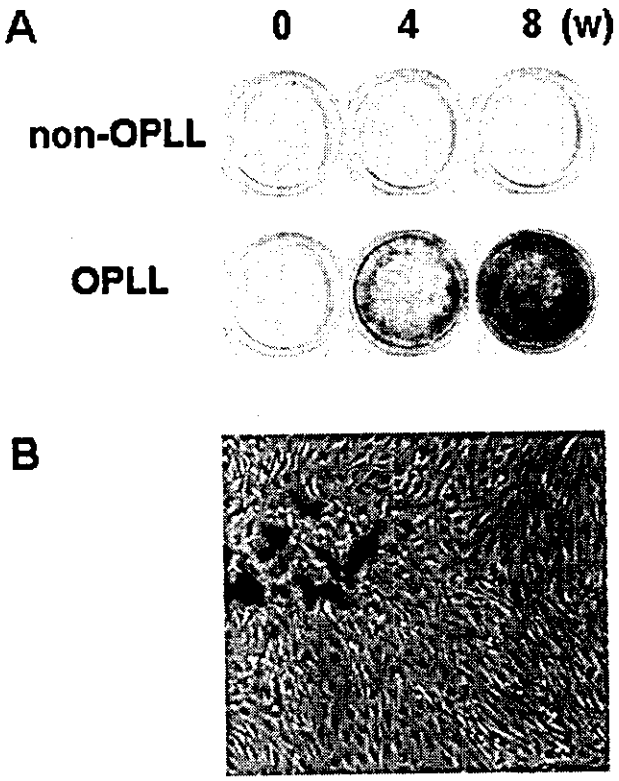

Fig. 1. Comparison of abilities to form mineralized nodules in OPLL and non-OPLL cells. (A) Cells were incubated in DMEM supplemented with 10% FBS. After cultures reached confluence, cells were maintained in osteogenic medium containing DMEM supplemented with 10% FBS, 50 μg/ml of ascorbic acid, and 5 mM β-glycerophosphate for the indicated time periods (0–8 weeks) and then stained with alizarin red. (B) A microscopic view of OPLL cells at 4 weeks (original magnification, × 40).

showed similar responses to mechanical stress as described below.

Expression of marker genes for osteoblast differentiation of spinal ligament cells before exposure to cyclic stretch

Before assessing the response to mechanical stress, we performed RT–PCR with primers specific for several marker genes for osteoblast differentiation of spinal ligament cells in the resting state (Table 3). No significant difference between non-OPLL and OPLL cells was found in alkaline phosphatase, collagen type I, BMP-2, BMP-4, or BMP receptors IA and II. On the other hand, osteopontin, BMP receptor IB, and osteocalcin were significantly higher in OPLL cells than non-OPLL cells.

Effect of uniaxial cyclic stretch on osteoblast differentiation in OPLL cells

To investigate the effect of cyclic stretch on osteogenic differentiation in OPLL cells, the expression of ALP, osteopontin, and osteocalcin was examined. In OPLL cells, cyclic stretch significantly increased the mRNA expression of ALP about 1.73-fold after stimulation for 9 h and osteopontin about 2.01-fold after stimulation for 3 h as compared with the cells maintained under the resting state (0 h), whereas non-OPLL cells exhibited no increase with stimulation. Even in control cultures, ALP mRNA expression was 1.72-fold greater in OPLL cells than non-OPLL cells, but osteopontin mRNA was almost equally expressed in both cells (Fig. 2). Osteocalcin was higher in OPLL cells than non-OPLL cells (Table 3); however, cyclic stretch failed to enhance its expression in both cells up to 9 h (data not shown).

Effects of Ca^{2+} channel blockers on stretch-induced ALP activity in OPLL cells

We next measured ALP activity in the cell layer (Fig. 3). ALP activity was 2.93-fold greater in OPLL cells than in non-OPLL cells without stimulation. After stimulation for 24 h, ALP activity in OPLL cells increased 3.0-fold, compared with control, whereas no change was observed in non-OPLL cells. To identify the possible signaling pathways involved in cyclic stretch-induced ALP activity, Gd^{3+}, a specific stretch-activated Ca^{2+} channel blocker, and specific voltage-dependent L-type Ca^{2+} channel blockers, diltiazem and nifedipine, were added to some culture media before stimulation at final concentrations of 50, 10, 1 μM, respectively. Application of these Ca^{2+} channel blockers suppressed stretch-induced ALP activity to control levels in OPLL cells. The increase in ALP activity was also inhibited in Ca^{2+}-free medium.

mRNA expression of the α1C subunit of voltage-dependent L-type Ca^{2+} channels

Because the involvement of voltage-dependent L-type Ca^{2+} channels in the signaling pathways that link mechanical stress to the above-mentioned biological responses was

Table 3
Expression of marker genes for osteoblast differentiation in human spinal ligament cells before exposure to cyclic stretch (assessment by RT–PCR)

Marker genes	Non-OPLL	OPLL
Alkaline phosphatase	1.26 ± 0.25	1.14 ± 0.24
Osteopontin	0.22 ± 0.04	0.95 ± 0.20*
Collagen type I	0.91 ± 0.13	0.84 ± 0.15
Bone morphogenetic protein 2	1.45 ± 0.26	1.02 ± 0.29
Bone morphogenetic protein 4	1.86 ± 0.43	2.32 ± 0.34
BMP receptor IA	1.16 ± 0.35	0.80 ± 0.18
BMP receptor IB	0.22 ± 0.04	0.59 ± 0.09*
BMP receptor II	0.47 ± 0.15	1.19 ± 0.28
Osteocalcin	1.27 ± 0.18	2.35 ± 0.51*

All values are the ratio against G3PDH and expressed as means ± SEM ($n = 10$). BMP, bone morphogenetic protein. * $p < 0.05$ vs non-OPLL cells.

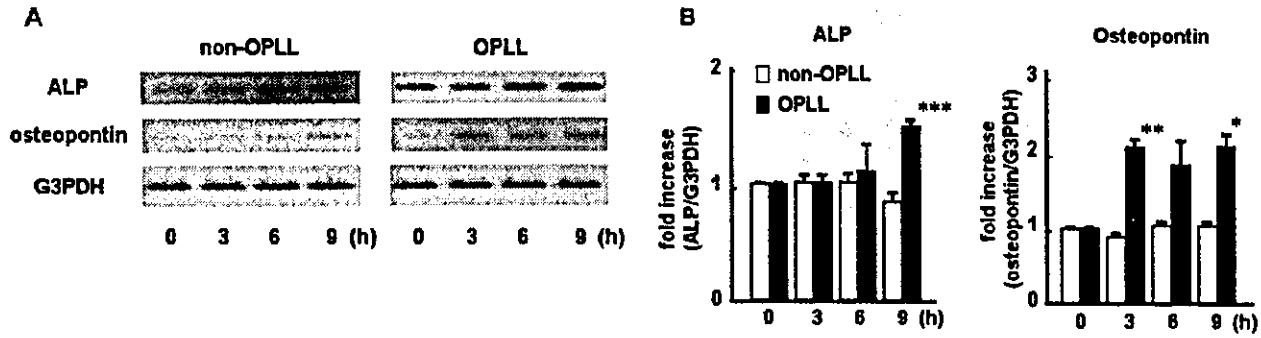

Fig. 2. Effects of uniaxial cyclic stretch on ALP and osteopontin mRNA expressions in OPLL and non-OPLL cells. Cells were subjected to uniaxial cyclic stretch for the indicated time periods (0–9 h) and then the mRNA of each gene was measured by quantitative RT-PCR. (A) The bands for RT-PCR products appeared upon agarose gel electrophoresis. (B) The densitometric quantification of the electrophoretic profile of each gene was normalized to the corresponding G3PDH signal. The fold increases of these mRNAs to control are shown from 10 different experiments. $*P < 0.05$ versus control; $**P < 0.01$ versus control.

