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２. 学会誌・雑誌等における論文掲載

掲載した論文（発表題目）	発表者氏名	発表した場所（学会誌・雑誌等名）	発表した時期	国内・外の別
A genome-wide association study identifies susceptibility loci for ossification of the posterior longitudinal ligament of the spine.	Nakajima M, Takahashi A, Tsuji T, Karasugi T, Baba H, Uchida K, Kawabata S, Okawa A, Shindo S, Takeuchi K, Taniguchi Y, Maeda S, Kashii M, Seichi A, Nakajima H, Kawaguchi Y, Fujibayashi S, Takahata M, Tanaka T, Watanabe K, Kida K, Kanchiku T, Ito Z, Mori K, Kaito T, Kobayashi S, Yamada K, Takahashi M, Chiba K, Matsumoto M, Furukawa KI, Kubo M, Toyama Y;Genetic Study Group of Investigation Committee on Ossification of the Spinal Ligaments, Ikegawa S	Nat Genet	2014	国外
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VIII　研究成果の刊行物・別刷

A genome-wide association study identifies susceptibility loci for ossification of the posterior longitudinal ligament of the spine

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Ossification of the posterior longitudinal ligament of the spine (OPLL) is a common spinal disorder among the elderly that causes myelopathy and radiculopathy. To identify genetic factors for OPLL, we performed a genome-wide association study (GWAS) in ~8,000 individuals followed by a replication study using an additional ~7,000 individuals. We identified six susceptibility loci for OPLL: 20p12.3 (rs2423294: $P = 1.10 \times 10^{-13}$), 8q23.1 (rs374810: $P = 1.88 \times 10^{-13}$), 12p11.22 (rs1979679: $P = 4.34 \times 10^{-12}$), 12p12.2 (rs11045000: $P = 2.95 \times 10^{-11}$), 8q23.3 (rs13279799: $P = 1.28 \times 10^{-10}$) and 6p21.1 (rs927485: $P = 9.40 \times 10^{-9}$). Analyses of gene expression in and around the loci suggested that several genes are involved in OPLL etiology through membranous and/or endochondral ossification processes. Our results bring new insight to the etiology of OPLL.

OPLL (MIM 602475) is a common disease caused by abnormal ossification of the posterior longitudinal ligament in the spinal canal, affecting 0.8–3.0% of Asians and 0.1–1.7% of European Caucasians[1]. The ossified ligament compresses the spinal cord and nerve root, causing serious neurological dysfunction[2]. Several lines of evidence suggest that genetic factors contribute to its etiology and pathogenesis[3–5]; affected sibling-pair linkage studies and candidate gene association studies have identified a number of genes or loci that are linked to OPLL susceptibility[6–8]. However, replication studies have failed to verify these results, even in the same ancestry groups as the original studies[9]. A GWAS using high-density SNP data has not yet been reported for OPLL.

To identify OPLL susceptibility genes, we conducted a GWAS in a Japanese population consisting of 1,130 individuals with OPLL and 7,135 controls (Online Methods). After quality control filtering of SNP genotyping data (Online Methods), 616,496 autosomal SNPs were examined for association with the Cochran-Armitage trend test. Analysis of population stratification with principal-component analysis (PCA) showed that all individuals in our study were Japanese

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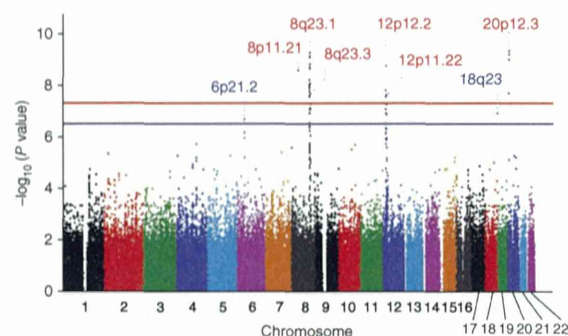

Figure 1 Manhattan plot showing the $-\log_{10}$ *P* value for each SNP in the GWAS. Values were plotted against their respective positions on the autosomal chromosomes. The red line represents the genome-wide significance threshold ($P = 5 \times 10^{-8}$). The blue line represents the threshold for the selection of SNPs for the replication study ($P = 5 \times 10^{-7}$).

(**Supplementary Fig. 1a**); however, there was a small proportion of samples that were separated from the major Japanese (Hondo) cluster[10] when PCA was performed using only the genotype information for the cases and controls in the study (**Supplementary Fig. 1b**). We used samples from the major Japanese cluster consisting of 1,112 cases and 6,810 controls (**Supplementary Table 1**) and generated a quantile-quantile plot (**Supplementary Fig. 1c**). We found that the genomic inflation factor (λ_{GC}) was 1.01, indicating that there was a low possibility of false positive associations resulting from population stratification. Twenty-six SNPs within six chromosomal regions at 8p11.21, 8q23.1, 8q23.3, 12p12.2, 12p11.22 and 20p12.3 had associations that reached the genome-wide significance threshold of $P < 5 \times 10^{-8}$ (**Fig. 1** and **Supplementary Table 2**). We further found an additional two loci (6p21.2 and 18q23) showing suggestive association with OPLL ($P < 5 \times 10^{-7}$; **Fig. 1** and **Supplementary Table 2**). To investigate additional susceptibility loci, we obtained whole-genome association results for imputed SNPs. We found that 895 SNPs in the 8 identified loci were associated at $P < 5 \times 10^{-7}$, and no other associated loci were uncovered. We also examined 12,228 SNPs on the X chromosome for association with OPLL and found no significant association.

