Association of Toll-Like Receptor 5 Gene Polymorphism with Susceptibility to Ossification of the Posterior Longitudinal Ligament of the Spine in Korean Population

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Objective: Ossification of the posterior longitudinal ligament (OPLL) has a strong genetic component. Specific gene polymorphisms may be associated with OPLL in several genes which regulate calcification in chondrocytes, change of extracellular collagen matrix and secretions of many growth factors and cytokines controlling bone morphogenesis. Toll-like receptor 5 (TLR5) may play a role in the pathogenesis of OPLL by intermediate nuclear factor-kappa B (NF-κB). The current study focused on coding single nucleotide polymorphisms (SNPs) of TLR5 for a case-control study investigating the relationship between TLR5 and OPLL in a Korean population.

Methods: A total of 166 patients with OPLL and 231 controls were recruited for a case-control association study investigating the relationship between SNPs of TLR5 gene and OPLL. Four SNPs were genotyped by direct sequencing (rs5744168, rs5744169, rs2072493, and rs5744174). SNP data were analyzed using the SNPStats, SNPAlyzer, Haploview, and Helixtree programs. Multiple logistic regression analysis with adjustment for age and gender was performed to calculate an odds ratio (OR).

Results: None of SNPs were associated with OPLL in three alternative models (codominant, dominant, and recessive models; p > 0.05). A strong linkage disequilibrium block, including all 4 SNPs, was constructed using the Gabriel method. No haplotype was significantly associated with OPLL in three alternative models.

Conclusion: These results suggest that Toll-like receptor 5 gene may not be associated with ossification of the posterior longitudinal ligament risk in Korean population.

Key Words: OPLL - TLR5 gene - Polymorphisms - Genetic association study.

INTRODUCTION

Ossification of the posterior longitudinal ligament (OPLL) is a pathological ectopic ossification of the paravertebral ligament that causes myelopathy due to chronic pressure on the spinal cord. Clinical study results strongly support the view that OPLL has a strong genetic component. However, the cause of this disease is still unknown. Application of gene analysis and molecular biology approaches has begun to clarify OPLL etiology and pathology.

Bone metabolism is regulated by hormonal or local factors in the bone microenvironment, and bone homeostasis is also influenced by the immune system. A critical element in this cross-talk is the inducible transcription factor nuclear factor-κB (NF-κB), which regulates gene expression during inflammatory and immune responses.

Toll-like receptors (TLRs) are part of the innate immune defense system, recognizing conserved patterns on microorganisms. Ten members of the human TLR family have been identified; several appear to recognize specific microbial products, including lipopolysaccharides, bacterial lipoproteins, peptidoglycans, and bacterial DNA. The TLR5s gene product is expressed in myelomonocytic cells, and recognizes bacterial flagellin, which is both a principal component of bacterial flagella and a virulence factor. The activation of this receptor mobilizes NF-κB and stimulates tumor necrosis factor-alpha (TNF-α) production. Therefore, TLR5 may play a role in the pathogenesis of OPLL by intermediate NF-κB.

To date, however, there are no available data on the role of...
**Methods**

**Subjects**

A case-control study was undertaken to investigate the relationship between OPLL and TLR5 polymorphisms. Unrelated patients with OPLL (n = 166; 96 males and 70 females) were recruited from the spine center of Kyung Hee University East-West Neo Medical Center, Korea. A healthy control group consisted of 231 subjects (115 males and 116 females) who were recruited among participants to a general health check-up program after confirming that they had no clinical evidence of OPLL or any other disorders. Experiments were conducted with the informed written consent of each participant. All procedures were carried out according to Declaration of Helsinki guidelines. Clinical data such as gender, age at disease onset, and the presence of bone erosion were obtained by reviewing medical records at the time of enrollment. Laboratory data included erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), and rheumatoid factor (RF) values. Plain radiograph, computed tomography, and magnetic resonance images of the cervical spine were obtained from all participants. The diagnosis of OPLL was based on previously reported criteria [18].

