenvironment created by bone-resorbing osteoclasts [Oursler, 1994]. TGF-β1 is important in the proliferation and differentiation of, as well as in matrix production by, osteoblasts [Bonewald, 1996], and it stimulates bone formation *in vivo* [Noda and Camilliere, 1989]. TGF-β1 also inhibits both the differentiation of osteoclasts and osteoclastic bone resorption [Pfeilschifter *et al.*, 1988; Takai *et al.*, 1998]. In addition, estrogen stimulates the production

of TGF-β1 by human osteoblastic cells [Finkelman *et al.*, 1992], and TGF-β1 contributes to estrogen-induced apoptosis of osteoclasts, resulting in reduced bone resorption [Hughes *et al.*, 1996]. TGF-β1 may thus mediate the local beneficial effects of estrogen on bone remodeling. Moreover, TGF-β1–deficient mice exhibit reduced bone mass [Geiser *et al.*, 1998], suggesting that bone mass is regulated by TGF-β1.

A single nucleotide deletion in intron 4 (1553-8delC) of *TGFB1* was shown to be more frequent in individuals with osteoporosis than in normal controls [Langdahl *et al.*, 1997]. This deletion was also associated with low bone mass in osteoporotic women and with increased bone turnover in both osteoporotic and normal women. Several other polymorphisms of *TGFB1* have been described [Cambien *et al.*, 1996; Grainger *et al.*, 1999], among which we focused on an 869T→C SNP (Leu10Pro) that affects the amino acid sequence of the signal peptide of the encoded protein. We showed that the serum concentration of TGF-β1 increased according to the rank order of 869T→C genotypes *TT* < *TC* < *CC* in control subjects as well as in individuals with osteoporosis [Yamada *et al.*, 1998]. The serum concentration of TGF-β1 was also shown to increase according to the rank order of genotypes *CC* < *CT* < *TT* for a –509C→T SNP, with the concentration in individuals with the *TT* genotype being twice than that in individuals with the *CC* genotype [Grainger *et al.*, 1999]. However, we did not detect a significant difference in serum TGF-β1 concentration among –509C→T genotypes [Yamada *et al.*, 2001b]. The transcriptional activity of the –509*T* allele of *TGFB1* was shown to be slightly greater than that of the –509*C* allele [Luedecking *et al.*, 2000]. However, it remains unclear whether differences in the circulating concentration of TGF-β1 among individuals with different *TGFB1* genotypes are reflected in the concentrations of this cytokine in the microenvironment of bone.

We examined whether the 869T→C SNP of *TGFB1* is related to BMD and genetic susceptibility to osteoporosis in postmenopausal women in two different regions of Japan [Yamada *et al.*, 1998]. In both regions, BMD for the lumbar spine was greater in women with the *CC* genotype than in those with the *TC* or *TT* genotypes. Multivariate logistic regression analysis with adjustment for age, height, body weight, years since menopause, and smoking status revealed that the frequency of the *T* allele was higher in subjects with osteoporosis than in controls in both regions. We also studied the association of the 869T→C SNP of *TGFB1* with BMD in community-dwelling Japanese individuals [Yamada *et al.*, 2001a]. BMD at the distal radius was lower in women with the *T* allele than in those with the *CC* genotype. Evaluation of BMD according to successive age groups revealed that, for women in their 70s, BMD for the distal radius was lower in those with the *T* allele than in those with the *CC* genotype, with the difference being 18% of the larger value. These observations therefore suggested that *TGFB1* genotype is a determinant of BMD, and that the *T* allele of the 869T→C SNP is a risk factor for susceptibility to osteoporosis in Japanese women.

We studied the relation of the 869T→C SNP of *TGFB1* to the prevalence of vertebral fracture in postmenopausal Japanese women [Yamada *et al.*, 2000b]. The frequency of vertebral fracture was higher in individuals with the *T* allele than in those with the *CC* genotype. Multivariate logistic regression analysis with adjustment for age, height, body weight, smoking status, and body fat and lean mass revealed that the frequency of the *T* allele was greater in the vertebral fracture group than in the control group. Analysis with adjustment for lumbar spine or total body BMD in addition to the above parameters also yielded an association without a significant change in the odds ratio. These data provide evidence for an association of the 869T→C SNP of *TGFB1* with the prevalence of vertebral fracture, with the *T* allele being a risk factor for this condition in postmenopausal Japanese women, and they suggest that the effect of *TGFB1* genotype on the prevalence of this condition is, at least in part, independent of BMD.

Whether genotype for the 869T→C SNP of *TGFB1* affects the response of individuals with osteoporosis to treatment with active vitamin D or HRT was examined in postmenopausal Japanese women who were followed up for measurement of lumbar spine BMD [Yamada *et al.*, 2000a]. In the control group, the annual rate of bone loss decreased according to the rank order of genotypes *TT* > *TC* > *CC*, with a significant difference apparent between individuals with the *CC* genotype and those with the *TT* genotype. In the group treated with active vitamin D, women with the *TT* or *TC* genotypes lost bone at a rate similar to that of untreated women with the corresponding genotypes, whereas individuals with the *CC* genotype responded to treatment with an annual increase in lumbar spine BMD of 1.6%. In the HRT group, lumbar spine BMD increased irrespectively of 869T→C genotype; although the annual gain increased according to the rank order of genotypes *TT* < *TC* < *CC*, there were no significant differences among genotypes. Multivariate regression analysis, with adjustment for age, height, body weight, time since menopause, lumbar spine BMD, and follow-up period, revealed that the annual increase in BMD in individuals with the *CC* genotype who were treated with active vitamin D was significantly different from the annual loss in BMD in controls with the same genotype, whereas no difference was apparent between controls and active vitamin D–treated individuals with either the *TC* or *TT* genotype. For all genotypes, the annual gain in BMD in the HRT group was significantly different from the annual loss in BMD in controls. These results suggested that the 869T→C SNP affects both the rate of bone loss in postmenopausal women and the therapeutic response to active vitamin D in postmenopausal women with osteoporosis.

