



Association between *BMP-2* and *COL6A1* gene polymorphisms with susceptibility to ossification of the posterior longitudinal ligament of the cervical spine in Korean patients and family members

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ABSTRACT. *COL6A1* and *BMP-2* genes have been implicated in ossification of the posterior longitudinal ligament (OPLL) susceptibility in Japanese and Chinese Han populations. However, no study has yet investigated the DNA of unaffected family members of patients with OPLL. This study investigated differences in genetic polymorphisms of *BMP-2* and *COL6A1* between Korean patients with OPLL and their family members (with and without OPLL). A total of 321 subjects (110 patients with OPLL and 211 family members) were enrolled in the study. Associations between two single nucleotide polymorphisms (SNPs) of the *BMP-2* gene (Ser37Ala and Ser87Ser) and two SNPs of *COL6A1* [promoter (-572) and intron 33 (+20)] with susceptibility to OPLL of the cervical spine were investigated between the two groups (OPLL+

and OPLL-). Of the 321 subjects, 162 had cervical OPLL (50.4%; 110 patients, 52 family members). There was a familial tendency of OPLL in 34 of the 110 families (30.9%). Allele and haplotype frequencies of the four SNPs in the *BMP-2* and *COL6A1* genes did not differ significantly between the OPLL+ and OPLL- groups, even when excluding participants over 50 years of age. This is the first report identifying SNPs of *COL6A1* and *BMP-2* in Korean patients and family members with OPLL. Although allele and haplotype frequencies were similar with those of a previous study in Japanese and Chinese patients, unaffected family members also showed similar rates of these SNPs in the present study. These results suggest that these SNPs may not directly influence the expression of OPLL.

Key words: Single nucleotide polymorphism; Genetic association; BMP-2; COL6A1; Ossification of the posterior longitudinal ligament

INTRODUCTION

Ossification of the posterior longitudinal ligament (OPLL) is a pathological ectopic ossification of the paravertebral ligament that causes myelopathy due to chronic spinal cord compression (Inamasu et al., 2006). Although OPLL can occur anywhere along the spinal axis, its most common location is the cervical spine, followed by the thoracic spine. The incidence increases with age, with most cases occurring after 40-50 years of age. Over the past several decades, a variety of genetic investigations, including pedigree studies, twin studies, and detailed molecular analyses, have documented many genes and loci of interest involved in mediating the molecular and genetic pathobiology of OPLL (Stapleton et al., 2011; Stetler et al., 2011). *NPPS*, *COL11A2*, *COL6A1*, *BMP-2*, and *TGF- β* have been implicated as notable genes, transcription factors, and cytokines in OPLL pathogenesis (Nakamura et al., 1999; Stapleton et al., 2011; Stetler et al., 2011).

Until now, however, no study has focused on genetic factors in family members of OPLL patients. Among these aforementioned genes of interest, the *BMP-2* and *COL6A1* genes were reported to be associated with OPLL in Japanese and Chinese Han populations, and were therefore selected for our study in a Korean population (Tanaka et al., 2003; Tsukahara et al., 2005; Kong et al., 2007; Wang et al., 2008). The primary aim of this study was to investigate the differences in genetic polymorphisms of *BMP-2* and *COL6A1* genes between OPLL patients and their family members (with and without OPLL).

MATERIAL AND METHODS

Disease criteria and subjects

This study was approved by the Institutional Review Board at Yonsei University College of Medicine, and informed consent was obtained from all participants prior to enrollment (3-2009-0135). A total of 321 subjects (110 OPLL patients and 211 family members) were enrolled in this study from May 2010 to February 2011. All participants underwent cervical

X-rays to document OPLL, as well as a health questionnaire, medical interview, and physical examination. Ethylene diaminetetraacetic acid (EDTA) anti-coagulated peripheral blood samples were obtained for DNA extraction from each participant. Genomic DNA samples were extracted from peripheral leukocytes using a standard procedure. All participants were classified as either OPLL+ or OPLL- according to cervical X-ray findings. We investigated the association of two single nucleotide polymorphisms (SNPs) (Ser37Ala, Ser87Ser) of the *BMP-2* gene and two SNPs (promoter (-572) and intron 33 (+20)) of the *COL6A1* gene with OPLL status in the two groups.