To confirm the associations at the eight loci, we selected the eight SNPs that had the smallest *P* value at each locus for the replication study. We genotyped an independent set of 548 Japanese individuals with OPLL and 6,469 Japanese controls (**Supplementary Table 1**), finding significant associations for 5 of the 8 SNPs, even after Bonferroni correction with $P < 6.25 \times 10^{-3}$ (0.05/8) (**Table 1** and **Supplementary Table 2**). Combining the results from the GWAS and replication study, six SNPs reached a genome-wide significance threshold of $P < 9.68 \times 10^{-9}$ (0.05/5,163,786) (**Table 1** and **Supplementary Table 3**). The Breslow-Day test for these six SNPs showed an absence of significant heterogeneity ($P > 0.05$) between studies. We carried out age- and sex-adjusted analyses using a logistic regression model and confirmed similar association after adjustment (**Supplementary Table 4**). We next conducted conditional analysis to adjust for the top SNPs at these six loci using the GWAS data. There were no independently associated signals in the six loci (**Supplementary Fig. 2**).

The most highly associated SNP in the meta-analysis, rs2423294 ($P_{combined} = 1.10 \times 10^{-13}$), was located in the 3′ flanking region of *HAO1* at 20p12.3 (**Fig. 2a**). *HAO1* is expressed primarily in the liver and pancreas and encodes hydroxyacid oxidase, which oxidizes 2-hydroxyacid. We identified 80 imputed SNPs in this region that reached a genome-wide significance threshold for association ($P < 5 \times 10^{-8}$); 71 highly associated SNPs ($P < 1 \times 10^{-9}$) were located in the 3′ flanking region of *HAO1* (**Fig. 2a**).

The associated region at 8q23.1 contained three genes—*RSPO2*, *EIF3E* and *EMC2* (**Fig. 2b**). *RSPO2* encodes a member of the R-spondin family of secreted proteins that are involved in β-catenin activation through canonical Wnt/β-catenin signaling[11], which is indispensable for osteoblastogenesis[12]. Reduced expression of R-spondin 2 has been reported in osteoarthritis-derived osteoblasts, and Wnt-dependent mineralization of osteoblasts is promoted by R-spondin 2 (ref. 13). *EIF3E* encodes a component of the eukaryotic translation initiation factor 3 (eIF-3) complex, which is required for several steps in the initiation of protein synthesis. The eIF-3 complex

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Table 1 Association of the six genome-wide significant loci

SNP ID	Allele (risk allele)	Chromosome	Genes in or near associated region[a]	Study	RAF Case	RAF Control	*P* value[b]	OR (95% CI)[c]	P_{het}[d]
rs2423294	T/C (T)	20p12.3	***HAO1***	GWAS	0.207	0.155	4.90×10^{-10}	1.43 (1.27–1.60)	
				Replication	0.203	0.155	4.00×10^{-5}	1.38 (1.19–1.62)	
				Combined[e]	0.205	0.155	1.10×10^{-13}	1.41 (1.29–1.55)	0.757
rs374810	A/G (G)	8q23.1	***RSPO2, EIF3E, EMC2***	GWAS	0.676	0.607	5.79×10^{-10}	1.35 (1.23–1.49)	
				Replication	0.675	0.615	8.88×10^{-5}	1.30 (1.14–1.49)	
				Combined[e]	0.676	0.611	1.88×10^{-13}	1.34 (1.24–1.44)	0.651
rs1979679	T/C (T)	12p11.22	***CCDC91***	GWAS	0.422	0.360	2.42×10^{-8}	1.30 (1.18–1.42)	
				Replication	0.426	0.364	4.75×10^{-5}	1.30 (1.14–1.47)	
				Combined[e]	0.423	0.362	4.34×10^{-12}	1.30 (1.21–1.40)	0.994
rs11045000	A/G (A)	12p12.2	***LOC100506393***	GWAS	0.523	0.454	1.74×10^{-9}	1.32 (1.20–1.44)	
				Replication	0.514	0.466	2.35×10^{-3}	1.21 (1.07–1.37)	
				Combined[e]	0.520	0.460	2.95×10^{-11}	1.28 (1.19–1.38)	0.281
rs13279799	A/G (G)	8q23.3	*LINC00536, EIF3H*	GWAS	0.378	0.317	8.96×10^{-9}	1.31 (1.19–1.44)	
				Replication	0.368	0.322	1.80×10^{-3}	1.23 (1.08–1.39)	
				Combined[e]	0.375	0.319	1.28×10^{-10}	1.28 (1.19–1.38)	0.416
rs927485	T/C (C)	6p21.1	*CDC5L, MIR4642, SUPT3H*	GWAS	0.176	0.134	2.10×10^{-7}	1.37 (1.22–1.55)	
				Replication	0.169	0.140	8.19×10^{-3}	1.25 (1.06–1.48)	
				Combined[e]	0.173	0.137	9.40×10^{-9}	1.33 (1.21–1.46)	0.372

RAF, risk allele frequency; OR, odds ratio; CI, confidence interval.
[a]Genes in the LD block of the associated SNPs are shown in bold. [b]Results from the Cochran-Armitage trend test. [c]Estimated for the risk allele from a 2 × 2 allele frequency table. [d]Results from the Breslow-Day test. [e]Calculated by the Mantel-Haenszel method.