**SNP selection and genotyping**

In the TLR5 gene region, four SNPs [NCBI SNP : rs5744168 (axon 6), rs5744169 (axon 6), rs2072493 (axon 6), and rs5744174 (axon 6)] were selected using online human SNP databases (http://www.ncbi.nlm.nih.gov/SNP). SNPs with unknown heterozygosity and minor allele frequencies below 5% were excluded. The locations of the three selected SNPs in the region of TLR5 are shown in Fig. 1. Genomic DNA was extracted using a commercially available kit (Qiagen, Valencia, CA, USA) from blood samples collected in EDTA. SNP genotyping was performed using direct sequencing with genomic DNA amplified using primers for each SNP. The samples were sequenced using an ABI Prism 377 automatic sequencer (Applied Biosystems, Foster City, CA, USA). Sequence data was analyzed using SeqManII software (DNASTAR, Madison, WI, USA). Hardy-Weinberg equilibrium (HWE) for four SNPs was assessed using SNPStats (http://bioinfo.iconcologia.net/index.php). A linkage disequilibrium (LD) block of polymorphisms was tested using Haploview version 3.32. The haplotypes and their frequencies were calculated by the EM algorithm.

**Statistical analysis**

All results are expressed as the mean ± SD. All analyses were conducted at the two-tailed 0.05 alpha level using SPSS 12.0 for Windows (SPSS, Chicago, IL, USA). The genetic association between the TLR5 polymorphisms and susceptibility to OPLL was analyzed. Multiple logistic regression analysis with adjustment for age and gender was performed to calculate an odds ratio (OR).

**Results**

The clinical characteristics of the 166 OPLL patients (96 male, 70 female) and 231 control subjects (115 male, 116 female) are shown in Table 1. The onset of clinical OPLL symptoms occurred at an average age exceeding 50 years. The mean age of symptomatic OPLL patients was 60.90 ± 11.01 years and of control subjects was 59.90 ± 12.84 years. The coexistence of OPLL and

| Number | Patients with OPLL | Controls |
|--------|--------------------|----------|
| Age (years, mean ± S.D.) | 60.99 ± 10.99 | 59.53 ± 14.17 |
| Sex distribution (n, male : female) | 96 : 73 | 115 : 118 |
| Involved number of spines | 2.44 ± 1.38 | |
| Type of OPLL (n) | | |
| Continuous | 25 | |
| Segmental | 31 | |
| Mixed | 23 | |
| Localized | 90 | |
| Associated conditions (n) | | |
| DISH | 4 | |
| OPL | 33 | |
| Myelopathy | 34 | |

TLR5 : Toll-like receptor 5, OPLL : ossification of the posterior longitudinal ligament, DISH : diffuse idiopathic skeletal hyperostosis, OLF : ossification of ligamentum flavum.
diffuse idiopathic skeletal hyperostosis (DISH) or ossification of ligamentum flavum (OLF) is not rare. The coexistence of OPLL with DISH was 2.4% (four of 166 patients) and OPLL with OLF was 19.8% (33 of 166 patients). The mean involved number of spines was 2.45 ± 1.39 segments.

Based on radiographic findings, OPLL has been classified into four types: 1) continuous type, in which ossification extends over several contiguous vertebrae; 2) segmental type, in which ossification is fragmented and located immediately behind each vertebral body with interruption at the intervertebral disc levels; 3) mixed type, which is a combination of continuous and segmental types of ossification; and 4) localized type, in which ossification is confined to the intervertebral disc space. In the present series, the prevalence of continuous type was 14.5% (24 out of 166), segmental type was 18.7% (31 out of 166), mixed type was 13.8% (23 out of 166), and localized type was 53% (88 out of 166).

The genotype distributions of TLR5 gene polymorphisms in the OPLL and control groups are shown in Table 2. The genotype frequencies of these SNPs did not show a significant association between OPLL and control groups in the codominant, dominant, and recessive models. Table 3 demonstrates the frequencies of the haplotypes composed of rs5744174 and rs2072493 among patients and controls. Three major haplotypes were evident. Similar to the single SNP evaluation, no significant differences in haplotype distribution between OPLL patients and controls were detected. The allele frequency of these SNPs was not also significantly associated with OPLL (Table 4).

**DISCUSSION**

Clinical studies have strongly suggested that OPLL is a multifactorial disease in which complex genetic and environmental factors interact. The involvement of genetic factors has been indicated by the higher incidence of OPLL in families and among twins than in the general population. Specific gene polymorphisms may be associated with OPLL in several collagen genes such as COL1A2 and COL6A1, which encode for extracellular matrix proteins.

