Doctoral dissertation

Ossification of the posterior longitudinal ligament (OPLL): Studies of the morphological characteristics-associated inflammatory mechanism of ectopic bone formation in spinal ligaments

後縦靭帯骨化症(OPLL):

脊椎靭帯における異所性骨化の形態学的特徴と炎症機序に関する研究

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Abstract

Ossification of the posterior longitudinal ligament (OPLL) is one of the pathological conditions that is characterized by the replacement of ligamentous tissue by ectopic new bone formation and primarily affects the spine. The pathogenesis of OPLL is multifactorial and remains to be fully elucidated. The focus of this dissertation was to explore morphological characteristics of ossification of the spinal ligaments, in particular, OPLL, while also making investigation on its possible link with an inflammatory mechanism.

In the first study, to clarify whether OPLL affects the sacroiliac (SI) joints, I investigated the morphological changes in SI joints in individuals with and without OPLL. This study demonstrated that the bony bridging and ankylosis of the SI joint occurred more frequently in the OPLL+ group compared with the OPLL− group. By contrast, the SI joint vacuum phenomenon was the main finding in patients with degenerative spinal disease without OPLL rather than SI joint ankylosis. Additionally, patients with OPLL conferred a high risk of SI joint intra-articular fusion, which is a hallmark of Ankylosing spondylitis (AS). Thus, this study revealed a novel SI joint characteristic in patients with OPLL. These findings also gave me a hypothesis that both OPLL and AS might share a similar tendency toward SI joint intra-articular fusion, and both diseases might have a similar etiology related to inflammatory-related enthesitis.

In the second study, given that OPLL and DISH share features of bone proliferation and ectopic ossification in the spinal ligament, and the relationship between them has attracted recent attention with many cases of coexistence of these two diseases being reported, I compared cervical OPLL patients with and without DISH and revealed that the concomitance rate of cervical OPLL accompanying DISH was 57.14%, which is remarkably high. I have also demonstrated two types of osteophytes in DISH (Flat and Jaggy types) and evaluated their relationship with serum hs-CRP levels. A high concentration of hs-CRP was associated with

the Flat type of ectopic bone formation in DISH, whereas a negative correlation was found between hs-CRP and the Jaggy type. These results suggest that the Flat type in DISH might be caused by an inflammatory pathogenesis rather than a degenerative process presented in the Jaggy type.

In the third study, following the previous work, which has described two ways of ossification growth in DISH, I recognized that these two types of new bone formation are also observed in OPLL and have been classified into plateau and hill shapes. Therefore, I further investigated the relationship between serum hs-CRP levels, spinal ligament ossification, and SI joint changes in OPLL. As a result, serum hs-CRP levels in the plateau-shaped group were significantly higher than those in the hill-shaped group. SI joint intra-articular fusion was the main finding in the plateau-shaped group and showed significantly higher hs-CRP levels compared to the anterior para-articular bridging, which more frequently occurred in the hill-shaped group. These findings suggested a possible inflammation mechanism that might contribute to the new bone formation in OPLL, particularly the plateau shape similar to the Flat type in DISH.

In the fourth study, based on previous findings that OPLL, DISH, and AS might share a similar stage of inflammation at the attachment site during progression, which contributes to the promotion of new bone formation, I explored a potential mechanism of inflammation by investigating the evidence of interleukin (IL)-17 expression in OPLL patients with and without DISH, and its relationship with ectopic bone formation in spinal ligaments as well as SI joint variations. The results showed that no significant difference in IL-17 levels was observed between patients with OPLL and controls. However, IL-17 levels were significantly higher in the DISH (+) group, especially in females. IL-17 levels were also related to the Flat type in the DISH (+) group, and high IL-17 levels were more often associated with type 4C SI joints. In addition, IL-17A expression was elevated in ossified ligament tissue and cells derived from

OPLL patients. Also, IL-17A stimulation promoted the proliferation and osteogenic differentiation of OPLL cells. Therefore, this study reveals the pathological and serological evidence of local inflammation contributing to paravertebral ossification of OPLL patients and thus identifies that IL-17A may be a potential target for the prevention and treatment of OPLL.

Taken together, this dissertation provides new insight into the variable morphological characteristics and proposes a novel inflammatory mechanism that might promote ectopic bone formation in spinal ligaments, in particular, OPLL.

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Contents

Chapter 1:

Introduction

Spinal ligament ossification is a type of heterotopic ossification that occurs throughout the spine. Ossification of the posterior longitudinal ligament (OPLL) is characterized by progressive ossification of the posterior longitudinal ligament (PLL), causing severe neurological problems, such as myelopathy and/or radiculopathy [1]. OPLL has also been associated with other paraspinal ligament disorders, such as diffuse idiopathic skeletal hyperostosis (DISH) and ankylosing spondylitis (AS) [2-9]. The radiographic appearance of both diseases is very similar, but the underlying pathology differs.

Previous study demonstrated that more than half of the patients with cervical OPLL had coexistent OPLL in the thoracolumbar region [10]. Cervical OPLL predominantly affects men at a ratio of 2:1 to 3:1 [11]. Conversely, patients with cervical OPLL coexisting in the thoracolumbar spine were significantly more often women. DISH also predominantly affects men at a ratio varying from 2:1 to 7:1 [12,13]. The middle to lower thoracic spine in DISH is frequently ossified and introduces bony ankylosis, predominantly in men [12]. I also found that patients with DISH carried bony bridging and ankylosis not only in the anterior longitudinal ligament (ALL) of the spine but also in the sacroiliac (SI) joint [14]. On the other hand, AS is a common rheumatic disease affecting the spine and SI joints, predominately in men, and sacroiliitis is its earliest manifestation [15,16]. However, the hallmarks of SI joint ossification and its variation in OPLL have not been clarified. In the first study, I investigated the morphological changes in SI joints in individuals with and without OPLL.

While there is no doubt that both spinal inflammation and new bone formation occur in AS, less is known about OPLL and DISH in this regard. Previous study determined that the serum level of high-sensitivity C-reactive protein (hs-CRP) in patients with OPLL was higher than in those without OPLL, which indicated that systemic inflammation might be an important factor in the pathogenesis of OPLL [17], and serum hs-CRP levels might be useful in detecting slight inflammation at ectopic bone formation sites in spinal ligaments. Although the

specificity of hs-CRP could not be evaluated regarding the pathogenesis of OPLL, this study raised suspicion of a possible common denominator in these three disease entities. In the second study, I characterize and clarify evidence as to whether the ectopic bone formations of DISH in patients with OPLL are caused by inflammatory or degenerative processes. Followed by the third study, where I investigated whether inflammation, spinal ligament ossification and SI joint changes are also linked in OPLL.

AS is well known as a rheumatic inflammatory disease that belongs to the spondylarthritis group (SpA), and tumor necrosis factor α (TNF-α) exerts a central role. Recently, the interest moved to other cytokines, namely interleukin 23 (IL-23) and interleukin 17 (IL-17). IL-17 is mainly produced by the helper T cell type 17 following IL-23 production. In fact, several clinical studies demonstrated the elevation of IL-17 and IL-23 levels in the serum of AS patients [18-21]. In addition, there is increasing evidence that IL-17A blockade can be effective in patients with active SpA [22]. Indeed, applying IL-17A blockers such as Secukinumab and Ixekizumab has been approved and proven to be effective anti-inflammatory strategies in the clinical management of AS, beyond the TNF-α inhibitor agent [23]. Given the hypothesis that both AS and OPLL might share a similar inflammatory pathogenesis, targeting IL-17 could lead to potential effective therapeutic strategies for the treatment of ectopic ossification of spinal ligaments in OPLL. Therefore, in the fourth study, I investigate the relationship between the severity and morphology of heterotopic ossification in the spinal ligaments including SI joints, and serum IL-17 levels in patients with OPLL with or without DISH, as well as a non-OPLL group.

This dissertation is based upon the following publications:

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Tran Canh Tung Nguyen, Yasuhito Yahara, Taketoshi Yasuda, Shoji Seki, Kayo Suzuki, Kenta Watanabe, Hiroto Makino, Katsuhiko Kamei, Kanji Mori, Yoshiharu Kawaguchi.
Morphological characteristics of DISH in patients with OPLL and its association with high-sensitivity CRP: Inflammatory DISH.
Rheumatology (Oxford). 2022 Oct 6;61(10):3981-3988.

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Journal of Clinical Medicine. 2023 Jan 2;12(1):349.

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Chapter 2:

Sacroiliac joint variation in patients with ossification of the posterior longitudinal ligament

Abstract

Objectives: Ossification of the posterior longitudinal ligament (OPLL) reveals heterotopic ossification in the spinal ligament. OPLL also tends to ossify ligaments and entheses throughout the body. However, the hallmarks of sacroiliac (SI) joint ossification and its variation in OPLL have not been clarified. Here, I investigated the morphological changes in SI joints in individuals with and without OPLL.

Methods: 240 age- and sex-matched patients (OPLL+, 120; OPLL−, 120) were included in the study. SI joint variations were classified into 4 types: Type 1, normal or small peripheral bone irregularity; Type 2, subchondral bone sclerosis and osteophyte formation; Type 3, vacuum phenomenon; and Type 4, bridging osteophyte and bony fusion. Type 4 was further divided into 3 subgroups as previously described. Interactions between the ossified spinal region in OPLL and morphological changes in the SI joint were evaluated.

Results: SI joint ankylosis occurs more frequently in patients with OPLL (51.7%) than in those without (non-OPLL) (33.3%). The SI joint vacuum phenomenon (49.2%) was the main finding in non-OPLL. SI joint ankylosis in OPLL was characterized by anterior bridging and intra-articular fusion. OPLL patients with multilevel ossification tend to develop degeneration and ankylosis of the SI joints.

Conclusions: OPLL conferred a high risk of SI joint ossification compared with non-OPLL, and patients with extensive ossification had a higher rate of SI joint ankylosis. Understanding SI joint variation could help elucidate OPLL etiology and clarify the phenotypic differences in the SI joint between OPLL and other spinal disorders.

Keywords: OPLL, sacroiliac joint, spinal ligament ossification.

Introduction

Ossification of the posterior longitudinal ligament (OPLL), diffuse idiopathic skeletal hyperostosis (DISH) and ankylosing spondylitis (AS) are the three most common diseases that are characterized by ossification of the spinal ligaments [1-8]. Although OPLL, DISH, and AS share the features of bone proliferation and heterotopic ossification in the spine, the hallmarks of bone proliferation in the spine are dissimilar. OPLL and DISH have been traditionally described as noninflammatory disorders, observed mainly in elderly males [9-11]. By contrast, AS is characterized by a chronic sterile inflammation of joints and entheses, localized primarily in the spine and sacroiliac (SI) joints, predominantly affecting men and young adults [12,13].

The SI joint is the largest axial joint in the human body, linking the spine and the pelvis [14,15]. It is stabilized by strong ligaments and ensures mobility between the sacrum and the iliac bone [15]. Those ligaments enclose the cartilaginous parts of the SI joints and represent a major site of entheses [16]. Degeneration of the SI joint is characterized by joint space narrowing, osteophytes, subchondral sclerosis, cysts, vacuum phenomena, and ankyloses [17,18]. SI joint and spinal ankylosis (so-called “bamboo spine”) are considered a hallmark of AS. Moreover, SI joint bridging was strongly associated with entheseal reactions in other parts of the body. The presence of SI joint bridging indicates an intensive general entheseal process in the skeleton [19]. Previous study showed that SI joint involvement in DISH is characterized by radiographic osteophytes, para-articular bony bridging, and coexistent osteoarthritis [20]. In contrast, intra-articular bony fusion, erosion, and sclerosis in the SI joint are frequently observed in AS [20]. Although patients with OPLL, DISH, and AS have a general tendency toward ossification in the spine and other skeletal entheses, I found no study that has addressed the morphological changes in the SI joint and their variation in patients with OPLL.

Therefore, the aims of the current study were 2-fold. One was to elucidate the variation in SI joint changes in a patient with OPLL. The second was to explore the association between

OPLL ossification severity and SI joint variation with the aim of better understanding the hallmarks of bone proliferation in the spine and SI joint in patients with OPLL.

Materials and methods

This study analyzed and compared the characteristics of SI joint variation in 2 different types of spinal disorders. The first included 120 patients (79 men, 41 women; mean age 71.4 years) with cervical OPLL, who were allocated to the OPLL+ group. The other included 120 age- and sex-matched patients (81 men, 39 women; mean age 71.3 years) with degenerative spinal disease without OPLL, who were allocated to the OPLL− group (Table 1). All patients were diagnosed in Toyama university hospital based on the findings of plain radiographs and computed tomography (CT) images of the whole spine. This study was approved by the ethics committee at Toyama University Hospital (Clinical research number 21–22). Patients provided written consent for participation in this analysis.

	OPLL+ group	**OPLL− group**	***P*-value**
Number of patients	120	120	—
Male (%) / Female (%)	79 (67.5%) / 41 (32.5%)	81 (67.5%) / 39 (32.5%)	0.78
Mean Age	71.4 ± 8.2	71.3 ± 8.1	0.96
Male/Female	70.6 ± 8.3 / 73 ± 7.9	70.8 ± 8.1 / 72.6 ± 8.2	—

Table 1. Demographic data of the patients with ossification of the posterior longitudinal ligament and the controls.

As previously reported, morphological changes of the SI joint were divided into 4 types based on CT findings: Type 1, normal or small peripheral bone irregularity; Type 2, subchondral bone sclerosis and osteophyte formation; Type 3, vacuum phenomenon of the SI joint; and Type 4, bridging osteophyte and bony fusion of the SI joint. Type 4 was further classified into 3 subgroups depending on the site of bony ankylosis, as previously described:

anterior para-articular bridging (Type 4A), posterior para-articular bridging (Type 4B), and intra-articular ankylosis (Type 4C) (Figure 1) [20]. CT images of the whole spine and SI joint were evaluated by 2 orthopedic surgeons (NTCT and YY).

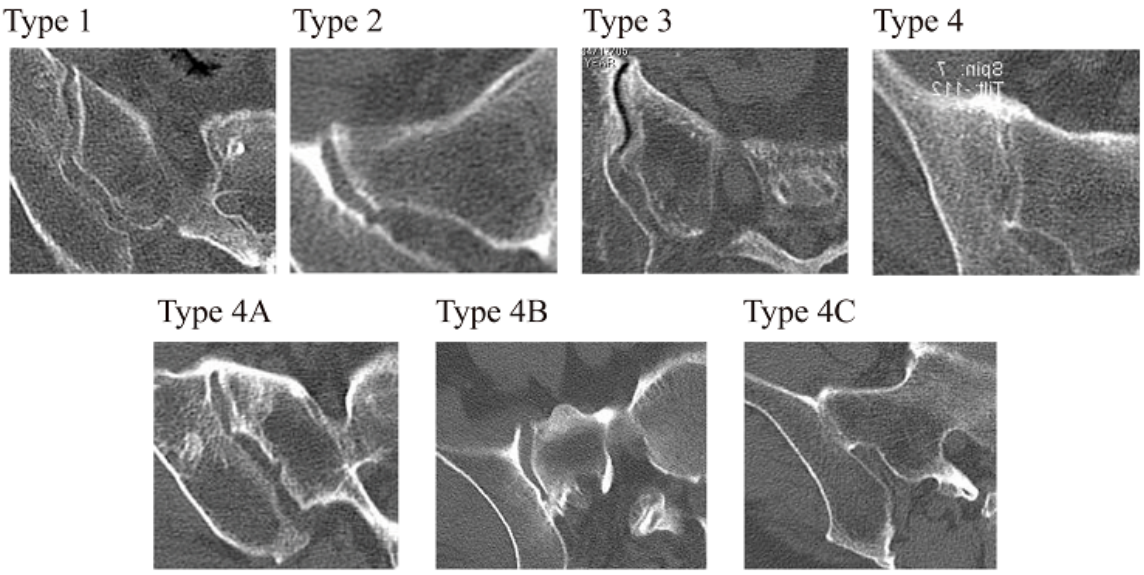
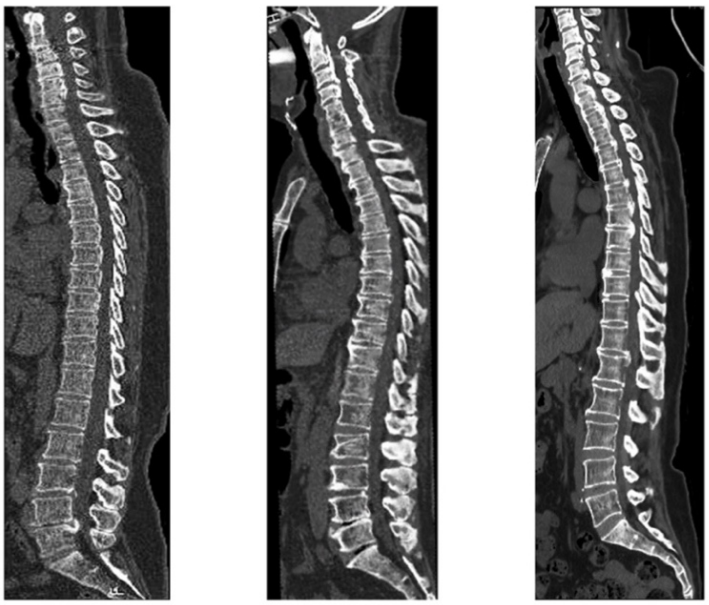


Figure 1. The morphological classification of SI joint variations. The morphological variations of SI joints were categorized into four types: type 1: normal or small peripheral bone nonunion similarity, type 2: subchondral osteosclerosis and osteophyte formation, type 3: vacuum phenomenon, and type 4: bridging osteophyte and bone fusion, which was further classified into three subtypes.

In patients with OPLL, the severity of the ossified lesions in the whole spine was evaluated using previously published ossification index (OS-index) [21]. In short, the index is determined by the sum of the number of vertebral bodies and intervertebral discs where OPLL is present. When the ossification area covers from the vertebral body level to the intervertebral disc level, the number of ossified lesions of each vertebral body level and intervertebral disc level is counted. The maximum OS-index is 14 in the cervical spine. The OS-index in the thoracic spine ranges from 0 to 24, and that in the lumbar spine from 0 to 11. The OPLL+ patients were also divided into 3 groups based on the severity of the ossification: Grade 1, OS-index <10; Grade 2, OS-index 10-19; Grade 3, OS-index ≥20 (Figure 2). The patients were

also divided into the following 2 groups according to the OPLL region: OPLL only in the cervical spine (C group) or in multilevel spinal regions (M group) [21]. The relationship was determined by comparing the region of OPLL and SI joint change.



Cervical OS-index	6	10	13
Thoracic OS-index	0	0	13
Lumbar OS-index	2	0	0
Whole spine OS-index	8	10	26
Grade	**Grade 1**	**Grade 2**	**Grade 3**

Figure 2. Representative sagittal computed tomography images of ossification of the posterior longitudinal ligament Grades 1, 2, and 3 of the OS-index. OS, ossification.

