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Original Article

Analysis of Notch1 protein expression in methotrexate-associated lymphoproliferative disorders

Takeshi Okatani,[1,2)] Midori Filiz Nishimura,[3)] Yuria Egusa,[3)] Sayako Yoshida,[3)] Yoshito Nishimura,[4,5)] Asami Nishikori,[3)] Tadashi Yoshino,[2)] Hidetaka Yamamoto,[2)] Yasuharu Sato[3)]

Methotrexate (MTX)-associated lymphoproliferative disorder (MTX-LPD) is a lymphoproliferative disorder in patients treated with MTX. The mechanism of pathogenesis is still elusive, but it is thought to be a complex interplay of factors, such as underlying autoimmune disease activity, MTX use, Epstein-Barr virus infection, and aging. The *NOTCH* genes encode receptors for a signaling pathway that regulates various fundamental cellular processes, such as proliferation and differentiation during embryonic development. Mutations of *NOTCH1* have been reported in B-cell tumors, including chronic lymphocytic leukemia/lymphoma, mantle cell lymphoma, and diffuse large B-cell lymphoma (DLBCL). Recently, it has also been reported that *NOTCH1* mutations are found in post-transplant lymphoproliferative disorders, and in CD20-positive cells in angioimmunoblastic T-cell lymphoma, which might be associated with lymphomagenesis in immunodeficiency. In this study, to investigate the association of *NOTCH1* in the pathogenesis of MTX-LPD, we evaluated protein expression of Notch1 in nuclei immunohistochemically in MTX-LPD cases [histologically DLBCL-type (n = 24) and classical Hodgkin lymphoma (CHL)-type (n = 24)] and *de novo* lymphoma cases [DLBCL (n = 19) and CHL (n = 15)]. The results showed that among MTX-LPD cases, the expression of Notch1 protein was significantly higher in the DLBCL type than in the CHL type ($P < 0.001$). In addition, among DLBCL morphology cases, expression of Notch1 tended to be higher in MTX-LPD than in the *de novo* group; however this difference was not significant ($P = 0.0605$). The results showed that *NOTCH1* may be involved in the proliferation and tumorigenesis of B cells under the use of MTX. Further research, including genetic studies, is necessary.

Keywords: methotrexate-associated lymphoproliferative disorders, other iatrogenic immunodeficiency-associated lymphoproliferative disorders, lymphoproliferative disorders arising in immune deficiency/dysregulation, *NOTCH1*

INTRODUCTION

Methotrexate (MTX) is a folate analog drug commonly used to treat autoimmune diseases, such as rheumatoid arthritis (RA).[1] MTX-associated lymphoproliferative disorder (MTX-LPD) is a lymphoproliferative disorder or lymphoma in patients treated with MTX.[2] In the revised 4th edition of the WHO Classification, it is included among other iatrogenic immunodeficiency-associated lymphoproliferative disorders (oii-LPD),[3] and in the 5th edition of the WHO Classification, it is classified in lymphoproliferative disorders/lymphoma arising in immune deficiency/dysregulation.[4]

Immunodeficient conditions under MTX use contribute to the development of LPD of various histological presentations, including reactive lymphocyte hyperplasia, low-grade B-cell lymphoma, diffuse large B-cell lymphoma (DLBCL), polymorphic B-cell lymphoproliferative disease, classical Hodgkin lymphoma (CHL), and rarely, T-cell LPDs/lymphoma.[5–8] Epstein-Barr virus (EBV) can be positive or negative for each subtype. Among these, the most commonly reported cases are DLBCL-type (35–60%) and CHL-type (12–25%).[9]

Although the pathogenesis of MTX-LPD is not yet fully elucidated, it is thought to be caused by a complex interplay of factors, such as autoimmune disease activity, MTX use, EBV infection, and aging.[10] Most MTX-LPD occurs in RA patients, and the incidence of LPD in RA patients is higher than in the general population. It has been reported that the risk of developing lymphoma increases with disease activity in RA, indicating that systemic inflammatory exposures may

