and walked supported at $2\frac{3}{4}$ years. His height at age 10 years was 137.6 cm (+0.4 SD), weight 36 kg (+0.7 SD), and OFC 53.5 cm (+0.3 SD). He had frontal bossing, mid-face hypoplasia, down-slanting palpebral fissures, a high-arched and narrow palate, coxa vara, muscular hypotonia, sensorineural deafness, and frequent complex seizures. Pigmentary dysplasia of the skin was present with normally pigmented areas surrounded by larger, hyperpigmented areas: ill-demarcated mottled patterns in the back, large-macular patterns in the abdomen, and linear patterns following the Blaschko's lines in the posterior thighs and legs [Ohashi et al., 1992] (Fig. 1). He could neither walk nor speak, and his developmental age was estimated at 9 months.

G-banded chromosome analysis of cultured peripheral blood lymphocytes from the boy showed mosaicism composed of cells with a supernumerary bisatellited marker chromosome and those with a normal karyotype (Fig. 2). Fluorescence in situ hybridization (FISH) was carried out using Vysis Prader–Willi/Angelman region probes that contains D15Z1 at the 15cen region (spectrum green), SNRPN at the 15q11-q13 PWS/AS critical region (spectrum orange), and PML at 15q22 (spectrum orange). The marker chromosome was double positive for D15Z1 and quadruple positive for SNRPN (Fig. 3A). One of chromosomes 14 was D15Z1 positive in both the abnormal and normal cell lines, and thus was interpreted a normal variant (Fig. 3A). FISH analysis using as a probe a clone spanning exons 12–14 of the *P* gene, distal to SNRPN [Lee et al., 1995] showed quadruple signals on the maker chromosome and a single signal on normal chromosomes 15 (Fig. 3B). His karyotype was thus interpreted as 47,XY,+idic(15)

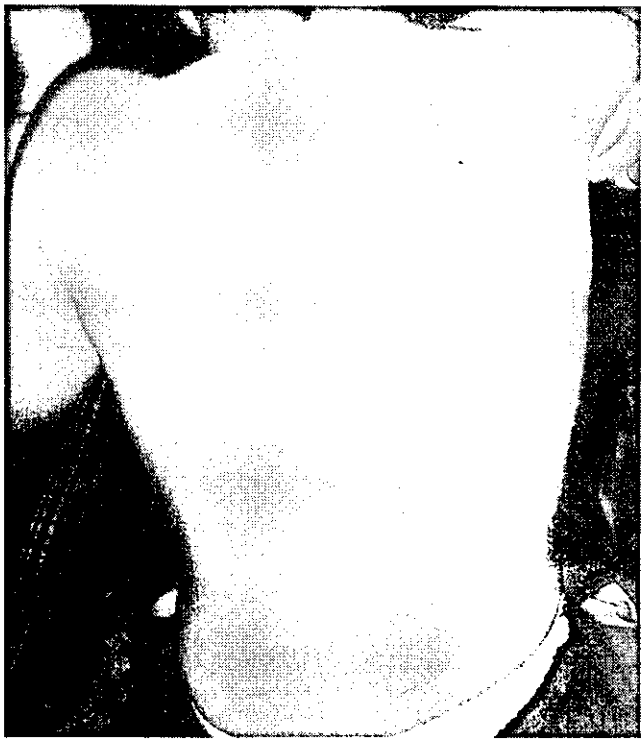
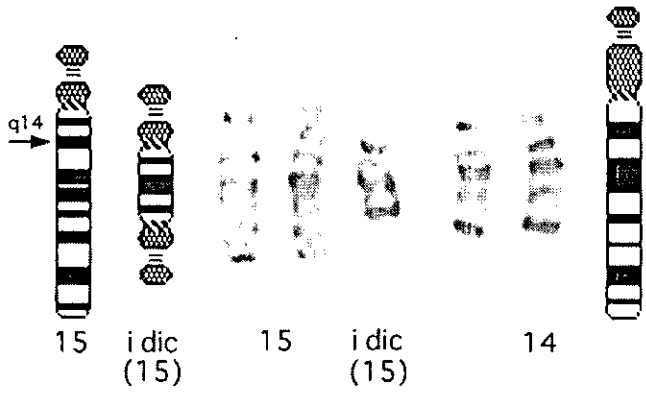


Fig. 1. The boy with mosaic patterns of skin hyperpigmentation.



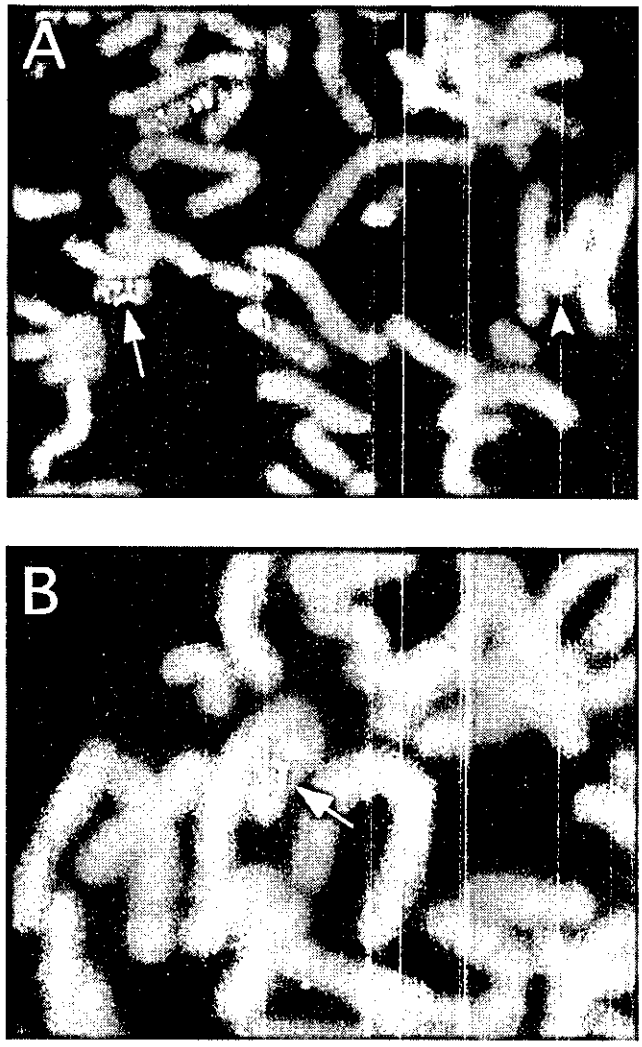


Fig. 3. Fluorescence in situ hybridization (FISH) analysis using Vysis PWS/AS region probe (**A**), and partial *P* gene probe (**B**). The inv dup(15) chromosome (arrow) is double positive for D15Z1 (green), quadruple positive for SNRPN (pink), and quadruple positive for the *P* gene (arrow). Probe D15Z1 cross-reacts with a normal chromosome 14 (arrowhead).

(pter→q14::q14→pter)[38]/46,XY[12].ish idic(15)(D15Z1++, SNRPN++++, P++++). G-banded chromosomes of his parents and sister were all normal. FISH analysis showed the presence of a variant chromosome 14 in both the mother and sister but not in the father. None of them had multicopies of SNRPN.

DISCUSSION

The boy we described had severe psychomotor retardation and developed complex seizures, common findings in the individuals with an additional inv dup(15) chromosome containing the PWS/AS critical region [Battaglia et al., 1997]. The 15q11-q13 region is known to be unstable, probably due to the presence of region-specific low-copy repeats, and to be frequently involved in structural rearrangements such as deletion, duplication, triplication, and formation of inverted duplication. He also had mottled and linear skin patterns of hyperpigmentation. The skin patterns may reflect different copy numbers of the *P* gene in his two cell lines, i.e., six copies in cells with an extra inv dup(15) chromosome versus two copies in those with a normal karyotype. This hypothesis is potentially supported by our previous finding in a woman with generalized hyperpigmentation associated with a 15q11.2-q14 duplication, including a duplication of the *P* gene [Akahoshi et al., 2001]. PWS or AS patients hemizygous for the *P* gene are usually hypopigmented, regardless of the composition of their intact *P* allele [Spritz et al., 1997]. There was a boy with a mosaic 15pter-q13 deletion and hypomelanosis of Ito [Pellegrino et al., 1995]. All these findings suggest that changes of the *P* gene copy number are related to abnormal skin pigmentation: its decreased copy number is associated with hypopigmentation, while increased number leads to hyperpigmentation. Further studies in individuals with a mosaic or nonmosaic supernumerary inv dup(15) chromosome containing the PWS/AS critical region may shed light on the correlation between the *P* gene copy number and skin pigmentation. However, the fact that the *P* gene is not imprinted, and its heterozygous mutations never result in detectable hypopigmentation seems to be in apparent contradiction with our gene-dosage hypothesis. Skin pigmentation is a quantitative trait determined by both polygenes and environmental factors. Obviously, there is a room left to learn about the function and metabolism of the melanocyte.

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We thank Professor Tadashi Kajii, Professor Toshiro Nagai, and Professor Takeo Kubota for their helpful suggestions and comments.

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American Journal of Medical Genetics 128A:170–172 (2004)

Rapid Publication

On the Reported 8p22-p23.1 Duplication in Kabuki Make-Up Syndrome (KMS) and its Absence in Patients With Typical KMS

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To the Editor:

Kabuki make-up syndrome (KMS) was described originally and independently by Niikawa et al. [1981] and Kuroki et al. [1981]. KMS was characterized by a distinctive facial appearance resembling the Kabuki actor's make-up, mild to moderate mental retardation, skeletal abnormality, postnatal growth retardation, and dermatoglyphic abnormality. (See a review by Matsumoto and Niikawa [2003].) Multiple organ involvement implies that KMS is a contiguous syndrome, but its cause remains unknown. Milunsky and Huang [2003] recently reported that all of six KMS patients they examined had an approximate 3.5-Mb duplication at 8p22-p23.1 demonstrated by comparative genomic hybridization (CGH) and fluorescence in situ hybridization (FISH) using four BAC clones (RP11-112G9, RP11-252K12, RP11-31B7, and RP11-92C1) as probes. They also suggested that a paracentric inversion, detected by RP11-122N11, separated from the duplicated region may contribute to the occurrence of the condition.