Statistical analysis

The data are presented as the mean value ± standard deviation. I employed Student's unpaired t-test for the statistical analysis of the difference in age and a 1-way analysis of variance followed by Tukey's *post hoc* test to compare the OS-index between the SI joint classification groups. I used the chi-squared test for the statistical analysis of the difference in sex, the type of SI joint variation between OPLL+ and OPLL− group, between the C group and the M group,

and between the OS-index grades. Excel statistical software (Statcel version 4; OMS, Tokorozawa, Japan) was used for the analysis, and $P < 0.05$ was considered as statistically significant.

Results

Both the OPLL+ and OPLL− groups presented SI joint degeneration, such as osteophyte formation (Type 2), SI joint vacuum phenomenon (Type 3), and SI joint fusion (Type 4). Type 1 involvement of the SI joint in both groups is a rare finding, and the majority of other cases were associated with SI joint degeneration more severe than Type 2 (Figure 3).

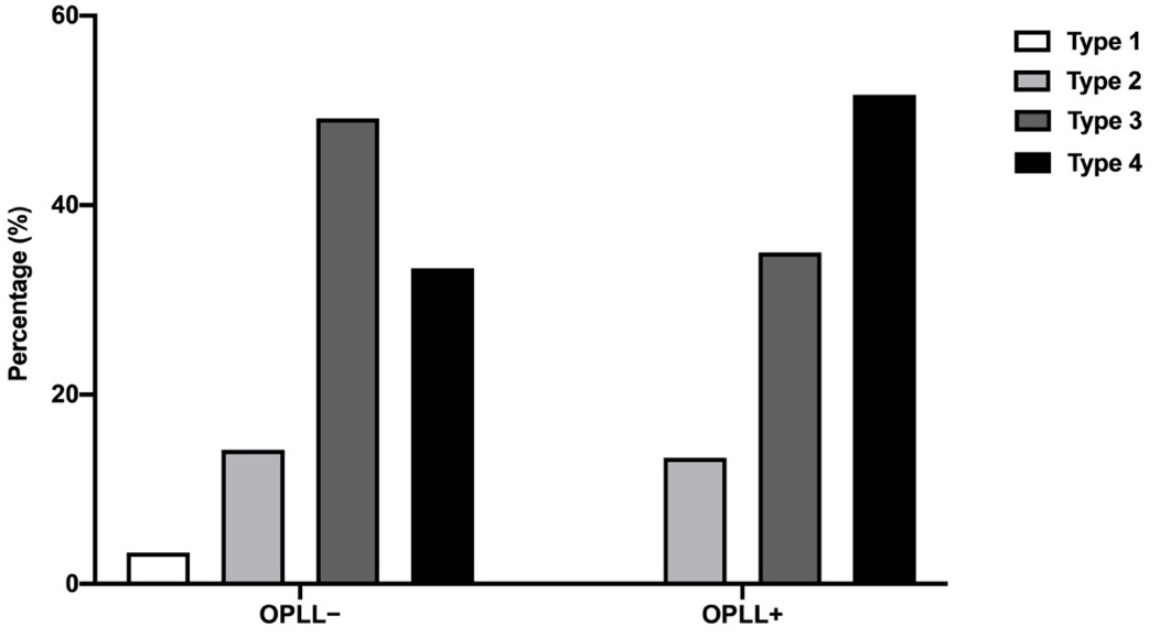


Figure 3. Prevalence of sacroiliac joint variation in patients with ossification of the posterior longitudinal ligament and controls.

There were significant differences in the prevalence of SI joint variation between the two groups ($P < 0.01$). Anterior and posterior bridging and fusion of the SI joint (Type 4) were detected frequently in the OPLL+ group (51.7%) compared with the OPLL− group (33.3%).

Conversely, the SI joint vacuum phenomenon (Type 3) was the most frequent change in the OPLL− group (49.2%)

In addition, there were significant differences in the ankylosis subclassification between the OPLL+ and OPLL− groups ($P < 0.001$). Anterior bridging of the SI joint (Type 4A) was the most common finding (82.9%) in the OPLL− group (Figure 4). On the other hand, anterior bridging (Type 4A; 33.9%) and intra-articular fusion (Type 4C; 56.5%) of the SI joint were mainly identified in the OPLL+ group.

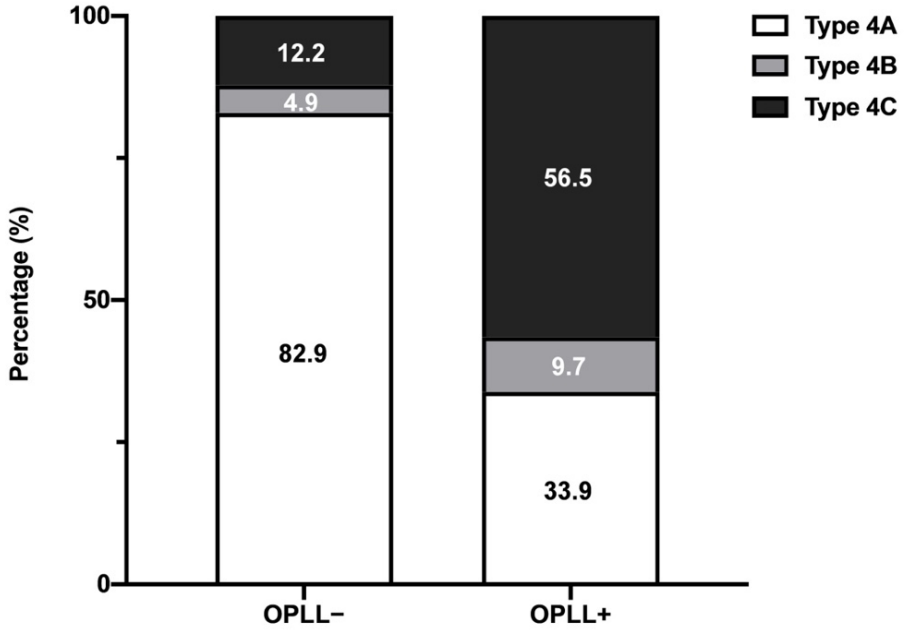


Figure 4. Prevalence of sacroiliac joint ankylosis in patients with ossification of the posterior longitudinal ligament and controls.

Approximately 60% of patients with cervical OPLL had coexistent OPLL in the other spinal regions (Table 2). Statistically significant differences were found in the SI joint classification and also in the Type 4 subclassification between the C group and the M group. The M group had a higher prevalence of SI joint Type 3 and Type 4 than the C group. Among those with Type 4 subclassification, no significant differences were found between the M group and the C group (Table 2).

SI joint classification	C group N = 45 (37.5%)	M group N = 75 (62.5%)	*P*-value
Type 1	0 (0%)	0 (0%)	<0.01
Type 2	12 (26.7%)	4 (5.3%)	
Type 3	16 (35.6%)	26 (34.7%)	
Type 4	17 (37.8%)	45 (60%)	
Subclassification	N = 17 (37.8%)	N = 45 (60%)	***P*-value**
Type 4A	8 (47.1%)	13 (28.9%)	0.3
Type 4B	2 (11.8%)	4 (8.9%)	
Type 4C	7 (41.2%)	28 (62.2%)	

Table 2. The relationship between the region of OPLL and SI joint variation. SI, sacroiliac; C, cervical; M, multilevel.

The average OS-index was 10.7 ± 6.2, and it ranged from 1 to 34. The OS-index of the whole spine appears to correlate with the morphological changes of the SI joint. Patients with OPLL who have SI joint fusion (Type 4) present a significantly higher OS-index compared with those with Type 2 (Figure 5).

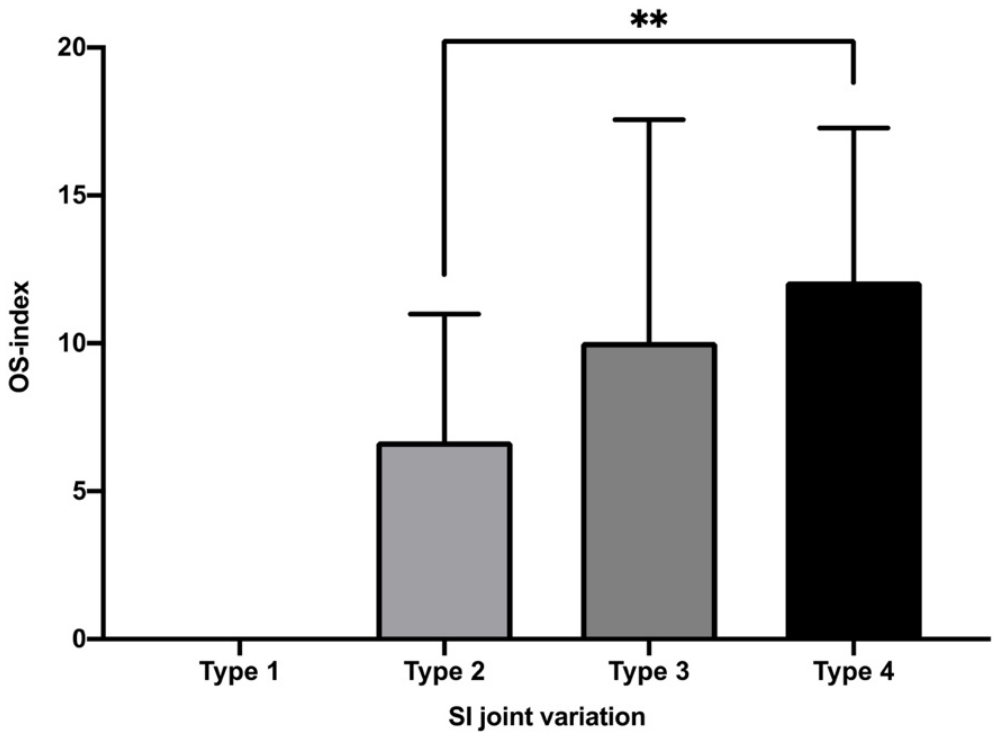


Figure 5. The OS-index of the whole spine is associated with SI joint variation. ***P* < 0.01; OS, ossification; SI, sacroiliac.

However, there were no differences between the OS-index and the subclassification of Type 4. I also found that the SI joint tended to show a high rate of bony bridging and intra-articular fusion in patients who had OS- index Grade 2 or Grade 3 (Table 3).

SI joint classification	OS-index			*P*-value
	Grade 1	**Grade 2**	**Grade 3**	
	N = 60 (50%)	N = 48 (40%)	N = 12 (10%)	
Type 1	0 (0%)	0 (0%)	0 (0%)	<0.001
Type 2	14 (23.3%)	2 (4.2%)	0 (0%)	
Type 3	28 (46.7%)	9 (18.8%)	5 (41.7%)	
Type 4	18 (30%)	37 (77.1%)	7 (58.3%)	
Subclassification	**OS-index**			***P*-value**
	Grade 1	**Grade 2**	**Grade 3**	
	N = 18 (30%)	N = 37 (77.1%)	N = 7 (58.3%)	
Type 4A	7 (38.9%)	14 (37.8%)	0 (0%)	0.17
Type 4B	2 (11.1%)	2 (5.4%)	2 (28.6%)	
Type 4C	9 (50%)	21 (56.8%)	5 (71.4%)	

Table 3. The relationship between OS-index Grade of the whole spine and SI joint variation. OS, ossification; SI, sacroiliac.

Discussion

The present study demonstrated that the bony bridging and ankylosis of the SI joint (Type 4) occurred more frequently in the OPLL+ group compared with the OPLL− group. By contrast, the SI joint vacuum phenomenon (Type 3) was the main finding in patients with degenerative spinal disease without OPLL rather than SI joint ankylosis. Additionally, patients with OPLL with multilevel ossification or a high OS-index had a strong tendency to develop degeneration and ankylosis of the SI joints. Thus, the current study revealed a novel SI joint characteristic in patients with OPLL. These findings could provide valuable insight for understanding the tendency for heterotopic ossification in the spine as well as in the SI joint in OPLL.

Given OPLL and DISH share the standard features of a generalized ossification tendency in the paraspinal ligaments, OPLL has recently been recognized as one of the clinical features of DISH, which is characterized by ossification along the anterolateral aspect of vertebral bodies and peripheral entheses. Historically, Resnick et al. showed that 50% of DISH cases were concomitant with OPLL [2], and a retrospective multicenter study from the Japanese Organization of the Study for Ossification of the Spinal Ligament reported the DISH prevalence in patients with cervical OPLL was 48.7%, with older age as a significant correlating factor [22]. Sato et al [23]. reported that OPLL plaque was contiguous with the ligamentous entheses to the vertebral body and to the deep layer of the PLL. Chen et al [24]. indicated that OPLL might actually be a type of enthesophyte, or enthesopathy, of DISH [25,26]. Given the sacroiliac joints are predominantly composed of fibrous connective tissues (fibrocartilage) and contain very little synovial fluid, these articulations can be considered entheses [27,28]. Entheses could therefore represent a site of endochondral ossification, resulting in para-articular bony ankylosis of the SI joint [29]. Dar et al. [19] studied 289 human male skeletons for the presence of SI joint bridging; they stated that SI joint bridging was strongly associated with “entheseal reactions” in other parts of the body. These features could explain why sacroiliac joint bridging occurs significantly more frequently in patients with OPLL as well as in DISH as a result of all-body entheseal reactions.

However, the current study showed that the SI joint fusion pattern was dissimilar between OPLL and DISH. Both anterior bridging (Type 4A; 33.9%) and intra-articular ankylosis (Type 4C; 56.5%) of the SI joint were mainly identified in patients with OPLL. On the other hand, Yahara et al. previously found that SI joint involvement in DISH was characterized by anterior bridging (Type 4A; 71.6%) rather than intra-articular ankylosis (Type 4C; 23%) [20]. OPLL and DISH share the features of bone proliferation and heterotopic ossification in the spine; however, the authors considered that the different underlying

mechanisms might partially affect the phenotype of the SI joint ankylosis, meaning these 2 diseases should be in different disease categories. Further study is warranted to understand the meaning of the phenotypic variation of the SI joint, and the data will provide a new way to recognize the characteristics of bone proliferation and ossification in OPLL as well as in other spinal disorders, such as DISH and spinal degeneration.

Enthesopathy had long been considered as a rheumatic or metabolic disorder [25,26], categorized as inflammatory or noninflammatory. AS is a type of spondyloarthritis, which is characterized by enthesitis of the spine and SI joint [12,30]. Sacroiliitis in AS includes joint erosions, joint space narrowing, sclerosis, and intra-articular ankyloses [20]. Given both AS and OPLL have a similar tendency toward SI joint intra-articular fusion, both diseases might have a similar etiology related to inflammatory-related enthesitis. Kawaguchi et al. previously found that the serum level of high-sensitivity C-reactive protein (hs-CRP) in the patients with OPLL was higher than that in individuals without OPLL, indicating that systemic inflammation might be an important factor regarding the pathogenesis of OPLL [31]. Other studies have reported that increased CRP was frequently observed in patients with painful axial AS and was correlated with both the activity and the functional severity of the disease [32,33]. Furthermore, elevated serum hs-CRP levels have occurred simultaneously with inflammation at the SI joint [34]. Therefore, some combination of systemic factors might act on these degenerative changes simultaneously in the SI joint and in the vertebral column in OPLL. These findings propose a hypothesis that there is some form of an inflammatory basis for the progressive bone formation in the spine and SI joint in OPLL. Further studies will be necessary to provide evidence that systemic inflammation leads to excessive bone formation in the spine and SI joint in OPLL. However, the insight about the interaction between inflammation and bone formation might help to understand its etiology and develop a novel anti-inflammatory intervention that has therapeutic effects on bone formation and the progression of OPLL.

A previous study indicated that the extent of OPLL in the whole spine is significantly associated with the extent of the OS-index [35]. The present study revealed that patients with a high OS-index tended to show a high rate of bony bridging and intra-articular fusion of the SI joint. This finding could suggest that SI joint degeneration reflects a general ossified tendency in the spinal canal and indicates the severity of the ossified lesions. In addition, the SI joint in the patients with OPLL at multiple spinal levels showed a greater tendency toward bony ankylosis compared with patients with OPLL only in the cervical region. Although the process of ossification remains unknown, it has been well documented that prognosis and surgical outcomes are poorer in patients with multiple-region OPLL than in patients with cervical lesions alone [36], and the increase in the area of ossified lesions affects the surgical results after laminoplasty [37,38]. Therefore, it is clinically important to clarify the factors associated with the extent of ossification not only in the whole spine but also in the SI joint. Thus, understanding the SI joint variation in OPLL and other forms of spinal disease could be, at least in part, helpful in its diagnosis and decision-making for the surgical strategy and in the development of novel therapeutics to prevent the bone formation and progression of OPLL.

This study had several limitations. First, the number of patients was small, and this is a retrospective and observational cross-sectional study. More extensive prospective studies are needed to substantiate these results and determine whether such SI joint findings correlate with the development of OPLL in a particular individual. Second, the relationship between systemic inflammation and OPLL regions or OS-index grade and SI joint degeneration was not evaluated. Furthermore, the presence of SI joint inflammation on magnetic resonance imaging was not determined. However, this study might provide a greater understanding of the pathological conditions with systematic inflammatory tendencies in human axial skeletons.

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Chapter 3:

Morphological characteristics of DISH in patients with OPLL and its association with high-sensitivity CRP: Inflammatory DISH

Abstract

Objectives: To characterize and clarify evidence as to whether the ectopic bone formations of diffuse idiopathic skeletal hyperostosis (DISH) in patients with ossification of the posterior longitudinal ligament (OPLL) are caused by inflammatory or degenerative processes.

Methods: Whole spine computed tomography (CT) and serum high-sensitivity C-reactive protein (hs-CRP) levels were obtained from 182 cervical OPLL patients (DISH+, n = 104; DISH–, n = 78). In the DISH+ group, ectopic bone formations were categorized into Flat and Jaggy types, then further divided into three subgroups: Group 1 (Jaggy-dominant pattern), Group 2 (Equivalence of pattern), and Group 3 (Flat-dominant pattern). Data were compared between the DISH+ and DISH– groups, and among the three subgroups.

Results: The upper thoracic spine was most affected by the Flat type, whereas the Jaggy type was more frequent in the middle and lower thoracic regions. There was no difference in hs-CRP levels between the DISH+ and DISH– groups. Among the three subgroups, hs-CRP levels in Group 3 (0.16 ± 0.09 mg/dL) were significantly higher than in Group 1 (0.04 ± 0.02 mg/dL) and Group 2 (0.08 ± 0.06 mg/dL). Higher levels of hs-CRP were associated with a greater number of vertebral units (VU) with Flat type formations ($\beta = 0.691$, $P < 0.0001$) and with a lesser number of VUs with Jaggy type formations ($\beta = -0.147$, $P = 0.036$).

Conclusion: The Flat type in DISH might be caused by an inflammatory pathogenesis rather than a degenerative process presented in the Jaggy type.

