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Nucleotide Pyrophosphatase Gene Polymorphism Associated With Ossification of the Posterior Longitudinal Ligament of the Spine

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ABSTRACT

Ossification of the posterior longitudinal ligament (OPLL) of the spine is a disease that causes paralysis by compressing the spinal cord. Based on the fact that the nucleotide pyrophosphatase (*Npps*) gene is responsible for ectopic ossification in *ttw*, an OPLL model mouse, the possibility was explored whether the human *NPPS* gene is associated with susceptibility to and severity of OPLL. First, we screened for single-nucleotide polymorphisms (SNPs) in the human *NPPS* locus using selected 25 OPLL patients with young onset (<35 years old) or severe ossification (>10 ossified vertebrae), and identified three novel SNPs in the locus. A case-control association study between 180 OPLL patients and 265 non-OPLL controls showed that one of these SNPs, IVS15-14T → C substitution, was more frequently observed in OPLL patients ($p = 0.022$), especially in those with severe ossification ($p < 0.0001$) and young onset ($p = 0.002$), than in controls. A stratified study with the number of ossified vertebrae in OPLL patients revealed that IVS15-14T → C substitution ($p = 0.013$) as well as young onset ($p = 0.046$) and female sex ($p = 0.006$) were associated with severe ossification. We conclude that the IVS15-14T → C substitution in the human *NPPS* gene is associated not only with susceptibility to, but also with severity of OPLL. (J Bone Miner Res 2002;17:138–144)

Key words: **single-nucleotide polymorphism, ossification of the posterior longitudinal ligament, *Npps*, *ttw* mouse, risk factor**

INTRODUCTION

OSSIFICATION OF the posterior longitudinal ligament (OPLL) of the spine is a disease that causes development of ectopic ossification of the ligament in the spinal canal (Fig. 1).[1–3] It was first described by Key in 1839,[4] and an autopsy report by Tsukimoto established the disease entity.[5] It is a common disease with an incidence of 2–4% in the middle and older population in Asian countries, whereas lower incidence has been reported in non-Asian countries.[6,7] OPLL is considered a collective result of heterogeneous etiopathologic factors affecting the spinal ligament; therefore, the clinical features, especially severity of ossification, of OPLL patients are variable. The average age of onset of clinical symptoms by OPLL is over 50 years old, and most OPLL patients have minimal ossification (one or two ossified vertebrae) exhibiting no or mild neurological symptoms with onset at older ages (Fig. 1B). On the other hand, some patients have massive ossification compressing the spinal cord that results in severe tetraparesis or parapa-

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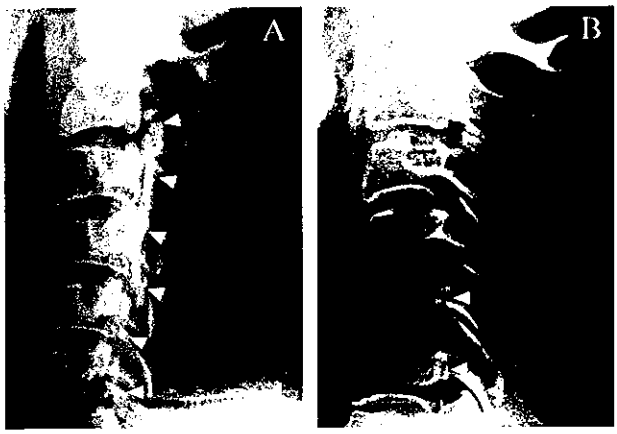

FIG. 1. X-ray of the cervical spine of two typical types of OPLL patients (A: 32-year-old female; B: 67-year-old male). The ossified posterior longitudinal ligament is indicated by arrows. Patient A showed severe tetraparesis in upper and lower limbs, which resulted from the compression of the spinal cord. Patient B exhibited only mild numbness in right fingers.

resis starting from a young age (Fig. 1A). Contribution of genetic factors to the etiology of OPLL have been implicated by epidemiological studies[6,8,9] and by association studies with variants around the HLA antigen region[10-12]; however, genetic components of this disease have not been completely understood. Several factors, including late onset of the disease and lack of a large family suitable for genetic linkage analysis, have hampered the genetic analysis of OPLL.

To investigate genetic factors involved in the etiology of OPLL, we studied *ttw* (*tiptoe walking*), a mouse model of OPLL that results from a naturally occurring mutation.[13] The *ttw* mouse is an excellent model for OPLL because it exhibits ossification of the spinal ligament similar to OPLL, with almost complete penetrance.[13,14] By the positional candidate approach, we have identified a nonsense mutation (Gly568stop) within the nucleotide pyrophosphatase (*Npps*) gene as the cause of the *ttw* condition.[15] The mutation results in truncation of the gene product, NPPS protein, and causes its dysfunction. Because NPPS is the ectoenzyme producing inorganic pyrophosphate (PPi),[16] a major inhibitor of tissue calcification and mineralization,[17] the dysfunction of NPPS in the *ttw* mouse may result in the decrease of PPi and lead to ossification of unwanted tissues.

The human counterpart of this gene, *NPPS*, has been cloned.[18,19] *NPPS* lies on chromosome 6q and is expressed in a variety of tissues including bone, cartilage, and ligament.[20,21] In a previous study, we characterized the genomic organization of the *NPPS* gene and examined its genetic variation in OPLL patients, to evaluate its possible involvement in the etiology of OPLL.[22] We identified a sequence variant with a one-base deletion in intron 20 of the gene and reported that this variant is less frequently observed in OPLL patients than in controls by a case-control association study. However, our subsequent study using a different population of OPLL patients with detailed information of phenotypes (n = 180, patients in Tokyo University Hospital) failed to replicate the association of this variation with severity of OPLL, as determined by the number of ossified vertebrae. Therefore, in this study, we again screened for single-nucleotide polymorphisms (SNPs) in the *NPPS* locus using OPLL patients with severe ossification and young onset in whom genetic influence seemed predominant. We identified three novel SNPs other than those reported previously[22]; among them an SNP in intron 15 (IVS15-14T → C) was significantly associated with not only susceptibility to but also severity of OPLL. This study further investigated risk factors including sex and age for severity of ossification in OPLL patients.

MATERIALS AND METHODS

Patients

A total of 180 OPLL patients (136 males and 44 females; ages 34–86 years old, 52.0 ± 9.2 years old, means ± SD) and 265 non-OPLL controls (194 males and 71 females; ages 29–88 years old, 53.4 ± 8.7 years old), who visited the orthopedic clinic at Tokyo University Hospital and gave written informed consent for the study, were investigated. All patients and controls were unrelated Japanese living on the main island of Japan. Diagnosis of OPLL was performed by specialists based on radiologic examination of the cervical, thoracic, and lumbar spines. All patients were symptomatic and required medical treatment including surgery. Spinal spondylosis, ankylosing spondylosis, disk herniation, and metabolic diseases associated with OPLL, such as hypoparathyroidism, were excluded by radiographic and biochemical examinations. Genomic DNA samples were extracted from peripheral leukocytes of OPLL patients and controls by a standard procedure and were subjected to polymerase chain reaction (PCR) amplification. The study protocol was approved by the ethical committee for human subjects of the University of Tokyo.

All phenotypic data of each patient, including severity of ossification and age, were recorded at the time of the patient's first visit to Tokyo University Hospital. All patients visited the hospital within 6 months after the onset of clinical symptoms. The severity of OPLL was determined by the number of ossified vertebrae on lateral X-ray films of the cervical, thoracic, and lumbar spines. In the lower cervical and upper thoracic area where OPLL lesions could not be detected on plain X-ray, a tomographic analysis was performed and the lesions were evaluated. Further analyses were performed in subgroups by dividing the entire OPLL population by sex, age, and severity of ossification. Patients at or below the average age (52.0 years old) were classified in the young group, and those older in the old group. Patients with more than 10 ossified vertebrae were classified in the severe group, and those with 10 or less than that in the mild group.

Identification of SNPs in the NPPS *gene*

To identify sequence variations associated with susceptibility to and severity of OPLL, 25 OPLL patients under 35 years of age or who had more than 10 ossified vertebrae

TABLE 1. GENOTYPIC AND ALLELIC DISTRIBUTIONS OF THE IVS15 T → C SNP IN OPLL PATIENTS AND NON-OPLL CONTROLS[a]

	Genotype			*Allele type (%)*				
Subjects (n)	*C/C*	*C/T*	*T/T*	*C*	*T*	χ^2	p *Value*	*Odds ratio (95% CI)*
OPLL								
All (180)	1	10	169	12 (3.3)	348 (96.7)	5.24	0.022	3.01 (1.1–8.1)
Male (136)	0	7	129	7 (2.6)	265 (97.4)	2.32	0.128	2.31 (0.8–6.9)
Female (44)	1	3	40	5 (5.7)	83 (94.3)	3.36	0.067	5.26 (1.6–17.6)
Control								
All (265)	0	6	259	6 (1.1)	524 (98.9)			
Male (194)	0	4	190	4 (1)	384 (99)			
Female (71)	0	2	69	2 (1.4)	140 (98.6)			

[a] Differences in allelic distribution between subpopulations of OPLL patients and corresponding controls were analyzed using χ^2 test.

were selected from the overall population. The screening of SNPs in the *NPPS* locus was carried out using DNA samples obtained from these patients in whom the influence of genetic factors seemed predominant. SNPs in *NPPS* were identified as previously described.[22] In this way, three novel SNPs in exon 9, intron 15, and intron 20 other than the SNPs reported in our previous study[22] were identified.