We analyzed a total of 26 Japanese and 2 Thai patients with KMS and 52 phenotypically normal controls regarding such duplication and inversion by FISH using 15 BAC clones covering 8p22-8p23.1 after obtaining written informed consent and with the approval by IRB of Nagasaki University. All of these patients were referred to us after making a definitive diagnosis of KMS. Their metaphase chromosomes were prepared for FISH from immortalized lymphoblastoid cell lines or peripheral blood lymphocytes according to standard protocols. Eight BAC clones (GS-77L23, RP11-245H16, RP11-5E15, RP11-399J23, RP11-403C10, RP11-589N15, RP11-252C15, RP11-45O16) selected from the UCSC Genome Browser version July 2003 (http://genome.ucsc.edu/cgi-bin/hgGateway?org=human) in addition to 7 clones (RP11-122N11, RP11-235F10, RP11-112G9, RP11-252K12, RP11-31B7, RP11-92C1, RP11-23H1) used by Milunsky and Huang [2003] were labeled with SpectrumGreen™-11-dUTP or Spectrum Orange™-11-dUTP (Vysis, Downers Grove, IL) and used as probes for FISH. FISH signals of all clones were carefully examined on both of metaphase chromosomes and interphase nuclei. Duplication was determined if signal intensity of probe A was much stronger than that of probe B in metaphases and two dots were surely observed in interphases using two-color FISH. At least 10 metaphases and 10 interphases were scored in each experiment.

None of the four clones reported to be duplicated in patients by Milunsky and Huang [2003] revealed any duplication in our 28 KMS patients examined (Fig. 1). All other clones, but RP11-122N11, also showed a single-copy signal, not duplicated (Fig. 1). The RP11-122N11 locus was reported to be inverted in six KMS patients and two of their mothers [Milunsky and Huang, 2003]. However, using RP11–122N11 as a probe we observed "duplicated" rather than "inverted" signals (Fig. 1). As the signal of two green signals for this probe looked similar in size and intensity (Fig. 1a), duplication is more likely. Of 22 KMS patients analyzed with this probe, 15 had a homozygous duplication and 7 a heterozygous one, and thus the allele frequency for the duplication is 88.5%. Similarly, of 52 normal persons, 40 and 12 had a homozygous and a heterozygous duplication, respectively, the frequency for the duplication being 84.1%, which is not statistically different in the patients by χ^2 analysis ($P = 0.47$). Thus, findings of RP11-122N11 signal were likely to be a polymorphism (Fig. 2A–D).

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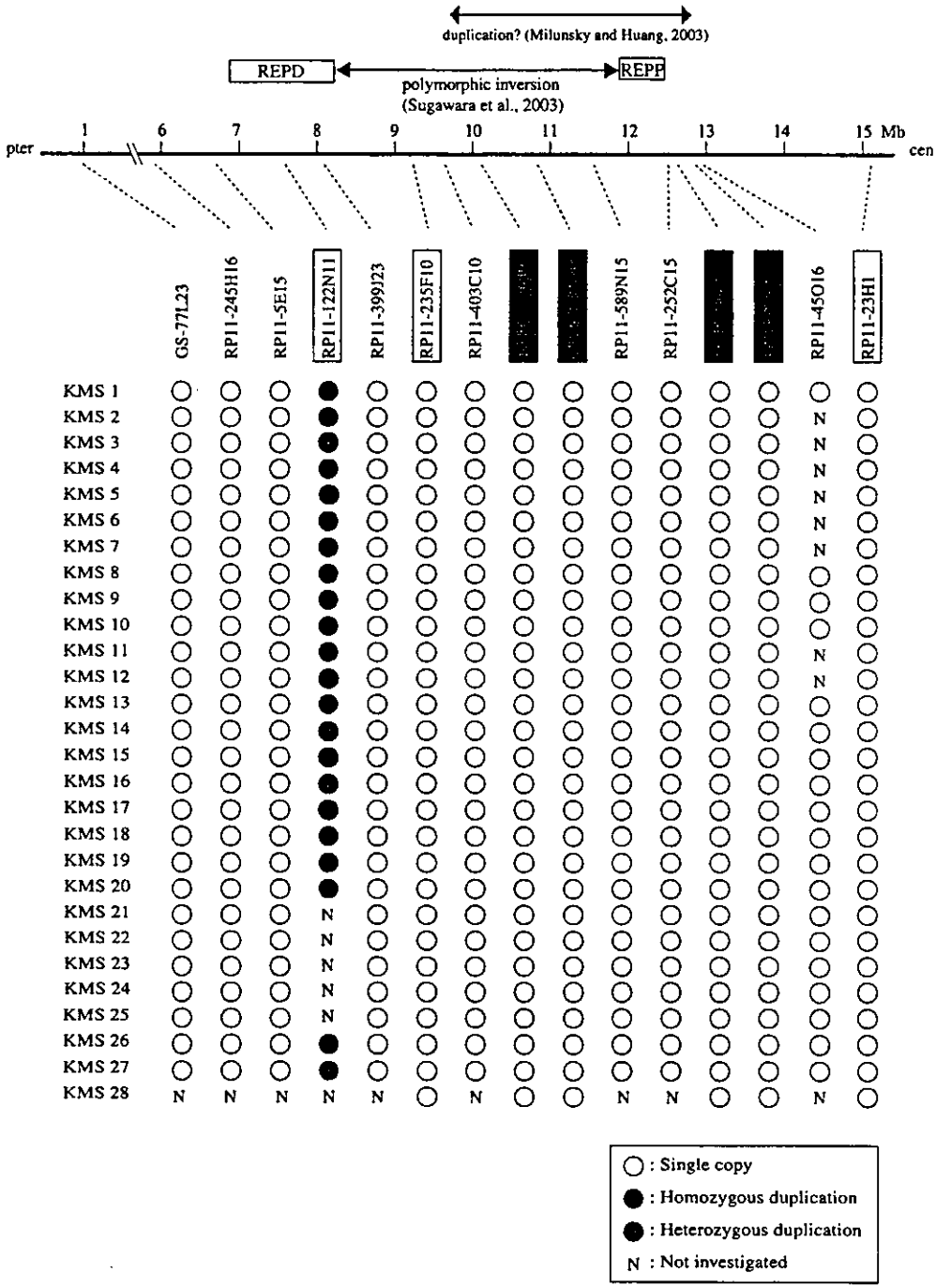

Fig. 1. Results of FISH analysis on 28 patients with Kabuki make-up syndrome (KMS). From top to bottom, a duplicated region reported by Milunsky and Huang [2003], polymorphic inversion reported by Sugawara et al. [2003], a scale from the 8p telomere to the centromere, BAC/PAC clones used for FISH study and their locations, and results of FISH studies in all patients. REPD, repeat distal; REPP, repeat proximal. BAC clones in square and grey square were those used for the study and reported to be duplicated, respectively, by Milunsky and Huang [2003]. Open, black and gray circles indicate a single-copy FISH signal, homozygous duplication, and heterozygous duplication, respectively. N, not investigated.

Unlike the data by Milunsky and Huang [2003], we were not able to detect any interstitial duplication at 8p22-8p23.1 in our series of 28 KMS patients. There must be some reasons for these discrepant results. The patient populations studied in two investigations may be different clinically. From our examination of the facial photographs of cases 1 and 2 in the report by Milunsky and Huang [2003], they may not have typical KMS and could be "8p23.1-p22 duplication syndrome." Alternatively, the discrepancy may be due to the complexity of the 8p23 region. We have constructed a comprehensive physical map covering low copy repeats (LCRs), and a common inverted region at 8p23. Although we did not incorporate the clone, RP11-122N11, to our previous map or evaluate it [Sugawara et al., 2003], we now assign it within one of LCRs

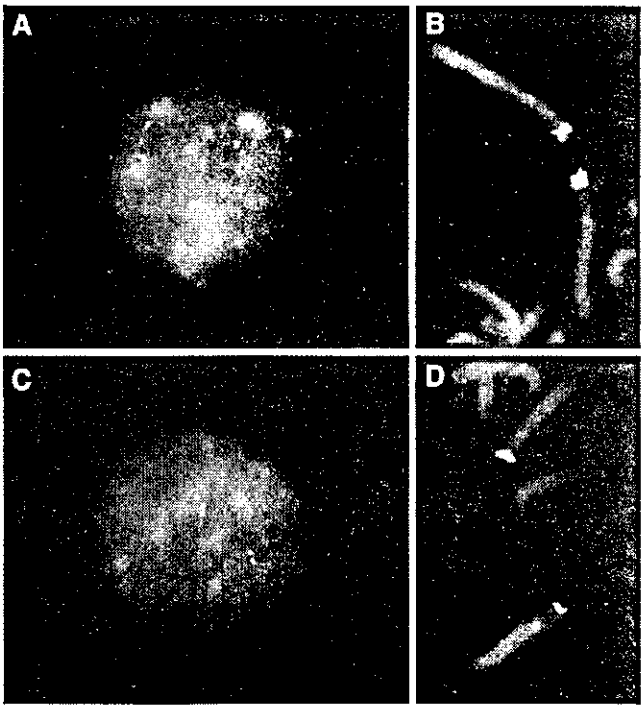

Fig. 2. FISH analysis using RP11-122N11 labeled with Spectrum-Green™ and RP11-235F10 labeled with Spectrum-Orange™ in normal controls, showing a homozygous duplication (**A**: An interphase nucleus and (**B**) metaphase chromosomes) and a heterozygous duplication (**C**: An interphase nucleus and (**D**) metaphase chromosomes).

according to both the UCSC database and our map. The clone would have shown seeming duplicated signals on both of homologous chromosomes, but the allele frequency of the RP11-122N11 duplication in the normal Japanese is 84.1%, not 100%. This implicates that this region has more complicated structure than expected, and should well be characterized.

In conclusion, our data suggest that the cause of KMS in most patients is still unknown, and further studies will be necessary absolutely.

