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Clinical Study

Unveiling the genetic variation of severe continuous/mixed-type ossification of the posterior longitudinal ligament by whole-exome sequencing and bioinformatic analysis

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ABSTRACT

BACKGROUND CONTEXT: Ossification of the posterior longitudinal ligament (OPLL) in the cervical spine is known as a rare, complex genetic disease, its complexity being partly because OPLL is diagnosed by radiological findings regardless of clinical or genetic evaluations. Although many genes associated with susceptibility have been reported, the exact causative genes are still unknown.

PURPOSE: We performed an analysis using next-generation sequencing and including only patients with a clear involved phenotype.

STUDY DESIGN/SETTING: This was a case control study.

PATIENT SAMPLE: A total of 74 patients with severe OPLL and 26 healthy controls were included.

OUTCOME MEASURES: Causal single-nucleotide variant (SNV), gene-wise variant burden (GVB), and related pathway

METHOD: We consecutively included the severe OPLL patients with continuous-/mixed-type and an occupying ratio of ≥ 40%, and performed whole-exome sequencing (WES) and bioinformatic analysis. Then, a validation test was performed for candidate variations. Participants were divided into 4 groups (rapidly-growing OPLL, growing rate ≥ 2.5%/y; slow-growing, < 2.5%/y; uncertain; and control).

RESULTS: WES was performed on samples from 74 patients with OPLL (rapidly-growing, 33 patients; slow-growing, 37; and uncertain, 4) with 26 healthy controls. Analysis of 100 participants identified a newly implicated SNV and 4candidate genes based on GVB. The GVB of *CYP4B1* showed a more deleterious score in the OPLL than the control group. Comparison between the rapidly growing OPLL and control groups revealed seven newly identified SNVs. We found significant association for 2 rare missense variants; rs121502220 (odds ratio [OR] = infinite; minor allele frequency [MAF] = 0.034) in *NLRP1* and rs13980628 (OR= infinite; MAF = 0.032) in *SSH2*. The 3

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genes are associated with inflammation control and arthritis, and *SSH2* and *NLRP1* are also related to vitamin D modulation.

CONCLUSIONS: Identification of unique variants in novel genes such as *CYP4B1* gene may induce the development of OPLL. In subgroup analysis, *NLRP1* and *SSH2* genes coding inflammation molecules may related with rapidly-growing OPLL.

Keywords: Arthritis; CYP4B1; NLRP1; Ossification of the longitudinal ligament; SSH2; Whole exome sequencing

Introduction

Ossification of the posterior longitudinal ligament (OPLL) is characterized by an ectopic ossification of the spinal ligaments, leading to myeloradiculopathy [1,2]. Since OPLL is frequent in elderly, East Asian males, the pathogenesis of the OPLL was thought to be an interaction between genetic susceptibility and environmental factors, such as eating habits, degeneration, and physical strain on the posterior longitudinal ligament [3–6]. Further complicating is that since OPLL is diagnosed by only radiological findings, any patient with calcification in the posterior longitudinal ligament can be diagnosed with OPLL regardless of clinical or genetic evaluation [7]. Among the patients diagnosed with OPLL, segmental- or localized-type OPLLs with a low occupying ratio (more available space for spinal cord) are usually clinically silent and require no treatments over the patient's lifetime, whereas continuous- or mixed-type OPLL, with high occupying ratio require special cautions as they may induce severe myelopathy and (in)complete cord injury. Recent investigators have suggested dividing OPLL into two categories based on the etiological point of view; either as a degenerative or genetic disease [8]. It is known that the longer the length of OPLL, the more likely it is to be a genetic disease [9].

Many genetic studies have suggested various causal genes, such as *COL6A1, HLA, BMP2, BMP4, NPPS, TGF-β1, ESR1, FGFR1, IL-1β, IL-15RA, IL-17,* and *RUNX2*, which differed from study to study [10–13]. Although these studies suggested that OPLL may be a genetic disease, still the exact causative genes are yet to be established [4]. One reason for the inconsistency of these studies' results may also be related to the disease's broad definition; it is possible that genetic and degenerative OPLL cases were analyzed together, contributing the inconsistent results.

Another reason may be that previous researchers attempted to uncover the causative genes using RNA expression analysis through microarray, low throughput sequencing of some targeted genes, or genome-wide association. Because of limitations of the methods employed, a substantial fraction of genetic contribution remains unexplored. Recently, whole-genome or exome sequencing has been employed to overcome the shortcomings of past studies. Of these methods, whole-exome sequencing (WES) evaluates all protein-coding genes and has revolutionized the discovery of genes in which variants cause rare diseases [14].