Association studies

To examine the association of the SNP with susceptibility to OPLL, a case-control association study was performed, comparing the allelic distribution between the patients and controls. In addition, to examine the impact of SNPs on severity of OPLL, the association between the SNPs and the number of ossified vertebrae was investigated in OPLL patients. These studies were performed not only in the whole population but also in the subgroups classified by sex, age, and severity of ossification. A 5-ng sample of DNA was used for PCR amplification of sequences containing the novel polymorphism. Because the SNP in intron 15 was a restriction fragment length polymorphism (RFLP) by *Afl*III enzyme, genotyping for it was performed by PCR-RFLP analysis. The primer sequences were 5′-TCAATTATG-TTTTATGAAAGGACTT-3′ and 5′-TTCAAGTCTAAA-ACTTTGCATATCA-3′. Genotyping for variances in exon 9 and intron 20 was performed by allele-specific oligonucleotide (ASO) hybridization and direct sequencing, respectively.

Statistical analysis

The χ^2 test was used to assess Hardy–Weinberg equilibrium and allelic frequency differences. Odds ratios (ORs) with 95% CIs were calculated as estimates of the relative risk. To compare the number of ossified vertebrae between two groups, distribution of values was first analyzed in each group using the f test. Between groups with normal distribution, parametrical analysis using Student's t-test (StatView-J 4.5; Stat View, Berkeley, CA, USA) was performed. When a group did not show normal distribution, nonparametrical analysis using Mann–Whitney U-test (StatView-J 4.5) was used. The simple regression analysis (StatView-J 4.5) was performed to evaluate association between age and number of ossified vertebrae. A value of $p < 0.05$ was considered to indicate statistical significance.

RESULTS

The screening of SNPs using OPLL patients in whom genetic influence seemed predominant ($n = 25$, <35 years old or >10 ossified vertebrae) identified three novel SNPs in the *NPPS* locus: C → T substitution at position 973 in exon 9; T → C substitution in intron 15 at position 14, upstream from the start of exon 16 (IVS15-14T → C); and a base-pair deletion in intron 20 at position 10, upstream from the start of exon 21. SNP in either exon 9 or intron 20 showed no significant difference of allelic distribution between OPLL patients and controls ($\chi^2 = 1.01$, $p = 0.316$ and $\chi^2 = 2.53$, $p = 0.282$, respectively), indicating that these two SNPs are not associated with susceptibility to OPLL. Hence, further association studies were performed on the IVS15-14T → C SNP. The distributions of genotype and allele type of this SNP in OPLL patients and controls are shown in Table 1. Deviation from Hardy–Weinberg equilibrium was not significant in any of the populations. The distribution of alleles was different between OPLL patients and controls ($\chi^2 = 5.24$, $p = 0.022$), and the OR for OPLL patients with minor C allele compared with that of controls was 3.01 (95% CI 1.1–8.1) in the overall population. The OR in female patients was higher than that in male patients. The allelic distribution, however, was not different between males and females in OPLL patients ($\chi^2 = 1.99$, $p = 0.158$) or in controls ($\chi^2 = 0.13$, $p = 0.716$). The average age also was not different between male and female patients (53.1 ± 0.8 and 50.4 ± 1.2 years old, respectively; $p = 0.097$). In addition, there was no significant difference of age between carriers of the minor C allele (homozygous CC genotype and heterozygous CT genotype) and noncarriers of C allele (homozygous TT genotype) (48.6 ± 2.9 and 52.7 ± 0.7, respectively; $p = 0.159$). These results indicate

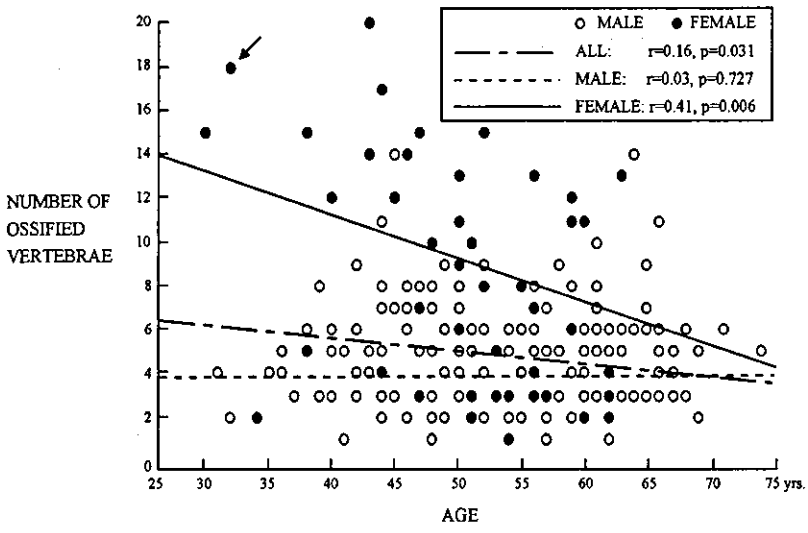

FIG. 2. Correlation between the number of ossified vertebrae and age in male (n = 136) and female (n = 44) OPLL patients. Data on each patient were recorded at the time of the first visit to the hospital, within 6 months after the onset of clinical symptoms. Arrow indicates the only patient with homozygous CC genotype.

that sex, age, and IVS15-14T → C SNP are not associated with one another in OPLL patients.

To learn the risk factors for severity of OPLL, we investigated the relationship of age and sex to the number of ossified vertebrae in patients (Fig. 2). The natural course of ossification in each individual is considered not to be regressive but progressive with aging, depending on environmental factors such as accumulated mechanical stress on the spine; however, the number of ossified vertebrae was, in fact, negatively correlated with age in all OPLL patients (p = 0.031). This negative correlation was more evident in female patients (p = 0.006), whereas in males the number of ossified vertebrae tended to increase slightly with age. It is therefore indicated that in OPLL patients there are both a younger onset subpopulation with severe ossification and an older onset subpopulation with mild ossification. These results imply that genetic factors may potently contribute to severity of ossification, especially in the female patients with young onset.

Hence, we further investigated the distributions of genotype and allele type of subgroups of OPLL patients classified by sex, age, and severity of ossification (Table 2). Allelic distribution was significantly different from that of controls in the severe and the young populations, but not in the mild or the old population. The minor C allele was more frequently observed in the severe (OR 11.21, [95% CI 3.3–38.3], $p < 0.0001$) and young (OR 4.44, [95% CI 1.6–12.7], p = 0.002) populations than in controls. In male patients the C allele was significantly frequent in the severe subpopulation (OR 12.48, [95% CI 1.3–117.7], p = 0.005), whereas in female patients it was frequent in the severe (OR 10.92, [95% CI 2.9–40.6], $p < 0.0001$) and the young (OR 9.7, [95% CI 2.8–33.1], $p < 0.0001$) populations. These results indicate that the association of IVS15-14T → C SNP with susceptibility to OPLL is particularly strong in OPLL patients with severe ossification or young onset.

Finally, we looked at the association of IVS15-14T → C SNP, sex, and age with severity of ossification in OPLL patients (Fig. 3). Patients carrying C allele (p = 0.013), of the female sex (p = 0.0003), and young onset (p = 0.046) showed a greater number of ossified vertebrae than noncarrier patients, of the male sex, and old onset, respectively. The association of IVS15-14T → C SNP was observed only in female patients. Females carrying C allele were all young onset and showed the greatest number of ossified vertebrae among all subgroups. In addition, the only case with homozygous CC genotype belonged to this subgroup (arrow in Fig. 2). In male patients, on the other hand, IVS15-14T → C SNP was not associated with severity of ossification either in the population as a whole or in the young and old populations individually.

DISCUSSION

Although OPLL is a multifactorial disease and its clinical features are variable, as shown in Fig. 1, previous studies on genetic backgrounds of OPLL were all case-control association studies that compared subjects with and without OPLL.[8–12,22] The present study is the first to investigate the disease's genetic backgrounds, taking severity of ossification as well as sex and age of OPLL patients into consideration. It is true that the natural course of ossification in each patient progresses with age, implying the contribution of environmental factors such as accumulated mechanical stress on the spine. However, this study clearly showed that there exists a subgroup with young onset and severe ossification among OPLL patients (Fig. 2). It is likely that the contribution of genetic factors is much stronger in this subgroup than in those with old onset and mild ossification. Using DNA samples from this subgroup of patients (<35 years old or >10 ossified vertebrae), we found three novel SNPs in the human *NPPS* gene that had not been identified in a previous report using common OPLL patients.[22] One of the SNPs, IVS15-14T → C substitution, was shown to be more frequently observed in these patients, especially in those with severe ossification and young onset, than in controls, suggesting the involvement of this SNP in susceptibility to the disease. A stratified study with severity of ossification in OPLL patients revealed that IVS15-14T → C

TABLE 2. GENOTYPIC AND ALLELIC DISTRIBUTIONS IN SUBPOPULATIONS OF OPLL PATIENTS[a]

	Genotype			*Allele type (%)*				
Subjects (n)	*C/C*	*C/T*	*T/T*	*C*	*T*	χ^{2b}	p *Value*[b]	*Odds ratio (95% CI)*[b]
Severe[c]								
All (22)	1	3	18	5 (11.4)	39 (88.6)	22.63	<0.0001	11.21 (3.3–38.3)
Male (4)	0	1	3	1 (12.5)	7 (87.5)	7.93	0.005	12.48 (1.3–117.7)
Female (18)	1	2	15	4 (11.1)	32 (88.9)	19.34	<0.0001	10.92 (2.9–40.6)
Mild[c]								
All (158)	0	7	151	7 (2.2)	309 (97.8)	1.54	0.215	1.98 (0.7–5.9)
Male (132)	0	6	126	6 (2.3)	258 (97.7)	1.54	0.215	2.03 (0.6–6.4)
Female (26)	0	1	25	1 (1.9)	51 (98.1)	0.25	0.618	1.71 (0.2–14.5)
Young[d]								
All (93)	1	7	85	9 (4.8)	177 (95.2)	9.22	0.002	4.44 (1.6–12.7)
Male (68)	0	4	64	4 (2.9)	132 (97.1)	2.39	0.122	2.65 (0.7–9.5)
Female (25)	1	3	21	5 (10)	45 (90)	19.31	<0.0001	9.7 (2.8–33.1)
Old[d]								
All (87)	0	3	84	3 (1.7)	171 (98.3)	0.36	0.546	1.53 (0.4–6.2)
Male (68)	0	3	65	3 (2.2)	133 (97.8)	0.94	0.333	1.97 (0.5–7.9)
Female (19)	0	0	19	0 (0)	38 (100)	0.44	0.509	1.05 (0.06–18.9)

[a] Differences in allelic distribution between subpopulations of OPLL patients and the overall controls (n = 265, shown in Table 1) were analyzed using χ^2 test.
[b] Compared with controls.
[c] Patients with more than 10 ossified vertebrae were classified in the severe group, and those with 10 or less than that in the mild group.
[d] Patients at or below the average age (52.0 years old) were classified in the young group, and those older in the old group.