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[1)]Department of Hematology, Hiroshima Red Cross Hospital & Atomic-bomb Survivors Hospital, Hiroshima, Japan, [2)]Department of Pathology and Oncology, Okayama University Graduate School of Medicine, Dentistry, and Pharmaceutical Sciences, Okayama, Japan, [3)]Department of Molecular Hematopathology, Okayama University Graduate School of Health Sciences, Okayama, Japan, [4)]Department of Medicine, University of Hawai'i, Honolulu, HI, USA, [5)]Department of General Medicine, Okayama University Graduate School of Medicine, Dentistry, and Pharmaceutical Sciences, Okayama, Japan
Corresponding author: Midori Filiz Nishimura, Department of Molecular Hematopathology, Okayama University Graduate School of Health Sciences, 2-5-1 Shikata-cho, Kita-ku, Okayama 700-8558, Japan. E-mail: midorifiliz-nishimura@okayama-u.ac.jp

play a role in the development of lymphoma and lymphoproliferative diseases.[11] Interleukin-6 (IL-6), an inflammatory cytokine that plays an essential role in the pathogenesis of rheumatoid arthritis, is likely a growth regulatory factor for B-cell malignancies in the hematopoietic system. IL-6 has also been reported to recruit myeloid-derived suppressive cells, which may suppress the T cell response to malignancy.[12] Other cytokines, such as IL-10, which is elevated in RA, are also known to function as growth factors for B-cell lymphomas.[13]

EBV is thought to play a role in lymphoma development through apoptosis protection and B-cell transformation.[14] Subsequent suppression of EBV-specific CD8 positive T cell function with MTX[15] and reduction in T-cell receptor repertoire in age-related immune-senescence[16] have also been reported to result in EBV-related LPD.

As mentioned above, various factors may be involved in the pathogenesis of MTX-LPD, and the pathogenesis is still not entirely elucidated.

Gion *et al.* analyzed the clinicopathological features and prognosis of DLBCL-type and CHL-type MTX-LPD[17] and reported that spontaneous resolution after discontinuation of MTX was observed in 81% (22 cases) with DLBCL type, while 76% (13 cases) with CHL type did not show spontaneous resolution of lesions despite discontinuation of MTX and required additional chemotherapy.[17,18] It was indicated that these differences in clinical course may be due to the escape of Hodgkin/Reed-Sternberg cells in CHL type MTX-LPD from monitoring immune cells via PD-L1 expression.[19] In addition, the most frequent genetic alteration of CHL is a copy number gain of 9p24.1 at the locus that includes PD-L1/L2, constituting up to 97% of all CHL.[20,21] These data indicate that there may be differences in the pathogenesis of MTX-LPD in different morphologies.

The Notch signaling pathway is a cell signaling system highly conserved among animals. The Notch pathway regulates cell proliferation, cell fate, cell differentiation, and other function. Mammals possess four different Notch receptors, Notch1, Notch2, Notch3, and Notch4.[22]

The Notch receptor is a single-pass transmembrane receptor comprised of an extracellular domain (Notch extracellular domain; NECD), a transmembrane domain (Notch transmembrane domain; NTMD), and an intracellular domain (Notch intracellular domain; NICD). During transportation of the Notch protein to the cell membrane, it undergoes cleavage by Furin in the Golgi apparatus, which splits the Notch receptor at the S1 section into two parts. These two regions form a heterodimer and are transported to the cell surface, having the external domain for ligand interaction. When a ligand binds to Notch, the receptor undergoes an S2 cleavage by the ADAM (a disintegrin and metalloproteinase) protease, followed by an S3 cleavage by γ-secretase, releasing the NICD into the cytoplasm. Once inside the cell, NICD can follow one of two pathways: Canonical Signaling (CS) or non-Canonical Signaling (non-CS). The primary pathway for NICD is CS, where it activates target genes (e.g., HES-1, HEY-1, Myc, Bcl-2, CyclinD1) resulting in cell proliferation and differentiation.

Among the *NOTCH* genes, *NOTCH1* and *NOTCH2* are known to be mutated in B-cell lymphomas. *NOTCH1* mutations are found in about 10% of chronic lymphocytic leukemia (CLL) cases[23] and have also been reported in mantle cell lymphoma.[24] *NOTCH2* mutations are found in about 25% of splenic marginal zone lymphomas.[25] *NOTCH1* or *NOTCH2* mutations have also been reported in DLBCL[26–28] and follicular lymphoma.[29] Recent research has indicated that mutations in *NOTCH1* are present in approximately 10% of DLBCL cases.[27] It has also been suggested that Notch1 might function as a driver in the pathogenesis of certain DLBCL cases.[28] To date, there are no published studies that have examined immunostaining of the NICD domain of Notch1 specifically in DLBCL.