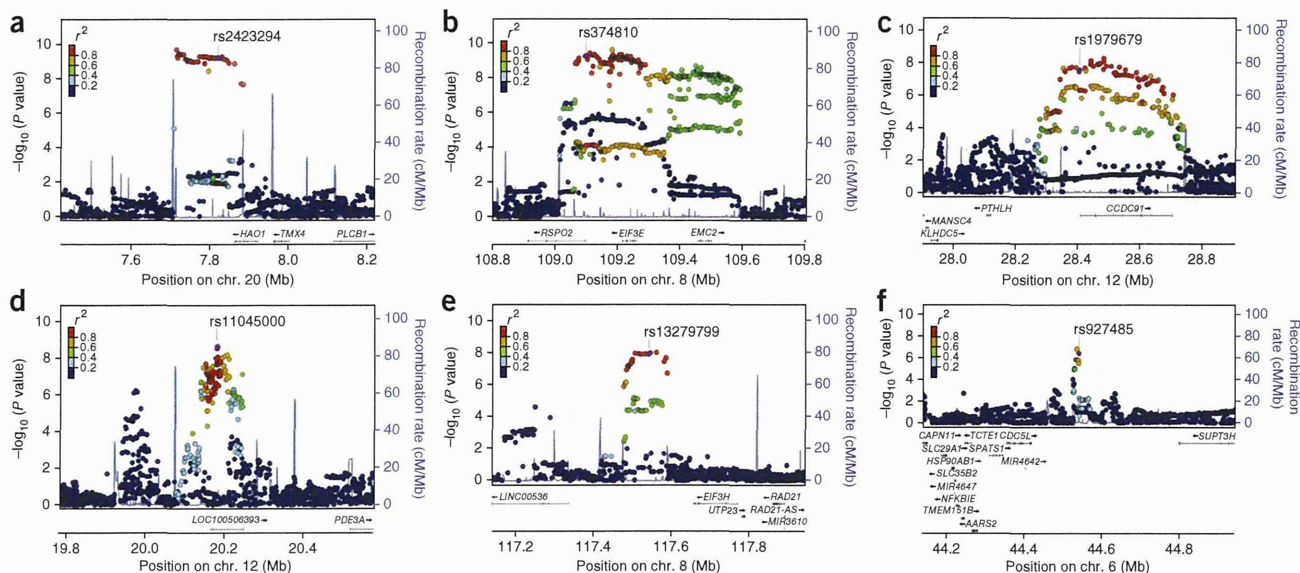

Figure 2 Regional association plots at six susceptibility loci for OPLL. Each plot shows $-\log_{10} P$ values against the chromosomal positions of SNPs in the specific region. The genotyped SNP with the strongest association signal in each locus is represented as a purple diamond; the other SNPs are colored according to the extent of LD with this SNP. Estimated recombination rates from the hg19/1000 Genomes Project March 2012 East Asian reference are shown as light-blue lines. (**a**) 20p12.3. (**b**) 8q23.1. (**c**) 12p11.22. (**d**) 12p12.2. (**e**) 8q23.3. (**f**) 6p21.1.

stimulates mRNA recruitment to the 43S preinitiation complex and scanning of the mRNA for recognition of the translation initiation codon[14]. *EMC2* encodes a component of the endoplasmic reticulum membrane protein complex[15]; however, its cellular function is unclear.

The chromosome 12p11.22 region contained *CCDC91* (**Fig. 2c**), which encodes a protein involved in the trans-Golgi network. Notably, the associated region was located adjacent to the *PTHLH* gene, which encodes a member of the parathyroid hormone (PTH) family. PTH, via its receptor PTH1R, regulates endochondral ossification, which is related to OPLL progression[16]. Associated SNPs at the 12p12.2 locus were in *LOC100506393* (**Fig. 2d**), which is predicted to encode a large intergenic noncoding RNA (lincRNA). *LOC100506393* consists of three exons and has no similarity to known orthologous transcripts. The associated region at 8q23.3 was located in a gene desert between *LINC00536* and *EIF3H* (**Fig. 2e**). *LINC00536* encodes a lincRNA of unknown function. *EIF3H* encodes a component of the eIF-3 complex, as does *EIF3E*. The fact that two of the six loci for OPLL harbor genes encoding proteins in the eIF-3 complex suggests that aberrations in this pathway might be key in OPLL pathogenesis.

The associated region at 6p21.1 was located in the gene desert between *CDC5L*, *MIR4642* and *SUPT3H* (**Fig. 2f**). The cell division cycle 5-like protein encoded by the *CDC5L* gene is a positive regulator of G2/M progression in the cell cycle[17]. CDC5L was also found to be a core component of a putative E3 ubiquitin ligase complex. This complex has been shown to have a role in pre-mRNA splicing from yeast to humans[18]. *MIR4642*, located in intron 14 of the *CDC5L* gene, encodes a microRNA whose target genes and functions are unknown. *SUPT3H* encodes a human homolog of Spt3, which is a *Saccharomyces cerevisiae* transcription factor required for the transcription of a number of RNA polymerase II–transcribed genes[19]. An association of bone mineral density in the lumbar spine with a SNP (rs11755164) 155 kb downstream of the *SUPT3H* gene has been reported[20], but this SNP was not in linkage disequilibrium (LD) with rs927485 ($D' = 0.013$, $r^2 = 0$).

Fifteen genes or loci have previously been reported to be associated with OPLL. We investigated the GWAS data for these reported genes but found no significant association (**Supplementary Table 5**). A genome-wide linkage study reported five loci that showed suggestive linkage after stratification by OPLL severity, age at diagnosis and associated diabetes mellitus[21]. One of the six SNPs that showed genome-wide significant association with OPLL, rs2423294 ($P_{\text{combined}} = 1.10 \times 10^{-13}$), was located in the linkage region at 20p12.3 (**Supplementary Fig. 3**). We stratified the cases from our GWAS according to the previous linkage study[21] and investigated the associations of the SNPs in the linkage regions. In all strata, the OR estimates for association increased after stratification (**Supplementary Table 6**). A stratum of subjects without diabetes mellitus showed genome-wide significant association at rs10486860 in the 7q22 linkage region ($P = 4.31 \times 10^{-8}$), suggesting that OPLL is genetically heterogeneous and that the locus is associated with a subtype of OPLL. Association of diabetes mellitus in individuals with OPLL and the contribution of abnormal insulin metabolism to OPLL have been reported[22].

To understand the functional roles of the loci and genes associated with OPLL, we first examined expression of the candidate genes within the LD blocks for the associated SNPs (**Fig. 2**). We assessed the mRNA expression of these six genes in human bone cells and fibroblasts by RT-PCR. *EIF3E*, *EMC2* and *CCDC91* were abundantly expressed in both cells, whereas *HAO1*, *RSPO2* and *LOC100506393* expression was absent in these cells (**Supplementary Fig. 4**). There was no difference in the expression of the genes in the two cell types. We then examined the expression of these genes in the ATDC5 cell line, a mouse model of endochondral ossification[23]. Expression of *Hoa1*, *Rspo2* and *Ccdc91* was lower during early stages of chondrogenesis, whereas expression of *Sox9*, the master gene for chondrogenesis, was higher; in later stages, the expression of cartilage matrix genes (*Acan* and *Col2a1*) was increased (**Supplementary Fig. 5**).