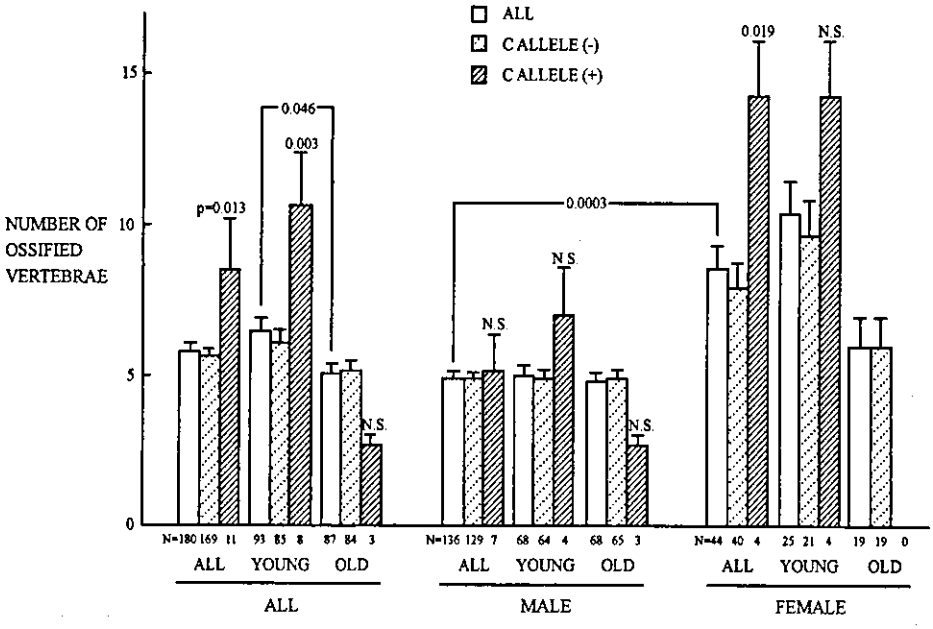

FIG. 3. The number of ossified vertebrae of OPLL patients in each subgroup classified by sex, age, and allelic type of IVS15-14T → C SNP. Data are expressed as means (bars) ± SEM (error bars) for the number of patients shown under each bar. The number above the bars is the p Value of the difference in the number of ossified vertebrae between carriers and noncarriers of the C allele, unless otherwise indicated by lines. n.s., not significant.

substitution, female sex, and young onset were associated with severe ossification, although these three factors were unrelated with one another.

In a previous study, we reported that a one-base deletion in intron 20 of the gene (IVS20-11delT) is more frequently observed in OPLL patients than in controls by a case-control association study, suggesting that this polymorphism is associated with susceptibility to OPLL.[22] Unfortunately, however, this comparison may be inappropriate because our subsequent reanalysis revealed that deviation of the genotype counts from Hardy–Weinberg equilibrium in controls (wild type/wild type 153, wild type/variant 130, variant/variant 49) was significantly large (χ^2 = 5.73, p = 0.017). When a case-control association study was performed in the statistically appropriate OPLL and control populations used in the present study in whom deviation from Hardy–Weinberg equilibrium was not significant, allelic distribution was found not to differ between OPLL

patients and controls ($\chi^2 = 0.80, p = 0.370$). The number of ossified vertebrae was also not different between carriers and noncarriers of the deletion variance (5.59 ± 0.33 vs. 6.15 ± 0.49, $p = 0.762$) in OPLL patients. These results indicate that IVS20-11delT SNP by itself is not related to susceptibility to or severity of OPLL, at least in the populations used in the present study. Our preliminary haplotypic analysis of IVS15-14T → C and IVS20-11delT SNPs, however, showed that patients with the C allele and without the deletion variance ($n = 5$) exhibited significantly greater ossification than patients of other haplotypes ($p < 0.05$, data not shown). Because it is possible that the association observed in this study is the result of linkage disequilibrium, we are planning to perform haplotypic analyses among several SNPs identified in the gene by increasing the number of subjects.

In addition to IVS15-14T → C SNP, both sex and age were associated with severity of ossification in OPLL patients. Because the allelic distribution was not correlated with either of the factors, they are likely to be independently related to severity of ossification. It is an interesting finding that ossification is more severe in female patients than in male patients, because the morbidity of OPLL is known to be 2- to 3-fold higher in males than in females.[3,6] This implies that the incidence and progression of ossification in OPLL patients are regulated by different factors. IVS15-14T → C substitution was associated with severe ossification, which was related to young onset of clinical symptoms; however, the genetic substitution was not directly associated with the onset age. This fact might indicate the existence of other factors affecting the onset of symptoms, and the most probable candidates may include the space of the spinal canal and the durability of the spinal cord.

Although IVS15-14T → C SNP in the human *NPPS* locus was shown to be the only sequence variation associated with both susceptibility to and severity of OPLL, the mechanism whereby this variation increases a risk for OPLL is currently unclear. The most likely mechanism may be that this intronic substitution affects the splicing mechanism because it lies near the exon–intron junction; the one-base substitution may yield an alternative transcript with abnormal function or affect the expression level of NPPS. The next task ahead of us will be investigating the relevance of the SNP to the production or function of NPPS.

Our results suggest that NPPS plays an important role in the etiology of OPLL, as it does in mouse[15]; however, its pathological mechanism causing ectopic ossification is unknown. The most likely explanation for this is an action of NPPS through the regulation of PPi because PPi is a major inhibitor of calcification and mineralization.[17] A recent report showed that ectopic calcification is also caused by a mutation of *ank* gene, whose product ANK protein is believed to be a transmembrane transporter that shuttles PPi between intracellular and extracellular compartments.[23] These similar phenotypes observed in the *ttw* and *ank* mouse strongly support the importance of endogenous PPi for the regulation of soft tissue calcification, although the former develops excessive hydroxyapatite and the latter calcium pyrophosphatase dihydrate (CPPD) deposition. Alternatively, NPPS could inhibit ectopic ossification through the regulation of bone anabolic factors such as insulin.[24] NPPS has been shown to be an inhibitor of insulin-receptor tyrosine kinase.[25] Association of NPPS with transforming growth factor β and fibroblast growth factors has also been suggested.[21] Further study is needed to clarify the regulatory mechanism of NPPS as a novel regulator of bone metabolism.

Results obtained in this study not only show the contribution of *NPPS* gene to the etiology of OPLL but also can possibly be applied to diagnosis and treatment of this disease. Postoperative courses of OPLL are not always satisfactory, one of the reasons for which is the recurrence or progression of ossification. Hence, some diagnostic marker to predict OPLL progression is useful to decide the surgical method including the range of decompression. The IVS15-14T → C SNP may be a strong candidate for this genetic marker. In addition, there is so far no conservative treatment to prevent the progression of ossification in OPLL patients. We believe that drugs to regulate the NPPS expression or function could possibly lead to the prevention of the condition.

When taken together, we first identified a sequence variation in the human *NPPS* gene that is associated with not only susceptibility to but also severity of OPLL. This gene might have a relatively small attributable risk fraction for this disease; however, identification of the disease-susceptibility and disease-severity gene could lead to a better understanding of the molecular pathogenesis of this disease. In addition, a human *NPPS* gene abnormality was recently reported to be associated with idiopathic infantile arterial calcification (IIAC), another disease with ectopic ossification.[26] Further studies on the functional relevance of the human *NPPS* gene abnormalities could provide new biological insights into the etiology of diseases with ectopic ossification.

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Association of Bone Metabolism Regulatory Factor Gene Polymorphisms With Susceptibility to Ossification of the Posterior Longitudinal Ligament of the Spine and Its Severity

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Study Design. A case–control association study and a stratified study investigating the genetic etiology for ossification of the posterior longitudinal ligament of the spine.

Objective. To determine the association of restriction fragment length polymorphisms of estrogen receptor, vitamin D receptor, parathyroid hormone, and interleukin-1α and -1β with susceptibility to ossification of the posterior longitudinal ligament of the spine and its severity.

Summary of Background Data. Contribution of genetic backgrounds to the etiology for ossification of the posterior longitudinal ligament of the spine has been suggested by epidemiologic studies.

Methods. Genomic deoxyribonucleic acid samples obtained from 120 patients (77 men and 43 women) with ossification of the posterior longitudinal ligament of the spine and 306 control subjects without the disorder (166 men and 140 women) were amplified by polymerase chain reaction, and polymorphism genotypes were determined by restriction endonuclease digestion. The distribution of genotypes was compared between patients with the disorder and control subjects. In addition, the severity of ossification was determined by the number of ossified vertebrae in patients with the disorder, and associations of the severity with age, gender, and genotypes were examined.