We sequenced the whole exomes of consecutive unrelated OPLL patients with continuous/mixed-type OPLL and high occupying ratio, as these cases are most likely to be due to genetics. Subsequently, we performed a case-control association study with the selected variants to identify new candidates involved in the pathogenesis of OPLL. We also compared variants by a growing rate of OPLL in the patients.

Materials and methods

Study design, setting, and participants

We retrospectively searched all patients diagnosed with OPLL of the cervical spine from 2008 to 2018 at *(Blind for review)* Hospital and reviewed their eligibility according to the inclusion criteria. This study's inclusion criteria were; Korean adults living in the Republic of Korea, presence of cervical OPLL, continuous- or mixed-type, and an occupying ratio of ≥ 40%. We contacted all patients who met the eligibility criteria and invited those satisfied with the inclusion criteria and agreed to participate in the study and obtained written consent from each participant to publish clinical and genetic data. The control group was limited to healthy volunteers in the same race and country without cervical OPLL as well as cancer or other genetic diseases. The control group confirmed the absence of OPLL by X-ray or computed tomography (CT). We explained the risks, benefits, and limits of WES analysis interpretation to the participants. This study was approved by the Institutional Review Board of *(Blind for review)* Hospital (reference #1810-046-977) and *(Blind for review)* Hospital (reference #2017-07-007).

We collected peripheral blood and detailed phenotyping data from individuals with OPLL. Among the OPLL group, judging by 3-dimensional CT or magnetic resonance imaging, we defined the rapidly growing group as the subgroup in which the length or depth of OPLL showed rapid growth of 2.5% or more per year; otherwise, we allocated patients to the slow-growing group. Patients were classified into the uncertain group if they did not have follow-up radiographic images that had passed more than two years from the initial pictures.

Exome capture and massively parallel DNA sequencing

We isolated genomic DNA, using standard techniques, from peripheral blood obtained from the patients with

OPLL and healthy controls. Exomes were captured using the Agilent SureSelectXT kit (Santa Clara, United-States). Sequencing was performed in the 100 samples on an Illumina HiSeq2500 (Illumina, San Diego, CA, USA) with paired-end mode for 100 or 150 base pairs reads. The mean coverage Depth was 101.25X, and the mapping rate was 99.4%. Sequence data in the form of BAM files were generated via the Picard data-processing pipeline and contained well-calibrated reads aligned to the GRCh37 human genome reference [15]. Samples across projects were then jointly called via the Genome Analysis Toolkit (GATK) best-practice pipeline for data harmonization and variant discovery [16]. This pipeline detected single-nucleotide variants (SNVs) and small insertion or deletion (indel) variants from whole-exome data. After completing WES, we compared the genomic data with clinical data such as the shape of OPLL, clinical course, and family history. The pathogenicity of the searched variants was evaluated according to the recent standards of the American College of Medical Genetics and Genomics (ACMG) [17].

Confirmation of variants

For the experimental validation of the candidate variants, we used an SNV type assay (Fluidigm, San Francisco, CA, USA). The genomic DNA flanking the interested SNV was amplified using polymerase chain reactions (PCRs) with specific target amplification (STA) primer set and Qiagen 2X Multiplex PCR Master Mix (Qiagen) in a 2.5-μL reaction volume, containing 50 ng of genomic DNA. The analysis was carried out using Fluidigm SNV genotyping analysis software (version 4.0.1; Fluidigm). SNV type assays were compared for all genotypes of final candidate variants of analysis workflow. The genotype was classified as either homozygous or heterozygous in alleles. Of the total 12 candidate variants, four were declared non-experimental. Except for 1, the remaining candidate variants were consistent between the WES and SNV type assay.

Statistical analyses

Participants were divided into 4groups (rapidly growing OPLL, slow-growing, uncertain, and control). Baseline characteristics, including age, sex, underlying disease, and clinical manifestations, were compared. The OPLL group was compared with the control, and later the rapidly growing group was compared with the control. We analyzed all non-synonymous variants inducing amino acid changes, and the process is depicted in Fig. 1. Our analysis plan consisted of 3 major genetic perspectives such as variant-, gene-, and pathway-wise evaluations. The common and rare variant-wise approach was conducted using Fisher's exact test with carrier frequency and sequence kernel association test-optical (SKAT-O) test with rare deleterious variants, respectively. The gene-wise analysis was performed using multiple logistic regression using gene-wise variant burden (GVB) and clinical variables [18–22]. We performed pathway enrichment analysis to identify the biological function of the candidate genetic markers. To calculate the pathogenicity of the variants, two *in silico* variant deleteriousness prediction scores were used: Scale-Invariant Feature Transform (SIFT; 0–1 range and variants with scores < 0.05 are deleterious) [23], and Combined Annotation Dependent Depletion (CADD); a PHRED-like scaled C-score of 10 indicates that a variant is predicted to be among the 10% most deleterious substitutions possible in the human genome and a score of 20 is indicative of the 1% most deleterious [24].