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Clinical Report

A Woman With 46,XX,dup(16)(p13.11 p13.3) and the ATR-X Phenotype

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We report a Japanese woman with 46,XX,dup(16)(p13.11p13.3), who closely resembled the phenotype of X-linked α-thalassemia/mental retardation syndrome (ATR-X, MIM # 301040). Although she never had α-thalassemia, she showed characteristic clinical features including severe mental retardation, characteristic facies and behavior. ATR-X is caused by mutations of the *ATRX* gene. Although the function of ATRX protein has remained unclarified, it is thought to be involved in the regulation of several genes. The only target gene identified so far is the *α-globin* gene at 16p13.3. Clinical similarity among patients with ATR-X and dup(16)(p13.11p13) may indicate that some target genes regulated by ATRX reside in the duplicated region between 16p13.11 and 16p13.3, and that these genes are abnormally upregulated in ATR-X differently from the *α-globin* gene.
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KEY WORDS: 16p duplication; α-thalassemia/retardation syndrome; ATR-X; ATR-16

INTRODUCTION

The α-thalassemia/mental retardation-16 syndrome (ATR-16, MIM# 141750) and X-linked α-thalassemia/mental retardation syndrome (ATR-X, MIM *300032) are two distinct syndromes that commonly feature α-thalassemia and mental retardation [McPherson et al., 1995]. ATR-16 is a contiguous gene syndrome, a condition associated with HbH disease showing mental retardation, genital abnormalities, and relatively non-specific dysmorphic features [Wilkie et al., 1990a], resulting from a deletion involving the tip of the short arm of chromosome 16 (16p13.3), where the α-globin genes (*HBA*) are located [Weatherall et al., 1981]. In contrast, ATR-X is an X-linked recessive disorder characterized by much more severe mental retardation, characteristic facies and behavior, and genital abnormalities [Donnai et al., 1991; Wilkie et al., 1991; Gibbons et al., 1995a]. The gene (*ATRX*) responsible for ATR-X has been assigned to Xq13.3 [Gibbons et al., 1995a; 1995b], and a number of studies have demonstrated several mutations of this gene in ATR-X patients [Picketts et al., 1996; Gibbons et al., 1997; Fichera et al., 1998; Wada et al., 2000]. The ATRX protein is a member of a subgroup of the helicase superfamily [Gibbons et al., 1995b; Villard et al., 1997], and is assumed to regulate the expression of several genes including *HBA*, which involve in the brain development [Gibbons et al., 1995c].

Here we describe a 32-year-old woman with 46,XX,dup(16)(p13.11p13.3), associated with a phenotype very similar to ATR-X. This suggests that some target genes of the ATRX protein may reside in a duplicated segment of the patient.

CLINICAL REPORT

A 32-year-old Japanese woman (Fig. 1A,C) has been a resident of Center for Severely Mentally Retarded Individuals since age 9 years. Her non-consanguineous parents, an elder brother, and a younger sister are all healthy. The patient was born at 35 weeks' gestation by vaginal delivery with a footling presentation. She had asphyxia, congenital hip joint dislocation, bilateral inguinal hernias, and a knot in the umbilical cord. Her birth weight was 1,650 g. She was in an incubator for 4 months. Her cry was weak and she sucked poorly during early infancy. She showed general hypotonia in infancy: she raised her head at age 8 months, crawled at 3 years, and stood at 5 years. She had epileptic seizures since age 3 years and was treated with anticonvulsants. A diagnosis of mental retardation complicated with epilepsy was made, and her development was markedly delayed.

At age 32 years, her height is 128 cm (−6.0 SD), her weight is 25 kg (−3.9 SD), and her OFC is 47 cm (−7.2 SD). She has a flat and mid hypoplastic face with prognathism, narrow and upward slanted palpebral fissures with hypertelorism, bilateral cataracts (extracted on the right side), low-set ears, a small crashed nose with a depressed nasal bridge, a shallow philtrum, full lower lip, widely spaced incisors, reversed articulation, hypersalivation, short neck, round back, and coxa vara. Developmental delay is severe with an estimated mental age of 6 months. She cannot speak. She repeats stereotyped behavior including breath holding, putting her hand to the back of the throat, and hitting her chin with her palm, often associated with emotional outbursts. Epileptic seizures are well controlled. She suffers from recurrent episodes of respiratory infection and feeding problems. She can sit and move by creeping, but her coordination is poor. She sometimes vomits blood due to gastroesophageal reflux. She can hold a spoon and a cup. She has developed secondary sexual characteristics and mammary hyperplasia. Menstruation has been irregular. The muscle tone of the trunk is weak, but her limbs are moderately spastic.

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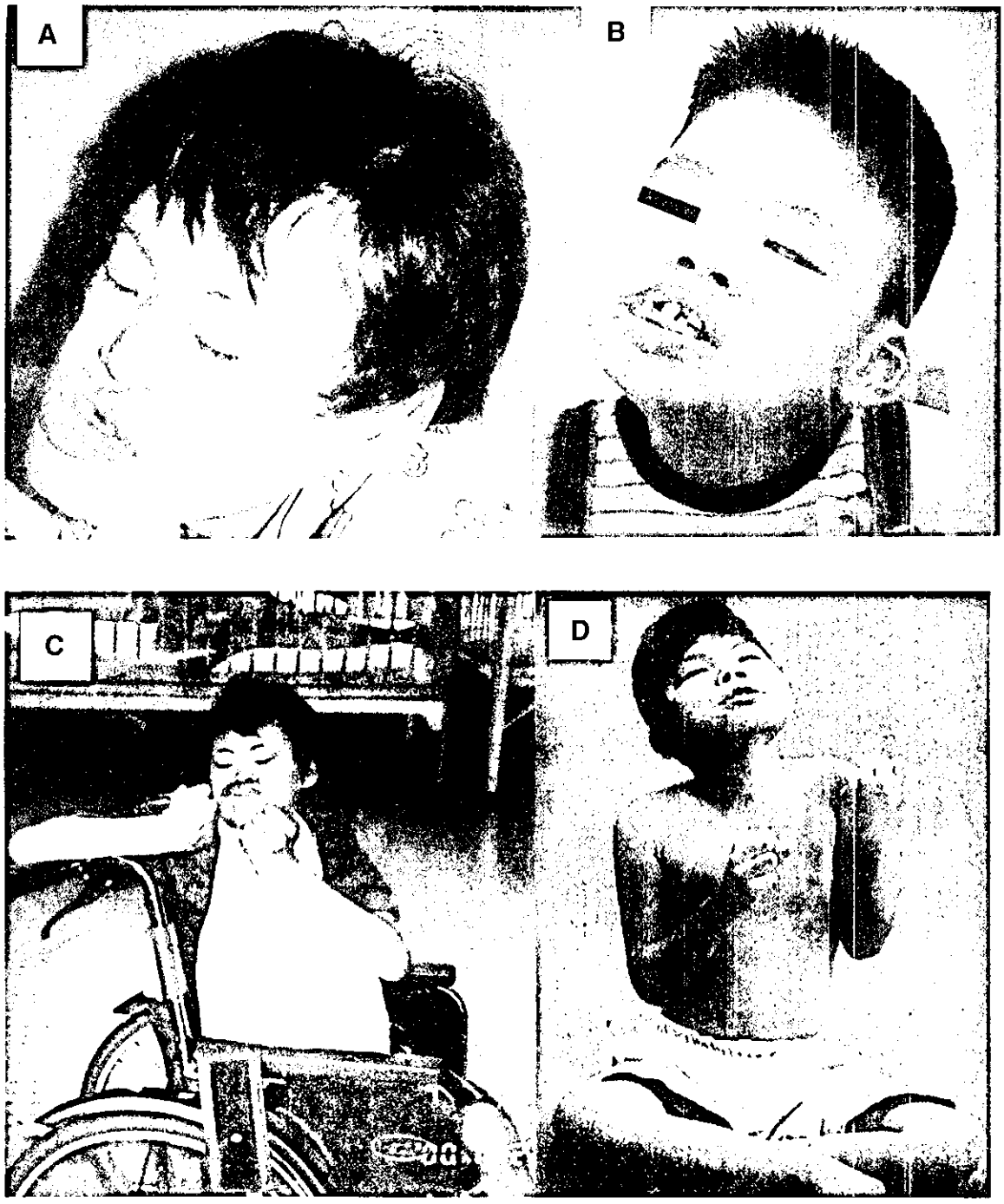

Fig. 1. The patient at age 32 years (**A**, **C**) and a boy with X-linked α-thalassemia/mental retardation syndrome (ATR-X) reported by Kurosawa et al. [1996] (**B**, **D**). Note typical posture with extreme emotion (C and D).

Routine laboratory tests showed no abnormalities, except for slightly decreased white blood count and serum IgG level. She had mild normocytic anemia: red cell count, 3.44×10^{12}/L; Hb, 9.9 g/dl; PCV, 0.311 L/L; MCV, 90.2 fl; MCH, 28.9 pg; and MCHC, 32.0%. Results of a screening for thalassemia were as follows: HbF was 0.5% (reference range < 1.0%), HbA2 that is decreased in α-thalassemia was 2.1% (2.5%–3.5%), the isopropanol test that detects unstable hemoglobins such as HbH was negative, and the glycerol lysis half-time that is prolonged in any type of thalassemia and some hemoglobinopathies was 75 sec (35–55 sec). HbH inclusion bodies in red cells, which characterize α-thalassemia including HbH disease, were absent. Isoelectrofocusing demonstrated no abnormal hemoglobins such as HbH or Hb Bart. Accordingly, α-thalassemia was unlikely to be present. The reduced level of HbA2 and slightly prolonged glycerol lysis time may have been attributable to the iron deficiency, which was suggested by a decreased serum ferritin level (7.4 ng/ml). Radiographic examination showed athelectasis in the midzone of the left lung, as well as scoliosis, costal neck, dislocation of the hip joints, and generalized bone atrophy. MRI of the brain demonstrated no remarkable change except microcephaly. The