Results. Estrogen receptor ($P = 0.007$) and interleukin-1β ($P = 0.001$) polymorphisms exhibited different distributions between patients with ossification of the posterior longitudinal ligament of the spine and control subjects in women, but not in men. In patients with the disorder, the severity of ossification was negatively correlated with age in women ($P = 0.013$), but not in men. Estrogen receptor polymorphism was associated with the severity only in women ($P = 0.001$).

Conclusions. The contribution of genetic backgrounds is likely to be stronger in women than in men with ossification of the posterior longitudinal ligament of the spine. Estrogen receptor polymorphism was associated with both initiation and promotion of the disorder, but interleukin-1β polymorphism was associated only with its initiation in women. [Key words: estrogen receptor, interleukin-1, OPLL, parathyroid hormone, polymorphism, vitamin D receptor] **Spine 2002;27:1765–1771**

Bone metabolism is regulated by a complicated network of systemic and local factors. Recent population-based case–control studies have shown how polymorphisms of these bone metabolism regulatory factor genes are associated with bone and cartilage disorders such as osteoporosis and osteoarthritis. These include genes for estrogen receptor (ER), vitamin D receptor (VDR), parathyroid hormone (PTH), interleukin-1 (IL-1), collagen Types 1, 2, and 11, calcitonin receptor, peroxisome proliferator–activated receptor-γ, HS-glycoprotein, and transforming growth factor (TGF)-β.[2–5,7,8,10,12,16,20,24,27,30,31,37,43,44,46]

Ossification of the posterior longitudinal ligament of the spine (OPLL) is a disease that causes ectopic ossification of the ligament to develop in the spinal canal.[33,34,42] Although OPLL causes severe neurologic symptoms caused by spinal cord compression, the mechanisms of OPLL initiation and promotion have not yet been identified. It is a common disease with an incidence of 2% to 4% in the middle-age and older populations of Asian countries, whereas lower incidence has been reported in non-Asian countries.[6,25] The disorder is believed to be a collective result of heterogeneous etiopathologic factors affecting the spinal ligament.

The clinical features of patients with OPLL are variable. The onset of clinical OPLL symptoms occurs at an average age exceeding 50 years, and most patients with OPLL have minimal ossification (one or two ossified vertebrae), exhibiting no or mild neurologic symptoms, with onset at older ages. On the other hand, some patients have massive ossification compressing the spinal cord that results in severe tetra- or paraparesis starting from a young age. It therefore is possible that there are

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Table 1. Primers for RFLP in Each Candidate Gene

Candidate Gene	Forward	Reverse	Product Size (bp)	Annealing Temperature (C)
ER	CTGCCACCCTATCTGTATCTTTC	TCTTTCTCTGCCACCCTGGCGTCG	629	57
VDR	AGCTGGCCCTGGCACTGACTC	ATGGAAACACCTTGCTTCTTCTCCC	265	56
PTH	CATTCTGTGTACTATAGTTTG	GAGCTTTGAATTAGCAGCATG	384	54
IL-1α	TGACAGAAATGTCTGGTGCC	ATGGTTTTAGAAATCATCAAG	346	55
IL-1β	CTCATATTCCTGGCTAGTTTTGCTGA	TTGAAAGCACAGTCGGGCATAC	345	56

ER = estrogen receptor; VDR = vitamin D receptor; PTH = parathyroid hormone; IL-1α and β = interleukin-1α and β.

different patient backgrounds in the initiation and promotion of OPLL. The contribution of genetic factors to OPLL susceptibility and its initiation has been reported by several epidemiologic studies,[9,25,38,40] and by association studies, with variants around the human leukocyte antigen (HLA) region.[17,26,35] Case–control association studies of candidate gene polymorphisms also have showed that collagen 11α2,[17,21,22] nucleotide pyrophosphatase (NPPS),[18,29] and transforming growth factor-β (TGF-β)[14] are involved in the initiation of OPLL. However, genetic components of this disease have not been completely determined. Several factors including late onset of the disease, lack of a large family suitable for genetic linkage analysis, and the probable polygenetic nature of the disease have hampered the genetic analysis of OPLL. In addition, genetic backgrounds of promotion or severity of ossification in patients with OPLL have hardly been investigated.

Patients with OPLL show an increase in systemic bone mineral density,[23] and sometimes are associated with some endocrinologic disorders, especially calcium metabolism disorders such as hypoparathyroidism and hypophosphatemic vitamin D-resistant rickets.[1,15,32,36,39] The authors' previous report on a calcium-loading study suggests that the action of vitamin D to resorb calcium from the intestine is impaired in patients with OPLL.[36] It has also been suggested that estrogen is related to OPLL because serum estrogen levels are elevated in some patients with OPLL, and the estrogen receptor (ER) of the spinal ligament cells from patients with OPLL has a higher affinity to estrogen than those from patients without OPLL.[28,45] Moreover, because inflammatory reaction of the spinal ligament was suggested to trigger the ossification, inflammatory cytokines such as IL-1 might possibly be involved in the initiation of the disease.[13] Hence, this study first investigated the association of polymorphisms of some candidate genes, PTH, VDR, ER, IL-1α, and IL-1β, all of which are known to be potent regulators of bone metabolism with susceptibility to OPLL, as determined by a case–control analysis between patients with OPLL and control subjects without the disorder. The authors also performed a stratified study to investigate the association of these gene polymorphisms with severity of ossification in patients with OPLL.

■ Materials and Methods

Patients. A total of 120 patients (77 men and 43 women; age, 32–78 years) with OPLL and 306 control subjects (166 men and 140 women; age, 31–80 years) without the disorder who visited the orthopedic clinic at Tokyo University Hospital and gave written informed consent for the study were investigated. All the patients and control subjects were unrelated Japanese living on the main island of Japan. Diagnosis of OPLL was made by specialists on the basis of radiologic examination of the cervical, thoracic, and lumbar spines.

All the patients were symptomatic and required medical treatment including surgery. Spinal spondylosis, ankylosing spondylosis, and disc herniation were excluded by radiographic examination. All the phenotypic data for each patient, including severity of ossification and age, were recorded at the time of his or her first visit to Tokyo University Hospital. All the patients visited the hospital within 6 months after the onset of clinical symptoms. The severity of OPLL showing how many levels the OPLL extends was determined by the number of ossified vertebrae on lateral radiographs of the cervical, thoracic, and lumbar spines. In the lower cervical and upper thoracic areas, where OPLL lesions could not be detected on plain radiograph, a tomographic analysis was performed and the lesions were evaluated. The study protocol was approved by the ethical committee for human subjects at the University of Tokyo.

Genomic DNA Analysis for Restriction Fragment Length Polymorphism (RFLP). Genomic DNA samples, extracted from peripheral leukocytes of patients with OPLL and control subjects by a standard procedure, were subjected to polymerase chain reaction (PCR) amplification. Genomic DNA (0.1 μg) was amplified by *Taq* DNA polymerase (PE Biosystem, Foster City, CA) using the sense and antisense primers for each factor (Table 1). The PCR products were digested by restriction endonucleases: *Xba*I, *Fok*I, *Bst*BI, *Iba*I and *Aba*I for ER, VDR, PTH, IL-1α and β, respectively. The digested products were analyzed by 1.2% agarose gel electrophoresis. The allele that could be digested by the enzyme was expressed in a lower case, and that which could not was expressed in a capital form. The frequency of specific genotypes was compared between patients with OPLL and control subjects, and the association between genotypes and the severity of ossification was investigated in patients with OPLL.

Statistical Analysis. The two groups were compared using Student unpaired t test. The χ^2 was used to assess Hardy-Weinberg equilibrium and genotype frequency differences. To compare the number of ossified vertebrae between the two groups, the distribution of values was first analyzed in each

Table 2. Background Data of OPLL Patients and Non-OPLL Controls

	All		Male		Female	
	OPLL	Controls	OPLL	Controls	OPLL	Controls
Number	120	306	77	166	43	140
Age (y)	32–78 (57.3 ± 0.6)	31–80 (58.1 ± 0.8)	32–78 (58.4 ± 0.7)	31–80 (59.3 ± 0.5)	33–77 (55.2 ± 1.0)	32–78 (56.7 ± 0.9)
Body weight (kg)	61.9 ± 1.1	58.3 ± 1.4	65.3 ± 1.2	63.3 ± 1.1	55.8 ± 0.6	52.3 ± 1.4
Height (cm)	159.1 ± 0.8	159.3 ± 0.8	163.3 ± 0.9	164.3 ± 0.7	151.5 ± 0.6	153.4 ± 0.5

Data of age, body weight, and height are expressed by the mean ± SEM.

group using the *F* test. Then, parametric analysis using Student *t* test was performed between groups with normal distribution. When a group did not show normal distribution, nonparametric analysis using the Mann–Whitney *U* test was applied. Multivariate logistic regression analysis was performed to adjust risk factors for gender, age, height, body weight, and each genotype. A simple regression analysis was performed to evaluate association between age and number of ossified vertebrae. The means of groups were evaluated by ANOVA, and the significance of the differences was determined by *post hoc* testing using the Bonferroni method. A *P* value less than 0.05 was considered to indicate statistical significance.

■ Results

Case–Control Association Study of ER, VDR, PTH, IL-1α, and IL-1β RFLPs

Table 2 shows the age, body weight, and height of the study participants, who were unrelated patients with OPLL (n = 120) and unrelated control subjects without the disorder (n = 306) living on the main island of Japan. Both populations were further divided into male and female subpopulations. No significant difference in age, weight, or height was seen between the OPLL and control groups in any of the subpopulations (all $P > 0.05$).