In general, the effect of active vitamin D on BMD is relatively small compared with that of HRT; however, our observations suggest that the apparently modest effect of active vitamin D on BMD is attributable to the inclusion of non-responders with the *TT* and *TC* genotypes, who constitute approximately 80% of the population. The molecular mechanism by which *TGFB1* genotype affects the response to active vitamin D therapy remains to be elucidated. Evidence suggests the existence of substantial cross talk between TGF-β signaling and signal transduction through the nuclear VDR [Yanagisawa *et al.*, 1999]. 1,25-Dihydroxyvitamin D_3 stimulates the production and release

of TGF-β in osteoblasts, and vitamin D deficiency results in reduced TGF-β concentrations in cortical bone [Finkelman *et al.*, 1991]. In addition, the amount of TGF-β stored in bone decreases with age [Nicolas *et al.*, 1994]. These observations suggest that the age-related decrease in the serum concentration of 1,25-dihydroxyvitamin D_3 may result in a similar reduction in the skeletal concentration of TGF-β, and a consequent increase in susceptibility to involutional osteoporosis.

Previous studies indicated that the –509C→T, and 869T→C SNPs of *TGFB1* are in linkage disequilibrium [Cambien *et al.*, 1996], suggesting the possibility that these SNPs cooperatively affect BMD and genetic susceptibility to osteoporosis. It was also possible that the association of the 869T→C SNP with BMD and the prevalence of osteoporosis was attributable to an effect of the –509C→T SNP on the transcriptional activity of *TGFB1*. We therefore investigated whether the –509C→T SNP of *TGFB1*, alone or in combination with 869T→C SNP, is associated with BMD and susceptibility to osteoporosis in postmenopausal Japanese women [Yamada *et al.*, 2001b]. Both lumbar spine and total body BMD were lower in individuals with the –509*TT* genotype than in those with the –509*CC* or the –509*CT* genotype. Multivariate logistic regression analysis with adjustment for age, height, body weight, time since menopause, smoking status, and fat and lean mass, revealed that the –509*TT* genotype was associated with susceptibility to osteoporosis. Analysis of combined genotypes revealed that lumbar spine BMD decreased as the number of *T* alleles increased. Individuals with both the –509*CC* and 869*CC* genotypes showed the highest BMD, and those with both the –509*TT* and 869*TT* genotypes showed the lowest BMD, with the difference in lumbar spine BMD between these groups being 38% of the larger value. We further studied the effect of the number of *T* alleles in the combined genotype on susceptibility to osteoporosis. Multivariate logistic regression analysis with adjustment for the same parameters revealed that the prevalence of osteoporosis was significantly greater among individuals with ≥3 *T* alleles than among those with ≤1 *T* allele. The –509C→T and 869T→C SNPs thus exhibited additive effects on BMD and on the prevalence of osteoporosis. These observations suggest that the effects of the –509C→T and 869T→C SNPs on BMD are, at least in part, independent.

CONCLUSION

In this review, I have summarized the candidate loci and polymorphisms in candidate genes related to bone mass or to predisposition to osteoporosis or osteoporotic fracture, both of which are major age-related disorders of the human skeleton. The studies described indicate the existence of a substantial genetic component of osteoporosis. However, despite the identification of several candidate genes related to osteoporosis, the replicability of such findings is poor, mainly because of the limited population size of the studies, the ethnic diversity of gene polymorphisms, and complicating environmental factors. Large-scale linkage analyses and population-based association studies in various ethnic groups are thus required to identify definitively the genes that determine bone mass or susceptibility to osteoporosis or osteoporotic fracture.

ABBREVIATIONS

BMD	=	Bone mineral density
TNF	=	Tumor necrosis factor
IL	=	Interleukin
CCR2	=	CC chemokine receptor 2
ERα	=	Estrogen receptor α
MMP-1	=	Matrix metalloproteinase–1
VDR	=	Vitamin D receptor
TGF-β1	=	Transforming growth factor–β1
SNP	=	Single nucleotide polymorphism
MCP	=	Monocyte chemoattractant protein
HRT	=	Hormone replacement therapy.

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ORIGINAL INVESTIGATION

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Association of the mitochondrial DNA 15497G/A polymorphism with obesity in a middle-aged and elderly Japanese population

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Abstract Although polymorphism of the mitochondrial DNA 15497guanine/adenine (Mt15497G→A) leads to the Gly251Ser amino acid replacement on human cytochrome *b*, it is unknown whether functional alteration of the mitochondrion is induced by the Gly251Ser replacement. To see if an association exists between the Mt15497G→A polymorphism and obesity, we examined differences in body size, body composition, and regional body fat distribution between the two genotypes in middle-aged and elderly Japanese individuals (825 women and 906 men). The Mt15497 genotype was determined with an automated colorimetric allele-specific DNA probe assay system using the polymerase chain reaction (PCR) method. The Mt15497G→A polymorphism was detected in 3.5% (n=60) of all subjects: 2.8% (n=23) among women and 4.1% (n=37) among men. After adjusting for age and smoking, we found that body weight, body mass index, waist and hip circumferences, fat mass, fat-free mass, intra-abdominal fat and triglycerides were significantly greater in women with the A allele compared with the G allele (p=0.001–0.025). For men, waist to hip ratio was significantly greater (p=0.032), and waist circumference, intra-abdominal fat and triglycerides had a trend to be significantly greater (p=0.062–0.087) in subjects with the A allele compared with the G allele. These data suggest that the Mt15497 polymorphism may be associated with obesity-related variables and lipid metabolism.