Keywords: DISH, OPLL, hs-CRP, inflammation.

Introduction

Diffuse idiopathic skeletal hyperostosis (DISH) is characterized by ossification of the anterior longitudinal ligament of the spine, as well as of various extraspinal ligaments in peripheral sites [1]. In 1976, Resnick and Niwayama proposed a pathological categorization of DISH as ligamentous ossification along the anterolateral aspects of at least four contiguous vertebrae, with relative preservation of disc height [1]. In DISH, the middle-to-lower thoracic spine is most frequently ossified, followed by the upper thoracic and lumbar vertebrae [1–4]. The pathogenic mechanisms leading to skeletal hyperostosis in DISH are poorly understood, and the etiology of the disease is still unknown. However, genetic, metabolic, endocrinologic, anatomic, environmental, and toxic factors have been suggested to be involved in the development of new bone formation in DISH [5,6].

DISH has been associated with other paraspinal ligament disorders, such as ossification of the posterior longitudinal ligament (OPLL) and ankylosing spondylitis (AS) [7–10]. Previous reports have suggested that DISH occurs concomitantly in cervical OPLL patients, with a prevalence of up to 50% [7–9]. These two diseases have been traditionally described as noninflammatory disorders, observed mainly in elderly males [2,11,12]. By contrast, AS is characterized by a chronic sterile inflammation of joints and entheses, localized primarily in the spine and sacroiliac joints (SIJ), predominantly affecting men and young adults [13,14]. Although DISH, OPLL, and AS share features of bone proliferation and ectopic ossification in the spine, their hallmarks of bone proliferation are dissimilar. Baraliakos et al. described two ways in which osteophytes grow; one growth pattern is more vertical. This pattern is prevalent in AS. The other is more horizontal and more frequently seen in DISH, which indicates that different mechanisms may play a role in ectopic new bone formation, such as inflammation and degeneration [15–17]. Yaniv et al. used computed tomography (CT) scans to evaluate the natural progression over ten years of these two types of bridging osteophyte formation in DISH;

a possible underlying inflammatory pathogenesis was indicated, rather than a degenerative one [18]. These findings are highly suggestive of a new hypothesis: those active local inflammatory or degenerative processes may contribute to the ectopic new bone formation in DISH. However, no scientific evidence for this has been found to date.

C-reactive protein (CRP) has been utilized in observational studies as a biomarker of low-grade inflammation [19]. Furthermore, high-sensitivity CRP (hs-CRP) is gaining popularity, as it permits the detection of lower levels of CRP. Previous study determined that the serum level of hs-CRP in patients with OPLL was higher than in those without OPLL, which indicated that systemic inflammation might be an important factor in the pathogenesis of OPLL [20], and serum hs-CRP levels might be useful in detecting slight inflammation at ectopic bone formation sites in spinal ligaments. Although OPLL is considered to be a partial phenotype of DISH [7]—and they may share a similar ossifying condition—I still do not know whether or not inflammation occurs in DISH.

In this study, I determined more precisely the characteristics of newly-formed ectopic bone in DISH using whole spine CT and classified two types: “Flat” and “Jaggy.” Further, I investigated its association with hs-CRP levels in order to explore the possibility that there is an inflammatory basis for bone formation in the spinal ligaments in DISH. It would provide clues that might help to better understand the etiology of ectopic bone formation in the spinal ligaments.

Materials and Methods

182 consecutive patients with cervical OPLL who were admitted to the Department of Orthopaedic Surgery at Toyama University Hospital, Japan between 2012 and 2014 were enrolled. Patients with comorbid cardiovascular diseases (CVD) such as coronary heart disease, hypertension, heart failure, valvular diseases, thromboembolic disease, stroke, or any

mention of CVD were excluded based on clinical examinations and medical history. All patients provided written, informed consent indicating their willingness to participate in the study, which was approved by the Ethics Committee at Toyama University Hospital. The diagnosis of DISH was based on CT scans of the whole spine, according to the Resnick criteria [1]. Patients who were diagnosed with DISH were allocated to the DISH+ group, while those without DISH were allocated to the DISH− group. Blood samples were collected from all participants on the morning of their hospital visit and were immediately stored at −80°C. Serum hs-CRP levels were measured by ultrasensitive latex-enhanced immunoassay using a BN ProSpec nephelometer (Dade Behring, Newark, NJ, USA) with a previously described method [20].

The morphology of the ectopic bony fusion formations in the anterior longitudinal ligament was categorized into two types, Flat and Jaggy, according to sagittal CT images, based on the approach of Baraliakos et al. [15–17] (Fig. 1). To differentiate between the two types, a 45° angle of the vertebral edge (VE) was set as the cut-off value. The VE is defined as the growth angle of ectopic bone formation to the anterior aspect of the vertebral body. The Jaggy type had at least one of the upper and lower VEs of new bone at an angle > 45°, whereas the Flat type had both upper and lower VEs of new bone at angles of ≤ 45°. Bony fusion types were evaluated by three orthopaedic surgeons (NTCT, YY, and HM) using CT images of the whole spine. Specifically, CT images of all participants were reviewed by two orthopaedic surgeons (NTCT and YY) who characterized the bony fusion types independently. If there was a difference in the classification, then the two observers discussed the differences until reaching a mutual consensus. Interobserver and intraobserver agreements were calculated as intraclass correlation coefficients (ICCs) by three orthopaedic surgeons (NTCT, YY, and HM) after evaluating bony fusion types in ten randomly patients. The prevalence of bony fusion types in each vertebral unit (VU), from C1 to S1, was also evaluated from sagittal images of whole

spine CT. A VU is defined as the area from the lower border of the upper vertebral body to the upper border of the lower vertebral body, according to the reports by Wanders et al. [21].

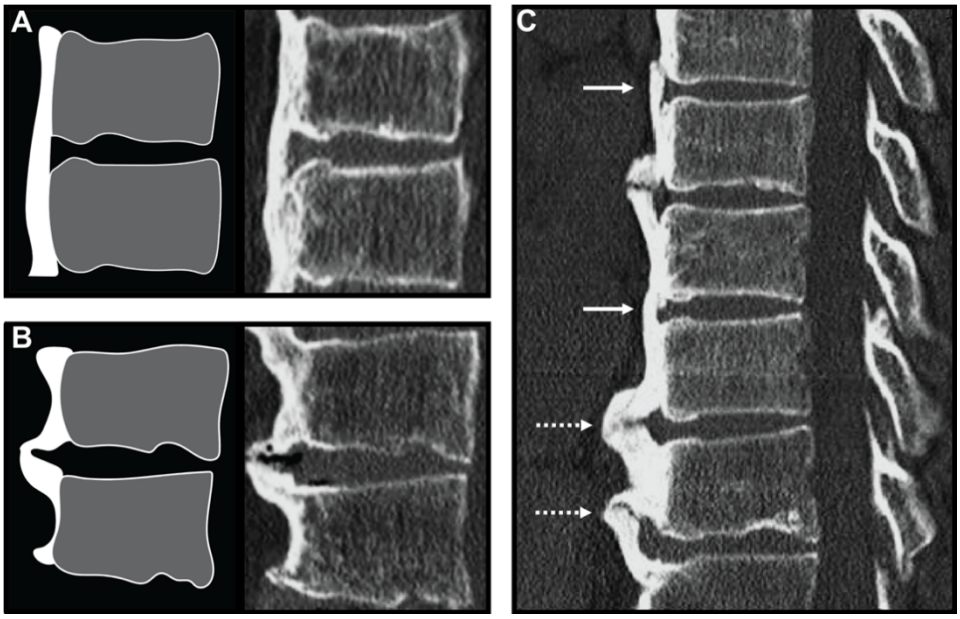

Figure 1. Representative morphological characteristics of new bone formation in DISH

(A) Flat, and (B) Jaggy, and (C) both types in a patient with DISH; Flat types are marked with solid arrows; Jaggy types are marked with dotted arrows.

These two types of ossification were shown to appear concomitantly in the same patient. Therefore, DISH patients were divided into three subgroups, according to the prevalence of bone formation types across VUs. Group 1 (Jaggy-dominant pattern) showed the Jaggy type of bone formation in ≥ 60% of VUs. Group 2 (Equivalence of pattern) showed Jaggy and Flat types in > 40% and < 60% of VUs. Group 3 (Flat-dominant pattern) showed the Flat type in ≥ 60% of VUs.

The data, including serum hs-CRP concentrations, were compared between the DISH+ and DISH– OPLL patients. In the DISH+ patients, data were also compared between the three subgroups. Furthermore, the relationship between hs-CRP and the number of VUs with Flat and Jaggy bone formation patterns were evaluated.

Statistical analysis

Age, height, weight, body mass index (BMI), serum hs-CRP concentration, and numbers of VUs were represented as mean ± standard deviation. The Student's unpaired t-test and one-way analysis of variance, followed by Tukey's post-hoc test, were used for statistical analysis of differences between the DISH+ and the DISH− groups, as well as for the three subgroups. The chi-squared test was used for statistical analysis of differences in gender. In the DISH+ group, Pearson's correlation was computed among hs-CRP levels and demographics (age, height, weight, BMI). Multiple linear regression analyses were performed to assess the associations of hs-CRP levels with number of Flat and Jaggy VUs. GraphPad Prism v9.0.1 software (GraphPad Software, San Diego, CA, USA) and Excel statistical software (Statcel version 4; OMS, Tokorozawa, Japan) were used for analysis. To clarify whether the results were lack of statistical power, post hoc power analyses were conducted using the G*Power 3.1.9.6 software program. A *P*-value of < 0.05 was considered statistically significant. ICC values were categorized as 0.00–0.20, poor agreement; 0.21–0.40, fair agreement; 0.41–0.60, moderate agreement; 0.61–0.80, strong agreement; and 0.81–1.00, almost perfect agreement.

Results

The baseline characteristics of patients, according to the presence or absence of DISH, are summarized in Table 1. Of 182 cervical OPLL patients, DISH was diagnosed in 104 (72 men and 32 women; mean age, 68.7 years). The comorbidity rate of DISH in patients with OPLL was 57.14%. There were 78 OPLL patients without DISH (40 men and 38 women; mean age, 63.9 years). Patients with DISH were significantly more likely to be elderly and to be male than those without DISH. However, there was no significant difference in serum hs-CRP concentration between the DISH+ group (0.09 ± 0.08 mg/dL) and the DISH− group (0.11 ± 0.16 mg/dL).

	DISH+	DISH−	*P*-value
Number	104	78	—
Age (years)	68.7 ± 9.6	63.9 ± 10.9	0.002
Sex (male/female)	72/32	40/38	0.01
Height (cm)	162.3 ± 8.7	160.0 ± 9.4	0.13
Weight (kg)	65.6 ± 16.1	61.8 ± 11.2	0.09
BMI (kg/m^2)	24.7 ± 5.0	24.1 ± 3.4	0.35
hs-CRP (mg/dL)	0.09 ± 0.08	0.11 ± 0.16	0.17

Table 1. Demographic data of OPLL patients with and without DISH

In the DISH+ group, a total of 1,125 VUs in 104 patients were evaluated. Coexistent Flat and Jaggy types were observed in 103/104 (99.04%) of the patients. The Jaggy type was observed in 51.64% of VUs (581/1,125); the Flat type was observed in 48.36% of VUs (544/1,125). The average number of VUs with the Flat type was 5.2 ± 3.7 (range, 0–19); the average number of VUs with the Jaggy type was 5.6 ± 2.7 (range, 1–14).

Between the three observers, average interobserver agreement was 0.899 ± 0.001. Intraobserver agreement was almost perfect, with ICC values of 0.964 (95% confidence interval (CI), 0.953–0.972), 0.892 (95% CI, 0.861–0.916), and 0.943 (95% CI, 0.926–0.956). These results indicate that my classification of the ectopic bone formation in DISH is simple to use, highly reliable, and reproducible.

To clarify the relationship between VU level and the occurrence of ectopic bony fusion types, I examined the distribution of the two types in DISH patients at each VU level, from C1 to S1. In the Flat type, the upper thoracic spine (T2/3–T5/6) was more affected than other VU levels; T4/5 (50.00%) was the most frequent terminal site of ossification (Fig. 2). The Jaggy type was predominant in the middle and lower thoracic spine (T7/8–T10/11), with the peak at T8/9 (73.08%) (Fig. 2). The thoracic level can represent a major site of ossification in patients with DISH.

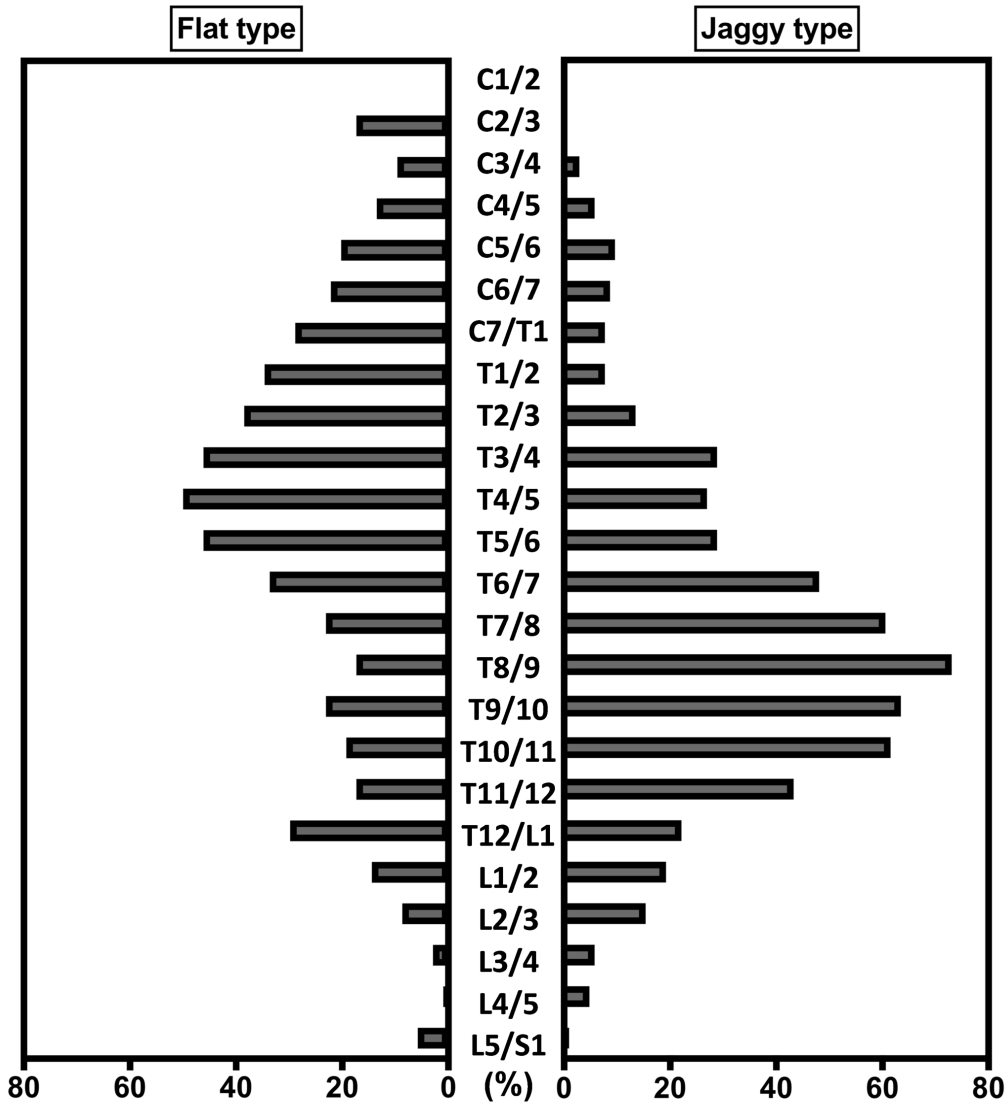

Figure 2. Prevalence of Flat and Jaggy types in each vertebral unit level

The demographic data of three subgroups are shown in Table 2, according to the prevalence of new bone formation types. No significant demographic differences were found between the subgroups; serum hs-CRP levels were not associated with age, height, weight, or BMI. The mean hs-CRP concentration was 0.04 ± 0.02 mg/dL in Group 1 (Jaggy-dominant pattern), 0.08 ± 0.06 mg/dL in Group 2 (Equivalence of pattern), and 0.16 ± 0.09 mg/dL in Group 3 (Flat-dominant pattern), which revealed a statistically significant difference between the three subgroups ($P < 0.0001$) (Fig. 3). Tukey's post-hoc analysis showed that patients with DISH from Group 3 (Flat-dominant) had significantly higher levels of hs-CRP, compared with

patients from Group 1 (Jaggy-dominant) and Group 2 (Equivalence of pattern) (Fig. 3). The post hoc power of one-way analysis of variance (calculated effect size 0.84, α = 0.05, total sample size 104, number of groups 3) was 1.00, thus suggesting that the significant difference finding might not be affected by the fact that I utilized a limited sample size.

	Group 1	**Group 2**	**Group 3**	***P*-value**
Number	47	25	32	—
Age (years)	70.4 ± 9.1	66.4 ± 10.1	68.0 ± 9.5	0.21
Sex (male/female)	32/15	20/5	20/12	0.36
Height (cm)	161.8 ± 9.3	163.2 ± 7.2	162.4 ± 9.3	0.85
Weight (kg)	64.2 ± 15.3	71.5 ± 14.1	63.3 ± 17.9	0.16
BMI (kg/m^2)	24.3 ± 4.3	26.9 ± 5.2	23.6 ± 5.4	0.06

Table 2. Demographic data of patients with DISH. Group 1, Jaggy-dominant pattern; Group 2, Equivalence of pattern; Group 3, Flat-dominant pattern.

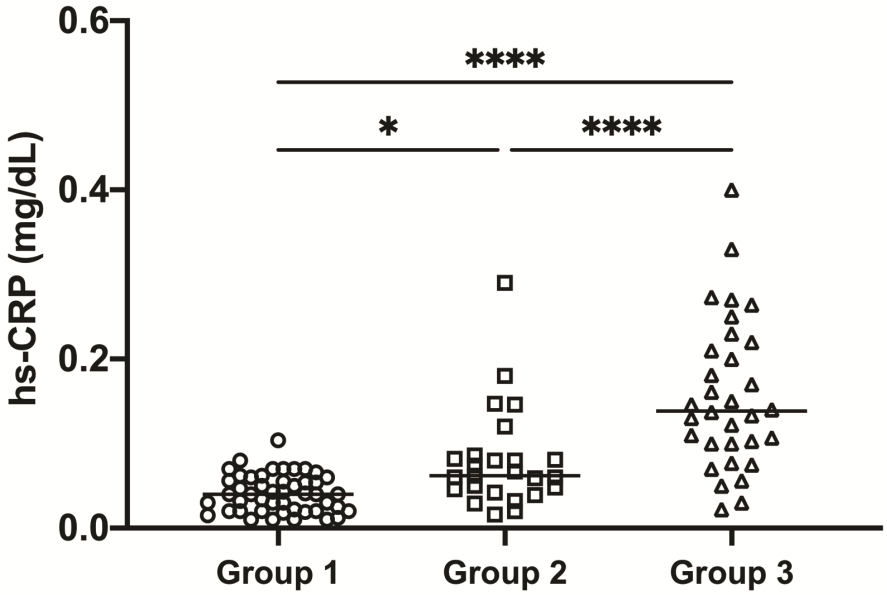

Figure 3. Comparison of serum high-sensitivity C-reactive protein levels between the three subgroups. Group 1 (Jaggy-dominant pattern); Group 2 (Equivalence of pattern); and Group 3 (Flat-dominant pattern). *, $P < 0.05$, ****, $P < 0.0001$.