suggested, we investigated the mRNA expression of the α1C subunit of voltage-dependent L-type Ca^{2+} channels and the effect of cyclic stretch on its expression in both cells. RT–PCR analysis showed that α1C mRNA was expressed and enhanced by cyclic stretch in OPLL cells with a significant difference. However, the expression of α1C mRNA was not enhanced by cyclic stretch in non-OPLL cells (Fig. 4).

Effect of cyclic stretch on the expression of the BMP and its receptors in OPLL cells

As described above, since BMPs appeared to participate in the development of OPLL, we examined whether the mRNA expression of BMP-2 and BMP-4 and BMP receptors (BMPR-IA, -B, -II) was affected by cyclic stretch in OPLL cells (Fig. 5). In OPLL cells, compared with control, cyclic stretch significantly increased the mRNA expression of BMP-2 about 1.72-fold after stimulation for 3 h, BMP-4 about 2.31-fold after 6 h, BMPR-IA about 2.01-fold after stimulation for 9 h, BMPR-IB about 2.51-fold after stimulation for 9 h, and BMPR-II about 1.62-fold after stimulation for 9 h. In contrast, these expressions were not changed by the same stimulation in non-OPLL cells. With the exception of BMPR-IA, there was also a significant difference in the expression of mRNA between OPLL cells and non-OPLL cells in control cultures.

Cyclic stretch-induced production of BMPs in OPLL cells

We next addressed the issue of whether the level of BMPs secreted into cell culture media was affected by cyclic stretch. Western blot analysis using antihuman BMP-2/4 polyclonal antibody demonstrated a 36-kDa BMP-2/4 band in conditioned medium from both cells. Compared with control, the level of BMP expression was enhanced in

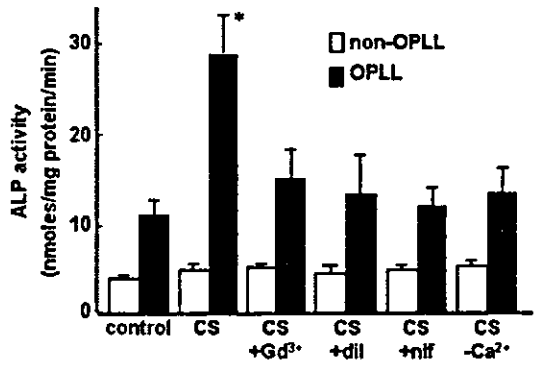

Fig. 3. Effect of Ca^{2+} channel blockers on stretch-induced ALP activity in OPLL and non-OPLL cells. Cells were treated with 50 μM Gd^{3+}, 10 μM diltiazem, 1 μM nifedipine, or Ca^{2+}-free medium for 90 min and then subjected to uniaxial cyclic stretch in the presence of each drug. After stimulation for 24 h, the ALP activity was measured as described under Materials and methods. Results are shown from 10 different experiments. Controls were cells maintained under resting state; CS, cyclic stretch; dil, diltiazem; nif, nifedipine; -Ca^{2+}, Ca^{2+}-free medium. $**P < 0.01$ versus control.

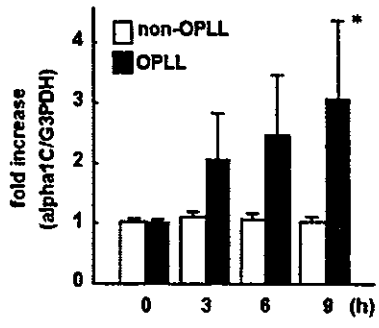

Fig. 4. Effect of uniaxial cyclic stretch on mRNA expression of α1C subunit of voltage-dependent L-type Ca^{2+} channels in OPLL and non-OPLL cells. Cells were subjected to uniaxial cyclic stretch for the indicated time periods and then mRNA was measured by quantitative RT–PCR. The densitometric quantification of the electrophoretic profile of α1C subunit mRNA was normalized to the corresponding G3PDH signal. The fold increases of mRNA to control are shown from 10 different experiments. Controls were cells maintained under a resting state; $P < 0.05$ versus control.

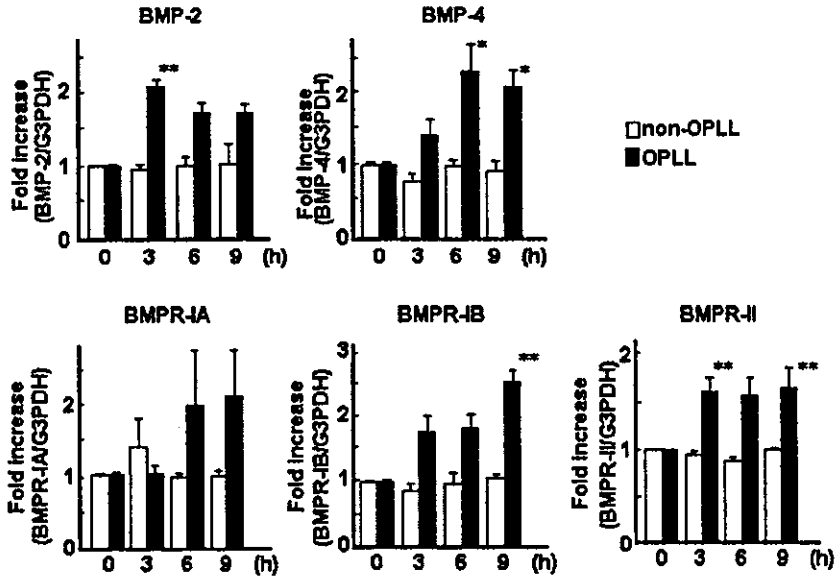

Fig. 5. RT–PCR analysis of BMP-2, BMP-4, and BMP receptors (BMPR-IA, -IB, -II) mRNAs in OPLL and non-OPLL cells. Cells were subjected to uniaxial cyclic stretch for the indicated time periods and then mRNAs were measured by quantitative RT–PCR. The densitometric quantification of the electrophoretic profile of each gene was normalized to the corresponding G3PDH signal. The fold increases of mRNA to control are shown from 10 different experiments. As control experiments cells were maintained under the resting state. $^{*}P < 0.05$ versus control. $^{**}P < 0.01$ versus control.

a time-dependent manner in OPLL cells, but not in non-OPLL cells. β-Actin as an internal standard for cell protein detected with anti-β-actin antibody did not change significantly by cyclic stretch in either kind of cell (Fig. 6).