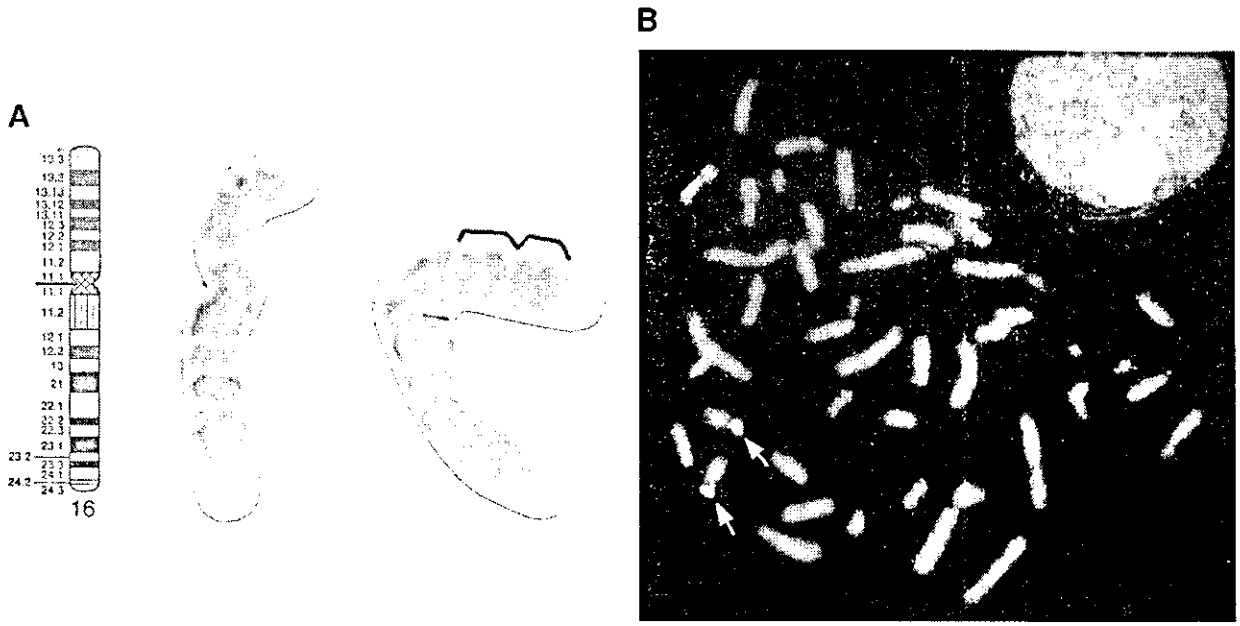

Fig. 2. Partial karyotype (A) and FISH study using a SOX8 probe (B) of the patient. Note a 16p13.11-16p13.3 duplication, and two normal SOX8 signals (arrows).

auditory brainstem evoked response (ABR) showed absent fifth wave with right (65 dBHL) and left (75 dBHL) click stimulation. Electroencephalography showed irregular waves, but no paroxysmal activity.

Cytogenetic Findings

GTG-banding chromosome analysis showed an extra material on 16p. Spectral karyotyping (SKY) demonstrated that this

TABLE I. Comparison of Clinical Manifestations Between the Present Patient and Three Disorders

	Disorders			
			dup(16p)	
	ATR-X	ATR-16	Present patient	Kokalj-Vokac et al. [2000]
Karyotype	N	N	dup(16)(p13.11p13.3)	ins(1;16)(q42;p13.1p13.3)
Developmental delay	+	+	+	+
Mental retardation	Severe	Mild ~moderite	Severe	Severe
Short stature	+	−	+	+
Microcephaly	+	+	+	+
Low-set ears	+	±	+	+
Non-symmetrical flat face	+	−	+	
Hypertelorism	+	+	+	+
Narrow palpebral fissures	+	−	+	+
Upword slanting palpebral fissures	+	−	+	+
Crashed nose	+	−	+	
Anteverted nares	+	+	+	+
Long philtrum	−	+	−	+
Thick and rolled-over lower lip	+	−	+	+
Widely-spaced incisors	+	−	+	
Prominent mandible	+	−	+	+
Self-hitting jaw by palm	+	−	+	
Putting their hands to the throat	+	−	+	
Characteristic posture	+	−	+	
Seizures	+	+	+	+
Hypotonia	+	+	+	+
Flexion contractures of proximal interphalangeal joints	+	−	+	+
Cataracts	+	−	+	
Inguinal hemias	+	±	+	+
Gastro-esophageal reflux	+	−	+	
Genital abnormalities	+	+	−	−
HbH disease	+	+	−	−

was derived from chromosome 16. High resolution banding indicated that the break points were located at 16p13.11 and 16p13.3 (Fig. 2A). Fluorescence in situ hybridization (FISH) analysis using a probe for the *SOX8* gene demonstrated a signal on each chromosome 16 (Fig. 2B), indicating no deletion or duplication of the gene. Thus, the patients karyotype was interpreted as 46,XX,dup(16)(p13.11p13.3).ish 16p13.3(SOX8 × 2). Her mother had a normal 46,XX karyotype, but karyotyping of her father was unavailable.

DISCUSSION

Previous study suggested that putative gene(s) for mental development must lie outside the 105-kb region spanning the α-*globin* gene complex, as hemizygosity for a 1-Mb segment from 16p13.3 is responsible for the ATR-16 syndrome [Wilkie et al., 1990a]. Pfeifer et al. [2000] speculated that *SOX8* is a candidate gene for the mental retardation phenotype of the syndrome, on the basis of their finding that SOX8 was deleted in a patient with ATR-16. However, as our FISH analysis using a SOX8 probe showed normal two-copy signals in our patient, her clinical manifestations are not attributable to deletion or duplication of SOX8.

Patients with ATR-16 show mild or moderate mental retardation, a broad forehead, downslanting palpebral fissures with epicanthus, a flat nasal bridge, retro-micrognathia, hypotonia, and talipes equinovarus [Wilkie et al., 1990a; Lamb et al., 1993; Lindor et al., 1997], whereas ATR-X patients show profound mental retardation, an asymmetrical flat face, hypertelorism, upslanting and narrow palpebral fissures, crashed nose, prognathism, widely spaced incisors, and thick and everted lower lip [Wilkie et al., 1990b; Gibbons et al., 1995a]. The woman we have described had features very similar to those of ATR-X, i.e., an asymmetrical flat face, upword slanting and narrow palpebral fissures, hyperterorism, crashed nose, thick and everted lower lip, and irregular teeth and prognathia, and short neck (Table I, Fig. 1A). Furthermore, she showed a characteristic posture and behavior that are diagnostic of ATR-X, including breath holding, putting hand into mouth, hitting the jaw with the palm, extreme emotions, autistic behavior, and head tilt and upward gaze. She also had severe mental retardation, spastisity, sensorineural deafness, gastroesophageal reflux, peripheral cyanosis, and cataracts. Almost all these features are identical to those seen in an ATR-X patient reported by Kurosawa et al. [1996] (Fig. 1B,D).

Reported patients with trisomy for 16p had low birth weight, severe developmental delay, psychomotor retardation, a round face, hypertelorism, narrow palpebral fissures, a depressed nasal bridge, anteverted nostrills, a thin upper lip, low-set ear, and hypotonia [Dallapiccola et al., 1979; Cohen et al., 1983; Jalal et al., 1989; Leonard et al., 1992; Carrasco Juan et al., 1997; Engelen et al., 2002]. In addition to them, a boy with 16p-trisomy reported by Kokalj-Vokac et al. [2000], whose duplicated region is very similar to that of our case, had a prominent mandible, characteristic posture of hands, flexion contractures of all proximal interphalangeal joints, and mild radial deviation of wrists, as also seen in our case and in patients with ATR-X (Table I). This suggests that the clinical features of ATR-X are similar to those of 16p-trisomy rather than those of ATR-16 due to16p-deletion.

In view of these findings above, it is likely that some target genes of ATRX protein reside within the 16p13.11-16p13.3 region neighboring the HBA cluster. It is also plausible that, although the HBA expression is downregulated by mutated ATRX, the expression of other target genes in this region involved in mental retardation, typical facies and behaviors of ATR-X is upregulated. Further precise analysis of duplicated regions in patients with 16p-trisomy, as well as an expression study of genes in this region, will help the understanding of a role of ATRX protein in its target genes.

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ORIGINAL ARTICLE

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Linkage and association analyses of the osteoprotegerin gene locus with human osteoporosis

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Abstract Osteoprotegerin (OPG), a secreted glycoprotein and a member of the tumor necrosis factor receptor superfamily, is considered to play an important role in the regulation of bone resorption by modifying osteoclast differentiation. Overexpression of OPG in mice has been reported to result in osteopetrosis, whereas targeted disruption of OPG in mice has been associated with osteoporosis. Accordingly, *OPG* could be a strong candidate gene for susceptibility to human osteoporosis. Here, we analyzed whether *OPG* is involved in the etiology of osteoporosis using both linkage and association analyses. We recruited 164 sib pairs in Gunma prefecture, which is located in the central part of Honshu (mainland Japan), for a linkage study, and 394 postmenopausal women in Akita prefecture, which is in the northern part of Honshu, for an association study. We identified two microsatellite polymorphisms in the linkage study, and six single-nucleotide polymorphisms (SNPs) in the *OPG* region for the association study. Although, no evidence of significant linkage between *OPG* and osteoporosis was found, a possible association of one SNP, located in the promoter region of the gene, was identified. A haplotype analysis with the six SNPs revealed that four major haplotypes account for 71% of the alleles in the Japanese population.

Key words Osteoprotegerin · Osteoporosis · Single-nucleotide polymorphism · BMD · Sib pair analysis

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Introduction

Osteoporosis is a systemic skeletal disease characterized by excessive bone resorption, typically in association with postmenopausal estrogen deficiency, which leads to low bone mass and microarchitectural deterioration with a consequent increase in bone fragility and susceptibility to fracture. Bone mineral density (BMD) is a complex trait that is influenced by multiple genes and environmental factors. Genetic factors have been estimated by twin studies to account for up to 80% of the variance in BMD (Giguere and Rousseau 2000). A number of candidate genes have been analyzed for involvement in the etiology of osteoporosis. These include, for example, the vitamin D receptor (Morrison et al. 1994), type I collagen (Grant et al. 1996), estrogen receptor (Kobayashi et al. 1996), interleukin 6 (Ota et al. 1999), and calcitonin receptor genes (Taboulet et al. 1998). Genome-wide screening of 330 DNA markers with 149 members of seven large pedigrees has been performed and several possible loci identified (Devoto et al. 1998). However, the contribution of these genes to the etiology of osteoporosis is still controversial, possibly because of racial difference, type I error, or misgenotyping (Morrison et al. 1997).