We evaluated the influence of specific variants on the development and rapid growth of OPLL by comparing the non-carriers and heterozygous/homozygous carriers of each variant and any other identified variants in total and determining the significance of any differences using Fisher's exact test. The association strength (risk) between carriers and non-carriers was the estimated odds ratios (OR) with 95% confidence intervals (CI). False discovery rate (FDR)-adjusted p-values were obtained with the Benjamini-Hochberg method due to multiple comparisons [25]. An FDR-adjusted p-value <.05 was considered significant. The validation test was performed on all candidate variants. For the comparison of allele frequency in various public databases, we used the Korean reference genome database [26], 1000 genome project phase 3 [27], and GnomAD v2.1.1. [28].

The GVB of each gene was computed using the geometric mean of the SIFT score for non-synonymous variants of the genes to evaluate the contribution of genetics to the development of OPLL. GVB score ranges from 0 to 1, with the more harmful genes presenting values closer to zero, and it was individually calculated using the geometric mean of the SIFT score for coding variants of the genes.

$$GVB_g = \begin{cases} 10^{-8}, \text{ If gene has nmLoF variant} \\ \left(\prod_{k=1}^{n} SIFT\ (v_k) \right)^{\frac{1}{n}}, \text{ else} \end{cases}$$

Where v_k is the k-th variant in the gene; n is the number of variants in gene g; nmLoF is the not-missense loss of function variant (Nonsense, Frameshift INDEL, Splice site).

Multiple logistic regression analyses examined the associations of GVB-adjusted baseline clinical factors in each treatment response outcomes and estimated the beta coefficient and 95% CI. The read quality of the sequences was assessed by checking the bam files for errors that may have occurred during the mechanical filtering process during pre-processing using the IGV Viewer (http://software.broadinstitute.org/software/igv/), and false-positive variants were removed. Four bioinformatics experts also conducted a final manual review to verify the results. Carriers were defined as heterozygous or homozygous carriers of altered alleles.

Within the gene regions in the Ensembl database, SNVs were annotated 0 to1 with the in-silico prediction and a