Effect of conditioned medium and anti BMP-2 antibody on ALP activity

Whether the secreted BMPs in conditioned medium actually functioned or not, we investigated the effect of the conditioned medium and anti-BMP-2/4 antibody on the ALP activity of OPLL cells. As shown in Fig. 7, treatment of OPLL cells with stretch-conditioned medium produced a 1.8-fold increase in ALP activity, and this effect was significantly blocked by anti-BMP antibody. In addition, the stretch-conditioned medium of OPLL cells failed to increase in ALP activity in non-OPLL cells (data not shown).

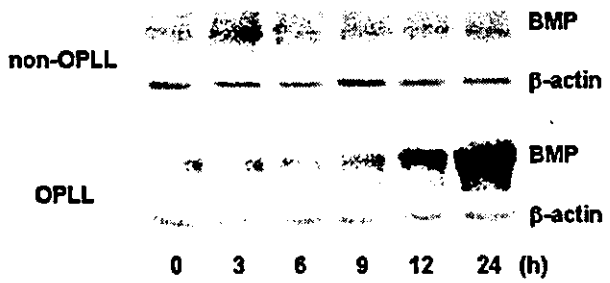

Fig. 6. Western blot analysis of BMP secretion induced by uniaxial cyclic stretch in OPLL and non-OPLL cell cultures. Cells were incubated with medium containing 1% FBS for 24 h and subjected to uniaxial cyclic stretch for the indicated time periods. The change in BMP protein was analyzed by Western blot using antihuman BMP-2/4 polyclonal antibody. β-Actin was shown as an internal standard of cell proteins detected with anti-β-actin monoclonal antibody. The data are representative of three experiments.

Discussion

Evidence is accumulating that OPLL cells show several different phenotypic characteristics for osteoblasts, while

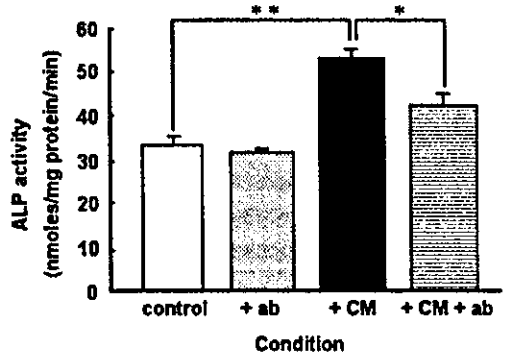

Fig. 7. Effects of conditioned medium and anti-BMP-2/4 antibody on ALP activity. Conditioned medium (CM) was prepared from the culture of OPLL cells subjected to uniaxial cyclic stretch for 9 h. The conditioned medium was incubated with or without the antibody against BMP-2/4 (ab) for 1 h and further incubated with protein G-agarose beads for 1 h. BMP-2/4 protein bound to beads was precipitated from the conditioned medium by centrifugation. OPLL cells were incubated with BMP-2/4-containing or BMP-2/4-depleted conditioned medium for 24 h and then the ALP activity was measured. Results are shown from 10 different experiments. $^{*}P < 0.05$. $^{**}P < 0.01$.

non-OPLL cells are fibroblastic. In our study, likewise, osteogenic medium induced in vitro mineralization in OPLL cell cultures, and the mRNA expression of both ALP and BMPs was greater than that in non-OPLL cells in control. Furthermore, some marker genes for osteoblast differentiation, such as osteopontin, osteocalcin, and BMP receptor IB, were higher in OPLL cells than non-OPLL cells. It is conceivable that the metaplasia of OPLL cells into osteogenic cells has already occurred in OPLL, consistent with other studies of OPLL pathogenesis [6,11–16]. This may explain why different responsiveness to cyclic stretch was observed between OPLL cells and non-OPLL cells. It is reasonable that non-OPLL cells would have the ability to respond to the same stimuli in a different manner.

There was also a difference in potentiality for mineralization in OPLL cells. Whether the ossification process of spinal ligaments is in an active state or not may reflect a difference in the osteogenic commitment of spinal ligament cells, possibly affected by the time of sample extirpation. Although, we cannot evaluate which condition is dominant on surgery, because OPLL usually progress very slowly and it requires a long time to detect changes in the size of ossified ligaments on X-ray, it could be presumed that populations of relatively high osteogenic potential cells are predominant in the active state compared with the terminal stage of ossification. The patient's age and gender seemed not to be involved, because progression of OPLL is frequently observed in elderly persons and our results did not show any correlation with the patient's gender.

It is well known that mechanical stress is converted to biochemical response in various cells, where it leads to an increase in gene expression and/or protein synthesis which help the cells to adapt to their microenvironment [23,24]. A lot of studies have also shown similar phenomena in in vivo or in vitro osteogenesis [25,26]. For example, an increase has been found in the level of ALP mRNA in mouse periodontal osteoblasts [27] and TGF-β1 and IGF-1 mRNA in human osteoblastic SaOS-2 cells [28] in response to mechanical stress. In the present study, we showed that the mRNA expression of ALP and osteopontin as well as ALP activity were significantly increased by uniaxial cyclic stretch in OPLL cells. These observations suggest that OPLL cells have a greater ability to differentiate into osteogenic cells in response to cyclic stretch than non-OPLL cells. Osteocalcin, a more specific marker for osteoblasts, was higher in OPLL cells than non-OPLL cells. However, the expression was hardly enhanced by cyclic stretch up to 9 h. It is estimated that the characteristics of OPLL cells are not same as those of osteoblasts or fibroblasts, but relatively osteogenic.

As a candidate for mechanotransuducers, stretch-activated Ca^{2+} channels (SA channels) have been identified in several cells [29,30]. The channel activity is blocked by Gd^{3+}; however, it is not completely selective for SA channels as it is also able to block voltage-dependent Ca^{2+} channels in pituitary cells [31], leaving an alternative possibility of their molecular identity as a family of voltage-dependent Ca^{2+} channels. Recently, the α1C subunit, cardiac isoform of L-type voltage-dependent Ca^{2+} channels has been shown to activate the SA channels in bone cells by using antisense oligodeoxynucleotides [29,32] Interestingly, the involvement of L-type Ca^{2+} channels in the process of endochondral mineralization has also been suggested [33]. The mechanism underlying the signaling pathway that converts mechanical stress into osteogenic response in OPLL cells still remains unclear, but present results show that the potent SA channel blocker Gd^{3+}, voltage-dependent L-type Ca^{2+} channel blockers diltiazem and nifedipine, and Ca^{2+}-free medium almost completely suppressed stretch-induced ALP activity. This suppression indicates that cycle stretch can stimulate ALP biosynthesis in OPLL cells by a mechanism mediated by Ca^{2+} influx from the extracellular space via mechanosensitive or voltage-dependent L-type Ca^{2+} channels. It is very interesting that nifedipine, which is commonly used for treatment of hypertension and ischemic heart disease, demonstrated an inhibitory effect on such osteogenic response to mechanical stress of OPLL cells. The relationship between the pathogenesis of OPLL and hypertension is not well known. Elucidation of this relationship may provide some contribution to clinical intervention against the progression of OPLL. In this study, we cannot determine which channel is predominant, but it concurrently leaves the possibility that the channels are closely related to one another in this process. Although it is unclear which channel is more important for the induction of ALP activity in response to cyclic stretch, it is conceivable that the α1C subunit is responsible, at least in part, for mediating these process, because mechanically enhanced α1C mRNA expression paralleled the increase in ALP in OPLL cells. Either way, further investigations are needed to clarify the mechanism involved in processes following Ca^{2+} influx.