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Introduction

Obesity probably develops through interaction of both genetic and environmental factors. Polymorphisms of leptin, the UCP family, and beta 3 adrenergic receptor genes are examples of some of the genetic factors predisposing individuals to obesity (Bouchard et al. 1988). Variations in mitochondrial (mt) DNA also have emerged as possible genetic factors that lead to a high BMI (Rowe et al. 1991). Hegel et al. (1997) and Kokaze et al. (2001) reported on the association between plasma triglycerides concentration and polymorphisms of mtDNA 16517 and 5178, respectively. They speculated that a polymorphism of mtDNA may partially alter the function of the mitochondrial β-oxidation of fatty acid. Moreover, qualitative change in the mitochondria may affect the function of the TCA cycle and energy consumption within skeletal muscle. Hence, functional alterations of mtDNA could facilitate development of obesity. Dionne et al. (1992) observed in a monozygotic twin study that the mtDNA D-loop *Kpn*I restriction site polymorphism was associated with weight gain after a 100 day over-feeding period. Other studies (Merriwether et al. 1995; Rowe et al. 1997) also suggest that mtDNA polymorphisms play a pivotal role in obesity.

Understanding the association between mtDNA polymorphisms and obesity may be helpful in preventing obesity-related chronic diseases. We detected a novel mtDNA nucleotide variation: polymorphism of mtDNA 15497 guanine/adenine (Mt15497G→A). Although Mt15497G→A leads to the Gly251Ser amino acid replacement on human cytochrome *b*, it is unknown whether a functional alteration of the mitochondrion is induced by the Gly251Ser replacement (Tanaka et al. 2002). Therefore, to elucidate an association between the Mt15497G→A polymorphism and obesity, we examined a relatively large sample size of

middle-aged to elderly Japanese individuals comparing body size, body composition, and regional body fat distribution of subjects carrying the G or A alleles of Mt15497.

Materials and methods

Subjects

The subjects were 825 women and 906 men who participated in the 2nd wave of examinations in the National Institute for Longevity Sciences-Longitudinal Study of Aging (NILS-LSA) from April 2000 to April 2002. These were randomly sampled, community-dwelling individuals aged 42 –82 years, stratified by age and gender, living in the neighborhood of the NILS. Details of the NILS-LSA have been described elsewhere (Shimokata et al. 2000). Physical characteristics of subjects are shown in Table 1. The aim and design of the study was explained to each subject before they gave their written informed consent. The study protocol was approved by the Committee on Ethics of Human Research of National Chubu Hospital and the NILS.

Determination of Mt15497 genotype

Mt15497 genotype was determined with an automated colorimetric allele-specific DNA probe assay system (Toyobo Gene Analysis, Tsuruga, Japan). In brief, the polymorphic region of the gene was amplified by polymerase chain reaction (PCR) with allele-specific sense (5′-TATTCTCACCAGACCTCCTXGG-3′ and 5′-ACTATTCTCACCAGACCTCCTXAG-3′) and biotin-labeled antisense (5′-GTGTTTAAGGGGTTGGCTAGG-3′) primers. The reaction mixture (25 µl) contained 50 ng of DNA, 5 pmol of each primer, 0.2 mmol/l of each deoxynucleoside triphosphate, 2.5 mmol/l $MgCl_2$, and 1 U of DNA polymerase (rTaq; Toyobo, Osaka, Japan) in rTaq buffer. The amplification protocol consisted of initial denaturation at 95°C for 5 min; 35 cycles of denaturation at 95°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 30 s; and a final extension at 72°C for 2 min. Amplified DNA was denatured with 0.3 M NaOH and then subjected to hybridization at 37°C for 30 min in hybridization buffer containing 35% formamide with allele-specific capture probes (5′- TCCTXGGCGACCCAGACAA-3′ or 5′- CTCCTXAGCGACCCAGACAAT-3′) fixed to the bottom of the wells of a 96-well plate. After thorough washing of the wells, alkaline phosphatase-conjugated streptavidin was added to each well and the plate was incubated at 37°C for 15 min with agitation. The wells were again washed, and after the addition of a solution containing 0.8 mmol/l 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium (monosodium salt) and 0.4 mmol/l 5-bromo-4-chloro-3-indolyl phosphate p-toluidine salt, absorbance at 450 nm was measured.

Anthropometric variables

Body weight was measured to the nearest 0.01 kg using a digital scale, height was measured to the nearest 0.1 cm using a wall-mounted stadiometer, and body mass index (BMI) was calculated as weight (kg) divided by height squared (m^2). Waist circumference and waist-to-hip ratio were used as the indices for body fat distribution in this study. The waist-to-hip ratio was calculated as a ratio of waist circumference measured at the level of the umbilicus to hip circumference.

Table 1 Descriptive characteristics of subjects (n=1731)

Variables	Women	Men
Number of subjects	825	906
Age (year)	60.1±0.4	60.8±0.3
Height (cm)	151.7±0.2	164.6±0.2
Body weight (kg)	52.6±0.3	62.7±0.3
Body mass index (kg/m^2)	22.9±0.1	23.1±0.1
Percentage fat mass (%)	30.8±0.2	21.3±0.1

Body composition by dual-energy x-ray absorptiometry

Whole-body fat mass, fat-free mass, and percentage fat mass assessed by dual-energy x-ray absorptiometry (QDR-4500, Hologic, Madison, OH, USA) were used as the indices for determining body composition. Transverse scans were used to measure fat mass and fat-free mass, and pixels of soft tissue were used to calculate the ratio (R value) of mass attenuation coefficients at 40–50 keV (low energy) and 80–100 keV (high energy) using software version 1.3Z.