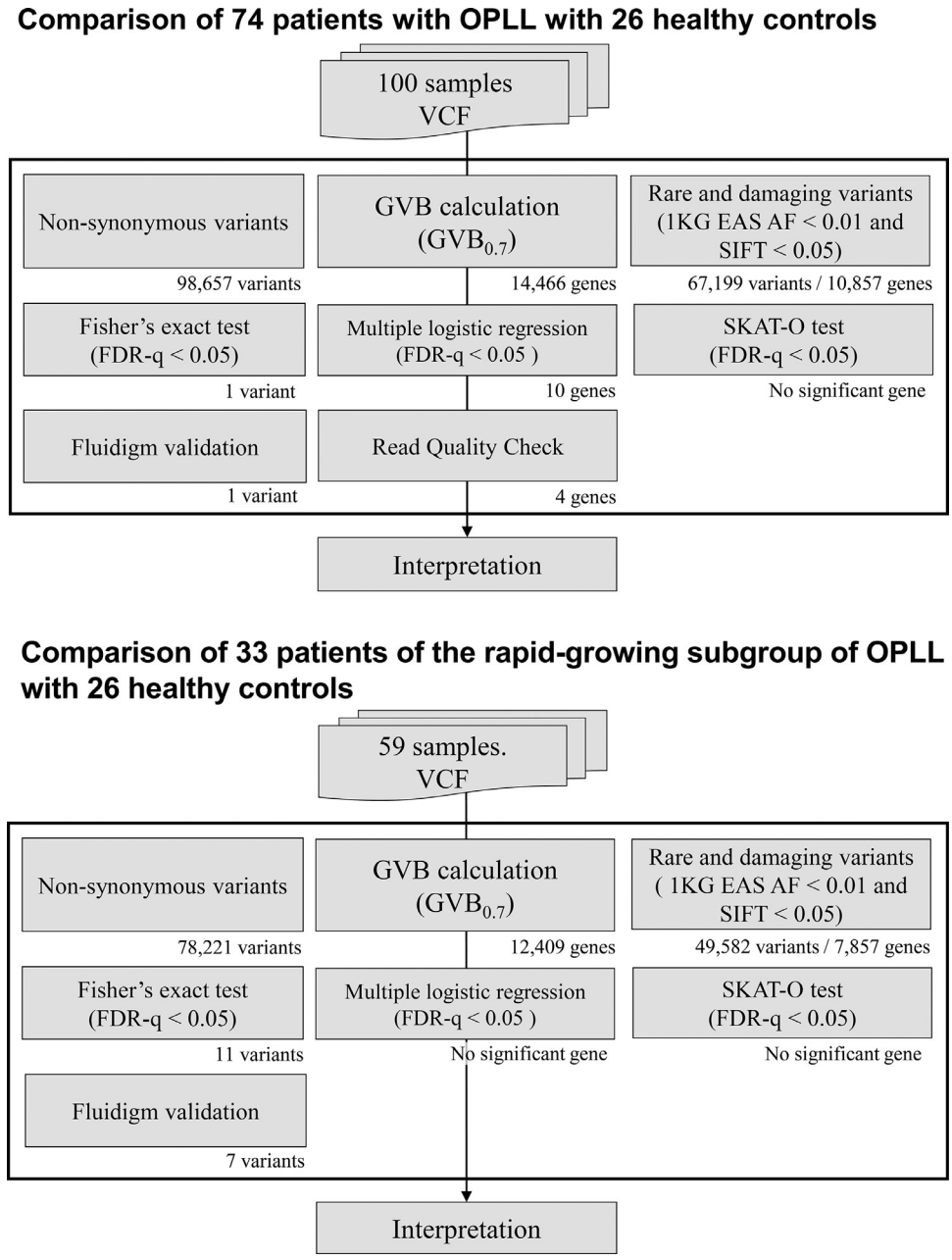

Fig. 1. Outline of the bioinformatic analysis process. Both variant- and GVB-centric analyses with rare variant association tests were performed. Abbreviations: VCF, variant call format; GVB, Gene-wise variant burden; FDR, false discovery rate; 1KG EAS AF, allele frequency for East Asian in 1000 Genomes project.

minor allele frequency (MAF). Burden testing on cases and controls was performed using SKAT-O [29] for accounting for a unidirectional effect. The test collapses the variant data within a region by summing the squares of score statistics for testing individual gene markers. For Burden testing, frequency based on each variant's MAF are usually used to establish rare deleterious variants (MAF < 0.01 & SIFT < 0.05). In this case, the individual squares of score statistics were weighted before they were summed.

We performed pathway enrichment analysis of the candidate genes identified by the method above. This method identifies biological pathways that are enriched in a gene list to a greater extent than would be expected by chance, and the most highly ranked enrichment terms for the input gene list provide knowledge about the list. WIKI [30] and Elsevier [31] pathway databases with molecular functions were used. All statistical analyses were conducted using R software (ver. 3.6.1; http://www.r-project.org/).

Results

We performed WES on samples obtained from 74 unrelated patients (rapidly growing, 33 patients; slow-growing, 37 patients; and uncertain, 4 patients) diagnosed with

Table 1
Baseline characteristics of the enrolled participants

Characteristic	OPLL				Control
	Rapidly-growing	Slow-growing	Uncertain	Total	
Number	33	37	4	74	26
Sex (M/F)	22/11	26/11	3/1	51/23	15/11
Age (SD)	60.24 (9.43)	62.42 (7.15)	60.93 (11.30)	61.37 (8.42)	42.27 (11.94)
Type of OPLL (Continuous/Mixed)	19/14	25/12	1/3	45/29	-
Upper involved vertebra (C2/C3/C4/C5)	22/9/1/1	29/3/4/1	3/1/0/0	54/13/5/2	-
Mean involved segments (SD)	4.52 (1.18)	5.11 (1.58)	4.25 (0.96)	4.80 (1.40)	-
Mean length of OPLL at baseline (SD)	62.70 mm (20.92)	75.94 mm (25.08)	77.53 mm (31.67)	70.12 mm (24.25)	-
Mean occupying ratio at baseline (SD)	53.22% (8.66%)	54.84% (9.54%)	47.13% (5.01%)	53.70% (9.05%)	-
Progression rate per year (SD)	5.88% (4.56%)	1.72% (5.83%)	NA	3.68% (5.64%)	-

Abbreviations: OPLL, ossification of the posterior longitudinal ligament; SD, standard deviation; NA, not available.

severe OPLL for the cervical spine and 26 healthy controls. All 100 subjects were of the Korean race and lived in the Republic of Korea. The baseline characteristics of the participant are described in Table 1. In the OPLL group, 67.1% were male, and the mean age was 61.37 years old (standard deviation [SD], 8.42). The upper involved vertebra was C2 in 54 patients, C3 in 13, C4 in 5, and C5 in 2, and the mean involved spinal segments was 4.8 (SD, 1.4). Among subgroups of the OPLL, the annual progression rates of the rapidly and slow-growing groups were 5.88% (SD, 4.56%) and 1.72% (SD, 5.83%) ($p < .01$), respectively. The rapidly and slow-growing groups showed similar occupying ratio at baseline (rapidly growing, 53.22%; slow-growing, 54.84%; $p = .46$), but significant difference in length of OPLL at baseline (rapidly growing, 62.7 mm; slow-growing, 75.9 mm; $p = .02$).