The genotype distributions of these RFLPs in the patients with OPLL and the control subjects are shown in Table 3. The genotypic frequencies for these RFLPs were not significantly different in any subpopulations from those expected for populations in Hardy–Weinberg equilibrium (all $P > 0.05$). Linkage disequilibrium among these RFLPs was evaluated by calculating haplotype frequencies according to the method of Hill[11] and Thompson et al.[41] None of the disequilibrium values for marker pairs differed significantly from zero (all $P > 0.05$), indicating that there was no significant linkage disequilibrium among these RFLPs. In the analysis of the overall populations (120 OPLL *vs.* 306 control), only the genotypic distribution of IL-1β RFLP was slightly, but significantly, different between the OPLL and control groups

Table 3. Genotype Distribution (%) of RFLPs in OPLL Patients and Non-OPLL Controls

		All		Male		Female	
Candidate Gene (restriction enzyme)	Genotypes	OPLL (n = 120)	Controls (n = 306)	OPLL (n = 77)	Controls (n = 166)	OPLL (n = 43)	Controls (n = 140)
ER (XbaI)							
	XX	7.5	3.3	2.6	3.0	16.3	3.6
	Xx	23.3	26.1	19.5	28.3	30.2	25.0
	xx	69.2	70.6	77.9	68.7	53.5	71.4
		$\chi^2 = 2.62$, $P = 0.121$		$\chi^2 = 1.88$, $P = 0.369$		$\chi^2 = 10.04$, $P = 0.007$	
VDR (FokI)							
	FF	43.4	43.1	42.8	43.4	44.2	42.9
	Ff	45.8	45.8	45.5	45.2	46.5	46.4
	ff	10.8	11.1	11.7	11.4	9.3	10.7
		$\chi^2 = 0.01$, $P = 0.977$		$\chi^2 = 0.01$, $P = 0.981$		$\chi^2 = 0.08$, $P = 0.960$	
PTH (BstBI)							
	BB	75.8	83.3	74.0	84.3	79.1	82.1
	Bb	24.2	16.7	26.0	15.7	20.9	17.9
	bb	0	0	0	0	0	0
		$\chi^2 = 3.18$, $P = 0.075$		$\chi^2 = 3.57$, $P = 0.061$		$\chi^2 = 0.21$, $P = 0.652$	
IL-α (IbaI)							
	II	0	0.3	0	0	0	0.7
	Ii	7.5	15.4	6.5	15.1	9.3	15.7
	ii	92.5	84.3	93.5	84.9	90.7	83.6
		$\chi^2 = 5.11$, $P = 0.078$		$\chi^2 = 3.57$, $P = 0.059$		$\chi^2 = 1.46$, $P = 0.483$	
IL-β (AvaI)							
	AA	32.4	18.2	23.2	16.8	41.6	19.6
	Aa	41.7	46.1	44.3	47.6	39.1	44.6
	aa	25.9	35.7	32.5	35.6	19.3	35.8
		$\chi^2 = 10.68$, $P = 0.042$		$\chi^2 = 4.62$, $P = 0.099$		$\chi^2 = 13.49$, $P = 0.001$	

ER = estrogen receptor; VDR = vitamin D receptor; PTH = parathyroid hormone; IL-1α and β = interleukin-1α and β.

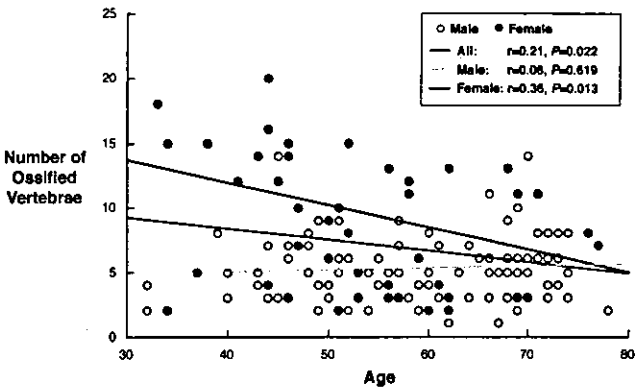

Figure 1. Correlation between the number of ossified vertebrae and age in the overall (n = 120), male (n = 77), and female (n = 43) populations of patients with ossification of the posterior longitudinal ligament of the spine. In the overall population with the disorder, the number of ossified vertebrae was negatively correlated with age. This negative correlation was more evident in women, whereas in men the number of ossified vertebrae was not significantly correlated with age. Data on each patient were recorded at the time of the first hospital visit, which was within 6 months of the onset of clinical symptoms.

(χ^2 = 10.68; P = 0.042), whereas those of ER, VDR, PTH, and IL−1α RFLPs were not. The populations were further analyzed by dividing the overall population into male and female subpopulations. In the male subpopulation (77 OPLL *vs.* 166 control), no RFLP, including that of IL-1β, showed a difference of distribution between the OPLL and control groups. In the female subpopulation (43 OPLL *vs.* 140 control), however, not only the RFLP of IL-1β (P = 0.001), but also that of ER (P = 0.007) exhibited a significant difference of distribution between the OPLL and control groups. These results suggest that genetic backgrounds of these factors affect the initiation of OPLL more strongly in women than in men.

Correlation of Gender and Age With Severity of Ossification in Patients With OPLL

To learn the risk factors for severity or promotion of OPLL, the relation of gender and age to the number of ossified vertebrae in patients with OPLL was investigated (Figure 1). The natural course of ossification in each individual is considered to be not regressive but progressive, with aging depending on environmental factors such as accumulated mechanical stress on the spine. However, the number of ossified vertebrae was, in fact, negatively correlated with age overall in the OPLL group (P = 0.022). This negative correlation was more evident in women (P = 0.013), whereas in men the number of ossified vertebrae tended to increase slightly, although not significantly, with age (P = 0.619). It is therefore indicated that among women there is a young onset subpopulation with severe ossification, suggesting that genetic factors may more strongly contribute to promotion of the ossification than environmental factors.

Correlation of ER, VDR, PTH, IL-1α, and IL-1β RFLPs With Severity of Ossification in Patients With OPLL

To study the contribution of these genes to severity or promotion of OPLL, the association of the RFLPs with the number of ossified vertebrae in patients with OPLL was investigated (Figure 2). In the analysis of the entire OPLL group (n = 120), none of the RFLPs showed significant association between genotypes and the severity of ossification (all $P > 0.05$). In the men as well, there was no association between genotypes and severity of ossification in any RFLPs (all $P > 0.05$). However, in the women, ER RFLP was associated with severity of ossification, and the X allele was related to severe ossification (P = 0.001), whereas other RFLPs, including IL-1β, were not.

■ Discussion

Although OPLL is a multifactorial disease whose clinical features are variable, previous studies on genetic backgrounds of OPLL were all case–control association studies that compared subjects with and those without the disease.[9,14,17,21,26,35,40] The current study therefore investigated the genetic backgrounds of the disease, taking the severity of ossification into consideration. The natural course of ossification in each patient with OPLL progresses with age, implying the contribution of accumulated mechanical stress on the spine. This study, however, clearly demonstrated that there exists a subgroup with young onset and severe ossification, especially among women (Figure 1). Hence, it is likely that the contribution of genetic backgrounds is stronger in women than in men. In fact, the case–control study showed the association of ER and IL-1β RFLPs with susceptibility to OPLL in women, but not in men. A stratified study in patients with OPLL also showed the association of ER RFLP with severity of ossification only in women.

It is interesting that ossification is more severe in female than in male patients with OPLL, although the morbidity of OPLL is known to be 2 to 3 times higher in men.[25,42] This indicates that men are more susceptible to OPLL than women, whereas women tend to have severer OPLL than men once it occurs. These facts imply that initiation and promotion of OPLL are regulated by different factors. In women, ER RFLP associated with both susceptibility to OPLL and its severity might contribute to the etiology of initiation and promotion of ossification, whereas IL-1β RFLP associated with susceptibility to OPLL, but not its severity, might be involved only in initiation of OPLL.

Another probable candidate gene whose polymorphisms reportedly are associated with OPLL is that of collagen 11α2, whose locus lies close to the HLA region on chromosome 6p.[17,21,22] Koga et al[17] reported that 4 of 16 polymorphisms identified in this gene were significantly associated with susceptibility to OPLL according to a case–control association study. However, no stratified study investigated the association of these polymor-