We next investigated the 63 genes within 1 Mb of the associated SNPs for differential expression in osteoblasts and fibroblasts. We examined the cell type–specific gene expression profile using the

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FANTOM5 SSTAR database[24]. The expression of four genes was increased by >2-fold in osteoblasts, whereas the expression of six genes was increased by >2-fold in fibroblasts (**Supplementary Table 7**). We examined the ten genes that showed differential expression in quantitative PCR (qPCR) experiments and confirmed increased gene expression of *RSPH9* and *STK38L* in osteoblasts (**Supplementary Table 7**), suggesting that these genes have a role in membranous ossification. The protein encoded by the *RSPH9* gene is a component of the radial spoke head in motile cilia and flagella[25]. The primary cilia machinery has a critical role in the Hedgehog signaling pathway in skeletal development and has been implicated in a dozen disorders known as skeletal ciliopathies[26]. *STK38L* encodes a serine/threonine protein kinase that controls the protein stability of the cyclin-dependent kinase inhibitor protein p21 through direct phosphorylation and inhibits G1/S progression through the cell cycle[27]. We further investigated expression of the 63 genes in mesenchymal stem cells (MSCs) from the spinal ligament. We compared gene expression between the MSCs obtained from subjects with and without OPLL[28] using a cDNA microarray and confirmed differential expression by qPCR. There was no significant difference in the expression of the genes within 1 Mb of the associated SNPs (data not shown).

To gain additional information regarding the function of the SNPs, we examined all publically available expression quantitative trait locus (eQTL) data (Genevar) obtained from the analysis of a lymphoblastoid cell line[29]. There was no evidence of a significant association between genotype and gene expression ($P > 0.05$ after adjustment for multiple testing). We applied literature-based pathway analysis, using GRAIL[30] to investigate the connections between genes in the OPLL-associated loci. We used 6 regions that had a significant genome-wide association as query regions, resulting in the analysis of 12 unique genes. We found a subset of two SNPs with a functional connection (GRAIL $P < 0.05$)—*EIF3E* (rs374810; $P = 0.020$) and *EIF3H* (rs13279799; $P = 0.021$)—with members of the translation initiation pathway.

In summary, through a GWAS and replication study in Japanese populations, we identified six new susceptibility loci for OPLL. One of these overlapped with a previously reported linkage region. Analyses of gene expression in and around the OPLL-associated loci suggested that *RSPH9* and *STK38L* might be involved in OPLL etiology through the membranous ossification process and that *HAO1*, *RSPO2* and *CCDC91* might be involved through the endochondral ossification process. Additional genetic and functional studies for the loci and genes identified by our study should aid in clarification of the etiology and pathogenesis of OPLL.

URLs. 1000 Genomes Project, http://www.1000genomes.org/; Genevar, http://www.sanger.ac.uk/resources/software/genevar/; GRAIL, http://www.broadinstitute.org/mpg/grail/; FANTOM5 SSTAR, http://fantom.gsc.riken.jp/5/sstar/Main_Page; EIGENSTRAT, http://genetics.med.harvard.edu/reich/Reich_Lab/Software.html; Minimac, http://genome.sph.umich.edu/wiki/Minimac; LocusZoom, http://csg.sph.umich.edu/locuszoom/; PLINK, http://pngu.mgh.harvard.edu/~purcell/plink/.

METHODS

Methods and any associated references are available in the online version of the paper.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

S.I. designed the project and provided overall project management. M.N. and S.I. drafted the manuscript. M.N. and M. Kubo performed the genotyping for the GWAS. A.T. analyzed the GWAS data. T. Tsuji, T. Karasugi, H.B., K.U., S. Kawabata, A.O., S.S., K.T., Y. Taniguchi, S.M., M. Kashii, A.S., H.N., Y.K., S.F., M. Takahata, T. Tanaka, K.W., K.K., T. Kanchiku, Z.I., K.M., T. Kaito, S. Kobayashi, K.Y., M. Takahashi, K.C., M.M., K.-I.F. and Y. Toyama managed the DNA samples and clinical data.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Subjects. The characteristics of each case-control group are shown in **Supplementary Table 1**. OPLL was diagnosed by experienced spinal surgeons from the participating hospitals on the basis of radiographic examination of the spine. Ectopic bone formation of the posterior longitudinal ligament in the cervical region of the spine was evaluated. A total of 1,660 Japanese individuals who had OPLL of more than two vertebra segments were included in this study. We used genome-wide screening data from subjects with 1 of 10 diseases (1,145 with cerebral aneurysm, 1,034 with chronic obstructive pulmonary disease, 811 with endometrial cancer, 1,020 with esophageal cancer, 935 with glaucoma, 1,442 with atopic dermatitis, 1,667 with epilepsy, 1,732 with Graves' disease, 612 with nephrotic syndrome and 1,016 with urolithiasis) unrelated to OPLL in the BioBank Japan Project[31], 978 volunteers from the Osaka-Midosuji Rotary Club in Japan and 887 healthy subjects from the PharmaSNP Consortium for the controls. All individuals gave written informed consent to participate in the study. This research project was approved by the ethical committees at the RIKEN Yokohama Institute.

Genotyping and quality control for GWAS. Genomic DNA was extracted from peripheral blood leukocytes using a standard method. For the GWAS, we genotyped case samples using the Illumina HumanOmniExpressExome BeadChip and control samples using the Illumina HumanOmniExpress BeadChip and the Illumina HumanExome BeadChip. After standard SNP quality control, which excluded SNPs with a call rate of <0.99, SNPs that deviated from Hardy-Weinberg equilibrium ($P < 1 \times 10^{-6}$ in controls) and non-polymorphic SNPs, a total of 616,496 autosomal SNPs were used for further analysis. For sample quality control, we evaluated cryptic relatedness for each sample with an identity-by-state method and removed samples that showed second-degrees relatedness or closer. To examine population stratification in this study, we performed PCA[32] using four reference populations from HapMap data as the reference, including Europeans (CEU), Africans (YRI), Japanese (JPT) and Han Chinese (CHB), with smartpca[32]. We generated the scatterplot, using the top two associated principal components (eigenvectors) to identify outliers who did not belong to the JPT/CHB cluster. Subsequently, we performed PCA using only the genotype information from the case and control subjects without HapMap data to further evaluate population substructure. After performing PCA, we selected 1,112 cases and 6,810 controls within the major Japanese (Hondo) cluster for subsequent analysis. We used the quantile-quantile plot of observed P values to evaluate the potential effect of population stratification.