Polymorphisms have been detected in the gene encoding nucleotide pyrophosphatase (NPPS), which is involved in regulation of calcification in chondrocytes and which may also be as-

### Table 2. Case-control association study of TLR5 gene in patients with OPLL

| SNP (Locus) | Genotype | Control n (%) | OPLL n (%) | Codominant OR (95% CI) | p | Dominant OR (95% CI) | p | Recessive OR (95% CI) | p |
|-------------|----------|---------------|------------|------------------------|---|----------------------|---|----------------------|---|
| rs5744168   | C/C      | 226 (97)      | 162 (95.9) |                        |   |                      |   |                      |   |
| (exon6)     | C/T      | 7 (3)         | 7 (4.1)    |                        |   |                      |   |                      |   |
| Arg392X     | T/T      | 0 (0)         | 0 (0)      |                        |   |                      |   |                      |   |
| rs5744169   | C/C      | 233 (100)     | 160 (100)  |                        |   |                      |   |                      |   |
| (exon6)     | C/T      | 0 (0)         | 0 (0)      |                        |   |                      |   |                      |   |
| Leu444Leu   | T/T      | 0 (0)         | 0 (0)      |                        |   |                      |   |                      |   |
| rs2072493   | A/A      | 122 (52.4)    | 80 (47.3)  |                        |   |                      |   |                      |   |
| (exon6)     | A/G      | 95 (40.8)     | 70 (41.4)  | 1.26 (0.92-1.71)       | 0.150 | 1.24 (0.83-1.85) | 0.290 | 1.67 (0.83-3.38) | 0.150 |
| Asn592Ser   | G/G      | 16 (6.9)      | 19 (11.2)  |                        |   |                      |   |                      |   |
| rs5744174   | T/T      | 6 (2.6)       | 2 (1.2)    |                        |   |                      |   |                      |   |
| (exon6)     | T/C      | 66 (28.3)     | 46 (27.2)  | 0.90 (0.61-1.35)       | 0.620 | 0.93 (0.60-1.45) | 0.760 | 0.53 (0.10-2.70) | 0.430 |
| Phe616Leu   | C/C      | 161 (69.1)    | 121 (71.6) |                        |   |                      |   |                      |   |

Note: p-Values were from logistic regression analyses with the codominant, dominant, and recessive models. *rs5744174: contig ref; T/C, Phe616Leu, TLR 5: Toll-like receptor 5, OPLL: ossification of the posterior longitudinal ligament, SNP: single nucleotide polymorphism

| Haplotype | OPLL | Control | OR | 95% CI | p |
|-----------|------|---------|----|--------|---|
| rs5744174/ (TA) | HAPI/ HAPI | 47 | 27.81 | 68 | 29.18 | Co-dominant | 0.890 | 0.660 | 1.190 | 0.425 |
| rs2072493 | HAPI/- | 86 | 50.89 | 125 | 53.65 | Dominant | 0.770 | 0.820 | 1.820 | 0.320 |
|           | HAPI/- | 36 | 21.30 | 40 | 17.17 | Recessive | 0.930 | 0.600 | 1.450 | 0.764 |
| HAPII (TG) | HAP2/ HAP2 | 19 | 11.24 | 16 | 6.87 | Co-dominant | 1.250 | 0.920 | 1.700 | 0.151 |
|           | HAP2/- | 70 | 41.42 | 95 | 40.77 | Dominant | 1.220 | 0.820 | 1.820 | 0.320 |
|           | HAP2/- | 80 | 47.34 | 122 | 52.36 | Recessive | 1.720 | 0.860 | 3.450 | 0.128 |
| HAPIII (CA) | HAP3/ HAP3 | 2 | 1.18 | 6 | 2.58 | Co-dominant | 0.860 | 0.580 | 1.270 | 0.448 |
|           | HAP3/- | 121 | 71.60 | 161 | 69.10 | Recessive | 0.450 | 0.090 | 2.270 | 0.336 |