Furthermore, the multiple linear regression analysis revealed a significant positive correlation between hs-CRP level and the number of VUs with Flat type ectopic bone growth ($\beta = 0.691$, $P < 0.0001$) (Table 3). In contrast, an inverse correlation between hs-CRP levels and number of VUs with the Jaggy type was also apparent ($\beta = -0.147$, $P = 0.036$) (Table 3).

	hs-CRP				
	Unstandardized coefficient		**Standardized coefficient**	**t**	***P*-value**
	B	**SE**	β		
(Constant)	0.034	0.016		2.205	0.030
Number of Flat VUs	0.014	0.001	0.691	10.021	<0.0001
Number of Jaggy VUs	−0.004	0.002	−0.147	−2.127	0.036

Table 3. Relationship (multiple liner regression) between serum hs-CRP level and number of VUs with Flat and Jaggy types. $P < 0.0001$; Adjusted $R^2 = 0.537$.

Discussion

Given that OPLL and DISH share features of bone proliferation and ectopic ossification in the spinal ligament, the relationship between them has attracted recent attention; many cases of coexistence of these two diseases have been reported [7–9]. In previous studies using whole spine CT, I demonstrated that more than half of patients with cervical OPLL had coexistent OPLL in the thoracolumbar region [22], and that >60% of the patients with OPLL had ossification of the ligamentum flavum, mainly in the thoracic spine [23]. Notably, the concomitance of DISH in these patients was also observed. In the present study, I compared cervical OPLL patients with and without DISH and revealed that the concomitance rate of cervical OPLL accompanying DISH was 57.14%**,** which is remarkably high.

Following previous work by Baraliakos et al. [15–17], I have demonstrated two types of osteophytes in DISH. One type includes osteophytes that are more vertically-oriented; the

other type includes more horizontally-oriented osteophytes; I have named them “Flat” and “Jaggy” types, respectively. These types were observed concomitantly in the anterior longitudinal ligament, mainly in the thoracic spine. As for segment level, the Flat type was predominant at the upper thoracic spine, whereas the Jaggy type was more frequently seen in the middle and lower thoracic regions, especially at the T8–9 level. It is interesting that the Jaggy type occurs at these sites. These findings are in keeping with previous studies that have investigated the regions of prevalence of ossification in DISH. Kagotani et al. found that ossification was observed mainly at middle-to-lower thoracic sites (T7/8–T9/10) [3]. Using whole spine CT assessments, Hiyama et al. and Hirasawa et al. both reported that T8–T10 was the most frequent level of ligamentous ossifications associated with DISH, and this may reflect a mechanical effect [2,4]. Degeneration in the thoracic spine has been shown to most frequently involve the lower thoracic segments, as the natural kyphosis curvature begins at almost the T8 level, leading to greater mobility and mechanical stress of the spine, due to flexion, extension, and rotation [24]. Thus, the pathogenesis of the Jaggy formation might be attributable to a degenerative process.

The Flat type’s bony fusion is similar to that found in AS, which is a frequent chronic inflammatory rheumatic disease that has the hallmarks of enthesitis of the spine (so-called “bamboo spine”) and SIJ. Chronic inflammation causes ossification those results in sacroiliitis and “syndesmophyte” formation in the spine, which is ossification without a sharp bone spur. In fact, the frequent occurrence of the Flat type in the upper thoracic spine indicates that mechanical stress is not an important role in its formation. Interestingly, the serum hs-CRP in Group 3 (Flat-dominant) was significantly higher than that in Group 1 (Jaggy-dominant) and Group 2 (Equivalence of pattern). Furthermore, serum hs-CRP levels were found to have a positive correlation with the number of Flat VUs and a negative correlation with the number of Jaggy VUs. Based on these results, I speculate that the Flat type might be caused by an

inflammatory rather than a degenerative process. The previous studies evaluated SIJ variations in DISH as well as OPLL and found that not only para-articular ankylosis but also intra-articular fusion (which is a hallmark of AS), were observed in the SIJ [25,26]. These findings and the present results are strongly suggestive that the pathogenesis of Flat type bony fusion in DISH might be associated with a local inflammation process and might share a similar mechanism of bone formation with AS.

Alternatively, another hypothesis suggest a mechanical effect on new bone formation in DISH, where vascular structure, acting as a natural barrier, and arterial blood flow and pressure may affect the progress of ossification [27]. Several researchers have reported that the presence of thoracic ossification is mainly contralateral to the aorta in DISH [28,29]. It has been reported that osteophytes at the cervical level are localized anteriorly (rather than lateral to the vertebral body), as arteries in the cervical area are located laterally [30]. Similarly, bone formation in the thoracic regions are thinnest, resulting in a flattening pattern, where segmental arteries run horizontally across the vertebral bodies [31]. In the present study, the Flat type was located predominantly in the upper thoracic region, where the aortic arch traverses downwards at the fourth thoracic vertebra [32]. Thus, it can be proposed that the aorta might play a role as a natural barrier; aortic pulsation might act to inhibit osteophyte development in the upper thoracic spine in DISH, leading to the Flat formation. Moreover, in the cervical region, the presence of the Flat type was observed more frequently than the Jaggy type. Therefore, it can also be proposed that the vertebral arteries and carotid arteries in the laterally and the trachea and esophagus in the anteriorly might act as a natural barrier to inhibit osteophyte development in the cervical spine in DISH, leading to the Flat formation as well. As a result of thinness, ankylosed spine, patients with DISH in the cervical spine are at greater risk of developing disc-level fractures. This finding was supported by several authors reporting that, in the cervical region, fractures through the disc space were most common in DISH patients [33,34]. In

addition, if the hypothesis that the vascular structure acting as a natural barrier is true, with the unlimited anteriorly bone formation might explain the ventral displacement of the trachea and esophagus, leading to the presentation of symptoms such as dysphagia and airway obstruction, which was reported to be observed in patients with DISH in the cervical spine [30]. However, all previous studies have based their evaluations only on the axial segment, not the sagittal view, and the evidence to support this hypothesis is also lacking.

Studies investigating the genetic components of DISH, OPLL and AS are in progress, and several susceptibility genes have been reported. It is well known that human leukocyte antigen B27 (HLA-B27) has the strongest correlation with AS; it is carried by 80%–95% of AS patients [35]. Given both AS and DISH might have a similar inflammatory pathogenesis, several authors have found an increased frequency of HLA-B27 among DISH patients; however, most studies did not confirm this association [36]. Recent studies have revealed that the cytokines interleukin 23 and interleukin 17 (IL-17) play important roles in the pathogenesis of AS, along with tumor necrosis factor. Inhibition of IL-17 has proven to be an effective anti-inflammatory strategy in the clinical management of AS [37]. Also, Dickkopf-related Protein 1, a natural inhibitor of Wnt signaling, has been suggested to play an essential role in regulating the bone remodeling and pathogenesis of DISH, OPLL, AS, and ossification of yellow ligament [38–40]. These observations, taken together with the current study's findings, suggest that establishing the inflammatory factors of DISH could lead to potential effective therapeutic modalities for the treatment of ectopic ossification in spinal ligaments. Future studies containing genome-wide analyses are warranted to confirm whether systemic and/or local inflammation is involved in the pathogenesis of DISH.

This study had several limitations. First, it is a retrospective and observational, cross-sectional study with a small number of patients. Second, serum hs-CRP levels were measured at only one time point. Third, elevations in hs-CRP level have already been shown to be

associated with not only CVD [41] but also metabolic syndromes [42] and other factors, for instance, obesity, smoking, or various disease states [43]. Herein, since I cannot exclude all the patients with numerous factors listed above, it is not evident whether inflammation occurs at the site of DISH or hs-CRP is just a marker of comorbid condition. Fourth, the study population was selected from patients with cervical OPLL. OPLL was previously considered specific to Asian peoples, whereas DISH is well known in Caucasians. Therefore, the results might not be a true representative of the general population. More extensive, prospective studies with larger datasets are needed to substantiate the curent study's results and to extend the diagnostic criteria for DISH. However, insight about the interaction between inflammation and bone formation may help us to understand the etiology of ectopic bone formation and to develop a novel anti-inflammatory intervention with therapeutic effects against the progression of DISH.

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Chapter 4:

Association of inflammation, ectopic bone formation, and sacroiliac joint variation in ossification of the posterior longitudinal ligament

Abstract:

Objectives: Ossification of the posterior longitudinal ligament (OPLL) is considered a multifactorial condition, and its connections with inflammation, as well as sacroiliac (SI) joint ankylosis, have been discussed recently. In this study, I investigated whether inflammation, spinal ligament ossification and SI joint changes are linked in OPLL.

Methods: The whole spinal computed tomography and serum high-sensitive C-reactive protein (hs-CRP) levels were obtained in 162 patients with cervical OPLL. Ossification lesion was categorized as plateau and hill shapes. Accordingly, patients were divided into plateau-shaped (51 males and 33 females; mean age: 67.7 years) and hill-shaped groups (50 males and 28 females; mean age: 67.2 years). SI joint changes were classified into four types and three subtypes, as previously described. Interactions among ossification shapes, hs-CRP levels, and morphological changes in the SI joint were investigated.

Results: The plateau shape was more common in the vertebral segments (59.5%), compared to the hill shape, which was predominant in the intervertebral regions (65.4%). Serum hs-CRP levels in the plateau-shaped group (0.11 ± 0.10 mg/dL) were significantly higher than that in the hill-shaped group (0.07 ± 0.08 mg/dL). SI joint intra-articular fusion was the main finding in the plateau-shaped group and showed significantly higher hs-CRP levels compared to the anterior para-articular bridging, which more frequently occurred in the hill-shaped group.

Conclusion: These findings suggested a possible inflammation mechanism that might contribute to the new bone formation in OPLL, particularly the plateau shape.

Keywords: OPLL, hs-CRP, sacroiliac joint, inflammation.

Introduction

Ossification of the posterior longitudinal ligament (OPLL) is characterized by the ectopic bone formation that occurs in the PLL due to hyperostotic changes [1]. The progression of these ossified lesions may lead to neural compression, which can cause serious symptoms, such as myelopathy and/or radiculopathy that require clinician attention. OPLL pathogenesis is multifactorial and remained to be fully elucidated. Consequently, there is no disease-modifying treatment; and thus clinical interventions are mainly focused on symptom relief, with severe cases managed through surgical treatment to relieve spinal cord compression.

OPLL has been related to several other entities, such as diffuse idiopathic skeletal hyperostosis (DISH) and ankylosing spondylitis (AS) [2–5]. DISH and OPLL are most frequently characterized by degenerative changes without the appearance of sacroiliitis [6–8], while AS is a common chronic inflammatory rheumatic disease that starts in the sacroiliac (SI) joints and spreads to the spine [9,10]. The radiographic appearance of both diseases is very similar, but with different underlying pathology. Recently, two types of new bone formation in the anterior longitudinal ligament (ALL) in DISH, as well as AS, have been reported [11–14]. One is thin, flat, vertical, and prevalent in patients with AS, and the other is thick, jaggy, horizontal, and more common in patients with DISH. The previous study classified the new bone formation in the ALL in DISH into flat and jaggy types [15]. The jaggy-type ossification is caused by a degenerative process, while the flat-type might be due to an inflammatory process similar to the “syndesmophyte” pattern in AS. Interestingly, these two types of new bone formation are also observed in OPLL and have been classified into plateau and hill shapes by Iwasaki et al. [16]. However, the author mainly focused on the surgical strategies but did not provide details and possible mechanisms of the lesions themselves.

Multiple studies have looked into associated factors with ossification development in OPLL. A close relationship between OPLL, as well as OPLL progression, and high-sensitivity

C-reactive protein (hs-CRP) has been proposed, suggesting an inflammatory mechanism might be related to OPLL pathogenesis [17]. The pathological bone formation has also occurred in the SI joint, causing SI joint ankylosis, including two forms. One is SI joint fusion through the auricular surface (intra-articular), which is characterized by articular inflammation and considered a manifestation of AS [10]. The other is SI joint bridging, but separate from the auricular component of the joint (para-articular), which was thought to be associated with DISH [18]. However, previous studies that reported the presence of SI joint intra-articular ankylosis in patients with DISH and those with OPLL [19,20] may suggest that the diseases have a pathogenetic route similar to AS. Although there is no doubt that spinal inflammation, SI joint intra-articular fusion and new bone formation both occur in AS [10,12], less is known about OPLL in this regard. Since the occurrence of the flat-type bony fusion in DISH might be related to inflammation, I hypothesize that the plateau shape of new bone formation in OPLL might also be related to the inflammation in the spinal column and perhaps inflammatory elsewhere in the SI joint.

The present study took advantage of the previous classification approach [16] and classified the new bone formation in OPLL into “plateau-shaped” and “hill-shaped.” Further, the characteristics of these two patterns and related factors, including serum hs-CRP levels and SI joint ankylosis, were investigated to elucidate the possible mechanisms that might promote the new bone formation in OPLL.

Materials and Methods

Patient selection

This study was approved by the Ethics Committee (Toyama University Hospital). All patients provided standard written informed consent to participate in the study. 162 patients who were admitted to the Department of Orthopedic Surgery at Toyama University Hospital, Japan from

2012 to 2014 were enrolled. Patients with comorbid cardiovascular diseases were excluded based on clinical examinations and medical history.

Measurement of serum hs-CRP level

Peripheral blood samples were collected from all patients on the morning of their hospital visit, and the serum was immediately frozen at −80°C until analysis. An ultrasensitive latex-enhanced immunoassay was obtained to measure the serum hs-CRP concentrations as previously described, using a BN ProSpec nephelometer (Dade Behring, Newark, NJ, USA) [17]. Results were expressed as milligrams per deciliter (mg/dL).

Radiographic assessment

OPLL diagnosis was based on plain radiograph observation and computed tomography (CT) imaging of the whole spine.

The ectopic bone formation shape in the PLL was categorized into the plateau and hill shapes using sagittal-view CT and based on the approach of Iwasaki et al. [16] (Figure 1). Briefly, the relatively narrow spinal canal without any localized massive ossification was assumed to present the plateau-shaped bone formation. Contrastingly, the hill-shaped ossified pattern is defined by a massive beak-shaped ossification that is localized to certain levels. Accordingly, patients were divided into two groups as follows: plateau-shaped and hill-shaped groups. The characteristics of these two patterns were assessed as per convention in literature [21], including the maximum ossified PLL thickness and its level, the space available for the spinal cord (SAC), and the occupying ratio (OR) of OPLL. OPLL types were also classified as continuous, segmental, mixed, or localized according to the Japanese Investigation Committee for Ossification of the Spinal Ligament criteria [22].

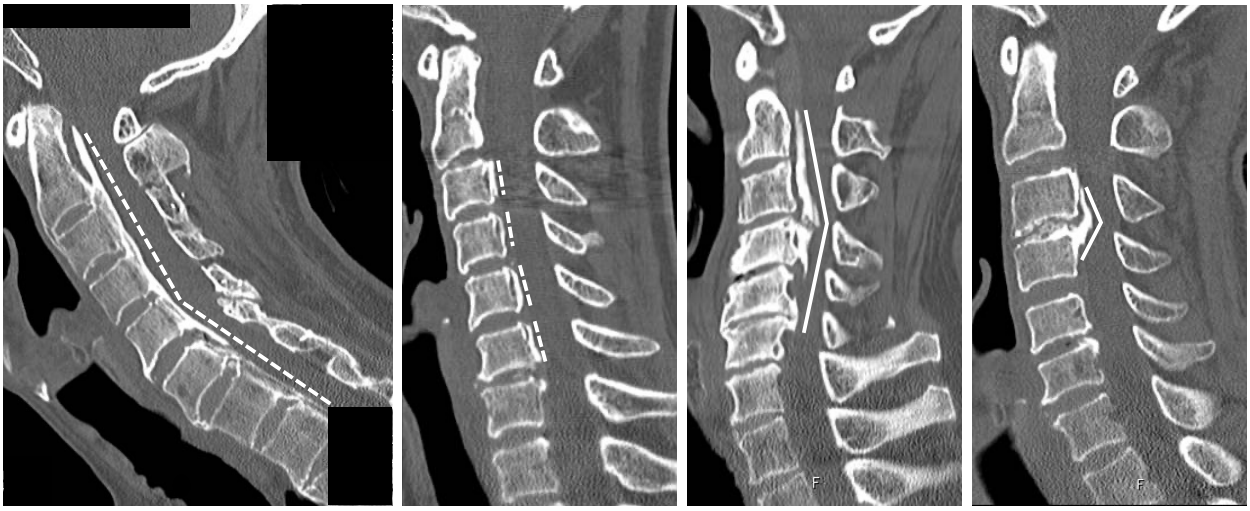

Figure 1. Representative OPLL type and OPLL shape. Plateau shapes are marked with dashed lines and indicate the relatively narrow spinal canal without any localized massive ossification. Hill shapes are marked with solid lines, defined by a massive beak-shaped ossification localized to certain levels [16]. The radiographs display (from left to right) plateau-shaped ossification in the continuous type OPLL, plateau-shaped segmental type of OPLL, hill-shaped ossification in a beak configuration, and hill-shaped circumscribed type of OPLL.

Morphological SI joint changes were categorized into 4 types based on the previously reported criteria as follows [19,20]: type 1: normal or small peripheral bone irregularity; type 2: subchondral bone sclerosis and osteophyte formation; type 3: vacuum phenomenon; and type 4: bridging osteophyte and bony fusion. Type 4 was further divided into three subgroups as follows: type 4A: anterior para-articular bridging; type 4B: posterior para-articular bridging; and type 4C: intra-articular ankylosis.

Two orthopedic surgeons independently evaluated the bone formation shapes and SI joint changes. Different opinions are discussed by the two examiners before making the final decision. Inter- and intra-observer agreements of the OPLL shapes classification were calculated as intraclass correlation coefficients (ICCs) using 20 randomly selected patients.

Data analysis and statistics

Serum hs-CRP levels were compared among two OPLL shape groups and four OPLL types, and the occurrences of OPLL shapes and types were evaluated. Furthermore, the interactions between serum hs-CRP levels, SI joint changes, and OPLL shapes were investigated.