In the replication study, we genotyped 548 individuals with OPLL using a multiplex PCR–based Invader assay (Third Wave Technologies). We recruited 6,469 controls within the Hondo cluster registered in BioBank Japan without OPLL for the replication study. The genotype concordance rate for the six SNPs in **Table 1** between samples genotyped using the Illumina HumanOmniExpressExome BeadChip and the same samples genotyped with the multiplex PCR–based Invader assay was 0.9997.

Statistical analysis. In the GWAS and the replication study, we assessed the association of each SNP with the 1-degree-of-freedom Cochran-Armitage trend test. We calculated OR values and their CIs from a 2 × 2 allele frequency table. We combined data from the GWAS and replication study using the Mantel-Haenszel method and examined heterogeneity between studies using the Breslow-Day test[33]. Regional association plots were generated using LocusZoom[34]. We carried out conditional logistic regression analysis with PLINK v1.07 (ref. 35), adjusting for the top SNPs for each locus to determine whether independently associated SNPs existed.

X-chromosome association study. We performed testing for association on the X chromosome for male and female genotypes separately. For male genotypes, we used 758 cases and 3,988 controls. After quality control as described above, we applied the χ^2 test to the 2 × 2 contingency table for 12,401 SNPs. For female genotypes, we used 354 cases and 2,822 controls and applied the 1-degree-of-freedom Cochran-Armitage trend test for 12,908 SNPs. We combined the male and female association test results for 12,229 SNPs using the inverse normal method[36].

Imputation. We performed genotype imputation within the GWAS using Minimac[37]. We used individuals from the 1000 Genomes Project (phased JPT, CHB and Han Chinese South (CHS) data; March 2012) as reference populations[38]. Because imputation errors are more serious when allele frequencies are low, SNPs with minor allele frequencies of <1% were excluded. SNPs with a low quality of imputation (Minimac software quality score of $R^2 < 0.9$) were also excluded. After quality control, 5,163,786 imputed SNPs were available for analysis. Association tests were performed for dosage with mach2dat[39].

Pathway analysis. We investigated connections between genes in the OPLL-associated loci with GRAIL[30]. We set the parameters as follows: genome assembly, release 22/hg18; HapMap population, JPT + CHB; functional data source, PubMed text (August 2012); gene size correction, off; gene list, all human genes within the database. The rs numbers of the six SNPs listed in **Table 1** were used as a query.

Gene expression analysis. MSCs from the spinal ligaments of individuals with and without OPLL were obtained as previously described[28]. ATDC5 cells (RIKEN Cell Bank) were cultured as previously described[23]. cDNA from MSCs, ATDC5 cells, dermal fibroblast and bone was synthesized for quantitative RT-PCR using the Sensiscript RT kit (Qiagen). Quantitative RT-PCR was performed with a StepOnePlus Real-Time PCR system (Applied Biosystems) using the QuantiTect SYBR Green PCR kit (Qiagen).

Microarray analysis. We analyzed mRNA expression in MSCs from the spinal ligaments of individuals with and without OPLL[28] using the SurePrint G3 Human Gene Expression 8x60K array (Agilent Technologies). For the transcripts whose levels were changed by >2-fold and had a false discovery rate–corrected Welch's t-test P value of <0.05, we verified the difference in expression by qPCR ($n = 8$ for individuals with and without OPLL).

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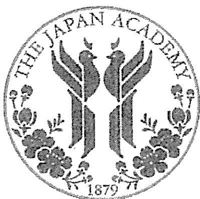
Genomic study of ossification of the posterior longitudinal ligament of the spine

Shiro Ikegawa



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Review

Genomic study of ossification of the posterior longitudinal ligament of the spine

By Shiro IKEGAWA*1,†

(Communicated by Tatsuo SUDA, M.J.A.)

Abstract: Ossification of the posterior longitudinal ligament of the spine (OPLL) is a common disease after the middle age. OPLL frequently causes serious neurological problems due to compression of the spinal cord and/or nerve roots. OPLL occurs in patients with monogenic metabolic diseases including rickets/osteomalacia and hypoparathyroidism; however most of OPLL is idiopathic and is considered as a multi-factorial (polygenic) disease influenced by genetic and environmental factors. Genomic studies for the genetic factors of OPLL have been conducted, mainly in Japan, including linkage and association studies. This paper reviews the recent progress in the genomic study of OPLL and comments on its future direction.

Keywords: OPLL, susceptibility gene, genetics, linkage study, association study, genome-wide association study

Introduction

Ossification of the posterior longitudinal ligament of the spine (OPLL; MIM 602475) is an intractable spinal disease. Spine is a columnar structure in the center of the body composed by spinal bones (vertebrae) and inter-vertebral discs. It is supported by spinal ligaments (flexible band-like structures), which include the anterior and posterior longitudinal ligaments and the yellow ligament (ligamentum flavum) of the spine. Ossification of the spinal ligaments is a group of disease that presents with ectopic (heterotopic) ossification in the spinal ligaments. Among them, the most serious disease is OPLL because the posterior longitudinal ligament runs behind the spinal column, anterior to the spinal cord within the spinal canal. The ossified posterior longitudinal ligament occupying the spinal canal eventually compresses the spinal cord and nerve roots (Fig. 1), leading to neurological disorders, including paresthesia, paralysis and bladder-bowel disturbance.

OPLL was first described more than 170 years ago.[1)] It has become well recognized after a report from Japan[2)] and is prevalent among Japanese,[3)] and hence has been studied very extensively in Japan. The Investigation Committee on the Posterior Longitudinal Ligament funded by the Japanese Ministry has been tackling this 'difficult' disease ('Nan-byo' in Japanese) since 1975.