Table 3. Haplotype frequencies of TLR5 gene polymorphism in patients with OPLL

| Block | Haplotype | OPLL | Control | model | OR | 95% CI | p |
|-------|-----------|------|---------|-------|----|--------|---|
| rs5744174/ (TA) | HAPI/ HAPI | 47 | 27.81 | 68 | 29.18 | Co-dominant | 0.890 | 0.660 | 1.190 | 0.425 |
| rs2072493 | HAPI/- | 86 | 50.89 | 125 | 53.65 | Dominant | 0.770 | 0.820 | 1.820 | 0.320 |
|           | HAPI/- | 36 | 21.30 | 40 | 17.17 | Recessive | 0.930 | 0.600 | 1.450 | 0.764 |
| HAPII (TG) | HAP2/ HAP2 | 19 | 11.24 | 16 | 6.87 | Co-dominant | 1.250 | 0.920 | 1.700 | 0.151 |
|           | HAP2/- | 70 | 41.42 | 95 | 40.77 | Dominant | 1.220 | 0.820 | 1.820 | 0.320 |
|           | HAP2/- | 80 | 47.34 | 122 | 52.36 | Recessive | 1.720 | 0.860 | 3.450 | 0.128 |
| HAPIII (CA) | HAP3/ HAP3 | 2 | 1.18 | 6 | 2.58 | Co-dominant | 0.860 | 0.580 | 1.270 | 0.448 |
|           | HAP3/- | 121 | 71.60 | 161 | 69.10 | Recessive | 0.450 | 0.090 | 2.270 | 0.336 |

TLR 5: Toll-like receptor 5, OPLL: ossification of the posterior longitudinal ligament
Table 4. Allele frequencies of TLR5 gene polymorphisms

|                | rs5744168 | rs5744174 | rs2072493 |
|----------------|-----------|-----------|-----------|
| Control (n = 226) | C 459 (98%) | T 388 (83%) | A 339 (73%) |
| T              | 7 (2%)    | C 78 (17%) | G 127 (27%) |
| OPLL (n = 169)  | C 331 (98%) | T 288 (85%) | A 230 (68%) |
| T              | 7 (2%)    | C 50 (15%) | G 108 (32%) |
| p value        | 0.5427    | 0.4568    | 0.4568    |
| OR (95% CI)    | 0.7211 (0.2506-2.0755) | 1.1579 (0.7868-1.7041) | 1.1579 (0.7868-1.7041) |

TLR 5 : Toll-like receptor 5, OPLL : ossification of the posterior longitudinal ligament

associated with OPLL [1]. However, the results of gene analysis studies are not always consistent. Involvement of many growth factors and cytokines, including bone morphogenic protein 2 (BMP2) and transforming growth factor-β (TGF-β) has been demonstrated in various histochemical and cytochemical analyses. Several transcription factors involved in cellular differentiation may also have a role.

Bone is actually in a constant state of dynamic turnover known as bone remodeling. It has been demonstrated recently that skeletal bone homeostasis can be a profound component of the immune system. Therefore, the term ‘osteoimmunology’ has been recently proposed to better explain the cross-talk between bone homeostasis and the immune system [2].

The transcription factor NF-kB participates as a critical element in the expression of a wide variety of genes that are involved in the regulation of immune system and inflammatory responses, proliferation, tumorigenesis, and survival. NF-kB is found in almost all animal cell types and is involved in cellular responses to stimuli such as stress, cytokines, free radicals, ultraviolet irradiation, oxidized low density lipoprotein, and bacterial or viral antigens [3]. NF-kB plays a key role in regulating the immune response to infection. Conversely, incorrect regulation of NF-kB has been linked to cancer, inflammatory and autoimmune diseases, septic shock, viral infection, and improper immune development [4,5]. Thus, NF-kB is a key molecule in the link between bone and immune response [5]. Results of a recent study suggest that NF-kB, stimulated by environmental factors involving PDGA-BB and TGF β1 in ligament cell, influences the osteoblastic differentiation of undifferentiated mesenchymal cells [6].

The TLR family fundamentally participates in pathogen recognition and activation of innate immunity. TLRs are highly conserved from Drosophila to humans, and share structural and functional similarities. They recognize pathogen-associated molecular patterns that are expressed on infectious agents, and mediate the production of cytokines necessary for the development of effective immunity. The various TLRs exhibit different patterns of expression [7].

The TLR5s gene product is expressed in myelomonocytic cells, and recognizes bacterial flagellin [8]. The activation of this receptor mobilizes NF-kB and stimulates TNF-α production [9]. It is likely that TLR5 plays a role in the pathogenesis of OPLL by intermediate NF-kB.

We evaluated the associations between TLR5 polymorphisms and susceptibility to OPLL in a Korean population. The results suggest that TLR5 does not contribute to a genetic susceptibility to OPLL, and that an innate immune response to bacterial flagellin from both Gram-positive and Gram-negative bacteria does not influence OPLL. It is possible that NF-kB may act through other immunological signaling pathway other than through a TLR5 response.

CONCLUSION

The present study suggests that TLR5 gene polymorphism and their major haplotypes are not associated with susceptibility to OPLL in Korean population.

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