Abdominal adipose tissue area by CT

The intra-abdominal fat area (IFA) and subcutaneous fat area (SFA) were measured at the level of the umbilicus (L4-L5) using computed tomography (CT) scans (SCT-6800TX, Shimadzu, Tokyo, Japan) carried out on subjects in the supine position. The IFA and SFA were calculated using a computer software program (FatScan, N2system, Osaka, Japan) (Yoshizumi et al. 1996). Firstly, a region of the SF layer was defined by tracing its contour on each scan, and the range of CT values (in Hounsfield units) for fat tissue was calculated. Total fat area was determined by delineating the surface having a mean CT value plus or minus two standard deviations, and the IFA was measured by drawing a line within the muscle wall surrounding the abdominal cavity. The SFA was then calculated by subtracting the IFA from the total fat area, and the IFA to SFA (I/S) ratio was determined. The intra-class correlation for repeated IFA determinations in our laboratory is 0.99.

Biochemical examination of blood

An antecubital blood sample was drawn from each subject after an overnight fast. Serum total cholesterol and triglycerides were determined enzymatically, serum high-density lipoprotein cholesterol was measured by the heparin-manganese precipitation method and fasting plasma glucose was assayed by a glucose oxidase method. Plasma insulin was measured in duplicate by radioimmunoassay. Glycosylated hemoglobin (HbA_{1c}) was measured by high performance liquid chromatography. Serum low-density lipoprotein cholesterol was estimated according to the Friedewald formula (1972).

Data analysis

Values are expressed as mean ± standard error (SE) in the tables. Quantitative data were compared by General Linear Model with age and smoking as covariates. Qualitative data were analyzed by the chi-square test. In each statistical analysis, probability values below 0.05 were regarded as significant. The data were analyzed with the Statistical Analysis System (SAS), version 8.2.

Results

The Mt15497G→A polymorphism was detected in 3.5% (n=60) of all subjects: 2.8% (n=23) of the women and 4.1% (n=37) of the men. In both sexes, age, smoking status, and height were similar between the Mt15497G→A genotypes (Table 2). To examine the gender's influence on the relationship between the Mt15497G→A genotypes

Table 2 Anthropometric variables, body composition and abdominal adipose tissue areas of subjects according to sex and Mt 15497G→A genotype

	Women			Men		
	G	A	*p* value	G	A	*p* value
Variables						
Number (%)	802(97.2)	23(2.8)	-	869(95.9)	37(4.1)	-
Age (year)	60.1±0.4	61.3±2.2	0.565	60.7±0.4	62.1±1.7	0.430
Smoking (%)	7.0	8.7	0.685	36.7	43.2	0.373
Anthropometric variables						
Height (cm)	152±0.3	153±1.2	0.462	165±0.2	166±1.1	0.263
Body weight (kg)	52.6±0.3	57.5±1.7	0.004	62.6±0.3	64.2±1.5	0.318
Body mass index (kg/m^2)	22.8±0.1	24.8±0.7	0.004	23.1±0.1	23.4±0.5	0.530
Waist circumference (cm)	83.0±0.3	88.0±2.0	0.014	85.0±0.3	87.4±1.3	0.087
Hip circumference (cm)	90.8±0.2	94.3±1.1	0.002	92.2±0.2	92.7±0.8	0.524
Waist to hip ratio	0.91±0.003	0.93±0.02	0.254	0.92±0.01	0.94±0.01	0.032
Body composition by dual-energy x-ray absorptiometry						
Percentage fat mass (%)	30.8±0.2	32.4±1.1	0.139	21.3±0.2	22.0±0.7	0.312
Fat mass (kg)	16.4±0.2	18.7±1.0	0.025	13.5±0.1	14.3±0.7	0.273
Fat-free mass (kg)	36.1±0.2	38.6±1.0	0.010	49.0±0.2	49.9±1.1	0.445
Abdominal adipose tissue area by computed tomography						
Intra-abdominal fat-area (cm^2)	63.7±1.5	84.4±8.6	0.017	93.7±1.7	110±8.5	0.065
Subcutaneous fat area (cm^2)	165±2.4	182±13.9	0.237	112±1.6	114±7.9	0.862
I/S ratio	0.40±0.01	0.48±0.05	0.122	0.86±0.01	0.99±0.07	0.062

I/S ratio: ratio of intra-abdominal and subcutaneous adipose tissue area. Data were adjusted for age and smoking

Table 3 Biochemical measurements according to sex and Mt 15497G→A genotype

	Women			Men		
	G	A	*p* value	G	A	*p* value
Variables						
Total cholesterol (mg/dl)	223.1±1.2	217.8±7.1	0.462	210.9±1.7	214.4±5.6	0.540
Triglycerides (mg/dl)	105.4±2.3	151.5±13.1	0.001	128.2±2.9	152.0±13.6	0.087
LDL cholesterol (mg/dl)	135.3±1.1	126.8±6.4	0.190	127.3±1.1	127.7±5.3	0.952
HDL cholesterol (mg/dl)	66.5±0.5	60.7±3.2	0.074	57.6±0.5	56.3±2.4	0.599
Glucose (mg/dl)	98.9±0.8	101.8±4.4	0.522	105.4±0.8	104.5±3.8	0.802
HbA_{1c} (%)	5.3±0.03	5.3±0.16	0.807	5.4±0.03	5.4±0.15	0.597
Insulin (μU/ml)	8.0±0.2	9.6±1.3	0.223	8.2±0.3	8.9±1.4	0.653

Data were adjusted for age and smoking

and anthropometric variables, body composition, abdominal adipose tissue areas, and biochemical blood parameters, we analyzed the data for men and women independently.

Anthropometric variables

For women, body weight, BMI, and waist and hip circumferences were significantly greater in subjects with the A allele than in those with the G allele (p=0.002–0.014). For men, waist to hip ratio was significantly greater in subjects with the A allele than in those with the G allele (p=0.032) and a trend toward significant difference was found in waist circumference (p=0.087). Although statistical significance was not achieved in any other variables (p>0.05), all measurement values were greater in subjects with the A allele than in those with the G allele.