Genetic variation associated with OPLL development

To estimate the impact of individual non-synonymous variants on the development and progression of OPLL, the OPLL groups and the control were compared using the Fisher's exact test in Table 2. In comparison with the OPLL and the control, rs759025747 in the *SPATA31A3* gene was significantly associated with OPLL development. A gene-wise analysis demonstrated 4 statistically significant genes associated with OPLL, such as *CYP4B1, ANAPC1, CELA3A*, and *SEMA4B* in Fig. 2. In the OPLL group compared to the control, the mean GVB of the *CYP4B1* decreased from 0.46 (SD, 0.51) to 0.23 (SD, 0.42), that of *ANAPC1* decreased from 0.81 (SD, 0.38) to 0.52 (SD, 0.47), that of *CELLA3A* decreased from 0.70 (SD, 0.15) to 0.64 (SD, 0.07), and that of *SEMA4B* decreased from 0.66 (SD, 0.13) to 0.61 (SD, 0.09). The GVB of *CYP4B1* and *ANAPC1* were much lower for the OPLL group than the control group. In pathway enrichment analysis, the cytochrome P450 pathway appeared to be the major enriched pathway for the variant-wise test of gene sets with eight candidate variants, which are denoted in Table 3. None of the different association tests showed a significantly increased burden of rare non-synonymous variants among the OPLL patients using SKAT-O test.

Genetic variation associated with rapid progression of OPLL

Significant associations were observed for seven missense variants involved in the rapid growth of OPLL such as, rs12150220 in *NLRP1*, rs139830628 in *SSH2*, rs4674941 in *DOCK10*, rs11540301 in *KRT6A*, rs34161108 in *DEPDC7*, rs141752962 in *GCFC2*, and rs17832431 in *FOCAD* (Table 2). All 7 genes were validated by the Fluidigm test. The identified variants in *NLRP1, SSH2, DOCK10, FOCAD, DEPDC7,* and *GCFC2* were the moderate rare variants (MAF < 0.05) compared to the data with the Korean reference database, 1000 genome project, and GnomAD. The OR of the variant in *DEPDC7* was 9.09 (1.11–426.1), and the OR of the other SNVs were infinite (MAF of the control, 0).

Review of previously proposed variations

Previously proposed causal genetic variations associated with OPLL did not demonstrate a statistical significance compared to this study (Table 4). *TGFB3* and *IL15RA* were frequent in the OPLL group compared to the control (OR of *TGFB3*, 3.43; OR of *IL15RA*, infinite), which did not show a statistical significance. SNV in *COL6A1, BMP4, FGFR1, CCDC91, RSPO2* and other genes were observed in only one case or not at all.

Discussion

Genetic OPLL has not been clearly defined yet. However, the more severe OPLL, the more probable it is a genetic disease. We hypothesized that the OPLLs which were continuous/mixed-type and an occupying ratio of ≥ 40% might belong to genetic OPLL, and searched for related genomic variations. This WES study identified several deleterious coding variants that could display promising associations in Korean OPLL with apparent

Table 2
Descriptive results of significant variants in a variant-wise test by Fisher's exact test

Comparison	Gene	Chr	Position	rsID	SIFT	CADD	Case			Control			FDR-q	OR (95% CI)	MAF KRGDB	MAF 1KGP EAS	MAF 1KGP ALL	MAF GnomAD Exome EAS	MAF GnomAD Genome EAS
							Ref	Het	Hom	Ref	Het	Hom							
OPLL vs. Control	SPATA31A3	9	40706227 G>A	rs759025747	0.03	1.94	62	12	0	26	0	0	0.0329	∞	0.092	NA	NA	0.151	0.0882
Rapidly-growing of OPLL vs. Control	DEPDC7	11	33049341 G>A	rs34161108	0.59	10.17	24	9	0	25	1	0	0.0323	9.09 (1.11-426.1)	0.054	0.070	0.046	0.049	0.054
	KRT6A	12	52881544 G>C	rs11540301	0.07	17.76	25	8	0	26	0	0	0.0070	∞ (1.55- ∞)	0.056	0.110	0.056	0.049	0.048
	SSH2	17	28004744 C>G	rs139830628	0.66	14.96	26	7	0	26	0	0	0.0145	∞ (1.27- ∞)	0.032	0.004	0.021	0.025	0.024
	NLRP1	17	5485367 A>T	rs12150220	0.13	2.99	27	6	0	26	0	0	0.0297	∞ (1.01-∞)	0.034	0.192	0.031	0.026	0.022
	DOCK10	2	225670901 G>C	rs4674941	0.19	12.78	26	7	0	26	0	0	0.0145	∞ (1.27-∞)	0.045	0.249	0.023	0.024	0.020
	GCFC2	2	75899101 T>C	rs141752962	0	24.8	27	6	0	26	0	0	0.0297	∞ (1.01-∞)	0.036	0.004	0.019	0.029	0.025
	FOCAD	9	20820329 A>G	rs17832431	1	0.002	27	6	0	26	0	0	0.0297	∞ (1.01-∞)	0.056	0.012	0.037	0.046	0.046