Data were presented as the mean ± standard deviation. The differences in the quantitative data were tested by the Student's unpaired t-test and the one-way analysis of variance, followed by Tukey's post-hoc test. Categorical data differences were measured by the chi-squared test. Statistical analysis was conducted with GraphPad Prism v9.0.1 software (GraphPad Software, San Diego, CA, USA) and Excel statistical software (Statcel version 4; OMS, Tokorozawa, Japan). Post hoc power analyses were conducted using G*Power version 3.1.9.6. *P*-values of < 0.05 were considered statistically significant. ICC values were categorized as poor (0.00–0.20), fair (0.21–0.40), moderate (0.41–0.60), strong (0.61–0.80), and almost perfect agreements (0.81–1.00).

Results

The baseline characteristics of enrolled patients are summarized in Table 1. Of the 162 patients with cervical OPLL, the plateau-shaped group was slightly predominant with 84 patients (51 males and 33 females; mean age: 67.7 years), whereas the hill-shaped group consisted of 78 patients (50 males and 28 females; mean age: 67.2 years). No significant difference was found in the demographic data between the two groups.

	Plateau-shaped	**Hill-shaped**	***P*-value**
Number of patients, n	84	78	—
Sex, n (%)			
Male	51 (60.7)	50 (64.1)	0.75
Female	33 (39.3)	28 (35.9)	
Age, year, mean ± SD	67.7 ± 10.4	67.2 ± 10.0	0.79
BMI, kg/m^2, mean ± SD	24.9 ± 4.0	25.5 ± 4.8	0.48

Max ossified PLL thickness level, n (%)			
C2	13 (15.5%)	1 (1.3%)	0.011
C2/3	10 (11.9%)	7 (9.0%)	
C3	6 (7.1%)	3 (3.8%)	
C3/4	11 (13.1%)	18 (23.1%)	
C4	9 (10.7%)	10 (12.8%)	
C4/5	3 (3.6%)	11 (14.1%)	
C5	9 (10.7%	7 (9.0%)	
C5/6	4 (4.8%)	8 (10.3%)	
C6	8 (9.5%)	5 (6.4%)	
C6/7	6 (7.1%)	7 (9.0%)	
C7	5 (6.0%)	1 (1.3%)	
Vertebral	50 (59.5%)	27 (34.6%)	0.002
Inter-vertebral	34 (40.5%)	51 (65.4%)	
Max ossified PLL thickness, mm, mean ± SD	0.55 ± 0.17	0.67 ± 0.17	<0.0001
C2	0.64 ± 0.11	0.60	0.003
C2/3	0.62 ± 0.17	0.63 ± 0.13	
C3	0.35 ± 0.10	0.49 ± 0.06	
C3/4	0.63 ± 0.15	0.69 ± 0.17	
C4	0.53 ± 0.16	0.67 ± 0.16	
C4/5	0.61 ± 0.04	0.73 ± 0.13	
C5	0.43 ± 0.12	0.62 ± 0.18	
C5/6	0.53 ± 0.21	0.69 ± 0.18	
C6	0.44 ± 0.14	0.58 ± 0.20	
C6/7	0.63 ± 0.15	0.73 ± 0.26	
C7	0.62 ± 0.17	0.60	
SAC, mm, mean ± SD	0.80 ± 0.20	0.66 ± 0.28	0.002
Occupying ratio, %, mean ± SD	40.9 ± 10.4	51.5 ± 14.4	<0.0001
≥60%, n (%)	2 (2.4%)	22 (28.2%)	<0.0001
<60%, n (%)	82 (97.6%)	56 (71.8%)	

Table 1. Demographic data and ossified lesion characteristics of patients with OPLL.

PLL, posterior longitudinal ligament; SAC, space available for the cord.

The OPLL shape evaluation revealed strong inter-observer agreement (ICC = 0.796, $P < 0.001$) and almost perfect intra-observer agreement (ICC 1 = 0.900, $P < 0.001$; ICC 2 = 0.813, $P < 0.001$), indicating substantial reliability.

The ossified lesion characteristics are also presented in Table 1. The plateau shape showed a lower maximum thickness, OR, and higher SAC rather than the hill shape. The ectopic new bone formation was seen at both vertebral and intervertebral levels. Interestingly, the vertebral segments were more affected than the intervertebral segments in the plateau-shaped group, with a prevalence of 59.5%. Conversely, the maximum thickness of ossified lesions occurred mainly in the intervertebral sites in the hill-shaped group (65.4%).

Figure 2 shows the relationship between OPLL shapes and types, wherein the OPLL shapes differed among the types. The more prevalent plateau shape was included in the continuous and segmental types, while the more predominant hill shape was found in the mixed and localized types.

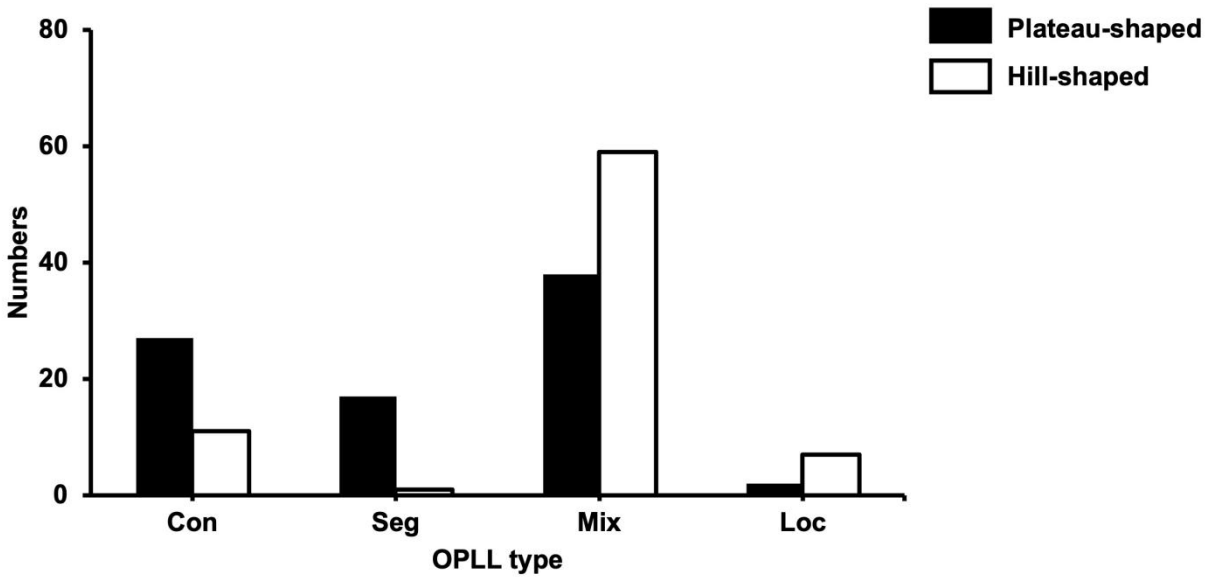

Figure 2. The relationship between OPLL type and OPLL shape. Con, continuous; seg, segmental; mix, mixed; loc, localized.

The associations between the OPLL shapes, SI joint variation, and hs-CRP levels were also investigated. The mean hs-CRP concentration was 0.11 ± 0.10 mg/dL in the plateau-

shaped group, and 0.07 ± 0.08 mg/dL in the hill-shaped group, yielding a statistical difference between groups (P = 0.004) (Figure 3A). Additionally, patients with the continuous type had significantly higher hs-CRP levels compared to patients with the localized type (P = 0.03) (Figure 3B).

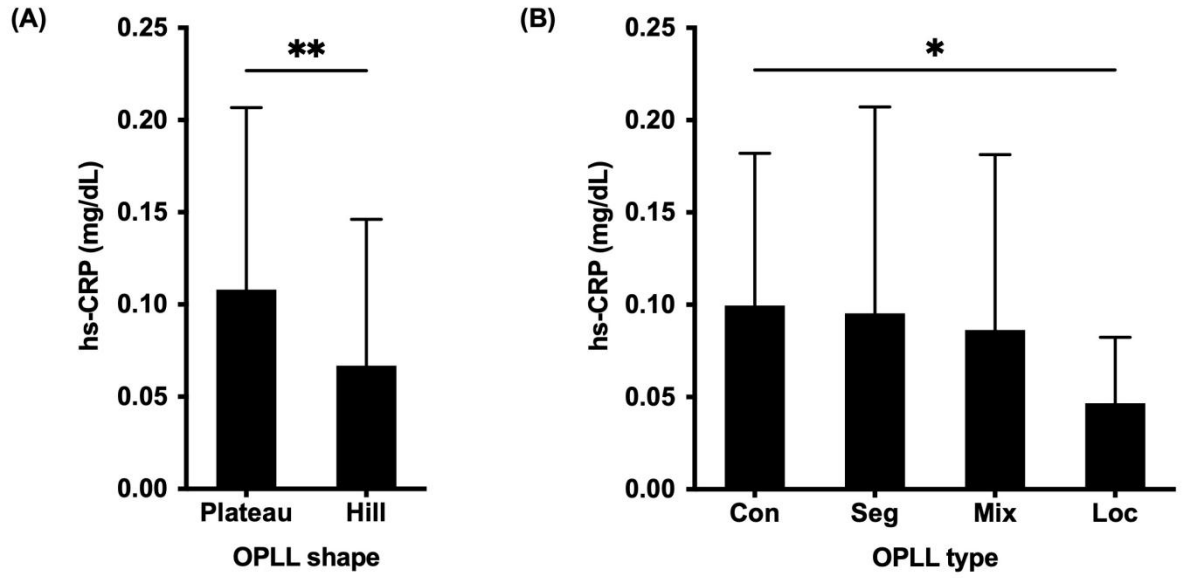

Figure 3. Comparison of serum high-sensitivity C-reactive protein levels between the OPLL types (A) and OPLL shapes (B). *P < 0.05; **P < 0.01. Con, continuous; seg, segmental; mix, mixed; loc, localized.

All patients with OPLL presented SI joint changes, such as osteophyte formation (Type 2), SI joint vacuum phenomenon (Type 3), and SI joint fusion (Type 4). No significant differences were found in the hs-CRP levels between patients with four types of SI joint variation (Figure 4A). However, the hs-CRP levels were significantly higher in patients with SI joint intra-articular fusion (Type 4C) compared to patients with SI joint anterior para-articular bridging (Type 4A) (P = 0.04) when compared between SI joint subtypes (Figure 4B).

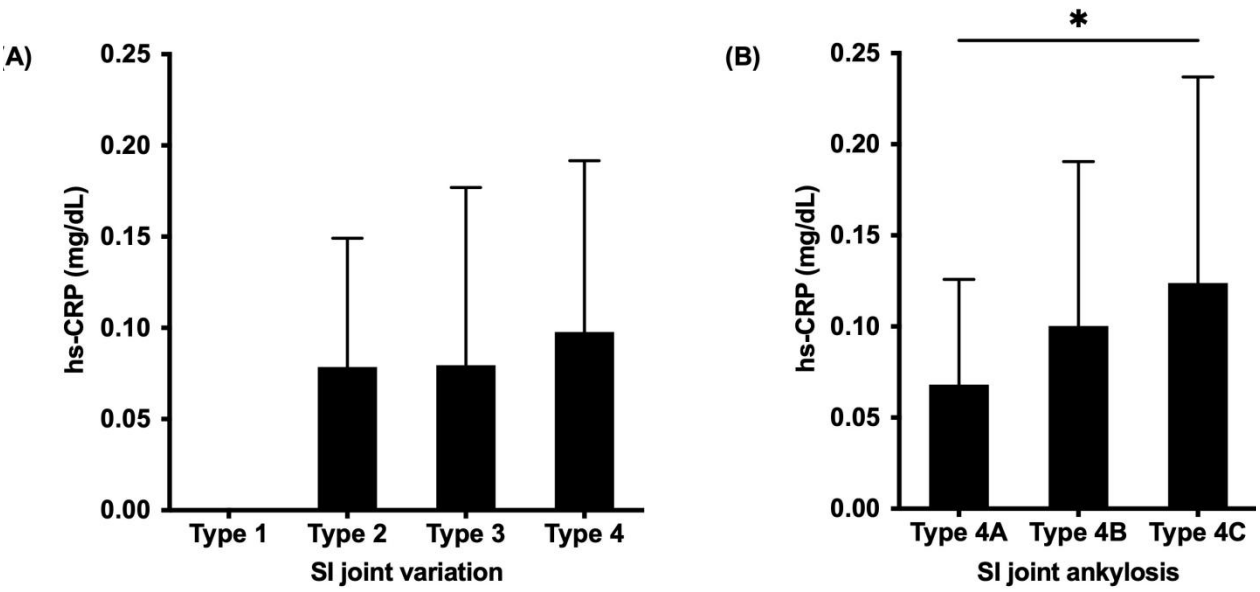

Figure 4. Comparison of serum high-sensitivity C-reactive protein levels between SI joint types (A) and subtypes (B) classifications [19,20]. *$P < 0.05$.

Consistent with the hs-CRP criteria, statistical differences were found between the SI joint ankylosis and OPLL shapes (Table 2). SI joint intra-articular fusion (Type 4C) was frequently detected in the plateau-shaped group (62.2%). Conversely, the SI joint anterior bridging was the most common change in the hill-shaped group (57.1%).

	Plateau-shaped	**Hill-shaped**	***P*-value**
SI joint variation	N = 84	N = 78	—
Type 1, n (%)	0 (0%)	0 (0%)	0.27
Type 2, n (%)	12 (14.3%)	13 (16.7%)	
Type 3, n (%)	35 (41.7%)	23 (29.5%)	
Type 4, n (%)	37 (44.0%)	42 (53.8%)	
SI joint ankylosis	N = 37	N = 42	—
Type 4A (Anterior), n (%)	10 (27.0%)	24 (57.1%)	0.03
Type 4B (Posterior), n (%)	4 (10.8%)	3 (7.1%)	
Type 4C (Intra-articular), n (%)	23 (62.2%)	15 (35.7%)	

Table 2. Occurrences of OPLL shapes and SI joint variation.

The post hoc power of the Student's unpaired t-test for the comparison of serum hs-CRP levels between the OPLL shapes (calculated effect size 0.44, $\alpha = 0.05$, total sample size 162) was 0.80. The post hoc power of the one-way analysis of variance for the comparison of serum hs-CRP levels between SI joint ankylosis (calculated effect size 0.30, $\alpha = 0.05$, total sample size 79) was 0.85. The post hoc power of the chi-squared test for the association between OPLL shapes and SI joint ankylosis (calculated effect size 0.68, $\alpha = 0.05$, total sample size 79) was 0.999. Thus, suggesting that the modest sample size in the present study was sufficient to detect the significant interaction effects in my findings.

Discussion

Although numerous classification systems for OPLL exist in academic circles, most of these systems describe and classify the shape of the ossification to provide more detailed information for diagnosis and optimize surgical guidance [23]. However, no classification addressed the key question that surrounds the underlying mechanism of ectopic ossification in OPLL. The present study assessed the morphological characteristics of new bone formation in OPLL and categorized them into plateau and hill shapes using spinal CT and utilizing the established morphological classification [16]. These two opposite appearance patterns have attracted recent attention by suggesting a difference in the ossification mechanism that might exist. Therefore, I further investigated its related factors to elucidate a part of OPLL ossification pathogenesis.

Previous studies have described two ways of osteophytes growth in DISH and AS, including one growth pattern that is more vertical and the other is more horizontal [11–14]. While horizontal growth is common in DISH, vertical growth is more prevalent in AS and indicates possible inflammatory pathogenesis. The present study suggests that spinal inflammation and plateau shape are linked in OPLL since the serum hs-CRP level in the

plateau-shaped group was significantly higher than that in the hill-shaped group. This result is consistent with my previous work and other studies [11–15], demonstrating a similar mechanism between the plateau shape OPLL, the syndesmophyte AS, and the flat-type DISH. Thus, this tendency for more vertically oriented bone formations might indicate underlying inflammatory pathogenesis in both diseases.

Another hint that supports this theory is the pelvic involvement, mainly the SI joint. The SI joint degenerative changes are common in DISH, which is characterized by joint space narrowing, osteophytes, subchondral sclerosis, cysts, vacuum phenomena, and ankyloses [24,25]. Moreover, the ankylosed SI joint typically involves its upper non-synovial portion characterized by para-articular bony bridging, while the lower synovial portion is spared [26,27]. Contrastingly, such a fusion in AS occurs due to synovitis with significant subchondral bone surface damage and erosions that present in the intra-articular fusion [26]. The recent study examined the SI joint variation in patients with OPLL using the whole spinal CT [20] and observed ankylosis not only in the entheseal sites but also in the synovial part of the SI joint with a high rate of the intra-articular fusion. Thus, morphological similarities of SI joint between OPLL and AS might be translated into pathogenic similarities at the onset of both conditions. The present study showed a significantly higher hs-CRP level in patients with SI joint intra-articular fusion compared to patients with anterior para-articular bridging. The serum hs-CRP level elevation has been shown to coincide with SI joint inflammation [28]. Additionally, increased CRP was frequently observed in patients with painful axial AS and was correlated with both disease activity and severity [29,30]. Interestingly, the current study showed a different SI joint fusion pattern between the plateau-shaped and hill-shaped groups. SI joint involvement in the plateau-shaped group was characterized by intra-articular fusion rather than anterior bridging, which was mainly identified in the hill-shaped group. These results, once again, strongly suggested that the different underlying mechanisms might partially

affect the OPLL ossification process, as well as the phenotype of SI joint ankylosis. Thus, the associations between the SI joint intra-articular fusion, hs-CRP levels, and plateau-shaped bone formation are in concert with the hypothesis that there is some form of an inflammatory basis for the progressive bone formation in the spine and SI joint in OPLL.

The analysis of the ossified lesion characteristics revealed that the plateau shape was highly associated with continuous and segmental types, whereas the hill shape more frequently occurred in patients with mixed and localized types. Additionally, the maximum thickness of the ossified PLL in the plateau-shaped group was prevalent at the vertebral segment and largest at the C2 level. Meanwhile, the maximum thickness was mainly observed in the intervertebral segment in the hill-shaped group, which is a site subject to degeneration. Moreover, the thickness and OR of the hill shape were significantly higher, and the SAC was significantly lower compared to that of the plateau shape. The mechanical stress has been believed to be involved in the formation and progression of OPLL [31], and the dynamic motion could lead to biochemical responses by inducing osteogenic differentiation in the ligament cells [32,33]. Consequently, ossifications actively grew in intervertebral levels to bridge gaps and stabilize the mobile segments. As previously reported, the jaggy-typed DISH is predominant in the T8/9 level, where the natural kyphosis curvature begins, indicating a relationship with mechanical stress due to spinal movement [15]. Moreover, hs-CRP levels were lower in the hill-shaped group. This result and the distribution tendency suggest that the degeneration process rather than the inflammation pathogenesis is related to the onset of the hill shape in OPLL.