Recently, it has been reported that *NOTCH1* is recurrently mutated in post-transplant lymphoproliferative disorders[30] and CD20-positive cells in angioimmunoblastic T-cell lymphoma,[31] suggesting that *NOTCH1* mutations may be associated with B-cell proliferation and lymphomagenesis in immunodeficiency.

We hypothesized that *NOTCH1* could be involved in the pathogenesis of MTX-LPD and investigated the extent of Notch1 protein expression in DLBCL and CHL types of MTX-LPD. We also compared the differences in Notch1 protein expression between *de novo* DLBCL and CHL.

MATERIAL AND METHODS

Case selection

Forty-eight patients with MTX-LPD [DLBCL-type (n = 24) and CHL-type (n = 19)] and 38 with *de novo* lymphomas [DLBCL (n = 19) and CHL (n = 15)] were included in this study. The de novo CHL patients included in this cohort consisted of 8/15 with nodular sclerosing type and 7/15 with mixed cellularity type. All 82 cases were retrieved from surgical pathology consultation files from the Department of Pathology, Okayama University. MTX-LPD cases were diagnosed between 2008 and 2022. *De novo* lymphoma cases were those diagnosed between 1995 and 2019 for which frozen specimens were present.

The typical histology of MTX-LPD included in this study is shown in Figure 1. Figure 1a shows DLBCL-type histology with the proliferation of large atypical lymphoid cells. In contrast, Figure 1b shows scattered large cells with one-to-two nuclei with well-defined nucleoli in a background of small lymphocytes and histiocytes.

Immunohistochemistry

Specimens were fixed in 10% formaldehyde and embedded in paraffin. Three-micrometer-thick sections were cut from paraffin-embedded tissue blocks and stained with hematoxylin and eosin. For histological subtyping, immunohistochemical staining was performed accordingly using antibodies against CD20 (clone: L26, 1:100; DAKO), CD3 (clone: LN10, 1:200; Novocastra Laboratories, Ltd., Newcastle upon

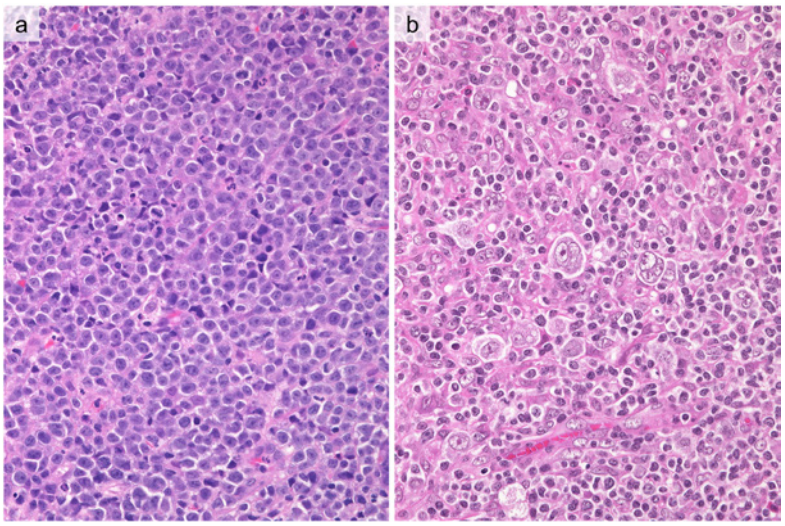

Fig. 1. Typical histology of MTX-LPD cases included in this study.
(***a***) DLBCL type: Diffuse proliferation of large atypical cells is observed (HE).
(***b***) CHL type: Scattered large atypical cells with distinct nucleoli are observed in a background of small lymphocytes. Reed-Sternberg cell-like cells are also present (HE).

Tyne, UK), CD5 (clone: 4C7, 1:100; Novocastra Laboratories, Ltd.), CD10 (clone: 56C6, 1:100; Novocastra Laboratories, Ltd.), CD15 (clone: Carb-3, 1:50; DAKO, Glostrup, Denmark), CD30 (clone: Ber-H2, 1:40; DAKO), and Ki-67 (clone: MIB-1, 1:2500; DAKO). In our cohort, in MTX-LPD or *de novo* cases displaying DLBCL morphology, there was diffuse proliferation of large atypical cells. These cells were CD20-positive, CD3-negative, and exhibited a high Ki-67 labeling index of over 30%. Cases of low-grade B-cell lymphoma that were CD5- or CD10-positive were excluded. Moreover, in both MTX-LPD and de novo lymphoma cases displaying CHL morphology, Hodgkin cells and Reed-Sternberg cells were CD20-negative, CD3-negative, CD30-positive, and CD15-positive. In CHL, due to the low number of atypical cells, accurate assessment was challenging. However, with Ki-67 staining, many of the Hodgkin and Reed-Sternberg cells were positive, indicating they were in the cell cycle.