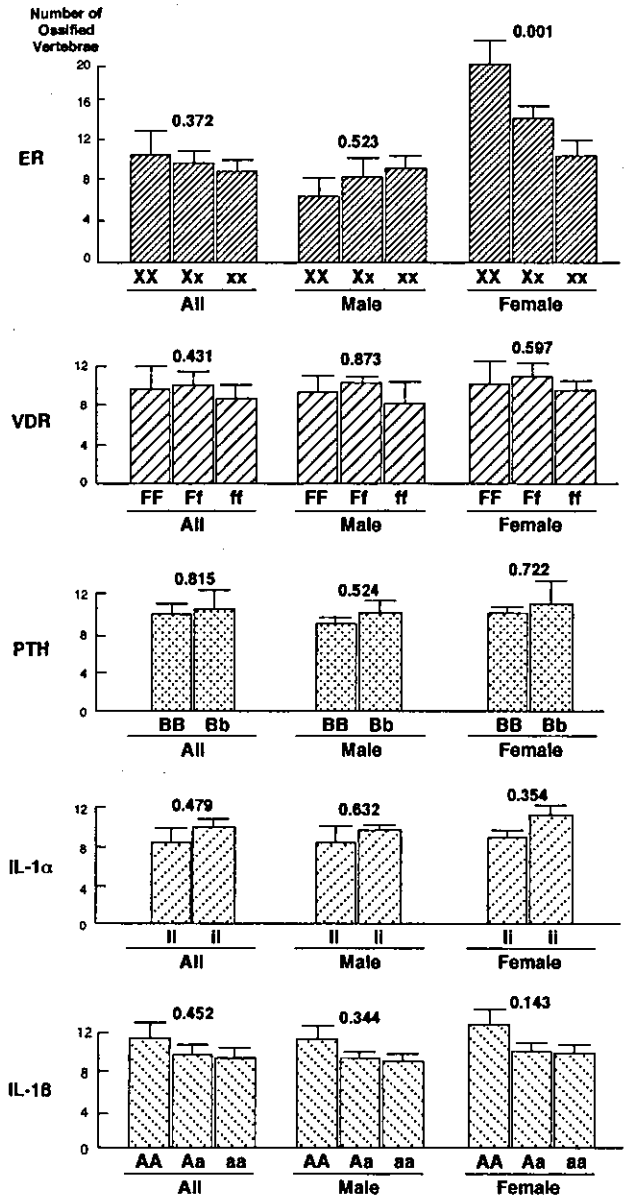

Figure 2. The difference in the number of ossified vertebrae among genotypes of estrogen receptor, vitamin D receptor, parathyroid hormone, and interleukin-1α and -1β restriction fragment length polymorphisms in the overall (n = 120), male (n = 77), and female (n = 43) populations of patients with ossification of the posterior longitudinal ligament of the spine. In the overall and male populations of patients with the disorder, none of the restriction fragment length polymorphisms was significantly associated with the severity of ossification. However, in women, only estrogen receptor restriction fragment length polymorphism was associated with severity of ossification, and the X allele was related to severe ossification ($P = 0.001$). Data are expressed as means (bars) ± standard error of means (error bars). The number above the bars is the P value of the difference in the number of ossified vertebrae among genotypes.

phisms with severity of ossification. The current authors preliminarily investigated the association of a reported collagen 11α2 gene polymorphism to show the highest association: T → A substitution at position −4 in intron 6, with severity of ossification in the patients with OPLL used in the current study (unpublished observation). No significant difference in the number of ossified vertebrae was seen in the results among genotypes ($P > 0.05$). This suggests that collagen 11α2 gene is not involved in severity of OPLL despite its significant association with susceptibility to the conditions. Here again, it was speculated that initiation and promotion of OPLL might be regulated by different genetic patient backgrounds. More interestingly, the authors' recent case–control study demonstrated that the association between the collagen 11α2 gene polymorphism and susceptibility to OPLL is much stronger in men than in women.[22] Conversely, the current study demonstrated that case–control associations of ER and IL-1β polymorphisms exist only among women. Another recent study also showed that an NPPS gene polymorphism is associated with OPLL much more strongly in women than in men.[18] The discrepancy in gender-specific associations among these studies indicates the difference in genetic backgrounds involved in the etiology of OPLL between the genders.

The mechanisms whereby the *Xba*I ER and *Aba*I IL-1β RFLPs associated with OPLL increase the risk for OPLL currently are unclear. On the basis of the fact that estrogen deficiency causes osteoporosis, estrogen is believed to be essential to maintain bone mass. However, the mechanism of estrogen signaling as an anabolic agent for bone formation remains unclear. It is possible that IL-1β, an inflammatory and bone resorptive cytokine, plays a role as a trigger of ectopic ossification, as reported previously, although the precise mechanism requires further elucidation.[13,19] In addition, the fact that neither of these polymorphic sites is located in exons but rather in introns indicates that these nucleotide variants do not directly lead to amino acid substitutions. The most likely mechanism may be the effect of these intronic substitutions on the splicing mechanism. The one base substitution may yield an alternative transcript with abnormal function or affect the expression level of proteins. The next task is to investigate the relevance of these polymorphisms to the production or function of ER and IL-1β.

Regarding the clinical utility of this study, the practical application of this genetic information on individuals to specific diagnostic or therapeutic modifications is a conceptual goal. The polymorphisms of genes such as ER and IL-1β might be a useful diagnostic marker to predict OPLL progression. Because there is yet no conservative treatment to prevent the promotion of OPLL, estrogen itself or regulators of its expression or function might possibly lead to prevention of the condition. In reality, however, such modification is an era away from the current generation. The legal, social, and ethical implications of this information are overwhelming.

The findings taken together demonstrate possible associations of ER and IL-1β genes with susceptibility to OPLL and/or its severity. These genes might have a relatively small attributable risk fraction for OPLL, but identification of the disease-susceptibility and -severity

gene could lead to a better understanding of the molecular pathogenesis of this disease, which is essential to the development of the epochal diagnosis and treatment. Further studies on the functional relevance of these gene abnormalities could provide new biologic insights into the etiology of diseases with ectopic ossification such as OPLL.

■ Key Points

- A case–control association study and a stratified study were conducted to investigate the involvement of gene polymorphisms of bone metabolism regulatory factors in susceptibility to and severity of OPLL.
- The contribution of genetic backgrounds was stronger in women with OPLL than in male patients.
- Among gene polymorphisms, that of estrogen receptor was associated with initiation and promotion of OPLL, and that of IL-1β only with its initiation in women.

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Large-Scale Screening for Candidate Genes of Ossification of the Posterior Longitudinal Ligament of the Spine

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ABSTRACT

Ossification of the posterior longitudinal ligament of the spine (OPLL) is the predominant myelopathy among Japanese, and is usually diagnosed by ectopic bone formation in the paravertebral ligament in Japanese and other Asians. To detect genetic determinants associated with OPLL, we performed an extensive nonparametric linkage study with 126 affected sib-pairs using markers for various candidate genes by distinct analyses, SIBPAL and GENEHUNTER. Eighty-eight candidate genes were selected by comparing the genes identified by complementary DNA (cDNA) microarray analysis of systematic gene expression profiles during osteoblastic differentiation of human mesenchymal stem cells with the genes known to be involved in bone metabolism. Of the 24 genes regulated during osteoblastic differentiation, only one, the alpha B crystalline gene, showed evidence of linkage ($p = 0.016$, nonparametric linkage [NPL] score = 1.83). Of 64 genes known to be associated with bone metabolism, 7 showed weak evidence of linkage by SIBPAL analysis ($p < 0.05$): cadherin 13 (*CDH13*), bone morphogenetic protein 4 (*BMP4*), proteoglycan 1 (*PRG1*), transforming growth factor beta 3 (*TGFb3*), osteopontin (*OPN*), parathyroid hormone receptor 1 (*PTHR1*), and insulin-like growth factor 1 (*IGF1*). Among these genes, *BMP4* (NPL = 2.23), *CDH13* (NPL = 2.00), *TGFb3* (NPL = 1.30), *OPN* (NPL = 1.15), and *PTHR1* (NPL = 1.00) showed evidence of linkage by GENEHUNTER. Only *BMP4* reached criteria of suggestive evidence of linkage. Because this gene is a well-known factor in osteogenetic function, *BMP4* should be screened in further study for the polymorphism responsible. (J Bone Miner Res 2002;17:128–137)

Key words: ossification of the posterior longitudinal ligament, linkage, affected sib-pair, candidate gene, cDNA microarray

INTRODUCTION

OSSIFICATION OF the posterior longitudinal ligament (OPLL) of the spine is a common hyperostotic disorder among Japanese and other Asian populations, where an estimated prevalence of 2–4% of the general population shows ectopic bone formation in the posterior ligament. Ectopic ossification compresses the spinal cord and leads to various degrees of neurological symptoms, from discomfort to severe myelopathy.[1] The average onset is about 50 years with male predominance of 2:1 and no regional difference of frequency in Japan. Despite the late onset, OPLL has a strong genetic background, as shown in the classical epidemiological study and by the estimated relative risk to siblings of about 10.[2] Because genetic determinants appear to play a crucial role in the etiology of OPLL, we began a large-scale screening for genetic loci associated with OPLL.

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Affected sib-pair linkage analysis of genome-wide screening is frequently applied to map loci of complex diseases, but it is difficult to narrow the responsive region to within 10 cM by nonparametric linkage study.[3] In addition, although a genetic loci has been determined, identification of the disease gene is difficult. An alternative approach that avoids this problem is linkage analysis of various candidate genes of interest.[4] We previously reported evidence of linkage in the HLA region of chromosome 6 in 91 affected sib-pairs, and showed a possible allelic association between variants of collagen 11A2 gene (*COL11A2*) and OPLL.[5] A nucleotide substitution at intron 6 (-4) in *COL11A2*, a T to A substitution most significantly associated with OPLL ($p = 0.0003$), was a functional variant resulting in altered splicing, which acts as a protective in the pathogenesis of OPLL.[6] The results indicate that the candidate approach appears to be beneficial to detect the causality of the disease. Because OPLL is a complex disease with several genetic factors other than *COL11A2* probably involved in the etiology, the more extensive candidate gene approach was performed in the present study. We selected a total of 88 candidate genes based on two separate investigations, of complementary DNA (cDNA) microarray profiles of systematic gene expression during osteoblastic differentiation and of genes known to be involved in bone metabolism.

A global view of gene expression in the entire genome during osteoblastic differentiation provides information on the interplay of genes relating to bone metabolism and also provides clues to genes in the molecular etiology of OPLL and related disorders. Twenty-four genes differentially regulated during osteoblastic differentiation were selected by this method and subjected to linkage analysis. Using the knowledge-based method, we also selected 64 candidate genes that are possibly involved in bone metabolism or related conditions. Recently, a skeletal gene database has been catalogued that provides information that is extremely useful in causal gene discovery in diseases of bone metabolism.[7] Microsatellite markers (mainly dinucleotide repeats), located close to or within the candidate genes, were obtained in the latest online information. With these markers, we performed nonparametric linkage study with 126 OPLL sib-pairs to identify genetic loci responsible in OPLL in Japan.