Classic degenerative spinal changes are also related to disc degeneration. Patients with OPLL comorbid with intervertebral disc degeneration (IVDD) are commonly observed in clinical practice. Luo et al. found a significant correlation between the ossification thickness and IVDD at corresponding levels in patients with OPLL [34]. Hanakita et al. suggested that growth factor secretion by a degenerated disc and mechanical stress imposed on ligaments can

promote the ossification in OPLL [35]. Other histological studies of the tip-toe walking mouse identified that the chondrocytes of the intervertebral disc herniation could migrate to ligaments and continue to proliferate, causing a PLL transformation into the cartilaginous ossification to reinforce the vertebral column [36,37]. Thus, the predominant occurrence of the maximum thickness level of hill shape in the intervertebral segment might be verified by the IVDD. However, because the IVDD in OPLL is usually mild, and the IVDD was not evaluated in the current study, this issue remains controversial.

The current study had several limitations. First, this was a retrospective and cross-sectional study in a single institute with a relatively small number of patients. Second, serum hs-CRP levels were measured at only one-time point, with inadequate follow-up. A prospective study with a larger sample size, frequent observation periods, and multiple time point evaluations of hs-CRP levels might be needed to verify my findings. Third, whether local inflammation occurs at the site of OPLL or not is not evident since I could not exclude all the patients with numerous factors other than cardiovascular diseases that might also affect the hs-CRP elevation, as well as I could not evaluate the specific inflammatory factors from the ossified OPLL lesions and the surrounding tissues. Further studies are planned to explore this aspect in the near future.

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Chapter 5:

Association between serum interleukin-17 levels and ectopic bone formation in OPLL patients with DISH

Abstract

Objective: To investigate the relationship between the severity and morphology of heterotopic ossification in the spinal ligaments, including sacroiliac (SI) joints, and serum interleukin-17 (IL-17) levels in patients with ossification of the posterior longitudinal ligament (OPLL) with or without diffuse idiopathic skeletal hyperostosis (DISH).

Methods: A total of 103 patients with OPLL (DISH (−), n = 50; DISH (+), n = 53) and 53 age- and gender-matched controls were included. The serum levels of IL-17 were analyzed, and the severity of ectopic ossification and the morphology of ectopic bone formation were evaluated. The SI joint morphological variations were categorized into four types. The expression of IL-17 in tissue and cells derived from OPLL patients, as well as the osteogenic differentiation ability of the OPLL cells, were also measured.

Results: No significant differences were found in serum IL-17 levels between the OPLL and control groups. However, the DISH (+) group showed higher IL-17 levels than the DISH (−) group, especially in female patients (P = 0.003). Additionally, IL-17 levels were positively correlated with the number of Flat vertebral units, meaning one of the characteristics of DISH ossification type (R^2 = 0.199, P = 0.012). IL-17 levels in type 4 were significantly higher in the DISH (+) group than in the DISH (−) group. IL-17RA expression was elevated in OPLL tissue and cells, and IL-17A stimulation promoted the proliferation and osteogenic differentiation of OPLL cells.

Conclusions: This study helps to elucidate the pathological mechanism of heterotopic ossification in the spinal ligaments and suggests the possibility of IL-17A as a potential target for the prevention and treatment of OPLL.

Keywords: IL-17, OPLL, DISH, sacroiliac joint, inflammation.

Introduction

Diffuse idiopathic skeletal hyperostosis (DISH) is characterized by ectopic new bone formation along the anterior longitudinal ligament. The new bone bridges four adjacent vertebrae, without significant loss of intervertebral disc height and degeneration of the facet or sacroiliac (SI) joints [1,2]. Ossification of the posterior longitudinal ligament (OPLL) occurs in the area of the posterior vertebrae and at the intervertebral disc levels [3]. DISH has an incidence rate of approximately 8.7% to 27.1%, while OPLL in Japan ranges from approximately 1.9% to 4.3%. Genetic and environmental factors have been suggested to contribute to the development, pathogenesis, and progression of both DISH and OPLL [4-8]. DISH and OPLL have similar characteristics, and many patients with OPLL have DISH. However, the pathogenesis of DISH and OPLL is not fully understood [9,10]. The classification of OPLL based on cervical lateral X-ray includes continuous, segmental, mixed, and localized types. It is both straightforward and user-friendly, enabling it to effectively illustrate the progression of ossification over time [11]. Recently, two pathological types in OPLL patients with DISH have been reported: the Jaggy type was thick, saw-toothed, and horizontal, which is likely to be caused by the degenerative process, and the Flat type was slender, flat, and vertical, which tends to correlate with inflammatory factors [12]. Several biomarkers, including phosphate and calcium metabolism factors, sclerostin, dickkopf-1, secreted frizzled-related protein-1, and fibroblast growth factor 23, have also been reported to be associated with the diseases [13].

The concept of seronegative arthritis was first introduced in 1974, which is now referred to as spondyloarthritis (SpA) [14]. SpA typically affects the spine, SI joints, entheses, and peripheral joints, resulting in stiffness. Similarly, SpA, DISH, and OPLL all tend to form new bones in the spine or in surrounding areas [15-18]. However, the newly formed bone in SpA manifests as osteophytes at sites where tendons and ligaments insert into adjacent bone membranes, a process that is not related to remodeling within the skeleton but rather is similar

to a response to injury. One characteristic of SpA, especially ankylosing spondylitis (AS), is the involvement of the pelvis, primarily the SI joint, which is also involved in DISH and in attachment in some patients with OPLL. In DISH, involvement of the SI joint typically concerns its upper non-synovial portion, whereas the lower synovial portion is not affected. Furthermore, it has been shown that the progression rate of new bone formation in SpA and DISH is similar [19,20]. Based on these findings, OPLL, DISH, and SpA share a similar stage of inflammation at the attachment site during progression, which contributes to the promotion of new bone formation.

Evidence suggests that SpA is triggered by pathological activation of the interleukin (IL)-23/17 pathway, and antibodies against IL-17A and Interleukin-17 receptor A (IL-17RA) have been used for treatment [21-24]. IL-17A and IL-17F are approximately 50% homologous, IL-23 not only induces the production of IL-17A but also triggers the activation of IL-17F, exacerbating the inflammatory response [25]. Synovial tissues of SpA patients contain cells with high expression of IL-17, which not only inhibits osteoblast activation and promotes bone loss in osteoporosis but also induces the expression of receptor activator of nuclear factor kappa-B ligand on osteoblasts, strongly inducing the differentiation of osteoclasts. The disruption of IL-17 homeostasis is associated with the progression of spinal and SI joint lesions in SpA patients [26,27].

This study aimed to explore the relationship between serum IL-17 levels in non-OPLL (controls) and OPLL patients, as well as the association between the degree and morphology of ectopic bone formation in the spine and SI joint variations and the IL-17 levels in OPLL patients with and without DISH. In addition, I obtained OPLL patient-derived tissue and cells to examine the expression of IL-17RA, and the effect of IL-17A on osteogenic differentiation.

Materials and methods

Patient selection

This was a retrospective study on patients with OPLL who attended the Department of Orthopaedic Surgery at the Toyama University Hospital from 2014 to 2018. Computed tomography (CT) images of the whole spine were used to evaluate the degree of ossification of OPLL and the presence of DISH at all levels of the spine, including the cervical, thoracic, and lumbar regions. DISH is defined by Resnick's criteria [28]. Radiological and biochemical examinations were performed to eliminate the possibility of metabolic diseases related to OPLL, such as hypertrophic disease, osteosclerosis, and hyperthyroidism. An age- and sex-matched group of patients with degenerative spine diseases (spinal stenosis, cervical spondylosis, lumbar disc herniation, lumbar spondylolisthesis) was recruited as the control group. The exclusion criteria for both the OPLL and control groups included SpA, inflammatory diseases, trauma, myocardial infarction, cerebral infarction, malignant tumors, and other illnesses. This study was approved by the Institutional Review Board of the Toyama University Hospital (R2015003).

Measurement of serum IL-17 levels

After centrifugation, serum samples were collected in the morning at the hospital visits and immediately stored at −80°C until analysis based on previous studies. The serum IL-17 levels were quantified using an enzyme-linked immunosorbent assay kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's protocol. The manufacturer reported a sensitivity of 0.051 pg/mL and an assay range of 0.2–15 pg/mL. Additional clinical serum tests were conducted, including alkaline phosphatase (ALPL), inorganic phosphate (Pi), calcium (Ca), high-sensitivity C-reactive protein (hs-CRP), and erythrocyte sedimentation rate (ESR).

Radiographic assessment

All patients with OPLL were classified into continuous, segmental, mixed, and localized types based on cervical CT according to the Japanese Investigation Committee on Ossification of Spinal Ligaments criteria. Spinal ossification assessment was conducted using the same methodology as previously reported [29]. The severity of OPLL was evaluated using the overall ossification index (OS index), which represents the combined ossification of the anterior longitudinal ligament, posterior longitudinal ligament, and ligamentum flavum [30].

The differences in serum IL-17 levels between patients with OPLL with or without DISH were also investigated. Based on previous experience, the morphology of heterotopic bone fusion of the anterior longitudinal ligament on sagittal CT images was categorized into two types: Jaggy type and Flat type using my previous classification criteria based on Baraliakos et al. approach [12,31,32]. The prevalence of bone fusion type in each vertebral unit (VU) was evaluated using sagittal CT images of the spine from C2 to S1. The VU was defined as the area between the lower boundary of the upper vertebral body and the upper boundary of the lower vertebral body. A 45° angle of vertebral edge (VE) was set as the cut-off value. The upper and lower VE of the new bone formation in the Flat type was less than 45°, while that in the Jaggy type was less than 45°. The bone fusion type was evaluated by three orthopedic researchers (ZY.H., T.C.T.N., and H.M.). In case of any discrepancy in the classification, the two observers discussed them until reaching a consensus.

Patients with DISH were classified into three groups according to the previously established criteria [12]. The first group (Jaggy-dominant) accounted for more than 60% of VUs with Jaggy morphology. The second group (Equivalence type) included patients with 40% < Jaggy VUs < 60%. The third group (Flat-dominant) consisted of patients with Flat-type VUs, accounting for more than 60%. I compared data among these three subgroups in patients with

DISH and examined the relationship between IL-17 levels and the number of VUs exhibiting Flat and Jaggy morphology.

The morphological variations of SI joints were categorized into four types based on CT scans to investigate the potential relationship between IL-17 levels and SI variations according to a previous research: type 1: nonossification or tiny peripheral bone irregularity, type 2: subchondral osteosclerosis and osteophyte formation, type 3: vacuum phenomenon, and type 4: bridging osteophyte and bone fusion, which can be further classified into three subtypes (type 4A: anterior para-articular bridging, type 4B: posterior para-articular bridging, and type 4C: intra-articular ankylosis). The distribution of these SI joint types was compared between OPLL patients with or without DISH to assess potential differences in SI joint changes between the two groups [19,20].

Tissue harvesting, histological and immunohistochemical staining

During the anterior cervical decompression surgery, the ossified posterior longitudinal ligament tissue samples were obtained in the patients with OPLL and cervical spondylotic myelopathy (CSM). The samples were fixed in 4% paraformaldehyde for 24 h at 4℃, decalcified in ethylenediaminetetraacetic acid decalcifying solution for 4–7 days at 4℃, embedded in paraffin, and sectioned at 6 um thickness. Hematoxylin and eosin (H&E) and safranin O staining were carried out routinely. Immunohistochemical (IHC) staining procedures were as follows: tissue sections reacted with a rabbit polyclonal anti-IL-17RA antibody (ab79056, Abcam, 1:200) overnight at 4℃ and goat anti-rabbit secondary antibody (ab150077, Abcam, 1:2000) for 1 h. The signal was then detected using peroxidase-labeled dextran polymer (EnVision FLEX, Dako) for 30 min at 37℃. Sections were incubated with a 3,3'-diaminobenzidine solution to visualize the chromogenic reaction, followed by nuclear counterstaining using hematoxylin.

Cell culture and osteogenic differentiation

The fragments will be minced and subjected to enzymatic digestion with collagenase. The cells were then filtered through a 70 μm pore diameter nylon mesh (Corning Gilbert, Glendale, AZ, USA) and centrifuged at 1600 rpm. The supernatant discarded. The resulting pellet was washed twice with phosphate-buffered saline (PBS; Sigma-Aldrich, St. Louis, MO, USA). Cells were cultured in a 10 cm culture dish with Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F12; Thermo Fisher Scientific) at 37°C in an atmosphere of 5% CO_2. The medium was supplemented with 10% FBS and 1% antibiotic mixture (100 U/mL penicillin and 100 mg/mL streptomycin); The cultures were incubated for 48 h, and the medium was refreshed. Then, cells derived from the explants were harvested from the dishes using 0.02% ethylenediaminetetraacetic acid and 0.05% trypsin for further passaging.

For osteogenic differentiation, the osteogenic medium was applied after the cells had reached 70-80% confluence at passage 3, consisting of 50 μg/ml ascorbic acid (A8960, Sigma), 10 mm β-minimal medium replacement with glycerophosphate (G9422, Sigma), and 100 nM dexamethasone (D4902, Sigma). To investigate the effects of the IL-17A factor on the osteogenic-related expression of cells, varying IL-17A concentrations of 0, 0.5, 5 or 50 ng/mL were added into the culture medium of both sham and osteoinduction groups. After 14 days of osteogenic induction and co-induction with IL-17A, real-time quantitative reverse transcription polymerase chain reaction (RT-qPCR), western blotting and immunofluorescence (IF) were performed to examine the osteogenic differentiation.

Quantitative RT-qPCR analysis

Total RNA was extracted using a PureLink RNA Mini Kit (Thermo Fisher Scientific, Waltham, MA, USA). cDNA synthesis was performed using a High-Capacity RNA-to-cDNA Kit

(Thermo Fisher Scientific, Waltham, MA, USA) with a GeneAmp PCR System 9700 thermal cycler (Thermo Fisher Scientific, Waltham, MA, USA). RT-qPCR analyses were performed using the iTaq Universal SYBR Green Supermix (Bio-Rad, Hercules, CA, USA) with the CFX Connect (Bio-Rad, Hercules, CA, USA). Primers for ALPL, osterix (SP7), runt-related transcription factor-2 (RUNX2), osteocalcin (OCN), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were purchased from Invitrogen (Carlsbad, CA, USA). Table 1 lists the primer sequences used for RT-qPCR. All experiments were performed in accordance with the manufacturer's instructions. The expression of the target genes was normalized to that of the GADPH reference gene and evaluated using the $2^{-\Delta\Delta Ct}$ method.

Gene	Direction	Primer sequence (5'- 3')
IL-17RA	Forward	AGGCTTGATGCTCTCGCTCTTC
	Reverse	CTCCTCTTCCTCCTCTTCCTCCAT
ALPL	Forward	GCTGTAAGGACATCGCCTACCA
	Reverse	CTCGTCACTCTCATACTCCACATCA
SP7	Forward	GCAAGAGGTTCACTCGTTCGGATG
	Reverse	TCAGGTGGTCGCTTCGGGTAAA
RUNX2	Forward	CACCACTCACTACCACACCTACCT
	Reverse	CTTCCATCAGCGTCAACACCATCA
OCN	Forward	AGGGCAGCGAGGTAGTGA
	Reverse	CCTGAAAGCCGATGTGGT
GAPDH	Forward	ACTTTGGTATCGTGGAAGGACTCA
	Reverse	CCAGTAGAGGCAGGGATGATGTT

Table 1. Human primer sequences used in RT-qPCR.

Western blotting

Cell lysates were prepared using RIPA Lysis and Extraction Buffer #89900 (Thermo Fisher Scientific, Waltham, MA, USA). Total protein concentration was determined using a BCA

assay (Thermo Fisher Scientific, Waltham, MA, USA). Thirty milligrams total protein was resolved by sodium dodecyl sulphate-polyacrylamide gel electrophoresis and proteins were transferred to a polyvinylidene fluoride membrane. The membrane was blocked in 5% skim milk and then incubated with primary antibodies (1:1000 dilution) for the following target proteins: alpha 1 chain of the type 1 collagen (COL1A1) (ab260043, Abcam), RUNX2 (ab236639, Abcam), SP7 (ab209484, Abcam). β-Actin (4970, Cell Signaling Technology, 1:2000) served as the loading control. Anti-rabbit IgG conjugated to horseradish peroxidase (7074, Cell Signaling Technology, 1:2000) was used as the secondary antibody. Band detection was performed using the Amersham ECL Prime detection reagent (RPN2232, Sigma-Aldrich) and a ChemiDoc MP touch chemiluminescence imaging system (Bio-Rad). The relative protein expression of β-actin was quantified by densitometric analysis using ImageJ software version 1.52 (National Institutes of Health).

Immunofluorescence staining

Cells were seeded on 4-well chamber slice, fixed with 4% paraformaldehyde and permeabilized in 0.1% Triton X-100 for 20 min. Subsequently, cells were incubated with primary anti-vimentin (ab167000, Abcam, 1:1000), anti-COL1A1 (ab260043, Abcam, 1:1000) or anti-SP7 (ab209484, Abcam, 1:1000) antibodies overnight at 4°C. This was followed by incubation with an Alexa Fluor® 488-conjugated secondary antibody (ab150077, Abcam, 1:2000) for 1 h. Nuclei were counterstained with 4,6-diamidino-2-phenylindole (DAPI, Abcam) for 5 min, and images were taken with a fluorescence microscope (Keyence Corporation).

Statistical analysis

Data are presented as means ± standard deviations. Statistical analysis was performed using GraphPad Prism 9 software (GraphPad Software Inc., San Diego, CA, USA) using unpaired two-tailed t-tests, ANOVA followed by Tukey–Kramer post hoc test, chi-square test, simple linear regression and Pearson's correlation, and Multiple linear regression analyses. $P < 0.05$ was considered significant.

Results

Baseline characteristics of the OPLL and control groups

The OPLL group consisted of 103 patients (52 men and 51 women) with a mean age of 68.0 ± 11.7 years (age range, 39–90 years). The control group included 53 age- and sex-matched patients with degenerative spine diseases (27 men and 26 women) with a mean age of 69.0 ± 14.1 years (age range, 30–88 years). No significant differences in demographic data, such as height, weight, and BMI, were observed between the two groups (Table 2).