OPLL is considered to be a 'genetic' disease. As in many other genetic diseases, the genetic aspect of OPLL is now being disclosed with a help of rapidly advancing genome science and technology. This paper briefly reviews the genomic study of OPLL and refers to its recent advance and future direction.

Epidemiology and Etiology

OPLL is a common disease. The incidence of OPLL is 0.8–3.0% in Asians and 0.1–1.7% in Caucasians.[3)] The most common site of OPLL is the cervical spine. The prevalence of radiographic cervical OPLL in a Japanese population-based cohort is 1.9%.[4)] Definite male predominance has been reported.[4),5)] The average age of onset is over 50 years. OPLL is a leading cause of myelopathy in Japan[4)] and hence is a serious problem in aging

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Abbreviations: OPLL: ossification of the posterior longitudinal ligaments of the spine; MIM: Mendelian Inheritance of Man; SNP: single nucleotide polymorphism; LD: linkage disequilibrium; GWAS: genome-wide association study.

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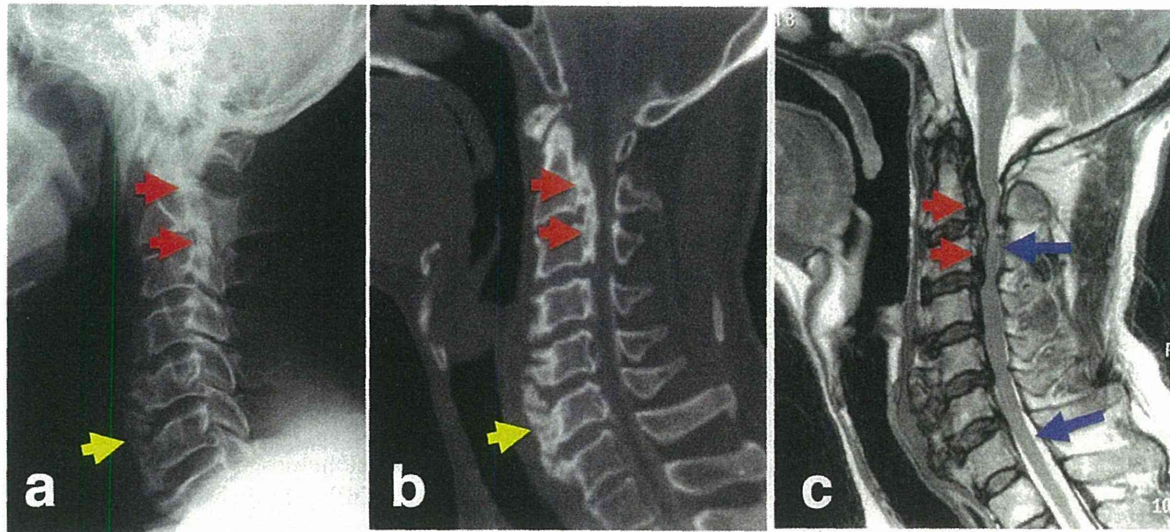

Fig. 1. Ossification of the posterior longitudinal ligament of the spine (OPLL). The lateral cervical spine in a patient with OPLL by a) plain radiograph, b) reconstruction computed tomography (CT) and c) magnetic resonance imaging (MRI). Red arrow: OPLL, yellow arrow: OALL (ossification of the anterior longitudinal ligament), blue arrow: spinal cord. Spinal cord is compressed by OPLL and became thin.

societies. OPLL is often complicated by ossification of other spinal ligaments and by diffuse idiopathic skeletal hyperostosis, which suggest that the OPLL patients have the intrinsic tendency for ectopic ossification.

Several lines of evidence suggest that genetic factors are likely to contribute to the etiology of OPLL.[6)–8)] In fact, genetic studies including affected sib-pair linkage studies and candidate-gene association studies have shown a number of genes/loci that link to OPLL susceptibility.[9)–11)] Some monogenic diseases present with OPLL. The list of the diseases associated with OPLL includes hypophosphatemic rickets/osteomalacia, including an autosomal dominant form (MIM 193100) caused by *FGF23* mutations, an X-linked dominant form (MIM 307800) caused by *PHEX* mutations, an X-linked recessive form (MIM 300554) caused by *CLCN5* mutations, and autosomal recessive forms caused by *DMP1* (MIM 600980) and *ENPP1* (MIM 173335) mutations. OPPL is also associated with endocrine disorders including hypoparathyroidism[12)] and acromegaly/gigantism.[13)] In these diseases, severe, early-onset OPLL occurs.

Most cases of OPLL are idiopathic, however. Many reports have suggested that idiopathic OPLL is a multi-factorial (polygenic) disease influenced by genetic and environmental (non-genetic) factors. Several clinical and environmental factors, including age, diabetes mellitus (DM), obesity, vitamin A-rich diet, exercise, and abnormal mechanical stress to the head have been considered as risk factors for OPLL.[13)–16)] On the other hand, OPLL is known to have a strong genetic predisposition. A study using 347 OPLL families reported that OPLL prevalence is 26% in parents and 29% in siblings of the OPLL probands.[17)] The association between OPLL and HLA haplotypes in 24 families with OPLL has been reported together with high prevalence of OPLL in the sibs who shared identical HLA haplotypes.[18)] As with other polygenic diseases, genetic studies based on the genome analysis of the patients, including linkage and association studies have been conducted and many genes/loci for OPLL susceptibility have been reported, mostly from Japan.