BMPs, a family of the TGF superfamily, cause ectopic bone formation in vivo [34] and play essential roles in embryogenesis and skeletal development [35]. Numerous studies have shown that BMP-2 stimulates osteoblast differentiation in human cells [36], cultured rat calvarial osteoblasts [37], ST2 bone marrow stromal cell lines [38] and also OPLL cells [15], leading to the notion that it is involved in the etiology of OPLL development. The question is whether these effects of BMPs are enhanced by mechanical stress in OPLL. In the present study, mRNA expressions of BMP-2 and BMP-4 and their receptors as well as the secretion of active BMP-2/4 into conditioned medium were significantly increased by uniaxial cyclic stretch in OPLL cells. Additionally, the evidence that stretch-conditioned medium increased ALP activity in OPLL cell cultures and this increase was reduced by anti-BMP2/4 antibody raises the possibility that stretch-induced BMP secretion promotes the osteogenic differentiation of OPLL cells. These results permit us to speculate that mechanical stress induces BMP synthesis as well as an increase in the mRNA level having biologic actions at or near their sites of origin via paracrine

or autocrine mechanisms. BMPs exert their effects via binding to two different types of serine/theronine kinase receptors, type I and type II. Two type I BMP receptors, i.e., BMPR-IA and BMPR-IB, transduce the signal after their activation by type II BMP receptor (BMPR-II) [39]. These receptors have shown a different expression pattern during bone formation [40] or in the differentiation status of bone cells [41]. In the present study, the expression pattern of BMPR-IB paralleled BMPR-II in response to cyclic stretch in OPLL cells, and the stretch-enhanced effect was more significant in the former. However, in control cultures, there was no difference between OPLL cells and non-OPLL cells in BMPR-IB mRNA expression, which may reflect the alternative ratio of type II to type I receptors in the state of OPLL cells, which possibly influences how mechanical signaling is perceived in OPLL cells. Thus, this mechanical up-regulation of BMPRs may also contribute to the promotion of the ossification process in OPLL. However, the mechanism of mechanical up-regulation of BMPs was not clear in this study. Recently, the presence of share stress-responsive elements was found in the promoter region of mouse BMP-2 [42] and mouse BMP-4 [43]. Further study of the transcriptional regulation of BMP response to mechanical stress will be required to clarify these possibilities.

In conclusion, the present study proved that mechanical stress plays a key role in the progression of OPLL, at least in part through the induction of osteogenic differentiation in spinal ligament cells and the promotion of the autocrine/paracrine mechanism of BMPs in this lesion. This study also suggests evidence for a role of Ca^{2+} influx in signal transduction of mechanical stress to the osteogenic response in OPLL cells. These effects of mechanical stress on OPLL cells should not directly result in ossification of ligament tissues; however, it is presumed to be an initial step of mechanically induced ossification processes.

Acknowledgments

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Genomewide Linkage and Linkage Disequilibrium Analyses Identify *COL6A1*, on Chromosome 21, as the Locus for Ossification of the Posterior Longitudinal Ligament of the Spine

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Ossification of the posterior longitudinal ligament (OPLL) of the spine is a subset of "bone-forming" diseases, characterized by ectopic ossification in the spinal ligaments. OPLL is a common disorder among elderly populations in eastern Asia and is the leading cause of spinal myelopathy in Japan. We performed a genomewide linkage study with 142 affected sib pairs, to identify genetic loci related to OPLL. In multipoint linkage analysis using GENEHUNTER-PLUS, evidence of linkage to OPLL was detected on chromosomes 1p, 6p, 11q, 14q, 16q, and 21q. The best evidence of linkage was detected near D21S1903 on chromosome 21q22.3 (maximum Z_{lr} = 3.97); therefore, the linkage region was extensively investigated for linkage disequilibrium with single-nucleotide polymorphisms (SNPs) covering 20 Mb. One hundred fifty positional candidate genes lie in the region, and 600 gene-based SNPs were genotyped. There were positive allelic associations with seven genes ($P < .01$) in 280 patients and 210 controls, and four of the seven genes were clustered within a region of 750 kb, ~1.2 Mb telomeric to D21S1903. Extensive linkage disequilibrium and association studies of the four genes indicated that SNPs in the collagen 6A1 gene (*COL6A1*) were strongly associated with OPLL ($P = .000003$ for the SNP in intron 32 [-29]). Haplotype analysis with three SNPs in *COL6A1* gave a single-point P value

of .0000007. Identification of the locus of susceptibility to OPLL by genomewide linkage and linkage disequilibrium studies permits us to investigate the pathogenesis of the disease, which may lead to the development of novel therapeutic tools.

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Introduction

Ossification of the posterior longitudinal ligament (OPLL [MIM 602475]) of the spine was first reported in Japan and is a common disorder among Japanese and other Asian populations (Matsunaga and Sakou 1997). The incidence of OPLL in Japan is 2%–4% of the general population >30 years of age, with a male predominance of 2 : 1 (Matsunaga and Sakou 1997). Heterotopic ossification of the spinal ligament is the specific feature of OPLL that causes compression of the spinal cord and leads to various degrees of myelopathy. Typical symptoms of OPLL are sensory and motor disturbance of the upper and lower extremities, abnormal reflexes, hyperresponsive deep reflexes, and bladder–bowel dysfunction. Various degrees of dysfunction, such as precise action and gait disturbance, lead to the restriction of activities involved in daily living and the deterioration of quality of life. Multiple etiologies for OPLL need to be considered because of the late-onset nature of the disease; however, the disease is, to some extent, genetically determined, as is demonstrated by the classic epidemiologic study and by the estimated relative risk of 10 for siblings of affected individuals (Terayama 1989; Sakou et al. 1991). OPLL is a high-bone-mass disease (or systemic hyperostosis), and patients with OPLL, especially women >60 years of age, have increased systemic bone mineral density (Yamauchi et al. 1999). Another ossification disorder, diffuse idiopathic skeletal hyperostosis (DISH), appears to be related to OPLL (Trojan et al. 1992). DISH is a common skeletal disease in middle-aged and elderly patients (with frequencies in individuals >50 years of age of 25% in men and 15% in women) in Western countries. The disease is characterized by ligamentous ossification of the anterolateral aspect of the spinal column, sometimes leading to bony ankylosis (Resnick et al. 1978). The extent of overlap of OPLL with DISH is uncertain; however, the pathophysiological mechanism of DISH may be similar to that of OPLL (Trojan et al. 1992; Weinfeld et al. 1997).