Genotyping and SNPs of *BMP-2* and *COL6A1* genes

Three milliliters of whole blood was collected in tubes containing EDTA (50 mM disodium salt), and genomic DNA was isolated with a Wizard® Genomic DNA Purification Kit (Promega Corporation, Madison, WI, USA). We analyzed two SNPs (Ser37Ala T/G and Ser87Ser A/G) in exon 2 of the *BMP-2* gene and two SNPs [promoter (-572) and intron 33 (+20)] in the *COL6A1* gene. Table 1 lists the details of these four SNPs and their primer sequences. The reaction was performed in a total volume of 50 µL, containing 200 ng genomic DNA, 1 µL 20 µM each primer, 8 µL mixture 2.5 µM of each dCTP, dTTP, dGTP, and dATP, 0.5 µL TaKaRa Ex Taq DNA polymerase, 5 µL 25 mM MgCl₂, 5 µL 109 LA PCR Buffer II (Mg²⁺ free), and up to 50 µL double-distilled H₂O. The thermocycling procedure was performed as follows: initial denaturation at 95°C for 5 min, 30 cycles of denaturation at 95°C for 30 s, annealing at 57°C for 30 s, extension at 72°C for 30 s, and a final extension at 72°C for 10 min. The expected sizes of the specific amplification products were 393 bp for the *BMP-2* gene, 181 bp for the *COL6A1* promoter, and 250 bp for intron 33 of the *COL6A1* gene.

Table 1. Information of 4 SNPs studied for *COL6A1*, *BMP-2* and the primers.

Gene	SNP location	Nucleotide substitution (M/m)	Primer sequence
<i>BMP-2</i>	Ser37Ala	T/G	Sense primer: 5'-CTCACGTCGGTCCTGTCC-3'
	Ser87Ser	A/G	Antisense primer: 5'-CCCTGCTCCATGCCTCAC-3'
<i>COL6A1</i>	Promoter (-572)	G/T	Forward: 5'-AAAAGAACGCGGAGATCTGA-3'
	Intron 33 (+20)	A/G	Reverse: 5'-GTCTGAAAACCGCGTGTCA-3'
			Forward: 5'-AGAATGTCACCGCCAGAT-3'
			Reverse: 5'-CCCTGGAGGTAACAGTGAGC-3'

M/m = major allele and minor allele.

Statistical analysis

The chi-squared test was used to determine whether individual variants were in Hardy-Weinberg equilibrium at each locus in the population. Allele and haplotype frequencies of each polymorphism in the two groups (OPLL+ and OPLL-) were compared using the chi-squared test with one degree of freedom (dominant model, recessive model, and allele frequency). The haplotype frequencies for multiple loci were obtained by the expectation-maximization method, and were estimated using the permutation test. In this study, haplotypes with frequencies less than 5% were excluded from further association analysis by the chi-squared test. The P values were 2-tailed and significance was set at $P < 0.05$. All statistical analyses were performed using SPSS v15.0 (SPSS, Chicago, IL, USA).

RESULTS

The mean age of participants was 45.7 years old (range, 13-88). Of the 321 participants, 148 were males, 173 were females, and 162 had cervical OPLL (50.4%; 110 patients and 52 family members). Of the 110 families, 34 had a familial tendency for OPLL (30.9%). Tables 2 and 3 list the allele and haplotype frequencies of the four studied SNPs.

Table 2. Comparative analysis of allele frequency of the 4 SNPs between OPLL+ and OPLL- groups.

Gene	BMP-2		COL6A1	
	Ser37Ala T/G	Ser87Ser A/G	Promotor (-572) G/T	Intron 33 (+20) A/G
Total No. (321)	78 (24.3%)	308 (95.9%)	104 (32.4%)	176 (54.8%)
No. of OPLL+ (162)	41 (25.3%)	157 (96.9%)	57 (35.2%)	91 (56.2%)
No. of OPLL- (159)	37 (23.3%)	151 (94.9%)	47 (29.6%)	85 (53.5%)
P value	>0.05* (P = 0.670)	>0.05** (P = 0.411)	>0.05* (P = 0.282)	>0.05* (P = 0.625)

Statistical analysis were performed between OPLL+ and OPLL- groups. *Chi-square test. **Fisher exact test.

Table 3. Haplotype analysis of BMP-2 and COL6A1 between OPLL+ and OPLL- groups (chi-square test).