Notch1 expression in nuclei was evaluated by immunohistochemical staining using an antibody against Notch1, which is an anti-NICD antibody of Notch1 (clone: OTI3E12, 1:50; Thermo Fisher Scientific, Massachusetts, USA).

The EBV was detected by *in situ* hybridization for EBER (Epstein-Barr-encoded RNA) (EBER-ISH) or immunohistochemical staining of latent membrane protein 1 (LMP-1; 1:50, CS1-4, Novocastra). EBER-ISH was determined to be positive when more than 50% of tumor cells showed staining in the nuclei.

Immunohistochemistry and *in situ* hybridization were performed using an automated Bond-III instrument (Leica Biosystems, Wetzlar, Germany).

Notch1 expression in nuclei of atypical cells was scored from 0 to 2 according to the following criteria[32] (Figure 2):
(1) Score 0, negative: No stained cells are observed, or no staining is observed in low magnification, but weak staining is observed at high magnification.
(2) Score 1, weak positive: Weak staining at weak magnification or weak/medium staining is recognized at high magnification for more than 25% of tumor cells.
(3) Score 2, strong positive: Strong staining is recognized at weak magnification for more than 25% of tumor cells.

Scores 1 and 2 were defined as positive and Score 0 as negative.

Statistical analyses

Statistical analyses were conducted using the Mann–Whitney U or Fisher's exact tests. Statistical significance was set at $P < 0.05$. All statistical analyses were conducted using R studio (version 4.2.2) software.

RESULTS

The demographics and clinical findings for MTX-LPD patients are summarized in Table 1. Of the 48 patients with MTX-LPD, 24 patients (50%) showed DLBCL type morphology, and 24 patients (50%) showed CHL type morphology. The mean age of the entire MTX-LPD cohort was 71.4 (range, 41–87) years, and was significantly higher in the DLBCL type group than in the CHL type group ($P = 0.002$). The proportion of females was higher than males in both morphological types, although there was no significant difference in the male-to-female ratio between the two morphological types ($P = 0.084$).

RA was the most common underlying disease for MTX-LPD, accounting for 41/48 (85.4%) of all MTX-LPD cases, 21/24 (87.5%) of DLBCL type cases, and 20/24 (83.3%) of CHL type. Among the sites of lesions excised or biopsied, lymph nodes were most frequently sampled, accounting for 38/48 (79.2%) cases of all MTX-LPD cases and 23/24 (95.8%) of CHL type cases. Among DLBCL type cases,