Body composition

For women, both fat mass and fat-free mass were significantly greater in subjects with the A allele than in those with the G allele (p=0.025 and 0.010, respectively). For men, although no significant difference was found in any measurement variables between the genotypes, all measurement values were greater in subjects with the A allele than in those with the G allele.

Abdominal adipose tissue area

For women, IFA was significantly greater in subjects with the A allele than in those with the G allele (p=0.017). For men, a trend toward significant difference was found in IFA (p=0.065) and I/S ratio (p=0.062).

We next examined the relationship of the Mt15497G→A polymorphism with biochemical parameters for lipid and glucose metabolism (Table 3). For women, triglycerides were significantly higher in subjects with the A allele than in those with the G allele (p=0.001). Plasma insulin was also 20% higher in subjects with the A allele compared with the G allele, but statistical significance was not achieved. For men, a trend toward significant difference was found in triglycerides (p=0.087).

Discussion

The mitochondrial oxidative phosphorylation system is a major source of energy utilization for cellular activities. Therefore, we tested a hypothesis that an association exists between the Mt15497 polymorphism and obesity-related measurement variables by examining a relatively large sample size of middle-aged to elderly Japanese men and women. Our data revealed that several obesity-related variables were significantly different between subjects having either the G or A alleles of Mt15497. It may be that increased efficiency of mitochondrial energy conservation at the cytochrome *bc*1 complex results in decreased energy consumption (Tanaka et al.2002). Another possibility is that inhibiting reduction in ubiquinone at the Qo site (one of the ubiquinone-binding sites of complex III) results in a reduced β-oxidation of fatty acid, which leads to fat accumulation. We are currently constructing cybrid clones carrying the Mt15497G→A polymorphism to determine whether the G251S replacement results in decreased activity of ubiquinol-cytochrome *c* reductase (complex III) or not. Instead, we carried out a molecular dynamic simulation to understand the effect of the G251S replacement to the molecular structure of cytochrome *b* in our database system http://www.giib.or.jp/mtsnp/search_mtSAP_3D_e.html. Andreu et al. argued that the G251D replacement due to the Mt15498G→A mutation is pathogenic because the presence of Asp instead of Gly should cause charge repulsion with Glu271, a residue at the Qp site (Andreu et al. 2000). This, in turn, would change the structure of the Qp site and impair hydroquinone binding.

For women, anthropometric variables, body composition and abdominal adipose tissue area were significantly associated with the Mt15497 polymorphism, whereas these associations were weaker in men compared with women. There is other evidence supporting the idea that the association of genetic variation with obesity is stronger in women than men (Borecki et al. 1993; Comuzzie et al. 1995). For instance, Comuzzie et al. (1995), in a Mendelian mixed model analysis for fat mass, incorporating genotype by gender interaction, reported that the major gene accounted for 37% of the total variance of fat mass in men compared with 43% in women. To our knowledge, however, little has been reported on gender's effect on the association between a mtDNA polymorphism and obesity.

It is well known that waist size and the amount of intra-abdominal adipose tissue are strongly associated with various risk factors for coronary heart disease [e.g., hypertension (Kanai et al. 1990; Matsuzawa et al. 1995), Type 2 diabetes (Yamashita et al. 1996; Macor et al. 1997) and Type 1 plasminogen activator inhibitor (Svendsen et al. 1996; Lindahl et al. 1998)]. Japan Society for the Study of Obesity (2002) defined "obesity disease" as not only the presence of obesity-related complications, but also their likely occurrence. In this case, "likely occurrence" means high-risk obesity as specified by an excess IFA (greater than 100 cm^2) measured by CT scan. Thus, individuals with high-risk obesity have a strong chance of suffering from obesity-related complications in the near future.

The results presented in Table 3 show a significant association in women and a trend toward significant association in men between high triglycerides levels and the Mt15497 polymorphism. In addition, plasma insulin level was 20% higher in women with the A allele compared to those with the G allele (see Table 3). Kokaze et al. (2001) found in their epidemiological study that the Mt5178A/C polymorphism was associated with the triglycerides level in Japanese women, and Gerbitz (1992) reported that impairment of ATP production by a mtDNA mutation caused insulin secretion defects and possibly insulin resistance as well. These reports suggest that mtDNA polymorphisms impair lipid metabolism and insulin secretion through a defect of mitochondrial function.

Detailed causes of the significant difference found in fat-free mass between the Mt15497 genotypes in women could not be clarified. Keightley et al. compared 10 cases of cytochrome *b* mutation and found that most of the patients in those studies presented with the predominant feature of severe exercise intolerance or hypertrophic cardiomyopathy (Keightley et al. 2000). Andreu et al. reported that the G251D replacement due to the Mt15498G→A mutation led to heart failure (histiocytoid cardiomyopathy) (Andreu et al. 2000). However, it is unknown how the Gly251Ser replacement by the Mt15497G→A mutation affects the human body. Finding of larger fat-free mass in subjects with the A allele of Mt15497 in our study is likely to indicate that increased fat mass indirectly affects the fat-free mass.

In conclusion, we have shown in a relatively large sample of middle-aged to elderly Japanese that significant associations exist between the Mt15497 polymorphism and body size, body composition, abdominal adipose tissue area, and lipid metabolism. Although these data suggest that the A allele of Mt15497 may be one of the important determinants of obesity, further studies are needed to validate our speculation.