	Control group (*n* = 53)	**OPLL group (*n* = 103)**	***P*-value**
Age, years	69.0 ± 14.1	68.0 ± 11.7	0.634
Gender, *n* (%)			0.957
Male, *n*	27	52	
Female, *n*	26	51	
Height (cm)	157.4 ± 10.3	158.4 ± 9.3	0.599
Male height (cm)	163.5 ± 8.3	165.1 ± 7.0	0.561
Female height (cm)	151.3 ± 8.5	152.8 ± 7.3	0.568
Body weight (kg)	60.8 ± 12.7	64.5 ± 14.9	0.283
Male weight (kg)	67.1 ± 10.7	68.4 ± 16.9	0.822
Female weight (kg)	54.5 ± 11.8	61.5 ± 12.1	0.114
Body mass index (kg/m^2)	24.4 ± 3.6	25.6 ± 4.7	0.297
ALPL (mg/dL)	213.27 ± 66.99	236.05 ± 91.57	0.239
Ca (mg/dL)	9.16 ± 0.40	9.09 ± 0.32	0.374

Pi (mg/dL)	3.43 ± 0.60	3.17 ± 0.53	0.034*
hs-CRP (mg/dL)	0.15 ± 0.18	0.25 ± 0.56	0.384
IL-17 (pg/dL)	0.36 ± 0.48	0.33 ± 0.56	0.711

Table 2. Demographic data and serum biomarkers of patients with ossification of the posterior longitudinal ligament (OPLL) and controls. *P < 0.05. ALPL, alkaline phosphatase; Ca, calcium; Pi, inorganic phosphate; hs-CRP, high-sensitivity C-reactive protein; IL-17, interleukin-17.

Serological characteristics of the OPLL and control groups

The serum IL-17 levels in the OPLL and control groups were 0.330 ± 0.563 pg/mL and 0.363 ± 0.479 pg/mL, respectively. No statistically significant difference in the serum IL-17 levels was observed between the two groups (P = 0.711). Furthermore, no significant differences in the serum ALP, Ca, and hs-CRP levels were observed between the two groups. The Pi levels in the OPLL and control groups were 3.17 ± 0.53 mg/dL and 3.43 ± 0.60 mg/dL, respectively. The Pi level in the OPLL group was significantly lower than that in the control group (P = 0.034) (Table 2).

Correlation between IL-17 levels and morphological characteristics of OPLL

Subsequently, the correlation between the serum IL-17 levels and the degree and morphological features of OPLL were examined. The OS index of the whole spine was 22.58 ± 16.02 (range, 2–75). No significant correlation was observed between the serum IL-17 levels and the total OS index (P = 0.35) (Figure 1A). Furthermore, the correlation between the maximum OPLL thickness and IL-17 levels was investigated in patients with OPLL (Figure 1C). The results indicated that IL-17 levels were positively correlated with the maximum OPLL thickness, suggesting that a higher IL-17 level might indicate thicker OPLL (r = 0.2777, P =

0.0115). Notably, in the continuous type, the IL-17 levels were lower (0.10 ± 0.13 mm), while in the localized type, the IL-17 levels were the highest (0.74 ± 1.06 mm), with a significant difference between the two groups (P = 0.029) (Figure 1B).

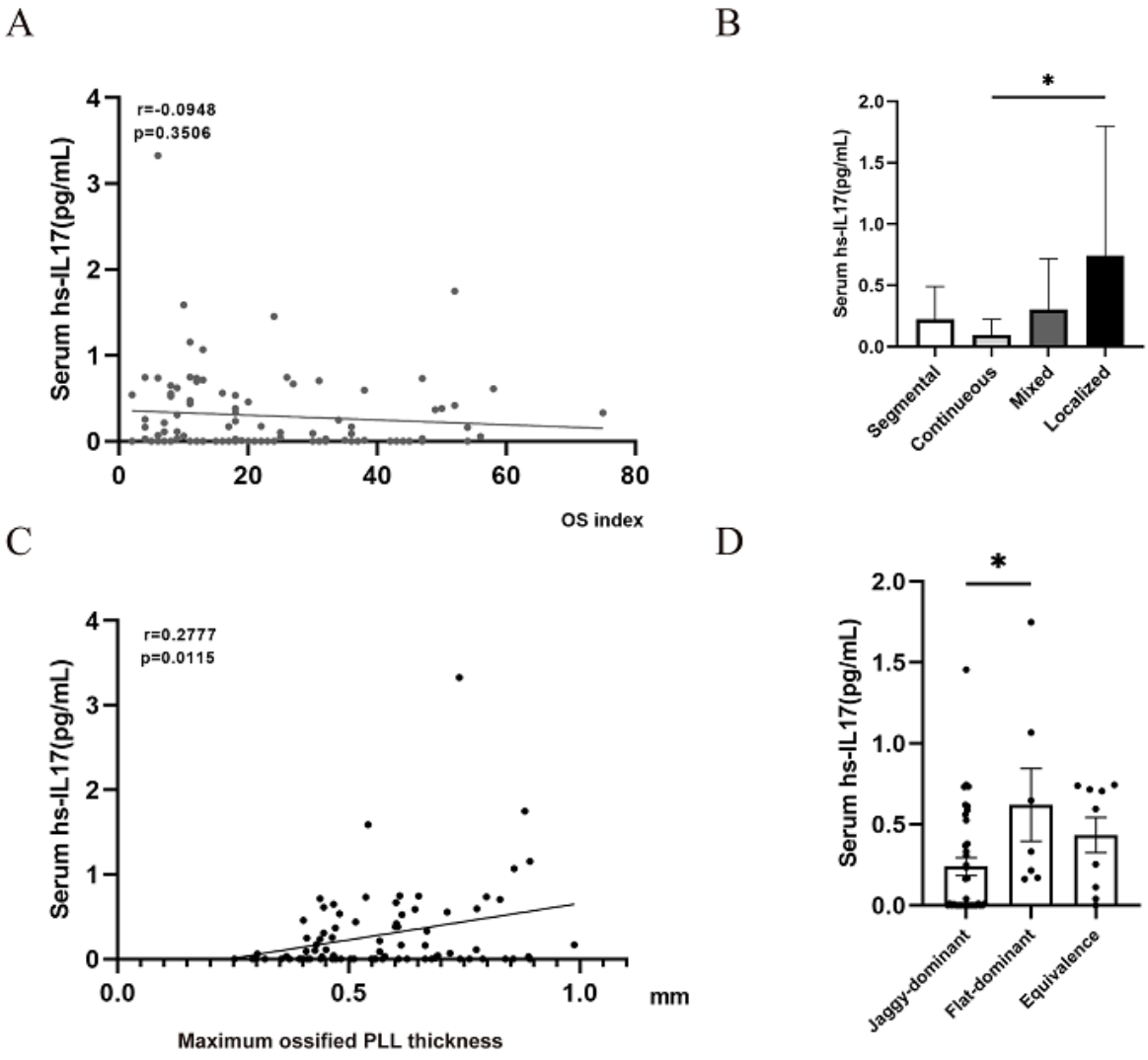

Figure 1. Relationship between the serum IL-17 levels and the degree of heterotopic ossification and morphological classification of OPLL and DISH. (A) Correlation between serum IL-17 and OS index. (B) Comparison of morphological characteristics of OPLL and serum IL-17 level. (C) Correlation between serum IL-17 and Maximum ossified PLL thickness. (D) Comparison of morphological characteristics of three subgroups and serum IL-17 level. The IL-17 levels were significantly higher in the Flat-dominant type than in the Jaggy-

dominant type. *$P < 0.05$. OS, ossification; IL-17, interleukin-17; PLL, posterior longitudinal ligament.

Baseline characteristics of the DISH (+) and DISH (−) groups in OPLL patients

Among the 103 patients with OPLL, 53 patients (27 males and 26 females, with a mean age of 72.1 ± 11.3 years) were diagnosed with DISH, while 50 patients (25 males and 25 females, with a mean age of 63.6 ± 10.7 years) did not have DISH. The presence of DISH was more frequent in older patients (P = 0.0002). However, no significant difference in other demographic data was observed between the two groups (Table 3).

	DISH (+) group (*n* = 53)	**DISH (−) group (*n* = 50)**	***P*-value**
Age (years)	72.1 ± 11.3	63.6 ± 10.7	0.0002***
Gender, *n* (%)			0.924
Male	27	25	
Female	26	25	
Height (cm)	157.3 ± 10.1	160.0 ± 8.7	0.241
Body weight (kg)	64.0 ± 15.9	65.4 ± 14.1	0.947
Body mass index (kg/m^2)	25.9 ± 5.8	24.9 ± 3.4	0.375
ALP (mg/dL)	234.06 ± 76.39	238.13 ± 105.92	0.829
Ca (mg/dL)	9.09 ± 0.29	9.09 ± 0.36	0.918
Pi (mg/dL)	3.16 ± 0.54	3.19 ± 0.51	0.772
hs-CRP (mg/dL)	0.28 ± 0.62	0.23 ± 0.51	0.719
IL-17 (pg/dL)	0.42 ± 0.39	0.24 ± 0.55	0.021*
IL-17 in males (pg/dL)	0.28 ± 0.28	0.34 ± 0.72	0.690
IL-17 in females (pg/dL)	0.45 ± 0.49	0.12 ± 0.21	0.003**
ESR (1 h) in females (mm/h)	19.30 ± 14.38	11.15 ± 6.38	0.026*
ESR (2 h) in females (mm/h)	38.78 ± 24.62	25.40 ± 11.87	0.037*

Table 3. Demographic data and serum biomarkers of the DISH (+) and DISH (−) groups. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. ALPL, alkaline phosphatase; Ca, calcium; Pi, inorganic phosphate; hs-CRP, high-sensitivity C-reactive protein; IL-17, interleukin-17; ESR, erythrocyte sedimentation rate.

Serological characteristics of the DISH (+) and DISH (−) groups

Table 3 also shows no significant differences in serum ALPL, Ca, Pi, and hs-CRP levels between the DISH (+) and DISH (−) groups. However, the serum IL-17 level in the DISH (+) group (0.42 ± 0.39 pg/mL) was higher than that in the DISH (−) group (0.24 ± 0.55 pg/mL) ($P = 0.021$). Moreover, the serum IL-17 level in female patients in the DISH (+) group (0.45 ± 0.49 pg/mL) was significantly higher than that in female patients in the DISH (−) group (0.12 ± 0.21 pg/mL) ($P = 0.003$). Additionally, the ESR (1h) in female patients with DISH (19.30 ± 14.38 mm/h) was significantly higher than that in female patients without DISH (11.15 ± 6.38 mm/h) ($P = 0.026$), and the same was observed for the ESR (2h) ($P = 0.037$).

Correlation between IL-17 levels and morphological characteristics of OPLL patients with DISH

A total of 53 patients with DISH were examined to investigate the relationship between VU level and morphological characteristics and IL-17 levels. A total of 1,219 VUs were analyzed from C2 to S1 to investigate the distribution of two types in patients with DISH. The IL-17 level in the Jaggy-dominant type was 0.27 ± 0.34 pg/ml, in the Equivalence type was 0.42 ± 0.31 pg/ml, and in the Flat-dominant type was 0.59 ± 0.63 pg/ml. Statistically significant differences were observed between the Jaggy-dominant and Flat-dominant types ($P = 0.036$) (Figure 1D). Additionally, table 4 reveals a significant positive correlation between IL-17 levels and the number of Flat VUs ($\beta = 0.344$, $P = 0.014$), but not significantly related to the

number and number of Jaggy VUs ($\beta = -0.213$, $P = 0.120$) based on multiple linear regression analysis.

	IL-17 levels				
	Unstandardized coefficient		Standardized coefficient	t	*P*-value
	B	S.E.	Beta		
Constant	0.376	0.145		2.599	0.012*
Number of Flat VUs	0.035	0.014	0.344	2.555	0.014*
Number of Jaggy VUs	−0.023	0.014	−0.213	−1.583	0.120

Table 4. Relationship between serum IL-17 levels and number of VUs with Flat and Jaggy types. $R^2 = 0.199$. *$P < 0.05$. IL-17, interleukin-17; VUs: vertebral units.

Correlation between IL-17 levels and SI joint variations

Data from 40 patients with DISH and 49 patients without DISH who had CT scans of the axial SI joint, and serum IL-17 analysis showed a significant difference ($P < 0.01$) in SI joint variation. Specifically, the DISH (+) group showed a significantly higher prevalence of anterior para-articular bridging (type 4A; 25%) and intra-articular ankylosis (type 4C; 27.5%) of SI joints than the DISH (−) group, whereas the prevalence of type 2 (36.7%) and type 3 (40.8%) of SI joints was more common in the DISH (−) group (Figure 2A). Among 89 patients, the IL-17 levels were significantly higher in type 4C (0.71 ± 0.46 pg/ml) than those in type 3 (0.20 ± 0.23 pg/ml) and type 4A (0.16 ± 0.25 pg/ml). No statistically significant differences were observed in the other groups (Figure 2B).

Serum IL-17 levels were lower in SI joint types 2 and higher in SI joint types 3 in the DISH (+) group (0.06 ± 0.13 pg/ml and 0.36 ± 0.25 pg/ml, respectively) than those in the DISH (−) group (0.46 ± 0.84 pg/ml and 0.18 ± 0.30 pg/ml, respectively). However, no significant difference was observed between the two groups. Furthermore, the serum IL-17 levels in SI

joint type 4 were significantly higher in the DISH (+) group (0.37 ± 0.42 pg/ml) than those in the DISH (−) group (0.02 ± 0.03 pg/ml), indicating that serum IL-17 levels may play an important role in the late SI joint variations in DISH (+) group (Figure 2C-E).

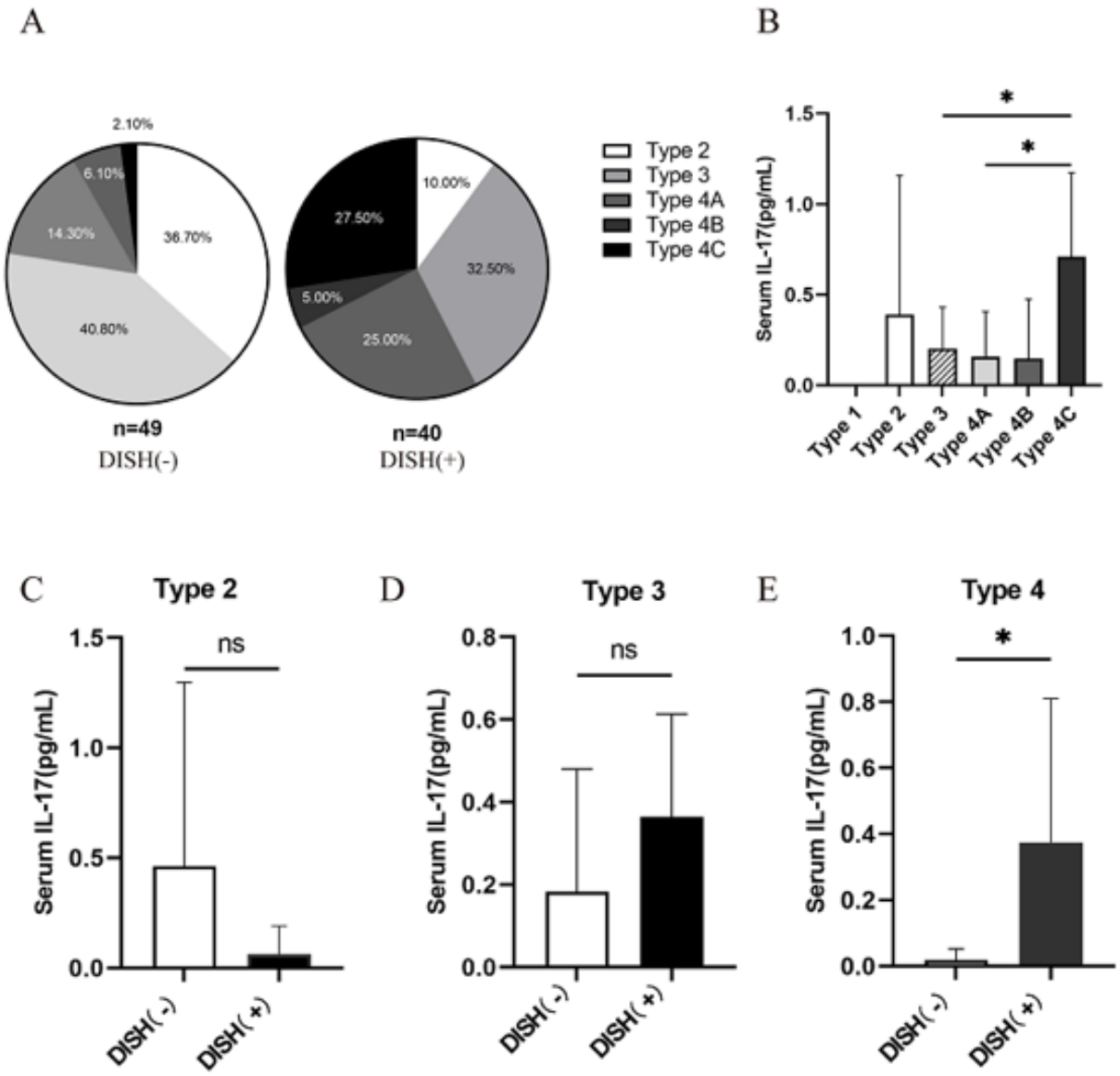

Figure 2. Relationship between the serum IL-17 levels and morphological classification of SI joint variations. (A) Prevalence of SI joint variation in the DISH (+) and DISH (−) groups. (B) The IL-17 levels were significantly higher in type 4C than in types 3 and 4A. (C–E) The IL-17 level in type 4 patients in the DISH (+) group was significantly higher than that in type 4 patients in the DISH (−) group. *$P < 0.05$. IL-17, interleukin-17.

Histological and immunohistochemical examination of posterior longitudinal ligament tissue

The posterior longitudinal ligament tissues were obtained from OPLL and CSM patients (Figure 3A, E). Compared to CSM sections (Figure 3F-G), in OPLL pathological sections (Figure 3B-C), irregular arrangement of fiber bundles, disappearance of elastic fibers, increase in collagen fibers, and accumulation of fibroblasts containing cells in the fibrocartilaginous layer in which cells were gradually replaced by chondrocytes were observed. In addition, vascular lumens can be seen in the ligament tissue, with inflammatory cells in the stroma. Fibrocartilage-like cells appeared and gradually evolved to calcify the cartilage layer (Figure 3B-C).