Because genetic factors appear to have a crucial role in OPLL, the use of molecular genetic studies is important to the understanding of the molecular etiologies of OPLL and will lead to the development of new therapeutics. Elsewhere, we reported linkage evidence in the HLA region of chromosome 6 in 91 affected sib pairs and identified the collagen 11A2 gene (*COL11A2* [MIM 120290]) as a possible candidate (Koga et al. 1998). Allelic association studies between OPLL and molecular variants in *COL11A2* demonstrated that a nucleotide substitution at intron 6 (−4), a T→A substitution, is significantly associated with OPLL, and the functional variant results in altered splicing, which is protective in the pathogenesis of OPLL (Maeda et al. 2001). Nakamura

et al. (1999) reported that the nucleotide pyrophosphatase gene (*NPPS* [MIM 173335]), which has been identified as causal in a mouse model of OPLL (Okawa et al. 1998), is also associated with OPLL.

To understand the whole picture of OPLL susceptibility, we conducted a genomewide linkage study in 70 Japanese nuclear families, comprising 169 subjects and 142 affected sib pairs. At least six potential loci were linked to OPLL, with evidence for linkage being particularly strong on chromosome 21. Linkage analysis alone cannot provide the necessary resolution to identify the underlying genee, especially in complex diseases. Fine mapping of the linked regions can be attempted using linkage disequilibrium analysis with SNPs. Because a recent study of asthma susceptibility found that the 1-LOD decrease in the support interval of the linkage likely contains the susceptibility locus (Van Eerdewegh et al. 2002), the interval of the 1-LOD decrease on chromosome 21, ~20 cM, was extensively studied by use of linkage disequilibrium mapping to pinpoint the locus of OPLL. When 600 SNPs of 150 genes were screened, significant associations were revealed between OPLL and molecular variants in collagen 6A1 (*COL6A1* [MIM 120220]). The disease-related linkage disequilibrium was also assessed by haplotype-based association study.

Subjects and Methods

Disease Criteria and Subjects

The ethics committees of Hirosaki University and Kagoshima University approved the study, and all patients gave written informed consent. OPLL was diagnosed by the observation of ectopic bone formation in the posterior longitudinal ligament during x-ray or computed tomography examinations of the cervical, thoracic, or lumbar portions of the spine. The disease status of OPLL in radiograms was classified into four types, according to criteria of the Investigation Committee on the Ossification of the Spinal Ligaments, the Ministry of Health, Labor, and Welfare, Japan: (i) segmental, (ii) continuous, (iii) mixed, and (iv) localized, circumscribed, or bridged. All four types of OPLL were examined in the present study.

The siblings of patients with OPLL were screened for excessive spinal bone formation by x-ray examination, and affected siblings, including asymptomatic individuals, were recruited (Koga et al. 1998; Furushima et al. 2002). Samples for DNA analysis were collected from a total 142 affected sib pairs from 70 Japanese families; 98 pairs were from Kagoshima, 40 pairs from Hirosaki, 2 pairs from Asahikawa, and 1 pair each from Wakayama and Okinawa. The family structure was as follows: 51 affected pairs, 12 affected trios, 5 affected quartets, 1 affected quintet, and 1 affected sextet. The affected siblings included 76 women and 93 men. For the allelic association study, 342 unrelated patients with OPLL (73 familial cases, including 70 probands used for linkage study and 269 sporadic cases) and 298 unrelated subjects without OPLL were recruited in Kagoshima and Hirosaki. All the subjects without OPLL were >60 years of age and had no signs of spinal ossification when examined by standard x-ray methods, thereby excluding excluding most unmanifested diseases.

Microsatellite Genotyping

Multiplex fluorescent genotyping was performed using ABI PRISM linkage mapping set version 2 (Applied Biosystems). Because several markers were not polymorphic in the Japanese population (Ikari et al. 2001), a set of 47 markers obtained from the Genome Database were added to the original set to fill in the gap (Onda et al. 2001). An extra sequence was attached to the 5′ end of the reverse primer to promote nontemplated addition of adenine for accurate genotyping (Brownstein et al. 1996). Four markers (D6S446, D9S1678, D17S938, and DXS8067) generated incomplete data on the first round of genotyping and were excluded from further analysis. Marker positions (in Kosambi centimorgans) were obtained from the Marshfield Medical Research Foundation (Broman et al. 1998). For chromosome 21, which demonstrated significant linkage when the framework marker set was used, markers were added for dense mapping that covered <5 cM in the region (markers D21S1904, D21S1884, D21S1258, D21S262, D21S1900, D21S1893, D21S1411, and D21S1903). Microsatellite genotyping was performed as described elsewhere (Furushima et al. 2002). All marker genotypes were checked for Mendelian inheritance, using the Checkfam program (Saito et al. 2002), and genotyping inconsistencies were corrected.

Affected-Sib-Pair Linkage Analysis

GENEHUNTER-PLUS (Kong and Cox 1997) and GENEHUNTER 2.1 (Kruglyak et al. 1996) were used to analyze the data on affected sib pairs. Multipoint analysis of the data from the genomewide scan and from the fine mapping was performed by weighting each family equally with GENEHUNTER-PLUS, a modified version of GENEHUNTER. GENEHUNTER-PLUS assumes a linear model for risk and thus provides more-accurate calculations of the variance than does the original program. GENEHUNTER 2.1 was used for estimates of the mean proportion of alleles shared identical by descent (IBD) and for exclusion mapping. The linkage exclusion was set at a LOD score of −2. GENEHUNTER 2.1 was also used to calculate information content. Allele frequencies of microsatellite markers were estimated in 64 unrelated Japanese subjects (Ikari et al. 2001). For dense mapping on chromosome 21, allele frequencies of the markers were estimated with 70 probands, to perform more stringent linkage analysis.

SNP Genotyping

SNPs were obtained from the two public databases: National Center for Biotechnology Information dbSNP and Institute of Medical Science-Japan Science and Technology Corporation database of Japanese SNPs. Gene-based SNPs were selected at 3- to 10-kb intervals to cover the gene. Twelve subjects with OPLL were genotyped for each SNP, to confirm frequencies of minor alleles, and SNPs with minor allele frequencies >0.08 were subjected to further analyses. SNPs in the coding region or regulatory region that may directly affect gene function were given priority, regardless of the minor allele frequencies. PCR analysis was performed with a standard protocol, except that a biotin-labeled primer was used when the pyrosequencing method was applied. SNPs were genotyped using either the pyrosequencing method (a real-time pyrophosphate DNA sequencing, on a PSQ96 Instrument [Pyrosequencing]) or direct sequencing (using BigDye terminator cycle sequencing on an ABI PRISM 3700 DNA analyzer [Applied Biosystems]).

Other Statistical Analyses

Allelic frequencies of polymorphisms in cases and controls were compared using a contingency χ^2 test. Haplotype frequencies for multiple loci were estimated using the expectation–maximization method with the Arlequin program, which is available at the Arlequin Web site or SNPAlyze program (Dynacom). Pairwise LD was estimated as $D = x_{11} - p_1 q_1$, where x_{11} is the frequency of haplotype A_1B_1, and p_1 and q_1 are the frequencies of alleles A_1 and B_1 at loci A and B, respectively. A standardized LD coefficient, r, is given by $D/(p_1 p_2 q_1 q_2)^{1/2}$, where p_2 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 $D' = D/D_{max}$, where $D_{max} = \min(p_1 q_2, p_2 q_1)$ when $D < 0$ or $D_{max} = \min(p_1 q_1, p_2 q_2)$ when $D > 0$ (Lewontin 1964).