Sib-pair linkage study

An Utah group conducted a sib-pair linkage analysis in 1998.[19)] It was the first genetic study of OPLL and one of the first successful studies in all the linkage studies of common diseases. The group examined 53 Japanese families by a non-parametric linkage analysis focusing on the HLA region and found a significant linkage on D6S276 ($P = 5.9 \times 10^{-6}$). Subsequently, by a candidate gene approach for positional candidates around the marker, an association with a SNP, IVS6-4T>A in *COL11A2* ($P = 4 \times 10^{-4}$) was found in an analysis using 129

Table 1. Previously reported OPLL susceptibility genes and their association in GWAS*

Gene	Chromosome	Report [1st author, journal, year]	No. subject [case/control]	P-value	
				Report	GWAS
IL-1β	2q14	Ogata, Spine 2002[24]	43/140	0.0012	0.025
RXRB	6p21	Numasawa, J. Bone Miner. Res. 1999[20]	134/158	0.0028	0.023
COL11A2	6p21	Koga, Spine 1996[9]	18/51	0.018	0.024
RUNX2	6p21	Liu, Clin. Orthop. Relat. Res. 2010[27]	82/118	0.034	0.065
ENPP1 (NPPS)	6q22-q23	Nakamura, Hum. Genet. 1999[10]	323/332	0.0029	0.030
ESR1	6q25	Ogata, Spine 2002[24]	43/140	0.0066	0.0091
IL-15RA	10p15	Kim, Cytokine 2011[28]	166/230	0.0028	0.18
GDF2 (BMP9)	10q11.22	Ren, PLoS One 2012[29]	450/550	1.3×10^{-9}	0.036
VDR	12q13	Kobashi, Spine 2008[25]	63/126	0.0073	0.025
TGFB3	14q24	Horikoshi, Hum. Genet. 2006[30]	711/896	0.00040	0.053
TGFB1	19q13	Kamiya, Spine 2001[23]	46/273	0.00020	0.32
BMP2	20p12.3	Wang, Eur. Spine. J. 2008[26]	57/135	1.6×10^{-5}	0.076
COL6A1	21q22	Tanaka, Am. J. Hum. Genet. 2003[11]	280/210	2.7×10^{-6}	0.41

*Nakajima *et al.* Nat. Genet. 2014.[37] IL: interleukin; RXRB: retinoic X receptor β; COL: collagen; RUNX: runt-related transcription factor; ENPP: ectonucleotide pyrophosphatase/phosphodiesterase; NPPS: nucleotide pyrophosphatase; ESR: estrogen receptor; VDR: vitamin D (1,25-dihydroxyvitamin D3) receptor; BMP: bone morphogenetic protein; TGFB: TGF (transforming growth factor)-β.

patients and 152 controls. *COL11A2* (MIM 120290) encodes one of the 3 α-chains of type XI collagen, a cartilage-specific minor collagen. The same group also reported an association with *RXRB* (MIM 180246) lying adjacent to *COL11A2* (P = 0.0028).[20] Functional impact of these genes on OPLL susceptibility and pathogenesis are unclear.

Tanaka *et al.* expanded the study by increasing the number of sibs to 99 pairs from 70 Japanese families and conducted a genome-wide linkage study.[11] A significant linkage at D21S1903 on 21q was found. They further conducted an association study of 150 candidate genes in a 20-Mb region around the marker using 280 OPLL patients and 210 controls, and found association of *COL6A1* ($P = 3 \times 10^{-6}$). *COL6A1* (MIM 120290) encodes one of the 3 α-chains of type VI collagen. Its functional impact on OPLL susceptibility and pathogenesis also remains unclear. Using the same cohorts, Furushima *et al.* conducted a linkage study for candidate genes selected based on expression profiles during osteoblastic differentiation of human mesenchymal stem cells and found suggestive evidence of *BMP4* (bone morphogenetic protein 4) linkage (MIM 112262) with OPLL.[21]

Independent from these studies, Karasugi *et al.* performed a large-scale genome-wide linkage study using 214 Japanese affected sib-pairs.[22] The number of the sib-pairs was more than doubled compared to the previous linkage studies; however, they could not replicate the previous results, nor find any new loci. However, in stratification analyses which included only definite (≧2 ossified vertebrae) cervical OPLL, they found several new loci showing suggestive evidence of linkage on chromosomes 1p, 2p, 7q, 16q, and 20p. Fine mapping using additional microsatellite markers detected the highest significant linkage ($P = 2.7 \times 10^{-4}$) at D20S894 on chromosome 20p12 in a subgroup that had no complication of DM. These results suggest the presence of genetic heterogeneity of OPLL.

Candidate-gene association study

Several groups, mainly in East Asia are working on candidate gene association studies, and a number of genes/loci associated with the OPLL susceptibility have been reported (Table 1). The list includes *NPPS* (nucleotide pyrophosphatase)/*ENNP1* (ectonucleotide pyrophosphatase/phosphodiesterase 1),[10] *TGFB1* (transforming growth factor β1),[23] ESR (estrogen receptor),[24] IL-1β,[24] *VDR* (vitamin D receptor),[25] *BMP2*,[26] *RUNX2*,[27] IL-15RA,[28] *BMP9*,[29] and *TGFB3*.[30] However, the results of these studies are not sufficiently convincing. The largest study to date is the one that examined 109 sequence polymorphisms in 35 candidate genes using a ~1,600 case-control cohort.[30] In addition, a few variants per gene, usually only one single nucleotide polymorphism (SNP), were examined to evaluate the gene, while tag SNPs for the linkage disequilibrium

Table 2. OPLL susceptibility loci identified by GWAS*

Chromosome	SNP ID**	P value	OR (95% CI)	Candidate gene
6p21.1	rs927485	9.40×10^{-9}	1.33 (1.21–1.46)	*RSPH9*
8q23.1	rs374810	1.88×10^{-13}	1.34 (1.24–1.44)	*RSPO2*
8q23.3	rs13279799	1.28×10^{-10}	1.28 (1.19–1.38)	
12p11.22	rs1979679	4.34×10^{-12}	1.30 (1.21–1.40)	*CCDC91*, *STK38L*
12p12.2	rs11045000	2.95×10^{-11}	1.28 (1.19–1.38)	
20p12.3	rs2423294	1.10×10^{-13}	1.41 (1.29–1.55)	*HOA1*

*Nakajima *et al.* Nat. Genet. 2014.[37] **The most significantly associated SNP in the loci. OR, odds ratio; CI, confidence interval; *RSPH9*: radial spoke head 9 homolog; *RSPO2*: R-spondin 2; *CCDC91*: coiled-coil domain containing 91; *STK38L*: serine/threonine kinase 38 like; *HOA1*: hydroxyacid oxidase 1.