Abbreviations: Chr, chromosome; Ref, reference allele carrier; Het, heterozygous alteration allele carrier; Hom, homozygous alteration allele carrier, FDR, false discovery rate; OR, odds ratio; CI, confidence interval; MAF, minor allele frequency; KRGDB, Korean reference genome database; 1KGP EAS, East Asian in 1000 Genomes project, phase 3; 1KGP ALL, all population in 1000 Genomes project, phase 3; GnomAD Exome EAS, East Asian in GnomAD exome; GnomAD Genome EAS, East Asian in GnomAD whole genome.

Fig. 2. Comparison of the gene-wise variant burden (GVB) between the OPLL and healthy control groups. Four genes show a statistically significant difference between the two groups ($p < .05$). GVB scores of CYP4B1 and ANAPC1 were much lower for the OPLL than the control group.

phenotypes. Major variants associated with the *CYP4B1* gene may induce the development of OPLL. In subgroup analysis, *NLPR1* and *SSH2* genes may related with rapidly-growing of OPLL. The 3 genes are associated with auto-inflammation, while *NLRP1* and *SSH2* are also related to vitamin D metabolism.

For OPLL cases where the diagnostic definition is ambiguous, it is essential to analyze patients showing an obvious phenotype of genetic disease. Among the four types of OPLL, continuous/mixed OPLL with a high occupying ratio usually requires surgery to prevent quadriplegia [32,33]. Until now, these four types of OPLL have been studied as a single condition [33]. Although clinical studies divided the OPLL into progressive (rapidly growing) and non-progressive (slow-growing) [5,34], none of the genetic/genomic studies excluded the patients with segmental/localized-type OPLL to unravel a genetic cause. We included only cases of continuous/mixed OPLL with a high occupying ratio to exclude degenerative disease, and the characteristics of OPLL in this study were as homogenous as possible. An epidemiological study demonstrated that OPLL frequently occurs in the mid-low cervical spine, such as C5 and C6, with only 4% occurrence in C2 for patients with OPLL; of these patients, 13% had continuous type, 4% mixed type, 73% segmental type, and 11% localized type [9]. Unlike the epidemiological data, 73.0% and 90.5% of the OPLL involved the C2 and C3, respectively, in this study.

The *CYP4B1* (Cytochrome P450 4B1) gene encodes a member of the cytochrome P450 superfamily of enzymes and has been implicated in various biological functions, including inflammation [35]. A recent analysis of differentially expressed genes demonstrated that *CYP4B1* was downregulated in both osteoarthritis and rheumatoid arthritis compared with the healthy control [36]. In this study, the GVB of *CYP4B1* was deleterious in the OPLL group

Table 3
Pathway enrichment analysis of identified candidate genes

Pathway	Term	p-value	Odds Ratio
WIKI pathway 2019	Nuclear Receptors in Lipid Metabolism and Toxicity WP299	.0245	40.40
	Nucleotide-binding Oligomerization Domain (NOD) pathway WP1433	.0303	32.52
	Oxidation by Cytochrome P450 WP43	.0448	21.86
Elsevier pathway	APC/C-FZR1 Complex	.0171	57.97
	Genes with Mutations Associated with Vitiligo	.0223	44.44
	NOD-like Receptors	.0237	41.67
	APC/C-CDC20 Complex	.0245	40.40
	Blau Syndrome	.0281	35.09
	Apoptotic Keratinocytes Clearance Recession in Systemic Lupus Erythematosus	.0340	28.99
	Dendritic Cell Activation in Systemic Lupus Erythematosus	.0405	24.24

compared with the control. Pathway enrichment test also showed that OPLL was associated with oxidation by cytochrome P450 (Identifier: WP33). This *CYP4B1* may induce arthritis and inflammation in the spine. Although the SNV in *SPATA31A3* was significantly frequent in the OPLL group and was not identified in the control group, the gene is a spermatogenesis associated gene. Because the function of this gene is not yet clearly known, we could not find a relation with the OPLL.