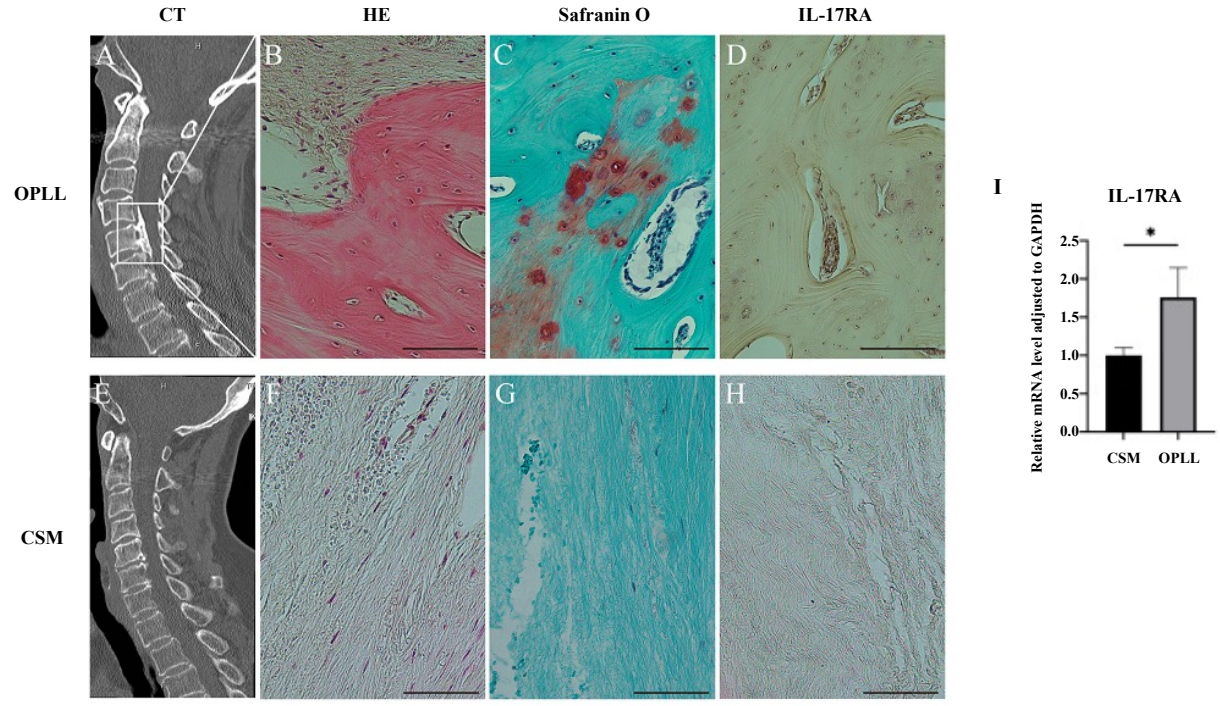

Figure 3. Imaging, histopathology and mRNA expression of IL-17RA in CSM and OPLL patients. (A, E) Computed tomography (CT) in patients with OPLL and CSM. (B-C, F-G) Hematoxylin and eosin (HE) staining and safranin O staining in patients with OPLL and CSM. (D, H) Immunohistochemical staining of IL-17RA in patients with OPLL and CSM. (I) RT-

qPCR analysis of IL-17RA expression in patients with OPLL and CSM. (scale bar: 200 μm). *$P < 0.05$. IL-17RA, Interleukin-17 receptor A.

IL-17RA is highly expressed both in vitro and vivo

IF staining of vimentin was used to determine the cell phenotype, and the results showed that most of OPLL cells were fibroblast-like phenotype (Figure 4A).

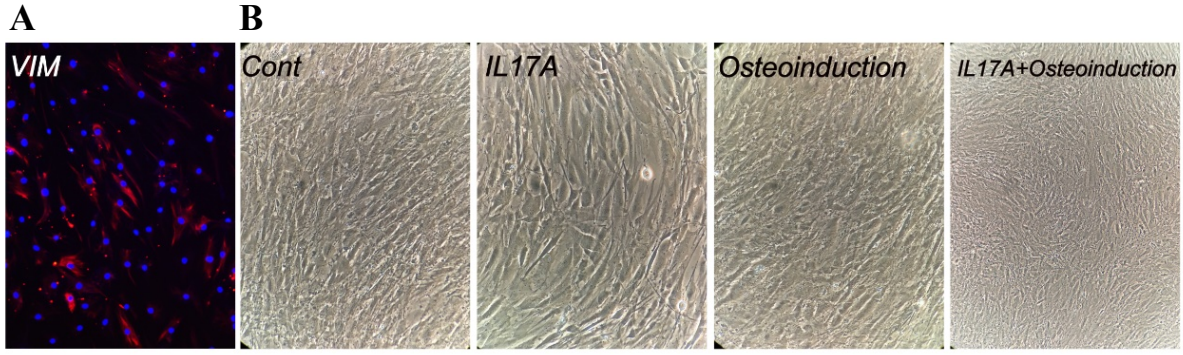

Figure 4. Cell culture and treatment. (A) Vimentin was visualized by immunofluorescence staining in ossified spinal ligament cells of OPLL patients. (B) Passage 3 of OPLL cells were induced by IL-17A or co-induce with osteogenic induction for 14 days. (n=6, scale bar: 200 μm). IL-17A, Interleukin-17A.

According to the IHC examination, IL-17RA expression was positively expressed in small amounts of the calcified cartilage layer in the tissue of CSM patients (Figure 3H), while it was highly expressed in chondrocytes and osteoblasts in the bone cavity in the tissue of OPLL patients (Figure 3D).

To confirm the expression of IL-17RA in cells, mRNA and protein levels of IL-17RA by RT-qPCR. IL-17RA expression was significantly increased in OPLL cells compared to controls (Figure 3I). These results were consistent with the expression of IL-17RA detected by IHC staining (Figure 3D, H). Overall, these results demonstrated that IL-17RA was highly expressed in OPLL cells and tissues.

IL-17A promotes osteogenic differentiation of OPLL cells

To confirm the osteogenic capacity of IL-17A, varying concentrations of IL-17A were added into the culture medium with or without osteogenic induction (Figure 4B). Over 14 days of the culture period, RT-qPCR, western blotting, and IF were performed to detect the expression of osteogenic-related markers (RUNX2, OCN, SP7, ALPL, COL1A1) in OPLL cells (Figure 5). The results show that the high dose of IL-17A stimulation (50ng/ml) significantly enhances the osteogenic activity of OPLL cells. With the highest dose of IL-17A stimulation, the increased RUNX2, ALPL, and COL1A1 expressions were observed only in the sham group, whereas the increased OCN and SP7 expressions were observed in both groups (Figure 5A, B). These results were consistent with the expression of COL1A1 and SP7 detected by IF staining (Figure 5C). Suggesting that IL-17A facilitates osteogenic differentiation of OPLL cells.

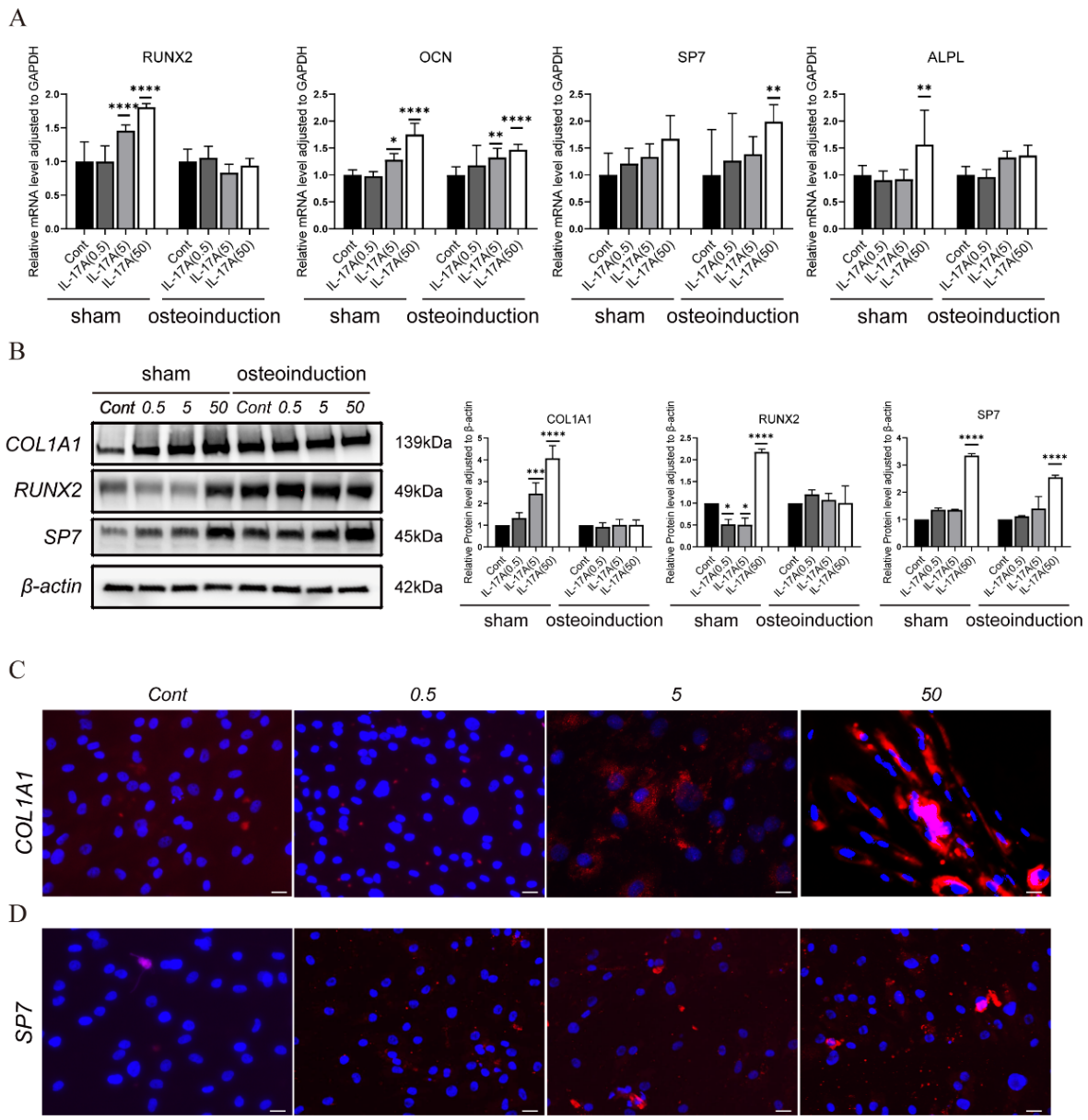

Figure 5. OPLL cells stimulated with varying concentrations of IL-17A with or without osteogenic induction, assessing osteogenic-related gene expression. (A) RT-qPCR analysis of RUNX2, OCN, SP7, and ALPL expression; (B) Western blot examination of COL1A1, RUNX2, and SP7 expression; (C, D) Immunofluorescence staining for COL1A1 and SP7 following IL17A induction at different concentrations. (n=3, scale bar: 50 μm). **P* < 0.05, ***P* < 0.01, ****P* < 0.001, *****P* < 0.0001. IL-17A, interleukin-17A; runt-related transcription factor-2, RUNX2; osteocalcin, OCN; osterix, SP7; ALPL, alkaline phosphatase; alpha 1 chain of the type 1 collagen, COL1A1; glyceraldehyde-3-phosphate dehydrogenase, GAPDH.

Discussion

Previous studies have shown that OPLL and DISH share similar characteristics in the spine and SI joint variations, and many cases have been reported where the two diseases coexist [33,34]. In the present study, the results showed that no significant difference in IL-17 levels was observed between patients with OPLL and controls. However, IL-17 levels were significantly higher in the DISH (+) group, especially in females. IL-17 levels may be related to the morphology and ossification of the anterior longitudinal ligament in the DISH (+) group, and high IL-17 levels were more often associated with type 4C SI joints. Therefore, this study reveals the spinal and SI joint features of patients with OPLL with or without DISH, providing valuable insights into the variable characteristics of ectopic bone formation of the spine and SI joints by the analysis of IL-17.

The immune microenvironment plays an important role in osteoblast proliferation and differentiation in OPLL [35]. Furthermore, the presence of OPLL with or without DISH can ultimately result in the development of degenerative cervical myelopathy (DCM). It is important to note that the distinct immune states and local immune microenvironment observed in OPLL patients might exert an influence on the progression of DCM in these individuals [36]. Interleukin has been identified as a key factor in the development of OPLL. Yayama et al. showed by suspension array and immunoblot analysis that levels of IL-6, IL-1A, fibroblast growth factor, and regulated upon activation, normal T cell expressed and secreted were significantly elevated in patients with OPLL [37]. Saito et al. also confirmed that IL-6 level plays an important role in the early osteoblastic differentiation process in the ossification of OPLL and in the induction of chondrocyte hypertrophy and apoptosis [38]. Additionally, at the genetic level, Wang et al. found five SNPs in IL-17RC in patients with thoracic OPLL, suggesting the existence of potential pathogenic mutations [39-41].

Previous research commonly believed that DISH is a non-inflammatory spinal joint disease that may be associated with mechanical, genetic, environmental, and metabolic factors [42-44]. Tenti et al. reported a significant increase in serum leptin and adiponectin levels in patients with DISH, whereas Okada et al. suggested that higher metabolic syndrome is related to DISH [45,46]. Inflammation has recently emerged as a potential factor associated with DISH, with indications that local inflammation may occur before the onset of new bone formation [17,18]. In symptomatic patients with DISH, spinal inflammation changes on MRI are common, and my previous research has also confirmed that hs-CRP may be related to the morphological features of DISH [12]. However, this study is the first to investigate the correlation between IL-17 and the extent and morphology of ossification in OPLL and DISH. IL-17 levels were significantly increased in female patients with DISH compared with male patients with DISH, possibly due to differences in hormones and gene expression.

In terms of morphology, according to previous work, ossification of the anterior longitudinal ligament is named "Flat" and "Jaggy" types [31]. The Flat type is similar to bone fusion found in AS, with characteristics similar to a "bamboo spine." The Jaggy type is more associated with mechanical stress and spinal degeneration. Interestingly, the serum IL-17 levels in the Flat-dominant group were significantly higher than those in the Jaggy-dominant group. Further, my previous study found that hs-CRP was elevated in the Flat type, compared to the Jaggy type. Based on these results, I speculate that the pathogenesis of the Flat type may be related to local inflammatory processes and has similarities in the ectopic spinal ossification mechanism with AS.

Unlike SpA, both patients with DISH and patients with OPLL with SI joint variations do not show signs of sacroiliitis, such as joint erosion or narrowing of the joint space [47, 48]. However, recently, frequent inflammatory spinal lesions on MRI in symptomatic patients with DISH have been reported, with more than half meeting the Assessment of Spondyloarthritis

International Society criteria, suggesting the possibility of local SI joint inflammation similar to that seen in SpA [49,50]. The results indicating a potential SpA-like mechanism of pathogenesis in patients with type 4 DISH. Therefore, this study provides insights into serum-level radiographic changes in SI joints associated with sacroiliitis caused by OPLL, DISH, and SpA, deepening my understanding of their pathogenesis and clinical implications.

IL-17A is a cytokine with multifunctional properties. It mediates numerous pathophysiological processes such as inflammatory responses [51,52], tumor immunity [53–55], and bone remodeling [56,57] by acting on different types of downstream cells and prompting the synthesis and secretion of various proinflammatory mediators, chemokines, and proliferative cytokines. Several pieces of experimental evidence indicate that IL-17A plays a significant role in promoting heterotopic ossification [58]. In addition, IL-17A could promote osteogenic differentiation of human mesenchymal stem cells [59]. In the present study, to clarify whether IL-17A is associated with OPLL in the cellular level, the expression of the IL-17RA gene and the molecular mechanisms that affect osteogenesis were investigated. As a result, I have found for the first time that IL-17RA levels were distinctly elevated in both OPLL tissues and cells. More importantly, IL-17A can promote the proliferation and osteogenic differentiation potential of OPLL patient-derived cells. These suggest that IL-17A-mediated inflammatory responses may play an essential role in the pathology of OPLL.

This study has some limitations. First, this was a retrospective study with a small sample size. Second, some patients with SpA (e.g., AS) were included to compare imaging and serum markers that may have better-elucidated correlations. Third, MRI was used to complement the study of SI joints, which may have provided more accurate results for the correlation between these three diseases. Fourth, whether IL-17 plays a local and confirmatory role and is responsible for immune response activation was not verified. Thus, detecting inflammatory cytokines (such as IL-6, IL-23, and TNF-a) from the tissues surrounding the ossification may

be necessary. This may provide more useful information that should be analyzed in future research.

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Chapter 6:

Concluding Remarks

The unifying theme of this dissertation is on identifying the mophological characteristics, novel mechanisms, and biomarkers of ectopic bone formation in spinal ligaments, inparticular Ossification of the posterial longitudinal ligament (OPLL).

In summary, I have identified sacroiliac (SI) joint variation in patients with OPLL. The results have shown that anterior bony bridging and ankylosis of the SI joint are more frequently observed among patients with OPLL and that OPLL patients with multilevel ossification or high ossification (OS)-index had a strong tendency to develop degeneration and ankylosis of the SI joints. Also, OPLL might share a similar inflammatory mechanism toward SI joint intra-articular ankylosis with ankylosing spondylitis (AS). Thus, clarification of the SI joint variation will lead to a better understanding of the etiology of OPLL and clarify the phenotypic differences between OPLL and other diseases that cause spinal ligament ossification.

Secondly, I have described two types of ectopic bone formation of diffuse idiopathic skeletal hyperostosis (DISH) (Flat and Jaggy types) in OPLL patients and evaluated their relationship with serum hs-CRP levels. A high concentration of hs-CRP was associated with the Flat type of ectopic bone formation in DISH, whereas a negative correlation was found between hs-CRP and the Jaggy type. These findings indicate that, in DISH, the Jaggy type of ossification is caused by a degenerative process, while the Flat type might be due to an inflammatory process similar to the ossification pattern in AS. This is a new finding in the pathogenesis of DISH; a pathological category of inflammatory DISH should be proposed. This study may enable the early development of therapeutic modalities for ectopic spinal ossification.

Additionally, I further described the detailed characteristic and related factors of these two opposite shapes of ossification in OPLL (plateau shape and hill shape). As a result, the associations between the elevation of serum hs-CRP levels, the occurrences of plateau shape of ossified lesions and SI joint intra-articular fusion found in the current study suggest a

possible inflammation mechanism for the progressive bone formation in the spine and SI joint in some cases in OPLL. However, all identified associations were weak, and thus, the role of inflammation in new bone formation in OPLL needs to be further investigated.

Therefore, I have evaluated the degree of heterotopic ossification and morphological differences between OPLL and DISH in the spine and SI joints and examined their relationship with serum IL-17 levels. The results suggest that IL17 is associated with the degree and morphological characteristics of ectopic ossification of the spine and SI joints in OPLL patients with DISH. And elevated serum IL-17 is more inclined towards Flat vertebral units and bridging osteophyte and bone fusion of the SI joint in OPLL patients with DISH. In addition, this is the first study discover the involvement and possible cellular mechanism of IL-17A in OPLL. These findings could be helpful in investigating the local inflammatory and pathological features of spinal heterotopic ossification and could serve as a foundation for future research. These findings provide a possible that OPLL has a diverse range of underlying pathogenesis. Suggesting that the pathogenesis research of OPLL should continue to focus on the inflammatory arm. Thus, this possible pathogenic pathway might have important future implications for the therapeutic intervention of OPLL.

The future direction of this research is to delve further into the involvement of IL17 in spine-associated heterotopic ossification at the cellular and molecular level, explaining the similarities and differences in the pathogenesis of OPLL, DISH, and AS. Another future direction is to seek potential therapeutic ways to control the levels of interleukin in OPLL to suppress ossification lesions using the IL-17 inhibitors which are widely applying in AS.