Results

Affected-Sib-Pair Linkage Analysis

A total of 70 Japanese families comprising 169 affected subjects and 142 sib pairs were included in the genome-scan data set. Multipoint Z_{lr} scores for all chromosomes (except the Y chromosome) are displayed in figure 1. The region with the most prominent evidence of linkage was on chromosome 21q22, with a maximum Z_{lr} score of 3.09 near marker D21S266. In addition to chromosome 21, five others—on chromosomes 1p, 6p, 11q, 14q, and 16q—showed evidence of linkage (defined as $Z_{lr} > 2.2$). Because the best evidence of linkage was observed with markers on chromosome 21q, high-resolution mapping was performed by adding eight markers. The fine mapping on chromosome 21 (fig. 2) indicated that the linkage peak ($Z_{lr} = 3.97$) was close to the q terminus near marker D21S1903. The highest two-point Z_{lr} score (3.69) was detected at D21S1903 on the q terminus, and the highest IBD sharing of 71% ($Z_0 = 0.04$; $Z_1 = 0.50$; $Z_2 = 0.46$) was observed at D21S262 on 21q22.

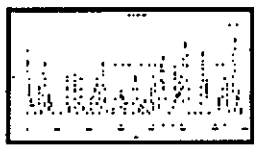

Figure 1 Genomewide linkage analysis of OPLL among 142 affected sib pairs. Summary of the framework genome scan for OPLL susceptibility loci showing the Z_{lr} scores that were calculated by GENEHUNTER-PLUS for all chromosomes. Dotted lines indicate chromosome boundaries, and the distance from the p terminus of chromosome 1 is shown on the *X*-axis.

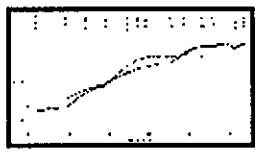

Figure 2 High-resolution mapping of chromosome 21. The solid line indicates the result of high-resolution linkage with 13 markers. The dotted line indicates the framework linkage result; asterisks (*) indicate the markers in the framework marker set. A Z_{lr} score of 3.97 is detected within the peak region close to the q terminus by fine mapping. The *Y*-axis indicates Z_{lr} score, and the *X*-axis indicates distance from the p terminus of chromosome 21.

Screening of Genes on the Linkage Region of Chromosome 21

The best evidence of linkage was detected with markers on chromosome 21q21; however, the

linkage region ($Z_{lr} > 2.2$) spans ~30 cM, and the 1-LOD decrease in the support interval was ~20 cM and contained 200 genes. To pinpoint the locus for OPLL, linkage disequilibrium analysis was performed using gene-based SNPs in the region. We first genotyped 600 SNPs of 150 genes in the linkage region from 96 selected patients and 96 controls. The selected patients consisted of 73 unrelated patients with family history and 23 patients with age at onset <45 years. The remaining 50 genes were not investigated, because there were no SNPs in the database or SNPs were not polymorphic in Japanese populations. Allelic association was assessed by χ^2 analysis with a contingency table. Seventy-four SNPs of 24 genes exhibited significant allelic associations ($P < .05$; data not shown). For the second screening, we genotyped the 74 SNPs in an expanded number of patients with OPLL ($n = 280$) and in control subjects without OPLL ($n = 210$). These patients included the initial screening sets. Fourteen SNPs of seven genes had allelic associations ($P < .01$) with OPLL (table 1). The most significant association with OPLL was observed with SNPs of *COL6A1*, which is located 1.2 Mb from the peak linkage marker, D21S1903. Because the four genes (*COL18A1* [MIM 120328], *PCBP3*, *COL6A1*, and *COL6A2* [MIM 120240]) that showed positive associations with OPLL are clustered within a 750-kb region in the vicinity of marker D21S1903, extensive SNP and pairwise linkage disequilibrium analyses were performed in the region. A total of 92 SNPs were analyzed for linkage disequilibrium structures in the region (fig. 3). Highly structured linkage disequilibrium blocks, as estimated with the D' statistic, were observed; for example, *COL18A1* was separated in two blocks, and the second block included *SLC19A1*. Because we genotyped gene-specific SNPs rather than SNPs in intergenic regions, we could not detect a broad scale of linkage disequilibrium blocks. The structures of pairwise linkage disequilibrium blocks were almost identical between patients and controls. SNPs of *COL6A1* that had the strongest association with OPLL were in a gene-specific linkage disequilibrium block; therefore, *COL6A1* was examined in more detail as the most likely candidate gene for OPLL. A total of 32 distinct SNPs—including the 4 SNPs used for the first screening, 25 additional dbSNPs, and 7 non-dbSNPs identified by direct sequencing—were genotyped in all the patients ($n = 342$) and controls ($n = 298$) (table 2). Among 7 SNPs identified in the present study, SNP31 at exon 35 led to a nonsynonymous change (serine to leucine), and other changes occurred in introns (table 2). Of 32 SNPs, 21 were statistically associated with OPLL ($P < .05$); of these, 4 had a particularly strong association ($P < .0001$ with OPLL; table 2). The most significant allelic association with OPLL was observed with a T→C substitution at intron 32 (-29) ($\chi^2 = 21.99$; df = 1; $P = .000003$; odds ratio = 1.82 [95% CI = 1.60–2.35]). A T→C substitution at exon 15 (+39), which is a silent substitution, had the most significant association with OPLL among six coding SNPs ($\chi^2 = 11.23$; df = 1; $P = .0008$; odds ratio = 1.49 [95% CI = 1.33–1.89]). For an explanation of numbers in parentheses that follow indications of location, see footnotes to table 2.

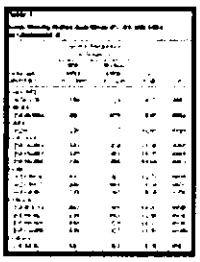

Table 1 Genes Showing Positive Associations ($P < .01$) with OPLL on Chromosome 21

Figure 3 Pairwise linkage disequilibrium analysis on the 750-kb region where candidate genes are clustered. The upper figure shows six genes, including four associated genes (shown in table 1) within 750 kb of the linkage region near the marker D21S1903. The lower figure shows pairwise linkage disequilibrium blocks in the region. Pairwise linkage disequilibrium between all pairs was evaluated by the D' statistic, and *COL6A1* linkage disequilibrium was analyzed with the r^2 statistic. SNPs with allele frequencies <.05 were excluded for the linkage disequilibrium estimate.