There was no SNV that showed a statistical difference between the OPLL and the control group, but 7 SNVs demonstrated a significant difference between the rapidly growing OPLL group and the control group. Among the 7 SNVs identified by comparing the rapidly growing OPLL and control groups, *NLRP1* and *SSH2* showed significant differences in allele frequency. Both genes are associated with inflammation and vitamin D modulation [37,38]. *NLRP1* (NACHT, LRR, FIIND, CARD domain and PYD domains-containing protein 1) was the first protein shown to form an inflammasome and a member of the nucleotide oligomerization domain (NOD)-like receptors family and regulates inflammasome activation, cellular apoptosis, and the innate immune system [39]. Variant in *NLRP1*, regardless of dominant or recessive, tend to be gain-of-function alleles that trigger inflammasome signaling with *IL1B* and *IL18* release [38]. Variants of this gene could induce auto inflammatory

Table 4
Review of previously reported genes that may be related to OPLL

Gene	Chr	Position	rsID	SIFT	CADD	OPLL			Control			p	OR (95% CI)
						Ref	Het	Hom	Ref	Het	Hom		
TGFB3	14	76447149_A>G	rs3917145	NA	0.13	65	9	0	25	1	0	.446	3.43
IL15RA	6	45390487_GGCGGC GGCGGCGGCTGC>-	None	NA	NA	68	6	0	26	0	0	.334	∞ (0.01-∞)
	10	6019437_G>A	None	0	23.30	74	0	0	25	1	0	.260	0 (0-13.7)
	6	45480034_C>G	None	0	29.60	73	1	0	26	0	0	1	∞ (0.01-∞)
GDF2	10	48414490_G>T	rs180821007	0.41	0.73	71	3	0	25	1	0	1	1.06
	10	48414152_G>A	None	0.06	14.59	73	1	0	25	1	0	.454	0.35 (0-27.95)
COL6A1	21	47419113_G>A	rs201227573	0.05	29.00	74	0	0	25	1	0	.260	∞ (0.01-∞)
	21	47406936_G>A	rs199842980	0.08	16.91	73	1	0	26	0	0	1	∞ (0.01-∞)
	21	47421948_G>A	rs373731596	0.17	25.5	73	1	0	26	0	0	1	∞ (0.01-∞)
	21	47410305_A>G	None	0.66	16.98	74	0	0	25	1	0	.26	0(0-13.7)
	21	47414111_A>G	rs768002460	0.12	20.1	73	1	0	26	0	0	1	∞ (0.01-∞)
	21	47423037_A>G	rs770703803	0.61	12.77	73	1	0	26	0	0	1	∞ (0.01-∞)
	21	47423510_G>A	rs527265374	NA	3.951	74	0	0	24	2	0	.066	0(0-1.84)
BMP4	14	54418717_T>C	None	0.4	12.00	73	1	0	26	0	0	1	∞ (0.01-∞)
ENPP1	6	132190576_A>G	rs201519006	0.01	26.60	73	1	0	25	1	0	.454	0.35 (0-27.95)
ESR1	6	152129221_C>T	rs79415092	NA	21.00	73	1	0	26	0	0	1	∞ (0.01-∞)
IL1B	2	113590255_C>T	None	NA	10.22	73	1	0	26	0	0	1	∞ (0.01-∞)
RSPO2	8	108913314_CT>-	None	NA	NA	73	1	0	26	0	0	1	∞ (0.01-∞)
IL17RC	3	9970030_C>T	rs143600903	0.21	35.00	73	1	0	25	1	0	.454	0.35 (0-27.95)
	3	9975136_C>T	None	NA	13.69	73	1	0	25	1	0	.454	0.35 (0-27.95)
FGFR1	8	38273490_G>C	None	0.02	16.73	74	0	0	25	1	0	.260	0 (0-13.7)
CCDC91	12	28459684_T>A	rs192440192	0	13.98	73	1	0	26	0	0	1	∞ (0.01-∞)

Abbreviations: Chr, chromosome; Ref, reference allele carrier; Het, heterozygous alteration allele carrier; Hom, homozygous alteration allele carrier, OR, odds ratio; CI, confidence interval.

disease with arthritis, such as systemic sclerosis, systemic lupus erythema, rheumatoid arthritis, and autoimmune thyroid disease [38–42]. The *SSH2* (Protein phosphatase Slingshot homolog 2) encodes a protein tyrosine phosphatase that plays a vital role in regulating actin filaments. This gene associated with neutrophil chemotaxis and excessive recruitment of neutrophils into healthy tissues could cause damage and inflammatory diseases such as asthma and arthritis [43]. An animal study presented that variations in SSH family genes suppressed osteoclast activity [44]. We searched the literature for an association between this disease and the other five SNVs, but unfortunately, found no relevant studies.