Gene	BMP-2		COL6A1		
	GG	TG	GA	GG	TG
Total No. (321)	78 (24.3%)	229 (71.3%)	135 (42.1%)	80 (24.9%)	96 (29.9%)
No. of OPLL+ (162)	41 (25.9%)	113 (69.8%)	69 (42.6%)	35 (21.7%)	55 (33.9%)
No. of OPLL- (159)	37 (23.3%)	116 (72.9%)	66 (41.5%)	45 (28.3%)	41 (25.7%)
χ^2 (d.f. = 1)	0.181	0.632	0.016	1.276	1.964
P value	>0.05 (P = 0.670)	>0.05 (P = 0.427)	>0.05 (P = 0.898)	>0.05 (P = 0.259)	>0.05 (P = 0.161)

BMP-2 gene polymorphisms

Of the 321 total participants, 78 had the Ser37Ala (T/G) SNP (24.3%), 41 of whom had cervical OPLL (52.6%) and 37 of whom did not. Of the 321 total participants, 308 had the Ser87Ser (A/G) SNP (95.9%), 157 of whom had cervical OPLL (50.9%). Among these 308 participants, 157 were OPLL+ (157/162, 96.9%) and 151 were OPLL- (151/159, 94.9%) (Table 2). There were no statistically significant differences in either SNP between the OPLL+ and OPLL- groups (P = 0.670, P = 0.411; chi-squared test or Fisher exact test, respectively) (Table 2).

COL6A1 gene polymorphisms

Of the 321 total participants, 104 (32.4%) had the G/T polymorphism in the COL6A1 promoter (-572), 57 of whom (54.8%) had OPLL. Among them, 57 were OPLL+ (57/162, 35.2%) and 47 were OPLL- (47/159, 29.6%). Of the 321 total participants, 176 (54.8%) had the A/G polymorphism in intron 33 (+20) of COL6A1, 91 (51.7%) of whom had OPLL. Of these 176 participants, 91 were OPLL+ (91/162, 56.2%) and 85 were OPLL- (53.5/159, 94.9%) (Table 2). Again, there was no statistically significant difference in either SNP between the OPLL+ and OPLL- groups (P = 0.282, P = 0.625) (Table 2).

Haplotype analysis

The GA and TA haplotypes of the *BMP-2* gene, and the TA haplotype of the *COL6A1*

gene showed frequencies less than 5%, and were thus excluded from further analyses. Of the five haplotypes that comprised the majority of genetic diversity in this study, no significant frequency differences in either gene were observed between the OPLL+ and OPLL- groups (Table 3).

Participants over 50 years old

To minimize confounding bias due to late-onset OPLL, we excluded all participants over 50 years of age, effectively excluding future OPLL patients from the OPLL- group. Of the 321 total participants, 153 were over 50 years old. Tables 4 and 5 list the allele frequencies of the four SNPs and the results of the haplotype analysis. As above, there was no statistically significant difference between the OPLL+ and OPLL- groups in allele or haplotype frequency (Table 5).

Table 4. Comparative analysis of allele frequency of the 4 SNPs between OPLL+ and OPLL- groups over 50 years old.

Gene	BMP-2		COL6A1	
	Ser37Ala T/G	Ser87Ser A/G	Promotor (-572) G/T	Intron 33 (+20) A/G
Total No. (153)	37 (24.2%)	151 (98.7%)	48 (31.4%)	84 (54.9%)
No. of OPLL+ (106)	26 (24.5%)	105 (99.1%)	36 (33.9%)	61 (57.5%)
No. of OPLL- (47)	11 (23.4%)	46 (97.8%)	12 (25.5%)	23 (48.9%)
P value	>0.05* (P = 0.881)	>0.05** (P = 0.521)	>0.05* (P = 0.300)	>0.05* (P = 0.323)

Statistical analysis were performed between OPLL+ and OPLL- groups. *Chi-square test. **Fisher exact test.

Table 5. Haplotype analysis of BMP-2 and COL6A1 between OPLL+ and OPLL- groups over 50 years old (chi-square test).

Gene	BMP-2		COL6A1		
	GG	TG	GA	GG	TG
Total No. (153)	37 (24.2%)	108 (70.6%)	65 (42.5%)	39 (25.5%)	46 (30.1%)
No. of OPLL+ (106)	26 (24.8%)	73 (69.5%)	41 (39.1%)	27 (25.7%)	35 (33.3%)
No. of OPLL- (47)	11 (22.9%)	35 (72.9%)	24 (50%)	12 (25%)	11 (22.9%)
χ^2 (d.f. = 1)	0.061	0.183	1.617	0.009	1.700
P value	>0.05 (P = 0.805)	>0.05 (P = 0.669)	>0.05 (P = 0.203)	>0.05 (P = 0.925)	>0.05 (P = 0.192)

DISCUSSION

OPLL likely stems from multifactorial causes (Smith et al., 2011). Based on the large number of genes associated with OPLL, it also appears that multiple genes are involved in both its inheritance and pathogenesis (Stapleton et al., 2011; Stetler et al., 2011). Genetic polymorphisms in several collagen genes (*COL11A2* and *COL6A1*), which encode for extracellular matrix proteins, have been associated with OPLL (Tsukahara et al., 2005; Kong et al., 2007). The *BMP-2* gene, which is a member of the TGF- β superfamily, acts as a potent stimulator of bone formation *in vivo* and has also been hypothesized to be involved in OPLL pathogenesis (Wang et al., 2008). Human nucleotide pyrophosphatase (NPPS) and its gene have been suggested to play a role in OPLL genetics (Nakamura et al., 1999). Wang et al. (2008) and Kong et al. (2007) reported that polymorphisms in *BMP-2* and *COL6A1* genes were associated with

OPLL. Among these proposed candidate genes, we chose to further investigate the *BMP-2* and *COL6A1* genes to test our hypothesis that they are associated with OPLL in Korean patients and their unaffected family members.