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Brief Research Communication

Association of Cholecystokinin-A Receptor Gene Polymorphisms and Panic Disorder in Japanese

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Several lines of evidence have suggested that naturally occurring alterations in cholecystokinin (CCK) systems could contribute to the development of panic disorder (PD). Among recent investigations, polymorphisms of the CCK and CCK-B receptor (R) genes were investigated, but the results were inconclusive. We recently cloned the genomic structures of human CCK-AR, and determined the transcriptional start site of the human CCK-AR gene. Two sequence changes were detected in the promoter region: a G to T change in nucleotide −128 and an A to G change in nucleotide −81 (GenBank database under accession number D85606). The frequencies of the genotypes and haplotypes of these two polymorphisms were compared in 109 Japanese patients with PD and 400 age- and gender-matched normal Japanese control subjects. The frequency of variant genotypes (−81A/G, −128G/T; G/G, G/T, and G/G, T/T) having variant haplotype (−81G/−128T) was significantly higher in PD than in controls ($P < 0.0001$, OR = 2.81, 95% CI = 1.74–4.39). The statistical differences between the haplotype distributions in the PD and control groups were highly significant: the frequency of variant haplotype (−81G/−128T) was higher in the former group than in the latter ($P < 0.0001$). This association was not affected by clinical characteristics such as age, gender, and age at onset of PD. In this study, the first to report the positive association of the CCK-AR polymorphisms and PD, haplotype analyses further strengthened the association based on our comparison of genotype distributions. The CCK-AR gene polymorphism may be involved in the neurobiology of PD.

KEY WORDS: panic disorder; cholecystokinin; CCK-A receptor; gene; polymorphism

Panic disorder (PD) is a common anxiety condition, characterized by unprovoked anxiety attacks distinguished by such symptoms as palpitations, chest pain, dyspnea, choking, tremors, faintness, and sweating, in addition to fear of dying, losing control, or going crazy [American Psychiatric Association, 1987]. The carboxy-terminal tetrapeptide of cholecystokinin (CCK-4) induces panic-like attacks when administered as an intravenous bolus in healthy volunteers, and in patients with PD [De Montigny, 1989; Bradwejn et al., 1991].

CCK is a classical gastrointestinal hormone and one of the most abundant neurotransmitter peptides in the brain. CCK receptor (R)s have been classified into two subtypes, CCK-A and CCK-B, on the basis of their affinities for a structurally and functionally related family of peptides that have identical COOH-terminal pentapeptide sequences but differences in sulfation at the sixth (gastrin) and seventh (CCK) tyrosyl residues [Wank, 1995]. Among recent investigations [Wang et al., 1998; Kennedy et al., 1999; Hamilton et al., 2001; Hattori et al., 2001a,b; Yamada et al., 2001] examined polymorphisms of the CCK and CCK-BR genes, but the results were inconclusive. There has been only one study to determine the CCK-AR gene polymorphism with no association [Kennedy et al., 1999], which was 5′ area of the 3′ untranslated region, and its functional role is unknown.

We recently cloned the genomic structures of human CCK-AR [Funakoshi et al., 2000], and determined the transcriptional start site of the human CCK-AR gene. Two sequence changes were detected in the promoter region: a G to T change in nucleotide −128 and an A to G change in nucleotide −81 [GenBank database under accession number D85606, Funakoshi et al., 2000]. Six genotypes, including a wild type (−81A/A, −128G/G) and five other variants, have been identified [Funakoshi et al., 2000; Shimokata et al., 2000]. The homozygote (−81G/G, −128T/T) showed a significantly higher percent body fat, although the real mechanism has not been clarified. In this study, we investigated a possible association between the CCK-AR gene and PD by evaluating the distribution of not only the genotypes but also the haplotypes of the two polymorphisms.

The subjects consisted of 109 Japanese patients with PD (64 males, 18–63 years old; 45 females, 21–71 years old), all of whom met DSM-III-R criteria for PD on the PD part of the Structured Clinical Interview for DSM-III-R (SCID) assessment. The age- and gender-matched control group consisted of 400 unrelated Japanese. The controls were employees and students in Kurihama National Hospital and in the Tokyo Metropolitan Institute of Gerontology. Nobody shows signs of

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psychiatric disorders (234 males, 20–62 years old; 166 females, 21–71 years old). The Ethics Committees of the National Alcoholism Center, Kurihama Hospital, and Tokyo Metropolitan Institute of Gerontology approved this study. Written informed consent was obtained from each subject. Genomic DNA was extracted from peripheral leucocytes.

Examination of the polymorphism in the promoter region of the CCK-AR gene was accomplished using a mismatch PCR-RFLP method [Funakoshi et al., 2000]. Briefly, a pair of primers (sense primer = 5′-GCATATGTACACATGTGTGT-AAAAAGCAGCCAGAC-3′, and anti-sense primer = 5′-GCC-CTTTCCTGGGCCAGACT-3′) were used to amplify the 103-bp product, which was subsequently digested with restriction enzyme *Hinf* I and fractionated by 12% polyacrylamide gel electrophoresis.

Statistical differences between PD and control subjects were assessed using Fisher's exact test. An odds ratio with 95% confidence intervals was calculated to evaluate the difference in genotype frequencies between the groups. Probability differences of $P < 0.05$ were considered statistically significant. To assess linkage disequilibrium between the two polymorphisms of the CCK-AR gene, we calculated the D value and its significance, using the ASSOCIAT program downloaded from the web site of Dr. J. Ott (ftp://linkage.rockefeller.edu/software/utilities/). All statistical computations were carried out using the Statistical Analysis System package, version 6.12 [SAS Institute Inc, 1988].

Comparison of the genotype and haplotype distributions of the CCK-AR gene −81A to G and −128G to T polymorphism in PD patients and control subjects (Table I) revealed frequencies among the controls that were quite similar to those reported in community-dwelling individuals. Three kinds of genotypes (−81A/A, −128T/T), (−81A/A, −128G/T), and (−81A/G, −128T/T) were not detected in the previous cohort studies [Funakoshi et al., 2000; Shimokata et al., 2000] and in the present study. Therefore, haplotype −81A/−128T was not present, either. These polymorphisms were in linkage disequilibrium (PD samples, D = 0.1495, $P < 0.0001$: controls, D = 0.0865, $P < 0.0001$). Both genotypic frequencies of distributions were in Hardy–Weinberg equilibrium.