Osteoprotegerin (OPG) is a secreted glycoprotein, which was independently identified by three laboratories (Simonet et al. 1997; Tsuda et al. 1997; Tan et al. 1997) and which is considered to be a member of the tumor necrosis factor receptor superfamily. Transgenic mice that overexpress OPG exhibit a generalized increase in bone density (Simonet et al. 1997). Two separate studies using OPG-deficient mice, which showed severe early onset of osteoporosis with increased osteoclast numbers, indicated that the function of OPG is to block osteoclast formation and bone resorption (Mizuno et al. 1998; Bucay et al. 1998). Consistent with this, OPG administration protected against the decrease in bone mass that occurs in ovariectomized rats, an animal model of postmenopausal osteoporosis (Simonet et al. 1997). From these findings, *OPG* appears to be one of the most attractive candidate genes responsible

for postmenopausal-type osteoporosis susceptibility. To investigate possible effects of genetic variations at the *OPG* loci, we performed a linkage study by a sib pair analysis, and an association study with postmenopausal women by identifying single-nucleotide polymorphisms (SNPs). A possible association with one of the studied SNPs located in the promoter region of the gene was detected: individuals with TT genotype in the osteoporosis group had significantly decreased bone mineral density (BMD) when compared with those with TC or CC genotypes.

Subjects and methods

Subjects

For the sib pair analysis, DNA samples were obtained from peripheral blood of 283 Japanese women from 131 families, comprising 164 sib pairs. To determine the frequency distribution of CA repeats in Gunma Prefecture 77 unrelated Japanese women were recruited and their DNA analyzed. All lived in the area, which is in the central part of Honshu (mainland Japan). Their ages ranged from 50 to 86 years old (mean 66.2 ± 7.1 years).

For the association study, DNA samples were extracted from peripheral blood of 394 postmenopausal Japanese women ranging in age from 66 to 92 (mean 73.2 ± 5.8 years) living in Akita prefecture, which is in the northern part of Honshu. No participant in either study group had medical complications or was undergoing treatment for conditions known to affect bone metabolism, such as pituitary disease, hyperthyroidism, primary hyperparathyroidism, renal failure, adrenal disease, or collagen disease, and none was receiving estrogen replacement therapy. To analyze the haplotype pattern of *OPG* in the Japanese population, 48 DNA samples from volunteers of both sexes ranging in age from 20 to 22, recruited in our medical school, were used. The ethics committee of the Asahikawa Medical College approved the protocol of this study. The nature, purpose, and potential risks of the study were carefully explained to all patients before they agreed to participate. All were volunteers and gave informed consent prior to the study.

Measurement of bone mineral density (BMD)

Instruments for BMD measurements were different between Gunma and Akita prefectures because we used the installed machine in each health check-up center. The BMD of the radial bone (expressed in g/cm^2) of each participant was measured by means of dual energy X-ray absorptiometry by using a DTX-200 (Osteometer MediTech, Hawthorne, CA, USA) instrument in Gunma, and with a DPX-L (Lunar Madison, WI, USA) instrument in Akita. In the sib pair linkage analysis, osteoporosis and osteopenia were defined as a decrease in BMD below 70% and 80%, respectively, of the mean in young adult women (cut-off values 0.333 = −2.6SD and 0.381 = −1.8SD), according to the general criteria recommended by the Japanese Society for Bone and Mineral Research (Orimo et al. 1996). On the other hand, in the association study with the Akita population, the analysis was performed on the quantitative phenotype, BMD. Consequently, factors (age, height, and weight) that would affect BMD values had to be adjusted. Thus, we used the following formula: body mass index (BMI) = body weight (kg)/body height^2 (m); adjusted BMD (adjBMD) = BMD − 0.0052 × (73.2 − age) + 0.0088 × (23.2 − BMI) (Ota et al. 2001).

Genotyping of microsatellite polymorphisms

A human genomic clone containing the *OPG* gene was identified by a P1-derived chromosome (PAC) Human Genomic polymerase chain reaction (PCR) Screening Kit (Incyte Genomics, Palo Alto, CA, USA), by using primer sequences derived from the 3′ portion of the gene. A fragment containing the CA repeat was identified by Southern blotting of PAC DNA digested by *Hae*III or *Sau*3AI with a $(GT)_{20}$ oligonucleotide probe, and then subcloned and sequenced. Two informative repeat sequences, named OPG1 and OPG2 and shown in Fig. 1, were identified. PCR primers were designed to flank the repeat sequences for the polymorphism analysis (Fig. 1). The PCR primers used were OPG1F (forward), 5′-GCACACACGCTCTGTTTC TC-3′; OPG1R (reverse), 5′-GGAGGGTGGTAACTTG GGAT-3′; OPG2F (forward), 5′-AGTCTGGGCAACA GAGCAAG-3′; and OPG2R (reverse), 5′-CTAGCCTGA TGAATTGTCATC-3′. Fluorescent-labeled primers were used for genotyping. PCR amplification was carried out as described below for genotyping except that the annealing temperature was 55°C. Electrophoresis was performed with an ABI 377 DNA sequencer; the data were extracted by using GeneScan Analysis software and analyzed by the Genotyper program (Applied Biosystems, Foster City, CA, USA).

a. OPG1

GCACACACGCTCTGTTTCTCTCTCTCTCTGTCTGTCTCTCT
CTCTGTGCATGTGAGTGCTT**TGTGTGTGTGTGTGTGTGTGT**
GTGTGTGTGTGTGTGGAATCAATATAGTAATAAGATATTTA
AAATTGTTAAATCCCAAGTTACCACCCTCC

b. OPG2

AGTCTGGGCAACAGAGCAAGATTTCAT**CACACACACACA**
CACACACACACACACACACATTAGAAATGTGTACTTGGC
TTTGTTACCTATGGTATTAGTGCATCTATTGCATGGAAC
TTCCAAGCTACTCTGGTTGTGTTAAGCTCTTCATTGGGT
ACAGGTCACTAGTATTAAGTTCAGGTTATTCGGATGCAT
TCCACGGTAGTGATGACAATTCATCAGGCTAG

Fig. 1a,b. Nucleotide sequences of the polymorphic repeats and their flanking region in the osteoprotegerin (*OPG*) gene locus. Sequences used for forward and reverse primers are *underlined*. CA (or GT) repeats are shown in *bold*. **a** *OPG1*. **b** *OPG2*

Search for single-nucleotide polymorphisms (SNPs)

A total of 23 primer sets were designed to amplify 12 kb of the *OPG* gene containing 1100 bases of the promoter region, all five exons and introns, and 840 base pairs of the 3′ flanking region. SNPs were detected by sequencing DNA samples from ten independent volunteers (20 alleles) on an ABI 310 sequencer (Applied Biosystems).

Genotyping of detected SNPs

Primers for each SNP were designed for the amplification refractory modification system (ARMS) technique (Newton et al. 1989). In all, six SNPs were analyzed: one in the 5′ untranslated region (5′UTR) (SNP1), one in exon 1 (SNP2), two in intron 2 (SNP3 and SNP4), and two in intron 3 (SNP5 and SNP6). ARMS primers were designed to amplify a region of about 250 bp. Sequences of the primers used are as follows: OPG5UTRA (forward), 5′-GGCTGC GGAGACGCACCCGCA-3′; OPG5UTRC (forward), 5′-GGCTGCGGAGACGCACCCGCC-3′; OPG5UTRAS (reverse), 5′-AGCATGGCATAACTTGAAAGC-3′; OPGE1K (forward), 5′-CGGGGACCACAATGAAC TAG-3′; OPGE1N (forward), 5′-CGGGGACCACAATG AACTAC-3′; OPGE1AS (reverse), 5′-GCTGTCTTCCA TAAAGTCAGC-3′; OPGi21C (forward), 5′-ATGCTAG AGTTTTGTGCATC-3′; OPGi21T (forward), 5′-ATG CTAGAGTTTTGTGCATT-3′; OPGi21AS (reverse), 5′-TTTCCTTTCTGAGTTAGCAGG-3′; OPGi22C (forward), 5′-ACTAAATTGCTTGGTATTTGCC-3′; OPGi22T (forward), 5′-ACTAAATTGCTTGGTATTTG CT-3′; OPGi22AS (reverse), 5′-TACAAAATCGTACAA AGACGT-3′; OPGi31G (forward), 5′-TCTCCCCAAAC AGTTTTGCG-3′; OPGi31A (forward), 5′-TCTCCCCAA ACAGTTTTGCA-3′; OPGi31AS (reverse), 5′-GTGCA CAATAAATGAAAAAAAGT-3′; OPGi32T (forward), 5′-CAGTTCCAGCATTGTTTAAT-3′; OPGi32C (forward), 5′-CAGTTCCAGCATTGTTTAAC-3′; and OPGi32AS (reverse), 5′-CTACTACCTATATTCATCT GA-3′. To confirm the reaction, a part of the β-globin gene was amplified as well, as a positive control. The PCR primers used were BGLOS (forward), 5′-ACACAACTGTG TTCACTAG-3′ and BGLOAS (reverse), 5′-CATGAGC CTTCACCTTAGGG-3′, which amplified a 360-bp region. After amplification, 3% agarose gel or 12% acrylamide gel electrophoresis were performed for genotyping. For some of the SNPs, the PCR products were sequenced to confirm the results obtained by the ARMS method. The PCR primers for sequencing were as follows: for SNP1 and SNP2, OPGSNP1F (forward), 5′-GCTCTCCCAGGGGACAGA CA-3′ and OPGSNP1R (reverse), 5′-AGACCAGGTGGC AGCAGCCT-3′; for SNP3 and SNP4, OPGSNP2F (forward), 5′-TAGCGTCTTTAGTTGTGGACT-3′ and OPGSNP2R (reverse), 5′-CCGGAACATATGTTGTCG TG-3′; and for SNP5 and SNP6, OPGSNP3F (forward), 5′-GTGTTAAGCTCTTCATTGGGTA-3′ and OPGSNP3R (reverse), 5′-AAATGGGAGTAATGGGTGTTTG-3′. PCR was performed in a volume of 12.5 μl containing 20 ng genomic DNA, 10 mM Tris HCl (pH 8.4), 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin, 200 μM deoxyribonucleotide triphosphates (dNTPs), 10 pmol of each primer, and 0.25 units of *Taq* polymerase. PCR amplification was performed with 30 cycles of 94°C for 30 s, 49°–62°C for 30 s, and 72°C for 30 s, depending on the region analyzed, with a final extension step of 5 min at 72°C in a Gene Amp PCR9600 System (Applied Biosystems). The amplified mixture was electophoresed in 1.5% agarose gel to isolate the fragment containing the PCR product. After that, the PCR product was extracted by using a Geneclean III Kit (Bio 101, Vista, CA, USA). The sequencing reaction and electrophoresis were performed with a BigDye Terminator kit (Applied Biosystems) following the manufacturer's protocol.