(LD) region containing the gene should be examined. Functional proof of the variants and/or genes through *in vitro* and/or *in vivo* experiments was missing and moreover, in most studies, the candidacy of the genes itself were not solid.

Only one exception was the study of *NPPS/ENNP1*.[10] It started by a mapping of the locus of *ttw* (*tiptoe walking*), a popular mouse model of OPLL[31] using parametric linkage analysis.[32] After identifying the disease gene for the autosomal recessive trait, *Npps*,[8] the human orthologous gene was examined for association. A few promising candidate variants were reported in *NPPS/ENNP1*;[10),33),34] however, their P-values were all not remarkable and no functional significance were presented for any of the variants. Loss of function mutations in *NPPS/ENNP1* causes idiopathic infantile arterial calcification (also known as generalized arterial calcification of infancy; MIM 208000)[35] and autosomal recessive form of hypophosphatemic rickets, type 2 (MIM 613312)[36] in man.

Genome-wide association study

The genome-wide sib-pair linkage analysis cannot pinpoint the causal gene. It only presents a 20–30 Mb of ambiguous regions that the gene can be located in. Therefore, after identification of a significant linkage, additional fine-mapping effort is always necessary, which needs extra time, cost and labor. Comparatively, a genome-wide association study (GWAS) is far faster, easier and cheaper. Therefore, the current method of choice for genome-wide screening of disease genes in the world is GWAS.

To identify susceptibility gene(s) for OPLL, Nakajima *et al.* conducted a GWAS using 8,265 Japanese subjects in collaboration with The Investigation Committee on the Posterior Longitudinal Ligament.[37] They successfully genotyped 616,496 SNPs on autosomes and 12,228 SNPs on X chromosome. By a whole-genome imputation using the GWAS data followed by a replication study using additional 7,017 subjects, they identified six susceptibility loci for OPLL (Table 2). One of the six loci identified by the GWAS on 20p12.3 overlapped to the previously reported linkage region,[22] reinforcing the level of evidence of the association. Thirteen genes that have previously been reported to be associated with OPLL had no significant association in the GWAS (P-value after a correction of multiple testing by the Bonferroni correction is $0.05/13 = 0.00038$) (Table 1).

They further stratified the case subjects of the GWAS according to the previous linkage study[22] and investigated the association of the SNPs in the regions.[37] Interestingly, odds ratios of the association increased in all strata, despite a significant decrease of sample numbers. A stratum of non-DM subjects showed genome-wide significant association at rs10486860 in the 7q22 linkage region ($P = 4.31 \times 10^{-8}$). These data further support the hypothesis that OPLL is genetically heterogeneous.

To identify the genes related to OPLL in the OPLL-susceptibility loci, Nakajima *et al.* analyzed gene expression in and around the loci.[37] They found several genes implicated in OPLL etiology and pathogenesis (Table 2). *RSPH9* (radial spoke head 9 homolog) on 6p21.1 and *STK38L* (serine/threonine kinase 38 like) on 12p11.22 may play a role in the membranous ossification process. These genes showed increased gene expression in osteoblasts compared to fibroblast in database analysis and quantitative PCR analysis. *HOA1* (hydroxyacid oxidase 1) on 20p12.3, *RSPO2* (R-spondin 2) on 8q23.1 and *CCDC91* (coiled-coil domain containing 91) 12p11.22 are implicated in the endochondral ossification process. Expression of these genes was decreased during early stage of chondrocyte differentiation of the ATDC5 cell, a mouse model of

endochondral ossification *in vitro*.[38] Further studies for identifying the OPLL susceptibility genes will be focused on these genes.

Future directions of the OPLL genomic study

The results of genome-wide linkage study and GWAS both indicate that OPLL is a genetically heterogeneous disease. This hypothesis is supported by the vast diversity of its clinical features, including location of the lesion (*i.e.*, cervical, thoracic, lumbar) and type of ossification (*i.e.*, continuous, segmental, mixed). By the stratification based on careful phenotyping of the patients, we can reduce the OPLL heterogeneity and hence expect to increase the chance to detect the association. Because the stratification is a trade-off between increase of purity of the population and decrease of the sample size, larger scale studies enrolling thousands of subjects will be necessary. Such studies will only become possible in a large-scale international collaboration work, which requires the definite phenotypic evaluation and common criteria based on the common understanding between collaborators. Phenotyping is more important in whole exome sequencing and whole genome sequencing, future promising approaches that enable us to identify the causal variants more robustly. In these studies, however, more samples are necessary, which further necessitate international collaborations.

After all, GWAS is a method of the positional cloning, not of identification of the causal gene. It merely tells us (if conducted successfully) the position of a functional fragment (a DNA sequence that increases the susceptibility to a disease by affecting its causal mechanisms) on a chromosome. The region defined by GWAS is far narrower than that defined by the linkage study, but still extends from tens to hundreds of kilo-bases region containing a lot of 'functional', potentially causal variants. We are usually not so lucky as to find a very easy-to-understand candidate causal variant like "a nonsense mutation in a gene that has been implicated in inhibiting osteogenesis (ex. BMPs)". The associated variants are mostly not in the genic regions but frequently in the gene deserts, as are in two among the six loci that Nakajima *et al.* identified.[37] There is a long and winding road before us. We are in need for methods efficiently and swiftly translating statistics (GWAS) to biology (functional study), but there is no king's road for it. Expression quantitative trait locus (eQTL) analysis is a promising approach, but the basis for conducting it in the OPLL research is very weak since the biological basis of OPLL is still unclear. We have to start from determining what cells or tissues are responsible for OPLL to conduct the eQTL analysis for OPLL. Is the problem in PLL itself or in near-by tissues, or via blood vessels? — no one knows. Now, we must integrate the genomic knowledge into the disease study. Biological studies after identification of susceptibility SNPs of OPLL by GWAS would lead us to the beginning of the end for understanding this mysterious disease as well as the mystery of ectopic ossification.

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