Review

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

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Abstract: Ossification of the posterior longitudinal ligament of the spine (OPLL) is 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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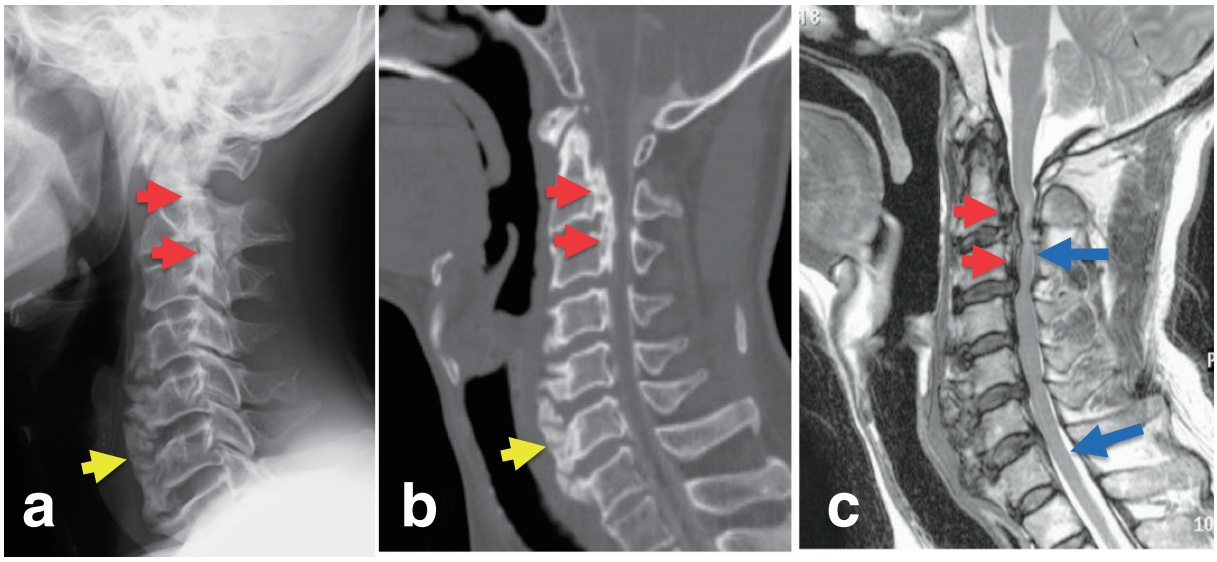

Fig. 1. Ossification of the posterior longitudinal ligament of the spine (OPLL). The lateral cervical spine in a patient with OPLL by a) plain radiograph, b) reconstruction computed tomography (CT) and c) magnetic resonance imaging (MRI). Red arrow: OPLL, yellow arrow: OALL (ossification of the anterior 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 ($\geqq$2 ossified vertebrae) cervical OPLL, they found several new loci showing suggestive evidence of linkage on chromosomes 1p, 2p, 7q, 16q, and 20p. Fine mapping using additional microsatellite markers detected the highest significant linkage ($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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Profile

Shiro Ikegawa is a Team Leader (Laboratory Head) of Laboratory for Bone and Joint Diseases, RIKEN Center for Integrated Medical Sciences. He was born in Nishinomiya in 1957. He graduated from the Faculty of Medicine, the University of Tokyo in 1983 and started his professional carrier as an orthopedic surgeon at the University of Tokyo where he managed the special clinic for patients with genetic skeletal diseases (1987–1995). He was a chief surgeon of National Rehabilitation Center for Disabled Children (NRCDC; known as 'Seishi Ryogo-en') in 1993 when he left the job and became a student of Department of Biochemistry, Cancer Institute. After once returned to NRCDC in 1994, he became Assistant Professor of Institute of Medical Science, the University of Tokyo in 1995 and obtained PhD from the University of Tokyo in 1996. He had the present position of PI in 2000 at the start of Japanese Millennium Project. His research interests have been in genetic aspects of monogenic and polygenic diseases affecting skeleton since the start of his research carrier. He is a 'gene hunter'. He has found disease genes of 17 monogenic skeletal diseases and susceptibility genes of 6 common bone and joint diseases, including osteoarthritis, lumbar disc herniation and idiopathic scoliosis (http://www.riken.jp/lab-www/OA-team/research.html). He was awarded the Raine Visiting Professorship in 2009 and the Basic Science Award of OARSI (Osteoarthritis Research Society International) and the JSHG (Japanese Society of Human Genetics) Award in 2012. Currently, he is a visiting lecturer/professor of 6 domestic and 4 foreign universities.