Statistical analysis

In an analysis using osteoporosis and osteopenia as the affected status, we analyzed three classes of sib pairs: (1) both sib unaffected (clinically concordant unaffected sib pairs); (2) one sib affected and the other not (clinically discordant sib pairs); and (3) both sibs affected (clinically concordant affected sib pairs). A nonparametric linkage analysis was performed by using the SIBPAL program (version 2.7) of the SAGE package (Case Western Reserve University, Cleveland, OH, USA). A significant increase in allele sharing (>0.5) for concordant pairs and/or a significant decrease in allele sharing (<0.5) for discordant pairs was considered evidence for linkage. Here, the term "allele sharing" means the proportion of shared alleles in a sib; thus, the value ranges from 0 to 1 and the expected value is 0.5.

For the association analysis, we compared BMD and adjBMD as a quantitative phenotype between genotype groups (TT vs. TC+CC and TT+TC vs. CC in SNP1; GG vs. GC+CC and GG+GC vs. CC in SNP2) by using both *t*-test (parametric) and the Mann-Whitney U test (nonparametric) from the statistical analysis system package (SAS Institute, Cary, NC, USA). A P value of < 0.05 was considered statistically significant.

Haplotype analysis

Haplotype frequency was estimated by the maximum-likelihood method by using a simplified version of the computer program GENEF (J-M Lalouel, University of Utah, unpublished). Procedures to generate the haplotype, are described in detail in Jeunemaitre et al. (1997). Briefly, two SNPs were chosen to generate a haplotype, followed by sequential inclusion of one SNP at a time. All haplotypes below a frequency of 1/4N, where N is the sample size, were automatically eliminated.

The strength of linkage disequilibrium (LD) was calculated by using Arlequin software for population genetic data analysis (http://anthropologie.unige.ch/arlequin). Pairwise LD was estimated as $D = x_{ij}\text{-}p_i p_j$, where x_{ij} is the frequency of haplotype A_1B_1, and p_1 and p_2 are the frequencies of alleles A_1 and B_1 at loci A and B, respectively. A

standardized LD coefficient, r, is given by $D/(p_1p_2q_1q_2)^{1/2}$, where q_1 and q_2 are the frequencies of the other alleles at loci A and B, respectively (Hill and Robertson 1968). Lewontin's coefficient D′ is given by D/D_{max}, where $D_{max} = \min[p_1p_2,q_1q_2]$ when $D < 0$ and $D_{max} = \min[q_1p_2,p_1q_2]$ when $D > 0$ (Lewontin 1984). An appropriate LD measure for association studies, d, is given by $d = D/p_1q_2$, where p_1 is the variant frequency and p_2 is the marker allele frequency (Kruglyak 1999).

Results

Sib pair linkage analysis

The frequency distribution of two microsatellite polymorphisms, OPG1 and OPG2, in the Gunma area was determined by using a randomly selected group of 77 unrelated Japanese women (Table 1). The polymorphic PCR products of OPG1 and OPG2 contained 16–23 and 15–21 repeats, respectively. The frequency of heterozygotes was calculated as 80.1% for OPG1 and 53.7% for OPG2. The total number of successfully genotyped sib pairs for the linkage analysis was 164 for OPG1 and 153 for OPG2. The results of the analysis for osteoporosis and osteopenia are shown in Table 2. No significant linkage of OPG1 or OPG2 to either osteoporosis or osteopenia was observed.

Table 1. Frequency distribution of CA repeat alleles in the *OPG* gene locus among 77 Japanese women

	OPG1		OPG2	
Alleles	Repeat no.	Freq.	Repeat no.	Freq.
A1	16	0.0001	15	0.0001
A2	17	0.2208	16	0.6474
A3	18	0.2662	17	0.1282
A4	19	0.2078	18	0.0641
A5	20	0.1169	19	0.1538
A6	21	0.1429	20	0.0000
A7	23	0.0455	21	0.0064

OPG, osteoprotegerin

Association analysis

A total of six SNPs (SNP1 to SNP6, see Subjects and methods) were detected in the *OPG* gene locus. The observed number of genotypes in each SNP site did not differ significantly from what would be expected from the Hardy-Weinberg equilibrium. Two SNPs (SNP1 and SNP2) out of six, located in the 5′-UTR and in exon 1, appeared to be more interesting in terms of OPG function. SNP1, either T or C at 223 bp upstream from the translation initiation site, could have some influence on promoter activity. SNP2, either G or C at the 9th base of the signal peptide coding sequence, changed the third amino acid from lysine to asparagine. Because of their possible role in OPG function and the limited amount of available DNA, we decided to genotype these two SNPs for the 394 samples from postmenopausal women of Akita Prefecture. Age, height, weight, BMI, BMD, and adjusted BMD in each genotype group are shown in Table 3 as mean ± SD. The analysis was performed with BMD and adjBMD as a quantitative phenotype between genotype groups (see Subjects and methods). In SNP1, individuals with the TT genotype showed significantly low BMD and adjBMD when compared with those of the TC or CC genotypes (Table 4) ($P = 0.028$ in BMD and $P = 0.021$ in adjBMD, by the Mann-Whitney U-test). Because our samples showed a nearly normal distribution, a *t*-test was also used, and it also indicated a significant difference ($P = 0.023$). This result may indicate that the allele with C in SNP1 has a protective effect with respect to osteoporosis. On the other hand, in SNP2, no significant result was obtained ($P = 0.561$ for BMD and $P = 0.369$ for adjBMD by the Mann-Whitney U-test; $P = 0.242$ by the *t*-test when GG was compared with GC+CC).

Table 2. Sib pair linkage

Locus	Status	Pairs	Mean	SD	SE	*t*-values	*P*-values
Osteoporosis							
OPG1	0	82	0.517	0.262	0.029	0.591	0.28
	1	49	0.470	0.259	0.037	0.807	0.21
	2	33	0.499	0.277	0.048	−0.020	0.51
OPG2	0	77	0.492	0.205	0.023	−0.324	0.63
	1	45	0.448	0.221	0.033	1.567	0.06
	2	31	0.447	0.185	0.033	−1.593	0.94
Osteopenia							
OPG1	0	44	0.516	0.252	0.038	0.425	0.34
	1	59	0.491	0.271	0.035	0.244	0.40
	2	61	0.495	0.268	0.034	−0.140	0.56
OPG2	0	41	0.483	0.216	0.034	−0.501	0.69
	1	57	0.492	0.200	0.026	0.313	0.38
	2	55	0.438	0.205	0.028	−2.231	0.99

Status: 0, concordant unaffected pairs; 1, discordant pairs; 2, concordant affected pairs. Criteria for diagnosis were bone mineral density (BMD) <70% (osteoporosis) and <80% (osteopenia) of the mean among young adult females. Mean is the average value of allele sharing. SD and SE are standard deviation and standard error, respectively, of the value of allele sharing

Table 3. Polymorphic status and clinical characteristics

	SNP1			SNP2		
	Genotype TT	TC	CC	Genotype GG	GC	CC
n	171	168	55	203	159	32
Age (years)	73.2 ± 6.1	73.1 ± 5.7	73.1 ± 5.9	73.0 ± 6.1	73.1 ± 5.7	74.2 ± 5.2
Height (cm)	144.7 ± 6.1	144.6 ± 5.9	145.4 ± 6.4	144.6 ± 6.2	144.4 ± 5.8	145.5 ± 6.0
Weight (kg)	49.1 ± 8.0	49.0 ± 8.2	50.3 ± 8.7	49.4 ± 8.0	48.9 ± 8.6	50.0 ± 7.7
BMI	23.4 ± 3.4	23.0 ± 3.2	23.4 ± 3.4	23.2 ± 3.4	23.0 ± 3.3	23.3 ± 3.5
BMD (g/cm^2)	0.296 ± 0.075	0.309 ± 0.075	0.314 ± 0.070	0.303 ± 0.074	0.307 ± 0.077	0.297 ± 0.059
adjBMD (g/cm^2)	0.297 ± 0.060	0.311 ± 0.058	0.312 ± 0.060	0.303 ± 0.058	0.308 ± 0.061	0.302 ± 0.060

BMI, body mass index; adjBMD, adjusted bone mineral density; SNP, single-nucleotide polymorphism

Table 4. Comparison of adjBMD between SNP1 genotype groups

	SNP1 genotype	
	TT	TC+CC
n	171	223
Age (years)	73.2 ± 6.1	73.1 ± 5.7
Height (cm)	144.7 ± 6.1	144.8 ± 6.0
Weight (kg)	49.1 ± 8.0	49.3 ± 8.3
BMI	23.4 ± 3.4	23.4 ± 3.3
BMD (g/cm^2)	0.296 ± 0.075*	0.310 ± 0.074
adjBMD (g/cm^2)	0.297 ± 0.060**	0.311 ± 0.059

Values are mean ± SD
* $P = 0.028$, ** $P = 0.021$ (Mann-Whitney U test, TT versus TC+CC)

Haplotype analysis

In order to find the OPG susceptibility haplotype for osteoporosis or other diseases in the future, a haplotype analysis was performed with all six SNPs, based on 37–48 Japanese DNA samples. Unfortunately, the amount of DNA collected from the 394 postmenopausal women of Akita Prefecture was not enough for genotyping these four additional SNPs. Thus, a haplotype association study could not be performed in the present study. Genotype and allele frequencies of these SNPs are shown in Table 5. As shown in Table 6, a strong linkage disequilibrium of variable degree was observed among these SNPs. Haplotype construction with these SNPs revealed that four major haplotypes accounted for 71% of the population (Table 7).