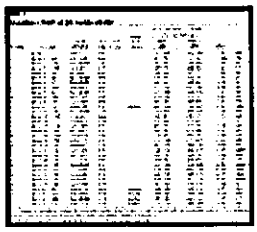

Table 2 Polymorphisms in *COL6A1* and Their Association with OPLL

Haplotype Analysis of COL6A1

Because multiple SNPs may act in combination to increase the risk of OPLL, haplotypes were constructed on the basis of maximum likelihood. Three SNPs in *COL6A1* (intron 21 [+18], intron 32 [−29], and intron 33 [+20]) that had highly significant associations with OPLL and were not in complete linkage disequilibrium with each other, as estimated using the r measure ($r^2 < 0.7$; fig. 4), were selected for haplotype analysis (table 3). Estimated haplotype frequencies of the four common haplotypes (H1–H4) of subjects with OPLL were compared with those of control subjects without OPLL. There was a significant difference in haplotype frequency between cases and controls: haplotype H2, which comprises all the minor alleles at each site, occurred at a higher frequency in patients with OPLL than in control subjects ($\chi^2 = 24.73$, df = 1, $P = .0000007$, odds ratio = 2.03 [95% CI = 1.76–2.70]); and haplotype H1, which comprises all the common alleles at each site, had a higher frequency in subjects without OPLL than in patients with OPLL ($\chi^2 = 11.64$; df = 1; $P = .0006$; odds ratio = 0.66 [95% CI = 0.59–0.84]). The global test also gave a statistically significant result ($\chi^2 = 26.73$; df = 4; $P = .000023$).

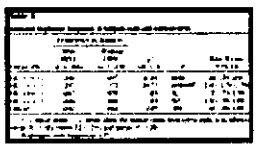

Table 3 Estimated Haplotype Frequency in Subjects with and without OPLL

Discussion

The linkage results of the genomewide scan with 142 affected sib pairs revealed that six loci, on chromosomes 1p, 6p, 11q, 14q, 16q, and 21q, confer susceptibility to OPLL ($Z_{lr} > 2.2$), and the most promising locus was on chromosome 21q22 ($Z_{lr} = 3.09$) (fig. 1). Dense mapping of chromosome 21 revealed increased evidence of linkage on 21q22 near D21S1903 ($Z_{lr} = 3.97$) that would exceed the threshold of significant linkage (fig. 2) (Lander and Kruglyak 1995). The linkage region was studied extensively through linkage disequilibrium mapping and haplotype analysis, to

narrow the susceptibility locus of OPLL.

Among five other susceptibility loci, chromosome 6 was of particular interest because previous studies reported that two candidate genes for OPLL (*COL11A2* and *NPPS*) are on that chromosome (Koga et al. 1998; Nakamura et al. 1999). In the multipoint linkage study of chromosome 6, the highest Z_{lr} score (2.22) and the best IBD sharing (59%; $Z_0 = 0.16$; $Z_1 = 0.50$; $Z_2 = 0.34$) was on 6p21, where *COL11A2* is located. On the other hand, the *NPPS* locus did not show any evidence of linkage ($Z_{lr} = 0.00$; mean IBD sharing 50.5%), confirming previous results of a single-point linkage analysis (Furushima et al. 2002).

The second most significant linkage ($Z_{lr} = 2.94$) was observed at chromosome 14, indicating that the chromosome 14q region may harbor another responsible gene. Notably, the linkage region at 14q is the widest linkage peak throughout the genome (43 cM; $Z_{lr} \geq 2.2$). Terwilliger et al. (1997) suggest that IBD sharing would be expected to decline gradually over adjacent markers when true linkage is present, resulting in a broad Z_{lr} score distribution. Because the best evidence of linkage was on chromosome 21, the 14q region was not pursued in the present study.

The genomewide linkage results indicated the best evidence of linkage to OPLL on chromosome 21; therefore, we proceeded to narrow the locus through a linkage disequilibrium study. We performed an association study with ~600 SNPs of 150 genes covering the 20-Mb linkage region of chromosome 21q. *COL6A1* is the gene most significantly associated with OPLL in the case-control comparison ($P = .04$–$.000003$) and the haplotype analysis ($P = .0000007$) (tables 2 and 3). In addition, pairwise linkage disequilibrium analysis revealed that *COL6A1* is in the gene-specific linkage disequilibrium block (fig. 3). Thus, susceptibility to OPLL could be accurately pinpointed to the *COL6A1* locus. Mutations in the genes that encode collagen VI subunits (*COL6A1*, *COL6A2*, and *COL6A3*) are causal of Bethlem myopathy (MIM 158810), a dominant form of myopathy, and Ullrich syndrome (MIM 254090), a recessive form of muscular dystrophy (Jobsis et al. 1996; Lamande et al. 1998; Pape et al. 1999; Camacho Vanegas et al. 2001; Scacheri et al. 2002) characterized by early childhood onset of proximal muscle weakness and contractures of multiple joints (Bethlem and Wijngaarden 1976; Mohire et al. 1988). In the present study, we did not detect any molecular variants in the key glycine residues of the triple-helix domain that result in Bethlem myopathy. *COL6A1* encodes the α_1 chain of type VI collagen, which is an extracellular matrix protein consisting of a short central triple helix flanked by two large globular domains. Because the SNP (the T→C substitution at intron 32 [-29]) that is most strongly associated with OPLL is near the branch site of the intron, that SNP may affect the lariat-shaped structure, thereby causing aberrant splicing. Our previous linkage and association studies identified *COL11A2* as a candidate for OPLL, and the functional intron 6 (-4A) polymorphism of *COL11A2* affects splicing of exon 6 (Koga et al. 1998; Maeda et al. 2001). RT-PCR analyses of *COL6A1* on cultured interspinous ligament cells from 10 patients with OPLL were performed, to study the splicing variants of *COL6A1*, as described in the analysis of *COL11A2* published elsewhere (Maeda et al. 2001). Thus far, we have not detected a splicing variant of *COL6A1* due to the intron 32 (-29) genotype (data not shown). The functional impact of the polymorphisms of *COL6A1* is uncertain, but *COL6A1* may lead to increased bone mass.

Two genes (*COL6A1* and *COL11A2*) encode extracellular matrix proteins, which may provide a scaffold for osteoblastic or preosteoblastic cells or chondrocytes that subsequently proceed to membranous or endochondral ossification (Zhang and Chen 2000; Gowen et al. 2003). Therefore, molecular variants of the extracellular proteins may be implicated in the ectopic ossification observed in OPLL.

The etiologies of all complex diseases potentially involve gene–gene and gene–environment interactions. In the case of OPLL, interactions of *COL6A1* and *COL11A2* must be taken into account in efforts to further understand the etiology of the disease. Elucidation of the molecular etiology of OPLL will lead to the development of new therapeutic approaches, not only for bone-forming diseases but also for diseases of bone loss, such as osteoporosis.