Previous genetic studies have suggested that OPLL develops via osteogenesis mediated by *IL17RC, BMP2, COL6A1, RSPO2* and *BMP4* [12,45–49]. Nakajima et al. identified 6 susceptibility loci for OPLL using genome wide association study (GWAS), and proposed *RSPO2* as a result of analyzing one of 6 loci [47,48]. Then, they performed expression analysis for some genes of 6 loci in their previous GWAS, and reported that *HAO1* may act an initiation factor and *RSPO2, HAO1*, and *CCDC91* may act progression of OPLL [46]. Our study also demonstrated a high frequency of these genetic variations in the OPLL group, but the difference did not reach a statistical significance. Variation in *HAO1* was not observed in both groups. The reason of difference between previous studies and ours may be related with strict case selection such as continuous/mixed type and ≥40% of occupying ratio, and new method of WES. Although GWAS is an efficient tool for genomic research, there are some limitations that it has still need strict corrected significance of multiple hypothesis test to avoid false positives and does not completely cover the entire coding region of genome.

This study suggests that auto-inflammation may be a critical process in OPLL and that *NLRP1, SSH2,* and *CYP4B1* may play a role in controlling disease progression. *NLRP1* is a well-known inflammasome-coding gene, and *SSH2* and *CYP4B1* are also related to inflammation. Especially, rapidly-growing OPLL may be close relation with inflammation related genes such as *NLRP1* and *SSH2*. A previous clinical study reported that OPLL patients showed a higher level of high sensitivity C-reactive protein (hs-CRP), a representative inflammatory biomarker, compared to healthy controls, and that the progression (rapidly growing) group showed higher levels of hs-CRP than the non-progression (slow-growing) group [34]. This trend in the inflammatory biomarker was concordant with the results of our genomic study. Another clinical study reported that non-steroidal anti-inflammatory drugs might be an effective medical treatment for the OPLL [50].

Limitations and strengths

The present study has some limitations that need to be acknowledged and addressed. First, the sample size of the study was quite small, conferring the study lesser statistical power. However, OPLL is a rare disease, and only a few of them accounted for genetic OPLL. In rare diseases, selection of patients with a clear phenotype may be important. Moreover, the cost of WES is still very expensive. Because of the high cost and strict inclusion criteria, the sample size could not but be small. Nevertheless, this study is the largest of the reported WES studies regarding OPLL genomics. The present research can be considered as a leap stone toward elucidating the genetics of the OPLL. Second, the effect of decompression/fusion surgery on OPLL progression was not considered during the process of dividing the rapidly-, slow-growing group. Of 74 OPLL patients, 71 patients underwent decompression/fusion during the study period. Three patients are being followed for reasons such as mild symptoms and fear of surgery. However, the effect of surgery may be small because the majority of the patients underwent surgeries. Third, all participants were of a single ethnic group, and it is difficult to generalize these results to the entire human population. However, OPLL is prevalent in Northeast Asia, including Korea and Japan, and genomic characteristics of Korean and Japanese are very similar. Thus, the results of this study may depict not only a genetic variation in the Korean population but also the genetic variation of the Northeast Asian-specific OPLL. Further research is warranted on other ethnic groups. Fourth, In including the control group, the number and proportion of patients and controls were different from those of general clinical studies because we tried to include the control group without OPLL as well as other genetic disease. Recruiting a genetically healthy control group was difficult because the participants were unaware of their genetic variation. Therefore, the control group mainly consisted of doctors who were aware of their genetic variations. It is not guarantee that the control group is free of other genetic disease, and missing to explicitly confirm the absence of genetic disorders in controls may be a weakness of this study. The mean age of the control group was substantially younger than that of the OPLL group. Even genetic OPLL can also be affected by age, which may be a weakness for this study. There may be errors due to differences in mean age, but the control group certainly did not have OPLL or major genetic disorders in themselves and their family. In addition, the GVB and SKAT-O test were adjusted age differences. Finally, variants outside of protein-coding regions were not considered and should therefore be analyzed future research.

Nevertheless, this study's strength is that the patients were homogenous with clear phenotypes, which may increase the accuracy of the analysis. Also, genomic variation was analyzed using the most up-to-date method, WES. We tried to identify the causal variations using all major analyzing methods, including variant and gene-wise tests. Moreover, Fluidigm validation experiment was conducted to confirm the candidate variants. As a result, we identified several variations that might play a crucial role in auto-inflammation and vitamin D metabolism. To the best of our knowledge, this study is the most extensive study using WES for the analysis of OPLL.

Conclusion

The identification of unique variants in *NLRP1, SSH2,* and *CYP4B1* suggests that auto-inflammation may play a role in the pathogenesis of OPLL. Further investigations of these variants are warranted to assess their potential roles as the molecular drivers of OPLL.

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Declarations of Competing Interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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