Discussion

In the current study, we investigated the role of OPG in the pathogenesis of osteoporosis by both linkage and association analyses. In the sib pair linkage analysis, two disease criteria (osteoporosis and osteopenia) were used to classify sib pairs. As shown in Table 2, even including osteopenia, it was possible to recruit only 61 affected sib pairs for OPG1 and 55 for OPG2. If λ_s is 3.0, a power to detect an effect of about 80% can be attained with 100 affected pairs (Risch 1990). However, the role of OPG in the pathogenesis of osteoporosis or osteopenia is not estimated to be very strong (Giguere and Rousseau 2000). Thus, the negative result of the present linkage analysis may be because the sample size was too small for detection, even if OPG has some role in the pathogenesis of osteoporosis.

In the association study, we analyzed the quantitative phenotype itself, BMD and adjBMD, which is considered a more powerful technique than a comparison between a disease group and a control group classified according to a quantitative variable (Duggirala et al. 1997). When a group of TT genotype in SNP1 was compared with a group of TC or CC genotype, significantly lower BMD and adjBMD values were identified by both parametric and nonparametric tests (Table 4), with marginal *P* values. SNP1, a T to C change 233 bp upstream from the translation initiation site, was located in the promoter region. Thus, this polymorphism could derive its functional significance by altering the level of promoter activity. A promoter assay with a reporter gene fusion construct could clarify this point. SNP2 changed the third amino acid (lysine to asparagine) of the signal peptide, which is necessary for OPG to be secreted from the cell. Lysine is a basic amino acid, while asparagine is an uncharged polar amino acid. In angiotensinogen, another secreted protein, a basic amino acid in the signal peptide was shown to drastically affect secretory kinetics (Nakajima et al. 1999a). Therefore, although we could not detect a significant disease association with SNP2, the point mutation could also influence OPG's secretory kinetics.

An association study with a haplotype analysis is a powerful tool for determining a genetic contribution to a common disease. Our analysis of the *OPG* gene revealed that four major haplotypes account for 71% of the population as reflected by the considerable linkage disequilibrium among the six SNPs (Table 6). With this information, another association analysis could be performed in the future with a larger sample size to have a reasonable power to detect an effect. Moreover, our finding of the haplotype profile of the gene is useful information for the study of the role of OPG in other disease conditions. In fact, since OPG was identified as a novel secreted protein involved in the regulation of bone density in 1997 (Simonet et al. 1997), several findings suggesting the relevance of OPG to other conditions have been reported. First, OPG-deficient mice had arterial calcification in the large arteries by 2 weeks of age (Bucay et al. 1998), indicating a possible role of OPG in diseases showing

Table 5. Genotype and allele frequencies of all six SNPs

Polymorphism	Nucleotide position	Genotype			No.	Allele frequency	
SNP1	−223[a]	TT	TC	CC		T	C
		18	22	8	48	0.6	0.4
SNP2	+9[a]	GG	GC	CC		G	C
		27	17	4	48	0.74	0.26
SNP3	IVS2-749G>T[b]	TT	TG	GG		T	G
		35	10	1	46	0.87	0.13
SNP4	IVS2-5C>T[b]	CC	CT	TT		C	T
		29	8	0	37	0.89	0.11
SNP5	IVS3-1059G>A[b]	GG	GA	AA		G	A
		35	9	2	46	0.86	0.14
SNP6	IVS3-915T>C[b]	TT	TC	CC		T	C
		16	21	8	45	0.59	0.41

[a]Nucleotide position is identified from the translation initiation site
[b]SNPs designated according to the mutation nomenclature in den Dunnen and Antonarakis (2000)

Table 6. Strength of linkage disequilibrium

Polymorphism		SNP1	SNP2	SNP3	SNP4	SNP5
SNP2	D′	0.90	...	...	...	...
	r^2	0.42				
SNP3		−0.65	−0.64	...	...	...
		0.06	0.03			
SNP4		0.53	−1	−1	...	...
		0.05	0.05	0.03		
SNP5		−0.78	−1	−1	−0.21	...
		0.09	0.08	0.04	0	
SNP6		0.81	1	1	−0.03	−1
		0.30	0.24	0.13	0	0.33

Table 7. Haplotype analysis of the *OPG* gene

SNP1 T→C	SNP2 G→C	SNP3 T→G	SNP4 C→T	SNP5 G→A	SNP6 T→C	Frequency
C	C	T	C	G	T	0.249
T	G	T	C	G	C	0.174
T	G	T	C	A	C	0.160
T	G	G	C	G	T	0.123
T	G	T	C	G	T	0.084
C	G	T	T	G	T	0.069
C	G	T	C	G	T	0.039
T	G	T	T	G	C	0.032
C	G	G	C	G	T	0.023
C	G	T	T	A	C	0.016
C	G	T	C	G	C	0.015
T	C	G	C	G	T	0.015
					Total	1.000

arterial calcification. In addition, an association of serum OPG levels with diabetes and cardiovascular mortality suggests the possibility that OPG may be a cause of vascular calcification (Browner et al. 2001). Second, expression of OPG is not restricted to bone; it is expressed in a variety of tissues and cell systems, such as heart, lung, kidney, placenta, liver, thyroid gland, spinal cord, and brain. In addition, it is expressed in various immune and hematological tissues and mesenchymal organs (Hofbauer 1999). Third, a possible role of OPG in the immune system was shown in experiments with OPG-deficient mice by Yun et al. (2001), who reported that OPG regulates B cell maturation and development.

Ethnic differences in the genetic background of diseases are sometimes observed. For example, although Grant et al. (1996) reported the association of osteoporosis with a polymorphic SP1-binding site in the collagen type Iα1 gene, we could not find the polymorphism in a study of Japanese individuals (Nakajima et al. 1999b). Thus, similar OPG studies should be performed with samples from other ethnic groups because OPG might also be related to osteoporosis in populations other than Japanese.

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ORIGINAL ARTICLE

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Haplotype analysis of the human collectin placenta 1 (*hCL-P1*) gene

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Abstract Collectins are a family of C-type lectins found in vertebrates. These proteins have four regions, a relatively short N-terminal region, a collagen-like region, an alpha-helical coiled coil, and a carbohydrate recognition domain. Collectins are involved in host defense through their ability to bind carbohydrate antigens on microorganisms. Type A scavenger receptors are classical-type scavenger receptors that also have collagen-like domains. We previously described a new scavenger receptor, collectin from placenta [collectin placenta 1 (CL-P1)]. CL-P1 is a type II membrane protein with all four regions. We found that CL-P1 can bind and phagocytize both bacteria and yeast. In addition to that, it reacts with oxidized low-density lipoprotein (LDL) but not with acetylated LDL. These results suggest that CL-P1 might play important roles in host defenses and/or atherosclerosis formation. One rational strategy to study the role of CL-P1 in these pathological conditions would be to perform a haplotype association study using human samples. As a first step for this strategy, we analyzed the haplotype structure of the *CL-P1* gene. By sequencing the *CL-P1* gene in ten Japanese volunteers, we identified five single-nucleotide polymorphisms (SNPs) with a minor allele frequency of at least 29%. To obtain SNPs in the 5′-upstream region of the gene, we screened a total of 20 SNPs described in the database and finally picked up one SNP for the present study. Thus, a total of six SNPs, one in the 5′-upstream region, two in intron 2, one in exon 5, and two in exon 6, were used to analyze the haplotype structure of the gene, with DNAs derived from 54 individuals (108 alleles). The analysis revealed that only two of six SNPs showed significant linkage disequilibrium ($r^2 > 0.5$) with each other. This haplotype information may be useful in disease-association studies in which a contribution of the *CL-P1* gene has been suspected, especially in immunological disturbance or atherosclerosis. Two SNPs in exon 6, both leading to amino acid substitutions, could be candidates for influencing disease susceptibility.

Key words Single-nucleotide polymorphisms · Linkage disequilibrium · Haplotype · Collectin · Japanese population · Host defense · Amino acid substitution · Atherosclerosis

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Introduction

Collectins are a family of proteins that contain a collagen-like region and a carbohydrate recognition domain (CRD) (Drickamer 1988). Collectins are found in vertebrates including avians (Laursen et al. 1998). There are four groups of collectins: a mannan-binding protein (MBP) group, two lung surfactant groups of surfactant protein A (White et al. 1985) and surfactant protein D (SP-D) (Persson et al. 1989), and a newly isolated group, collectin liver 1 (Ohtani et al. 1999) and collectin placenta 1 (CL-P1) (Ohtani et al. 2001). Collectins are known to be involved in innate immunity through various mechanisms, for example, by activation of the complement pathway (Kawasaki et al. 1989), by opsonization via collectin receptors (Schweinle et al. 1989; van Iwaarden et al. 1992), and by amplification of phagocytosis by macrophages (Pikaar et al. 1995). Interestingly, the type A scavenger receptor (SR-A) also contains a collagen-like domain (Kodama et al. 1990), which could bind and destruct pathogens by endocytosis and phagocytosis. In fact, recent data with SR-A1 knock-out mice (Suzuki et al.

1997) support the possibility that scavenger receptors also have a role in innate immunity.