MATERIALS AND METHODS

Study subjects

OPLL was diagnosed by the occurrence of heterotopic bone formation in the posterior longitudinal ligament on X-ray examination by expert orthopedic surgeons. A total of 126 affected sib-pairs were recruited from various university hospitals in Japan, including 85 from Kagoshima, 37 from Hirosaki, 2 from Asahikawa, 1 from Okinawa, and 1 from Wakayama. The affected sibships comprised 54 pairs, 16 trios, and 4 quartets of OPLL siblings. The average age of the participants was 59.1 years: 59.5 years for males and 58.6 years for females. Because of the late-onset nature of the disease, we could not collect data from the parents of the sibs. For the allelic association study, 250 unrelated OPLL cases and 200 non-OPLL controls were collected in Kagoshima. All non-OPLL controls were over 60 years old and diagnosed by the absence of ossification on spinal X-ray examination. This study was approved by the Ethical Committees of Kagoshima University and Hirosaki University, and all participants gave written informed consent.

cDNA microarray analysis

Human mesenchymal stem cells (hMSC) are pluripotent cells with the ability to differentiate into osteogenic, chondrogenic, tendonogenic, adipogenic, and myogenic lineages.[8] We used gene expression profiling during osteoblastic differentiation of hMSC as a model of the ossification process in OPLL. Cells provided by Poietics (BioWhittaker, San Diego, CA, USA) were cultured for 24 h in the presence or absence of 0.1 μM dexamethasone, 10 mM β-glycerophosphate, and 0.05 mM ascorbic acid (osteogenetic supplement [OS]), and harvested to investigate differences in gene regulation at a very early stage. Total RNA was extracted from cultured cells with Trizol Reagent according to the manufacturer's protocol (GIBCO BRL, Rockville, MD, USA), and subjected to analysis with an messenger RNA (mRNA) purification kit (Amersham Pharmacia Biotech, Tokyo, Japan), which yielded 2–3 μg of mRNA from 10^7 cells. Isolated mRNA was reverse transcribed with Cy3 or Cy5 labeled random 9-mers (Operon Technologies, Alameda, CA, USA). Labeled probes were hybridized with cDNA microarray, which included 8502 cDNA elements corresponding to 8372 unique annotated genes and 130 ESTs from unique unnamed gene clusters prepared by Incyte Pharmaceuticals (Palo Alto, CA, USA). The microarrays were scanned in both Cy3 and Cy5 channels with Axon GenePix scanners (Axon Instruments, Foster City, CA, USA) with 10 μm resolution. Incyte GEM Tools software (Incyte Pharmaceuticals) was used for quantification of image analysis. The area surrounding each element image was used to calculate the local background subtracted from the total element signal. Because the fluorescent signals of the empty spots were below 400, genes with signals above 500 were considered expressed. The relative expression level of a gene in a differentiated hMSC to that in an undifferentiated hMSC is the expression ratio. An expression ratio of 3 was set as cut-off.

Search for genetic markers and genotyping

Candidate genes were selected as described above, and microsatellite markers located close to or within the genes were selected mainly by searching online information [National Human Genome Research Institute (NHGRI) (http://www.nhgri.nih.gov/), National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/) (GeneMap'99 and Map Viewer)]. Markers with heterozygosity above 60% in the Japanese population were selectively subjected to linkage analysis. The distances between the genes and the markers averaged 1.7 cM.

Polymerase chain reaction (PCR) and genotyping of microsatellites

PCR was performed in a 96-well plate in a final volume of 6 μl, containing 5 ng of genomic DNA, 200 nM dNTPs, 1.5 mM $MgCl_2$, 0.6 μl 10× PCR buffer, 1 μM mixed primer (one of the primers was fluorescent labeled), and 0.15 U *Taq*Gold DNA polymerase (PE Biosystems, Tokyo, Japan). After denaturation for 12 minutes at 95°C, 35 cycles (15 s at 94°C, 15 s at 55°C, 30 s at 72°C), plus 10 minutes extension, were performed with a GeneAmp PCR System 9700 (PE Biosystems). Pooled PCR products were electrophoresed on a DNA sequencer (model 377; PE Biosystems). DNA fragment size was determined with GENESCAN (version 3.1) software, and genotyping was carried out with GENOTYPER software (version 2.5) (PE Biosystems). Information of primer sequences and PCR conditions may be obtained from the authors on request.

Affected sib-pair linkage analysis

Evidence for linkage was assessed by the nonparametric affected sib-pair method. Two programs, SIBPAL (http://darwin.cwru.edu/pub/sage.html) and GENEHUNTER (http://linkage.rockefeller.edu/soft/gh/), were used.[9,10] Nonparametric linkage with the affected sibling was performed by identical descent method with SIBPAL of the SAGE package. SIBPAL is a test of mean that measures the mean proportion of shared IBD alleles and compares it with that expected under null hypothesis (50%). The test statistic has a standard normal distribution under the null hypothesis and, because the alternative hypothesis of linkage is given when IBD sharing is over 50%, the test is one sided, and accurate p values can be obtained by use of a one-sided t-test, as implemented in the SIBPAL program. Single-point linkage was also examined with the GENEHUNTER program, which performs a likelihood ratio test for allele sharing in affected sib pairs, and calculates a nonparametric linkage score (NPL; nonparametric LOD [$\log_{10}$ of the odds ratio] score) using possible triangle constrains.[10] Under the null hypothesis an NPL score is a normally distributed statistic with a mean of zero and a variance of one. Because of the late onset of OPLL, the parents of sibs could not be analyzed. IBD analysis was performed by estimating the parental genotype using the allelic frequencies of the reference controls. Such linkage results are extremely sensitive to the allelic frequencies of the reference population. In general, the allelic frequencies of microsatellite markers obtained through public databases were determined with whites, and could not directly be applied to Japanese. The heterozygosity of each microsatellite marker was determined with 64 Japanese subjects.

NPPS, *a candidate gene identified in TWY mouse*

The human PAC library based on the PCR protocol (Incyte Pharmaceuticals) was screened for the nucleotide pyrophosphatase gene *NPPS*. Two clones (131F15 and 223P18) were identified, both containing the entire coding region of *NPPS*. To obtain a microsatellite marker in *NPPS*, the clone 131F15 was digested with *Bam*HI and *Eco*RI followed by Southern blot analysis with a CA repeat probe (10 repeats). A positive band (4-kb) was excised and subcloned into pBluescript. The insert with a positive signal was then digested with *Bam*HI and *Hin*dIII and Southern blot analysis with the same probe was performed again. The 1-kb fragment with a positive signal was subcloned and subjected to sequencing. Sequencing was performed with BigDye Terminator cycle sequencing with a 377 ABI Prism automated DNA sequencer (PE Biosystems). We identified two dinucleotide repeats in the PAC clone. One microsatellite was difficult to genotype. The other microsatellite (CA repeats) was denoted NPPS1, and the heterozygosity of the marker was estimated as 0.67.

Single-strand conformational polymorphism (SSCP) analysis

SSCP analysis was performed with a GeneGel Excel 12.5/24 kit (Amersham Pharmacia Biotech). A 4-μl aliquot of the PCR product plus 2 μl of sample buffer were mixed thoroughly, denatured at 95°C for 5 minutes, and applied to the premade gel. The running conditions for GeneGel Excel 12.5/24 were 600 V, 25 mA, and 15 W, for 80 minutes. A PlusOne DNA Silver Staining Kit (Amersham Pharmacia Biotech) was used for detection. The SSCP variant was further verified by direct sequencing.

Other statistics

Comparisons of the allelic or genotypic frequency of the polymorphisms between cases and controls were performed by use of the contingency χ^2 test. We also computed statistical power as the type II error complement defined as 1-β for testing negative evidence against an existing association as described.[11]

RESULTS

Selection of OPLL candidate genes by cDNA microarray analysis

Human mesenchymal stem cells (hMSC) have the ability of multipotent differentiation. Especially in the presence of glycerophosphate, dexamethasone, and ascorbic acid (an osteogenetic supplement [OS]), the cells differentiate into an osteoblastic lineage. Because the heterotopic ossification observed in OPLL is a result of both endochondral and intramembranous ossification mechanisms,[12] osteoblastic differentiation of hMSC is an appropriate model for the ossification process observed in OPLL. The temporal pattern of gene expression during osteoblastic differentiation at an early stage (24 h post-OS) was examined by cDNA microarray, covering over 8000 distinct cDNA sequences of human genes. Because the genetic causality in OPLL would be a gene regulated at a very early stage of differentiation, we isolated mRNA 24 h after OS induction. A typical pattern of differential expression is depicted in Fig. 1, where the expression level of the gene is shown by difference in color. A total of 6500 genes were observed with positive

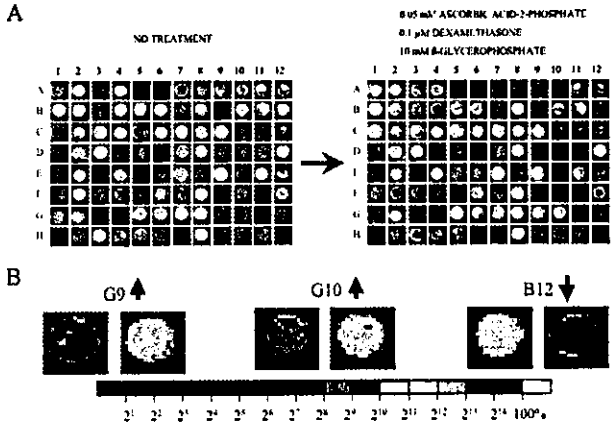

FIG. 1. cDNA microarray analysis of hMSC before and after osteoblastic induction. Cells were cultured for 24 h in the presence or absence of 0.1 μM dexamethasone, 10 mM β-glycerophosphate, and 0.05 mM ascorbic acid (OS), and prepared for mRNA labeling. Differences in gene expression profile during osteoblastic differentiation then were investigated by the use of cDNA microarray prepared by Incyte Pharmaceutical. mRNA was labeled with Cy3 (without OS induction) and Cy5 (with OS induction) and hybridized with cDNA microarray. The microarrays were scanned in both Cy3 and Cy5 channels with Axon GenePix scanners. The area surrounding each element image was used to calculate a local background that was subtracted from the total element signal. Because the fluorescent signals of empty spots were below 400, genes with signals above 500 were considered expressed. (A) A typical example of microarray data. Differential expression between mRNA from uninduced cells (left) and induced cells (right) is depicted by color gradient. (B) Genes showing differential expression: the up-regulated genes (G9 and G10) and the down-regulated gene (B12) are shown.