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Electronic-Database Information

URLs for data presented herein are as follows:

- Arlequin, http://anthropologie.unige.ch/arlequin (for the Arlequin program) First citation in article
- Center for Medical Genetics, Marshfield Medical Research Foundation http://research.marshfieldclinic.org/genetics/ (for genetic linkage maps) First citation in article
- GenBank, http://www.ncbi.nlm.nih.gov/Genbank/ (for gene sequences) First citation in article
- Genome Database, The, http://www.gdb.org/ (for primer design of microsatellite genotyping) First citation in article
- Institute of Medical Science–Japan Science and Technology Corporation database of Japanese SNPs, http://snp.ims.u-tokyo.ac.jp/ (for dbSNP selection) First citation in article
- NCBI dbSNP Home Page, http://www.ncbi.nlm.nih.gov/SNP/ (for dbSNP selection) First citation in article
- Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/ (for OPLL, Bethlem myopathy, Ullrich syndorome, *COL11A2, COL6A1, COL6A2,* and *NPPS*) First citation in article
- Primer 3, http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi (for designing PCR primers) First citation in article
- Pyrosequencing Technical Support, SNP Primer Design, http://techsupport.pyrosequencing.com/v2/index.asp (for designing sequencing primers for pyrosequencing) First citation in article

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Significance of Bone Formation Markers in Patients With Ossification of the Posterior Longitudinal Ligament of the Spine

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Study Design. Serum concentrations of bone formation markers were correlated with the type, location, and progression of ossification of the posterior longitudinal ligament.

Objective. To determine the relation between bone formation markers and ossification of the posterior longitudinal ligament.

Summary of Background Data. Few reports have correlated bone formation markers with ossification of the posterior longitudinal ligament.

Methods. In this study, 43 patients with cervical ossification of the posterior longitudinal ligament and myelopathy underwent laminoplasty. The patients were observed for more than 10 years, after which plain radiographs and tomograms of the cervical region were taken. The radiographs were selectively performed to address thoracic and lumbar ossification of the posterior longitudinal ligament. Serum concentrations of bone formation markers (intact osteocalcin, osteocalcin, carboxyterminal propeptide of human type 1 procollagen, and bone-specific alkaline phosphatase) were measured and correlated with these radiographic studies.

Results. A positive correlation was observed between intact osteocalcin, osteocalcin, and carboxyterminal propeptide of human type 1 procollagen in patients with combinations of cervical, thoracic, or lumbar ossification of the posterior longitudinal ligament.

Conclusions. Serum concentrations of intact osteocalcin, osteocalcin, and carboxyterminal propeptide of human type 1 procollagen may reflect the activity of general ectopic bone formation in patients with ossification of the posterior longitudinal ligament. [Key words: bone formation markers, bone-specific alkaline phosphatase, carboxyterminal propeptide of human type 1 procollagen, intact osteocalcin, ossification of the posterior longitudinal ligament, osteocalcin] **Spine 2003;28:378–379**

Patients with ossification of the posterior longitudinal ligament (OPLL) exhibit a genetic predisposition toward a generalized hyperostosis.[9] The relation between several bone formation markers (intact osteocalcin, osteocalcin, carboxyterminal propeptide of human type 1 procollagen, and bone-specific alkaline phosphatase) and radiologically confirmed cervical, thoracic, and lumbar OPLL was evaluated.

Methods

Participants and Radiographic Analysis. In this study 43 patients with cervical laminoplasty were followed for an average of 14 years (Table 1). All the patients had OPLL documented on radiographs of the cervical spine and tomograms. A selected subset demonstrated radiographic evidence of thoracic and/or lumbar OPLL.

Biochemical Markers. Serum concentrations of four bone markers (Table 1)[1,2,6,10] were obtained from all the patients at the time of their final examination. The marker concentrations were correlated with the patients' radiographic evidence of OPLL. The intra- and interassay coefficient of variations for all the bone formation markers were less than 4% and 6%, respectively.

Statistical Analysis. Serum concentrations of the markers were expressed as mean ± standard deviation. Differences between groups were analyzed for statistical significance using the Student's t test (unpaired). A P value less than 0.05 was considered statistically significant.

Results

OPLL was found only in the cervical spine in 20 patients. In another 23 individuals, cervical, thoracic and/or lumbar OPLL was present (Table 1). I-OC, OC and PICP showed significantly higher concentrations in patients with OPLL in the cervical, thoracic and/or lumbar spine compared with those exhibiting cervical OPLL alone (Table 2). B-ALP, however, showed no significant correlation with OPLL (Table 2).

Discussion

Generalized spinal hyperostosis develops in patients with ossification of the posterior longitudinal ligament.[8] The current study was conducted to evaluate the relation between the characteristics of OPLL and the serum levels of bone formation markers.

Several reports document the relationship between OPLL and bone formation markers.[3,4] However, they have not been analyzed for OPLL involving multiple spine levels. In this study, OPLL found in the cervical, thoracic, and lumbar regions was correlated with greater

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Table 1. Patients Data and Results of Serum Analyses of Bone Formation Markers

Number of patients	43
Average age	68 (range, 51–89)
Sex	
Male	28
Female	15
Average JOA score	
Preoperatively	9.16 ± 3.19
Postoperatively	13.6 ± 2.71
OPLL level	
Cervical	20
Cervical, thoracic, and/or lumbar	23
Serum concentrations of bone formation markers	Average
I-OC (sandwich enzyme immunoassay)	4.5 ± 2.4 ng/mL
OC (two-site immunoradiometric assay)	6.2 ± 2.6 ng/mL
PICP (radioimmunoassay)	117 ± 40.7 ng/mL
B-ALP (enzyme immunoassay)	23.3 ± 6.4 U/L

osteosynthetic activity attributed to significantly higher concentrations of intact osteocalcin (I-OC), osteocalcin (OC), and carboxyterminal propeptide of human type 1 procollagen (PICP) than found in patients exhibiting cervical OPLL alone. Thus, the serum concentrations of I-OC, OC, and PICP may reflect the activity of general ectopic bone formation in patients with OPLL. In contrast, bone-specific alkaline phosphatase (B-ALP) was not correlated with OPLL in the current study. Several studies have indicated that B-ALP is an early marker and OC a late marker in osteoblast differentiation.[5,7] Therefore, the stage of osteoblast differentiation may be associated with the activity of general ectopic bone formation in patients with OPLL.

Table 2. Correlation Between Bone Formation Markers and OPLL Combination

Bone Formation Markers	Cervical (n = 20)		Cervical, Thoracic and/or Lumbar (n = 23)
I-OC (ng/mL)	3.6 ± 1.61	*	5.44 ± 2.68
OC (ng/mL)	5.23 ± 1.76	*	7.17 ± 3.02
PICP (ng/mL)	102.9 ± 32.6	*	132.4 ± 43.6
B-ALP (U/L)	22.1 ± 4.68		24.6 ± 7.68

* $P < 0.05$.

■ Key Points

- The concentrations of I-OC, OC, and PICP were significantly higher in patients with cervical, thoracic, or lumbar OPLL.
- Elevated serum concentrations of I-OC, OC, and PICP reflected the heightened ectopic bone formation seen in patients with OPLL.

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Association Between Polymorphism of the Transforming Growth Factor-[beta]1 Gene With the Radiologic Characteristic and Ossification of the Posterior Longitudinal Ligament

[Diagnostics]

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Outline

Graphics

Abstract

Study Design. A study was conducted to examine the relation between the transforming growth factor-[beta]1 (TGF-[beta]1) polymorphism (T->C transition in the signal sequence) and ossification of the posterior longitudinal ligament (OPLL).