Recently, we cloned and characterized the human *CL-P1* gene (*hCL-P1*), and found that it was a membrane-type collectin that functions as a scavenger receptor. The major tissue expression of the gene was endothelial cells but not in monocyte–macrophage lineage cells. We also found that CL-P1 was able to bind and phagocytose bacteria and yeast as well as oxidized low-density lipoprotein (Ohtani et al. 2001). With these results, we hypothesized that this gene could play an important role in the human innate immunity system and/or atherosclerosis formation. To study this possibility, we are collecting human samples that are susceptible to infection, especially in childhood, and also samples from patients with atherosclerosis without lipid profile abnormalities. Haplotype association studies would be the best choice for this purpose. Here, we report the haplotype structure of the *CL-P1* gene obtained from six single-nucleotide polymorphisms (SNPs) that were genotyped with 108 alleles in Japanese subjects.

Subjects and methods

Identification of SNPs

To identify useful SNPs in the gene region, we sequenced amplified DNAs derived from ten Japanese volunteers (20 alleles). Polymerase chain reaction (PCR) primers were chosen at approximately 500-bp intervals, with about an 80-bp overlap (Shinohara et al. 2001). A total of 16 primer sets were designed based on the information obtained from a public genome database (AC022545, AP000925, AP001005, http://www.ncbi.nlm.nih.gov/) to amplify all 10 exons; 5′-flanking regions; and introns 1, 4, 6, 7, and 9. Other introns were sequenced in the regions near exon–intron boundaries. After amplification, a sequencing reaction was performed and analyzed on an ABI 310 genetic analyzer (PE Biosystems, Foster City, CA, USA). SNPs were detected by alignment of the sequencing data of ten individuals. To obtain SNPs in the 5′-upstream region, we analyzed 20 SNPs described in the publicly available SNP database of The SNP Consortium Ltd (TSC; http://snp.cshl.org/index.html) with 20 Japanese alleles.

SNP genotyping

SNPs were genotyped using three PCR-based methods: single-strand conformational polymorphism (SSCP), SNaPshot, and sequencing. PCR was performed in a volume of 12.5μl containing 20ng genomic DNA, 75mM Tris HCL (pH 8.8), 20mM $(NH_4)_2SO_4$, 0.01% Tween 20, 1.5mM MgCl2, 200μM deoxyribonucleotide triphosphates (dNTPs), 10pmol of each primer, and 0.25 units of Taq polymerase. PCR amplification was performed through 30 cycles of 94°C for 30s, 49°–62°C for 30s, and 72°C for 30s, depending on the region analyzed, with a final extension step of 5min at 72°C in a Gene Amp PCR9700 System (PE Biosystems). For SSCP analysis, each 2.5μl of PCR products were mixed with formamide dye and heat denatured, and then applied to polyacrylamide gel electrophoresis. After electrophoresis, the gel was silver stained (Wako Pure Chemical, Tokyo, Japan) to detect polymorphic bands. A SNaPshot primer extension method was performed following the manufacturer's instructions (PE Biosystems). To obtain a purified template for SNaPshot and sequencing, we removed dNTPs and primers from amplified genomic DNA by means of ExoSAP-IT (USB, Cleveland, OH, USA). In the case of the SNaPshot method, reaction mixtures were electrophoresed on an ABI 310 genetic analyzer and data were analyzed with GeneScan software (PE Biosystems). For haplotype analysis, a total of 54 DNA samples (108 alleles) of healthy volunteers, recruited in our medical school, were genotyped. All of them were medical students and gave their informed consent. The Ethics Committee of Asahikawa Medical College approved this study.

Haplotype analysis

Estimation of haplotype frequency was performed by the maximum-likelihood method using SNPAlyze (DYNACOM, Yokohama, Japan, http://www.dynacom.co.jp/).

Results and discussion

By sequencing the *CL-P1* gene region of ten Japanese volunteers (20 alleles), we identified five SNPs, two in intron 2 (SNP2, SNP3), one in exon 5 (SNP4), and two in exon 6 (SNP5, SNP6; Fig. 1, Table 1). In consideration of their importance in gene regulation, 20 SNPs were screened by sequencing the 5′-upstream region described in The SNP Consortium Ltd database. SNP1 was shown to be useful for our purposes. A total of six SNPs were used for genotyping and haplotype analysis. Genotype and allele frequencies of six SNPs and their location in the gene are shown in Table 1. All genotype frequencies conformed to Hardy-Weinberg equilibrium. Genotyping of 54 individuals (108 alleles) revealed that all SNPs are quite common, the minor allele frequency being at least 29%.

Of six SNPs, three (SNP4–6) are located in the coding region, one silent mutation is located in exon 5 (SNP4: T267T), and two missense mutations are located in exon 6 (SNP5: S522P, SNP6: G606S; Table 1). As shown in Fig. 1, the SNP5 in exon 6 is in the collagen-like domain that has a characteristic Gly-X-Y repetitive pattern, in which X and Y can be any amino acid but are frequently prolines or hydroxyprolines. SNP5, either serine or proline, located in the X position, could have some affect on the stability of collagen structure. In fact, in the *MBP* gene, which belongs to one of the collectin groups and is known to have a significant role in innate host immunity, three relatively common mutations in the homotrimeric region have been reported to be associated with invasive pneumococcal disease (Roy

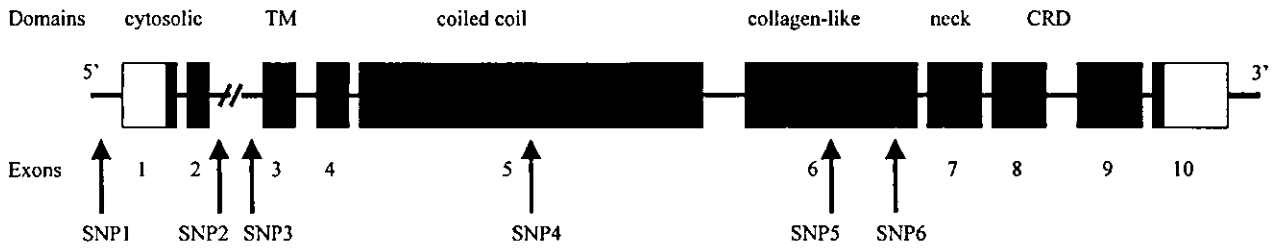

Fig. 1. Relationship between genomic structure and the location of single-nucleotide polymorphisms (*SNPs*) in the human *CL-P1* gene. *Vertical rectangles* and *horizontal lines* represent exons and introns, respectively. Coding regions are shown with *filled rectangles*. *Arrows* indicate appproximate position of each SNP. Functional domains are indicated *above the gene*. *TM*, Transmembrane domain; *CRD*, carbohydrate recognition domain

Table 1. Genotype and allele frequencies of six SNPs in Japanese subjects

Polymorphism	Nucleotide position	Amino acid	Genotype			Allele		Allele frequency	
SNP1 (5' upstream region)	−4078[a]		TT	TG	GG	T	G	T	G
			22	24	8	68	40	0.63	0.37
SNP2 (intron 2)	IVS2+814G>A[b]		GG	GA	AA	G	A	G	A
			21	24	9	66	42	0.61	0.39
SNP3 (intron 2)	IVS2−1619A>G[b]		AA	AG	GG	A	G	A	G
			24	24	6	72	36	0.67	0.33
SNP4 (exon 5)	800+1C>T[b]	T267T	CC	CT	TT	C	T	C	T
			28	21	5	77	31	0.71	0.29
SNP5 (exon 6)	1563+1C>T[b]	S522P	CC	CT	TT	C	T	C	T
			27	23	4	77	31	0.71	0.29
SNP6 (exon 6)	1815+1A>G[b]	G606S	GG	GA	AA	G	A	G	A
			23	27	4	73	35	0.68	0.32

SNP, Single-nucleotide polymorphism
[a]Nucleotide position is identified from the translation initiation site
[b]The designation of other SNPs follows the recommendation of mutation nomenclature in Dunnen and Antonarakis (2000)

et al. 2002). Although two of the three mutations, those in codons 54 and 57, located in the Gly position, the remaining mutation in codon 52 is located at the X position, which exchange the amino acid cysteine for arginine. In terms of SNP6 in exon 6, either glycine or serine is located in the neck domain. Hydrophobic amino acids in the neck domain contribute to form an alpha-helical coiled coil. A coiled-coil structure can be recognized by its characteristic heptad repeat pattern a-b-c-d-e-f-g, in which "a" and "d" are hydrophobic residues. Each alpha-helix interacts with a neighboring CRD (Sheriff et al. 1994). Human MBP and SP-D have such heptad repeats in the neck domain (Zhang et al. 2001). However, in the case of hCL-P1, the neck domain of 17 amino acid residues does not have a characteristic heptad structure. Even so, both collagen-like domain and CRD have a collaborative role in recognizing pathogens, and an amino acid alteration in the neck domain might still have some influence.

The result of linkage disequilibrium (LD) analysis is summarized in Table 2. Among the SNPs, only SNP4 and SNP5 showed significant LD (r^2 = 0.823). Haplotype frequencies among 108 alleles were calculated (Table 3). The top eight haplotypes with frequencies over 5% accounted for 67% of all haplotypes.

CL-P1 is a member of the collectin family, which is considered to play significant roles in innate immunity. Classical collectins are soluble, but CL-P1 is membrane bound.

Table 2. Analysis of linkage disequilibrium for all possible two-way comparisons among six SNPs

		SNP1	SNP2	SNP3	SNP4	SNP5	SNP6
SNP1	*D'*		0.040	0.659	0.144	0.245	0.208
	r^2		0.001	0.119	0.005	0.014	0.012
	P		0.822	0.000	0.534	0.287	0.279
SNP2	*D'*			0.003	0.226	0.145	0.003
	r^2			0.000	0.032	0.013	0.000
	P			0.822	0.040	0.236	0.822
SNP3	*D'*				0.325	0.437	0.092
	r^2				0.092	0.167	0.008
	P				0.001	0.000	0.351
SNP4	*D'*					0.907	1.000
	r^2					0.823	0.195
	P					0.000	0.000
SNP5	*D'*						1.000
	r^2						0.195
	P						0.000

CL-P1 might bind and control not only bacteria and yeasts, but also modified LDLs in the vascular space. The collagen-like domains in human and mouse CL-P1, which have the highest identity (96%) described to date, may play the most important role in these biological functions (Ohtani et al. 2001).

In conclusion, these six polymorphisms, their haplotype, and the state of LD will the useful for investigation of a possible relationship between genetic variation at the