signals in undifferentiated hMSC. mRNA levels of 24 genes were found to be down-regulated or up-regulated more than 3-fold on osteoblastic differentiation: 12 genes were down-regulated and 12 genes were up-regulated (Table 1). Twenty ESTs were also identified but, because their function was undetermined, they were not pursued in the linkage analysis. The 12 up-regulated and 4 down-regulated genes were further subjected to the *Taq*Man real-time PCR semiquantitative protocol. We confirmed that all of the 12 up-regulated and 4 down-regulated genes were regulated in the expected directions during osteoblastic differentiation (data not shown).

OPLL candidate genes through knowledge-based search

Knowledge-based candidate genes for OPLL were selected by the following criteria: genes catalogued in the skeletal gene database, genes with functional roles in bone formation, and genes of which the gain or loss of function is related to bone formation, all according to cell or animal model studies. Sixty-four distinct genes were identified, as summarized in Table 2. Recently, a skeletal gene database was established that provides important information for gene discovery in bone and mineral disorders.[7] The two animal models for OPLL are well studied: the TWY mouse[13,14] and the Zucker fatty rat.[15,16] Because the genetic causality in these animals has been identified as the nucleotide pyrophosphatase gene[17] and leptin receptor gene,[18] respectively, these genes were also examined.

Evidence of genetic linkage with OPLL

The nearby or intragenic microsatellite markers of the candidate genes were identified by searching online information as described above. The heterozygosities of all of the markers were above 0.6 in the Japanese population. The distance between the genes and the markers was 1.7 cM in average (details are shown in Tables 3 and 4). These markers were tested for linkage with 126 OPLL sib-pairs. The results of affected sib-pair linkage analysis are shown in Tables 3 and 4. Among the 24 genes regulated during osteoblastic differentiation, only one, the alpha B crystallin gene, showed evidence of linkage ($p = 0.016$, NPL = 1.83) (Tables 3 and 5). In the knowledge-based selection of candidate genes, 7 genes showed significant evidence of linkage with p values less than 0.05 by SIBPAL (summarized in Table 5). The best evidence of linkage by SIBPAL was observed with marker D16S3091, located 2.9 Mb from *CDH13* ($p = 0.005$). By GENEHUNTER, the strongest linkage was detected with marker D14S276 (NPL = 2.23), located 1.3 Mb from *BMP4*. Among the 7 genes (Table 5), *BMP4* (NPL = 2.23), *CDH13* (NPL = 2.00), *TGFb3* (NPL = 1.30), *OPN* (NPL = 1.15), and *PTHR1* (NPL = 1.00) showed positive evidence of linkage (NPL above 1.0) by GENEHUNTER. The linkage result with *BMP4* (NPL = 2.23) was "suggestive evidence of linkage," according to the guideline described by Kruglyak and Lander.[19]

Animal models for OPLL and genetic causality

Two animal models for OPLL are well established, the TWY mouse and the Zucker fatty rat.[13–16] The causality in the TWY mouse, which exhibits an autosomal recessive trait, was found in the nucleotide pyrophosphatase gene *Npps*, which functions in the nucleotide phosphodiesterase reaction.[17] Further study showed that human *NPPS* is also involved in OPLL by revealing an allelic association with an intron 20 polymorphism (denoted as IVS20-11delT).[20] Human *NPPS* locates on chromosome 6q22. We identified a polymorphic microsatellite (denoted NPPS1) in the PAC clone containing the entire *NPPS*. The marker NPPS1, with heterozygosity of 0.67, was tested for linkage analysis. Other markers nearby were identified and tested for linkage, as summarized in Table 6. No evidence of linkage was obtained within the *NPPS* locus ($p = 0.20$ for NPPS1, $p = 0.24$ for D6S457; Table 6). We also performed an allelic association study between OPLL and the polymorphisms in *NPPS*. As shown in Fig. 2, SSCP screening of the intron 20/exon 21 junction of *NPPS* revealed very complicated patterns giving six distinct SSCP bands. Because of this complexity in the SSCP analysis, all of the genotype was determined by direct sequencing to avoid genotype errors. Each polymorphism was subjected to an allelic association study with 250 OPLL patients and 200 non-OPLL controls. No evidence of association with IVS20-11delT was ob-

TABLE 1. GENES REGULATED DURING OSTEOBLASTIC DIFFERETIATION OF HUMAN MESENCHYMAL STEM CELLS BY cDNA MICROARRAY ANALYSIS

Expression ratio		*Candidate gene*	*Locus*	*AccessionNum*[a]
Up-regulation[b]				
1	+5.9	Crystallin, alpha B	11q22.2	AL038340
2	+5.3	Sortilin 1	1p21.3	NM_002959
3	+4.9	RNA helicase-related protein	17q21.2	AW192642
4	+4.6	Collagen, type VIII, alpha 1	3q12.2	NM_001850
5	+4.0	Leptin receptor	1p31	U52914
6	+3.9	Adrenomedullin	11p15.4	NM_001124
7	+3.6	Heat shock 105kD	13q14.2	NM_006644
8	+3.6	Phosphatidylinositol-(4,5)-bisphosphate 5-phosphatase	17p	U45974
9	+3.3	Protein tyrosine kinase 2 beta	8p21.1	U43522
10	+3.2	Microseminoprotein, beta	10q11.2	NM_002443
11	+3.2	Prepropeptide specific to rod photoreceptor	18p	D63813
12	+3.0	Integrin, alpha 5	12qcen	X06256
Down-regulation[c]				
1	−7.7	Tumor necrosis factor, alpha-induced protein 6	2q31	M31165
2	−6.0	Stromal cell-derived factor 1	10q11.1	U19495
3	−5.4	Methylene tetrahydrofolate dehydrogenase	14q21.3	NM_006636
4	−4.0	A disintegrin and metalloproteinase domain 12	10q26.3	NM_003474
5	−3.8	Insulin-like growth factor binding protein 3	7p13	M31159
6	−3.6	Human GOS2 protein gene	1q32.2	M69199
7	−3.5	Plasminogen activator inhibitor, type II	18q21.3	J02685
8	−3.3	Plasminogen activator, urokinase	10q24	D11143
9	−3.2	Insulin-like growth factor binding protein 5	2q	L27560
10	−3.0	P311 protein	5q14.3	NM_004772
11	−3.0	Antigen identified by monoclonal antibodies 4F2	11q13	J02769
12	−3.0	Solute carrier family 21 (organic anion transporter)	12p12	U21943

[a] Accession Number of GenBank database (NCBI).
[b] +, up-regulated genes after 24 h of OS induction.
[c] −, down-regulated genes after 24 h of OS induction.

served in the current samples (allelic association: $\chi^2 = 0.813$, df = 1, $p = 0.367$; genotype association: $\chi^2 = 2.20$, df = 1, $p = 0.138$). Furthermore, we computed the statistical power defined as the type II error complement ($1-\beta$) for testing negative evidence against the occurrence of an association. The power in the current study was calculated to be 0.919, sufficient to supersede the association evidence provided by Nakamura et al. [20]

The Zucker fatty rat is characterized by progressive obesity with hyperalimentation, which is a recessive trait. OPLL or OALL (ossification of anterior longitudinal ligament) is frequently observed in Zucker fatty rats. A molecular variant of the leptin receptor has been identified as the genetic causality in Zucker fatty rats.[18] The leptin receptor was also identified as an up-regulated gene during osteoblastic differentiation, indicating that the gene is closely related to bone metabolism (Table 1). No evidence of linkage with the marker (D1S230) located close to the leptin receptor was observed ($p = 0.474$) (Table 3).

DISCUSSION

OPLL is a hyperostotic disease with a pathology almost opposite that of osteoporosis, a typical bone loss disease. Osteoporosis was observed in OPLL subjects with low frequency compared with subjects of similar ages[21]; therefore susceptibility genes for OPLL might be involved in protective effect for osteoporosis. In the present study, we performed a linkage study using a candidate gene approach to identify genetic loci relevant to OPLL. As shown in Tables 3 and 4, the candidate genes and the marker distances are generally less than 3 cM, which should be sufficiently close for linkage study as a candidate gene analysis. Because the relative power and accuracy of linkage tests varies with the methods used, we applied two different programs, SIBPAL from the SAGE package and GENEHUNTER. SIBPAL calculates the excess allele sharing, comparing it with the null hypothesis under no linkage by *t*-statistics. Thereby accurate *p* values can be obtained. GENEHUNTER is a likelihood method that calculates the LOD score, which is generally a more powerful linkage analysis than the probability test. However, estimate of linkage by GENEHUNTER is known to be conservative in cases of incomplete information.

OPLL causal genes should be regulated at a very early stage of induction, so mRNA was prepared from hMSC 24 h after OS induction, and the gene expression profile was compared with mRNA of untreated cells. Because of the