TABLE I. Genotype and Haplotype Frequencies of the −81A to G and −128G to T Polymorphisms in Patients With Panic Disorder and Controls

	Polymorphisms		Panic disorder	Controls
	−81	−128	N (%)	N (%)
Genotype[a]			N = 109	N = 400
	A/A	G/G	48 (44.0%)	238 (59.5%)
	A/G	G/G	13 (11.9%)	71 (17.8%)
	A/G	G/T	36 (33.0%)	75 (18.8%)
	G/G	G/G	1 (0.9%)	6 (1.5%)
	G/G	G/T	9 (8.3%)	6 (1.5%)
	G/G	T/T	2 (1.8%)	4 (1.0%)
OR (95% CI)[b]			2.81 (1.74–4.39)	
Haplotype[c]			N = 218	N = 800
	A	G	145 (66.5%)	622 (77.8%)
	A	T	0 (0.0%)	0 (0.0%)
	G	G	24 (11.0%)	89 (11.1%)
	G	T	49 (22.5%)	89 (11.1%)

[a]Percentages may not total 100 due to rounding. Three genotypes (−81A/A, −128T/T), (−81A/A, −128G/T), and (−81A/G, −128T/T) were not present. $P < 0.0001$ (df = 5), $P < 0.0001$ (with −81G/−128T haplotype vs. without −81G/−128T haplotype, df = 1) when analyzed by Fisher's direct test.

[b]Ratio of odds (genotypes with −81G/−128T haplotype/genotypes without −81G/−128T haplotype) and 95% confidence interval.

[c]Haplotype (−81A/−128T) was not detected. $P < 0.0001$ when analyzed excluding −81A/−128T haplotype (df = 2), $P < 0.0001$ when compared between subjects with and without −81G/−128T haplotype (df = 1).

The frequency of variant genotypes (−81A/G, −128G/T; G/G, G/T, and G/G, T/T) having variant haplotype (−81G/−128T) was significantly higher in PD than in controls ($P < 0.0001$, OR = 2.81, 95% CI = 1.74–4.39). The statistical differences between the haplotype distributions in the PD and control groups were highly significant: The frequency of variant haplotype (−81G/−128T) was higher in the former group than in the latter ($P < 0.0001$; Table I).

Stratification of the PD samples and controls with respect to age and gender did not alter these relationships. Nor did the age at onset of PD affect the distributions of the CCK-AR gene polymorphisms (data not shown).

The frequencies of both the variant genotypes and haplotypes of the −81A to G and −128G to T polymorphisms of the CCK-AR gene were higher in our PD group than among our control subjects, suggesting that this gene is involved in the development of PD.

CCK-AR is expressed in specific brain regions such as the amygdala, nucleus tractus solitarius, posterior nucleus accumbens, ventral tegmental area, hypothalamus, substantia nigra, hippocampus, area postrema, and raphe nucleus, whereas CCK-BR is widely distributed throughout the central nervous system [Wank, 1995]. The expression patterns of these receptors overlap in the brain, and the cross-reactivity of each antagonist could not be excluded in pharmacological studies. Therefore, the functional differences of these two receptors remain unclear. Recently, we developed CCK-AR, BR, and ARBR gene knockout (−/−) mice and found that CCK-AR and BR may exert opposite influences on anxiety-related behaviors [Miyasaka et al., 2002a,b]. These evidences suggest that CCK-AR might be involved in induction of panic like attacks, although CCK-4 is a ligand of CCK-BR.

Our research has focused on two neighboring polymorphisms in the 5′ regulatory region of the CCK-AR gene, which shares the region involved in the regulation of the human CCK-AR promoter function [Takata et al., 2002]. We have examined CCK-AR gene polymorphisms in 50 patients with gallstone and 300 patients with diabetes mellitus before the establishment of RFLP method [Funakoshi et al., 2000]. We found one case with G to A in the intron 1, and another case C to G in the exon 3 without change in amino acid (Thr). The polymorphisms of promoter region (between −351 and +176) were also examined and no polymorphisms besides −81A to G and −128G to T were detected. Therefore, although various kinds of CCK-AR polymorphisms have been reported [Inoue et al., 1997; Tachikawa et al., 2001, Okubo et al., 2002], these may occur sporadically.

Although our recent investigation using the STC-1 murine neuroendocrine cell line showed that neither the −81A to G nor the −128G to T polymorphism affects luciferase activities [Takata et al., 2002], limitations in the experimental conditions suggest that those findings should be interpreted as inconclusive, because no human cell lines have been available. In a recent examination of the correlation of demethylation of the CCK-AR gene and its expression, we found significantly higher gene expression when the methylation level of the gene was low [Matsusue et al., 1999; Miyasaka et al., 2002a,b]. We observed many GC-rich segments in the CCK-AR promoter region, and the nucleotide position at −128 was methylated. Thus, a G to T replacement at the −128 position might be capable of altering CCK-AR gene expression.

In this study, the first to report the positive association of the CCK-AR polymorphisms and PD, haplotype analyses further strengthened the association based on our comparison of genotype distributions.