Several population-based epidemiological studies in Japan have reported a prevalence of OPLL ranging from 1.5 to 2.4% (Okamoto and Yasuma, 1967; Izawa, 1980; Matsunaga and Sakou, 2011), although most of these studies were conducted over 30 years ago and were based on low-quality plain films. A Japanese nationwide multicenter survey of 347 subjects with OPLL reported an incidence in first-degree relatives of 23%, and of 22% in other relatives (Terayama, 1989). In the present study, the prevalence of OPLL in family members of affected subjects was 24.6% (52/211 family members), which is significantly higher than reports of the general population. In our study, 34 of the 110 families (30.9%) exhibited a familial tendency toward OPLL, which is similar to the incidence of familial tendency in studies of Japanese populations (Terayama, 1989). Nonetheless, the mode of inheritance of OPLL remains unclear (Terayama, 1989; Hamanishi et al., 1995). Unfortunately, because we did not determine the complete pedigree of each enrolled family, we cannot further speculate about the mode of inheritance.

The present study is the first comparative report of SNPs of the *COL6A1* and *BMP-2* genes between patients with OPLL and their family members. Our results differed from that of a previous study investigating the SNPs of these genes in Japanese and Chinese Han patients with OPLL. Whereas this previous study used age-matched unaffected volunteers as the control group, the present study used unaffected family members of OPLL patients as the control. Although the frequencies of the “G” allele of *BMP-2* Ser37Ala (T/G), the “T” allele of the *COL6A1* promoter, and the “G” allele of *COL6A1* intron 33 were similar to those reported in previous studies (Kong et al., 2007; Wang et al., 2008), unaffected family members in our study also exhibited similar allele frequencies (Table 2). This finding raises doubt as to the significance of these four SNPs for OPLL expression.

In this study, we excluded all family members over 50 years old to minimize any confounding bias, as OPLL usually develops in the fifth or sixth decade of life (Choi et al., 2011). However, results were not changed after this exclusion, indicating that the unaffected family members over 50 years old exhibit similar frequencies of the four SNPs as the OPLL+ group (Table 4). These results suggest that family members of OPLL patients may have a similar genetic makeup regardless of OPLL expression, at least with respect to the four SNPs studied here. A recent proteomic analysis of the osteogenic process and osteoblastic differentiation revealed several proteins that appear to be directly related to the osteogenic pathway. However, the process of osteogenic differentiation is still not completely understood at either the cellular or molecular level. Proteomic techniques may improve the study of phenotype alterations, and thus the understanding of osteogenic induction (Giusta et al., 2010). Given results of the present study, further investigations beyond SNP analyses, including proteomics and other environmental influences, are merited.

This study has several limitations. First, OPLL diagnosis was based on plain films of the cervical spine alone. We have previously demonstrated that patients with symptomatic cervical OPLL can have concurrent ossification of the thoracic ligament (OLF), which has also been associated with *BMP-2* and *COL6A1*. Thus, the possibility of concurrent ossification of other spinal regions may have influenced our results. The prevalence of OLF without cervical OPLL is unknown, although a Japanese study reported a prevalence of OLF of 6.2% in men and 4.8% in women (Wiseman et al., 2002). Second, we studied only four SNPs of the *BMP-2* and *COL6A1* genes, and other genes have been implicated in OPLL, including the *NPPS*, *COL11A2*,

and *BMP4* genes. Nevertheless, our results demonstrating similar SNP frequencies between OPLL patients and unaffected family members may influence the direction of future research.

In conclusion, this is the first report investigating SNPs of *COL6A1* and *BMP-2* genes in Korean patients with OPLL and their family members. Although the frequencies of the “G” allele of *BMP-2* Ser37Ala (T/G), the “T” allele of the *COL6A1* promoter, and the “G” allele of *COL6A1* intron 33 were similar to those reported in previous studies, unaffected family members in our study also exhibited similar allele frequencies. Our results suggest additional pathobiological causes of OPLL expression. Further molecular and functional studies are necessary to examine this hypothesis.

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