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日本人の長寿要因

Q 日本人が世界的にみて長寿である要因として、どのようなものが考えられるか。

（静岡県　I）

A 日本人の平均寿命は平成一三年度では男性七八・〇七歳、女性八四・九三歳である。平均寿命の諸外国との比較は、国によって統計作成期間が異なるので厳密な比較は困難であるが、日本人の寿命が世界一の水準であることはほぼ間違いない。

では、日本人の平均寿命がなぜ長いのか、これに対する答を出すには、さまざまな国に住む集団を対象にして、数多くの長寿要因に関する詳細な国際的比較研究を行うことが必要であろう。しかし、そのような研究の実施は難しく、問に対する明確な答は今のところ出されていない。ここではいくつかの可能性のある長寿要因を述べてみる。

まず、日本における医療制度の充実と社会的な長寿要因の存在である。日本人の乳幼児の死亡率は諸外国に比べて低い。小児医療が充実しており、乳幼児の健康が、そして生命が手厚く守られている。また、国民皆保険制度の存在や高齢者に対する医療制度が比較的整備されていることも重要であろう。老人健診などの健康診断も広く実施されて、健康増進や病気の早期発見、早期治療につながっている。

日本人は高齢になっても勤労意欲が高く、また実際に社会参加率が高い。高齢者の社会参加が寿命の延長につながっているということを示す研究結果も出されている。日本の社会が比較的平等で、貧富の差が少ないことも長寿要因となっているかもしれない。米国のような自由競争社会では劣悪な健康状態を強いられている貧困層が存在し、国民全体の平均寿命を短くしている。また、日本では諸外国に比べ学校教育が充実している。教育によって国民全体の健康に関する知識や関心が高まっていると思われる。

日本人の食事や運動、入浴などのライフスタイルが長寿に適していることも考えられている。日本には独特の食習慣がある。先進諸国中で脂肪摂取量が飛び抜けて少なく、米飯を中心として炭水化物の摂取が多い。また、魚の摂取が多いことも特徴である。豆腐や納豆、味噌などの大豆製品の摂取が多く、これらは動脈硬化の進行を防ぐには理想に近い食習慣である。またカテキンやビタミンCなどの抗酸化物質が多く含まれる緑茶の摂取は、動脈硬化や癌を防いでいる可能性がある。高齢になっても社会参加を続けていることで運動量を保つことができている。清潔好きも重要な要因であろう。毎日入浴し、身の回りを常に清潔に保っている。このことが感染症の予防につながっていると推測される。

最後に、日本人の遺伝素因についても考える必要がある。日本人と共通の祖先を持つモンゴロイドの人たちは氷河期にベーリング海峡を渡り新大陸に移り住むことができた。白人の祖先は暑さと皮膚癌を引き起こす紫外線に耐えられず、黒人の祖先は寒さに耐えられなかった。モンゴロイドだけが極寒の北極圏に住むイヌイットからアマゾンの熱帯雨林地帯に住むインディオまで、あらゆる環境に適応し生き残ってきた。この適応力の高さが現在の日本人の長寿命につながっているのかもしれない。

しかし、長く生きられることを単純に喜んではいられない。高齢になるほどアルツハイマー病やパーキンソン病など慢性に経過する老年病に罹患する患者数が増えてくる。これらの疾病は直接、死につながるわけではないが、長期にわたって慢性的に進行し、人格を崩壊させたり寝たきりにさせたりして、精神的・肉体的に本人や周囲の人々を苦しめる。単なる長生きの「長命」ではなく、元気で健康な生き生きとした長生きである「長寿」を目指す長寿医療の推進が、これからの日本にはぜひとも必要である。

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解　　説

特集

老年症候群

加齢変化と老年症候群

Physical and mental changes with aging and geriatric syndrome

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Key word　高齢者　老化　加齢変化　老年症候群

Ⅰ．老化と加齢変化(図1)

人は誰でも老いてくると多かれ少なかれ心身の衰えを自覚する．加齢に伴う心身の変化を一般に「加齢変化」と呼んでいる．しかし実生活の中で顕著な症状や障害を持たないままに天寿を全うする人がいる一方で，高齢者に特有な症状や疾患で苦しむ人は，加齢とともに加速度的に増えていく．高齢になるほど拡大するこの個体差は何に起因するのだろうか．

加齢に伴う身体機能変化の原因は概念的に次の3つに分類される．

(1)加齢に伴って必然的に起こる機能低下

(2)長い人生において環境や生活習慣から受けてきた好ましくない影響による機能低下の加速

(3)疾患・障害による機能の悪化

(1)は人類としての種の遺伝子に組み込まれた，万人に認められる普遍的・不可逆的な老化として従来「生理的老化」と呼ばれてきた．しかし近年，遺伝疫学研究がさかんになり，生来獲得されている遺伝子多型もまた，加齢変化の個体差に影響を与えることがわかってきた．遺伝子によって規定された，内因的・必然的な加齢変化にも「個体差」があることが判明したのである．

理想的な老化像は概念的には最適な遺伝子多型の組み合わせを持ち，有害な環境要因・生活習慣に一切暴露しないことであろう(理想的老化，図1A)．

しかし実際には誰しもがいくつかの老年病にかかわる遺伝子多型を持ち，長年の生活習慣や環境要因への影響を受けるために理想的老化よりも加速された老化を受けることになる．老化の危険因子が少なければ，生理機能の加齢に伴う低下は緩やかに進み，日常生活に不自由を感じることがほとんどなく，天寿を全うする(図1B)．

老化に伴う機能低下は糖尿病，高血圧症，心臓病，脳血管障害などの高齢者特有の疾患(老年病)の易罹患性を高める．老年病関連の遺伝子多型が集族し，好ましくない生活習慣・環境要因が多いと老年病のリスクは増大する．老年病は多くの場合慢性的な機能低下を伴うために発病ごとに機能は悪化する(図1C)．

集団を対象とした疫学的研究では，高齢者における経年的な機能低下を「必然的な老化」，「個体差としての老化」，「疾患の影響」に分類することが可能であろうが，個体レベルでこの3つの変化を判別することは困難である．臓器に認められる

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