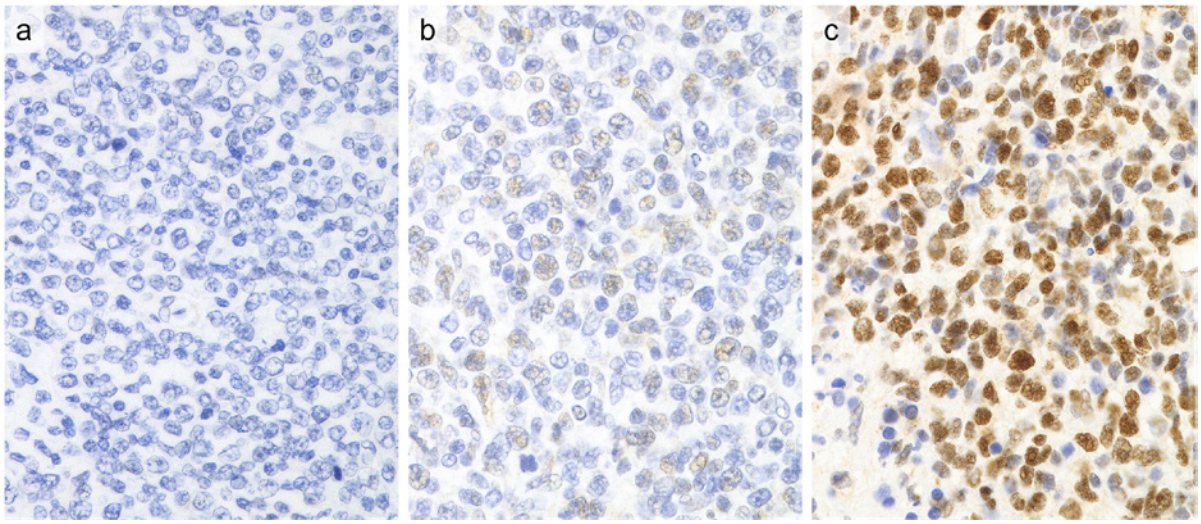

Fig. 2. Evaluation of Notch1 antibody expression in nuclei.
(***a***) The nuclei of atypical cells showed no staining and were assessed as Score 0. (***b***) The nuclei showed weak positivity and were assessed as Score 1. (***c***) The nuclei showed strong positivity and were assessed as Score 2. For both Score 1 and Score 2, positivity was defined when more than 25% of tumor cells showed nuclear staining.

Table 1. Demographics and clinical findings for MTX-LPD

	MTX-LPD (n = 48)		
	DLBCL type (n = 24)	CHL type (n = 24)	*P*
Age, mean (range)	76.1 (62–87)	66.8 (41–82)	0.002*
Sex (male:female)	3:21	8:16	0.084
Underlying condition n (%)			
Rheumatoid arthritis	21 (87.5)	20 (83.3)	—
Hashimoto disease	1 (4.2)	0	—
SAPHO syndrome	0	1 (4.2)	—
Crohn disease	0	1 (4.2)	—
Unknown	3 (12.5)	2 (8.3)	—
Excised or biopsied site n (%)			
Lymph nodes	15 (62.5)	23 (95.8)	—
Axillary	6 (25.0)	3 (12.5)	—
Cervical	4 (16.7)	8 (33.3)	—
Subclavicular	0	3 (12.5)	—
Mediastinal	0	1 (4.2)	—
Inguinal	3 (12.5)	4 (16.7)	—
Peribronchial	1 (4.2)	0	—
Retroperitoneum	0	3 (12.5)	—
LN of unknown site	1 (4.2)	1 (4.2)	—
Soft tissue	2 (8.3)	0	—
Skin	2 (8.3)	0	—
Stomach	2 (8.3)	0	—
Tonsil	1 (4.2)	0	—
Gingiva	1 (4.2)	0	—
Orbit	1 (4.2)	0	—
Liver	0	1 (4.2)	—
Presence of EBV infection†			
Present	15 (62.5)	22 (91.7)	0.011*
Absent	8 (33.3)	1 (4.2)	

†The presence of EBV infection was evaluated by EBER in situ hybridization or LMP-1 immunostaining in proliferating atypical lymphocytes, and 23 cases each of DLBCL type and CHL type were evaluated. Significance was calculated using the Mann–Whitney U test. Fisher's exact analysis was used for the statistical analysis of nominal scales. *$P < 0.05$.

15/24 (62.5%) were sampled from lymph nodes, while 9/24 (37.5%) were sampled from extranodal sites such as soft tissue, skin, stomach, and others. The positive rate of EBER *in situ* hybridization was 15/24 (62.5%) for DLBCL type cases and 22/24 (91.7%) for CHL type cases, with the CHL type showing a significantly higher positive rate ($P = 0.011$).

Table 2 compares the demographics and clinical findings between MTX-LPD and de novo lymphoma by morphology. When comparing groups exhibiting DLBCL morphology, there was no significant age difference observed between MTX-LPD cases and *de novo* cases ($P = 0.239$). However, there was a significantly higher proportion of females in the MTX-LPD group ($P < 0.001$). On the other hand, when comparing groups with CHL morphology, the MTX-LPD group was significantly older than the *de novo* group ($P = 0.032$), and also had a higher proportion of females ($P = 0.017$).

From the positivity of tumor cells due to EBER staining, examining the presence or absence of EBV infection, in both the groups showing DLBCL or CHL morphology, the infection rate was higher in the MTX-LPD group (both $P < 0.001$).

Regarding the site of origin, in MTX-LPD, 21% of cases were extranodal lesions (37.5% in DLBCL morphology and 4.1% in CHL morphology).

Table 3 compares Notch1 protein expression in the nuclei of tumor cells between groups exhibiting DLBCL or CHL morphology, categorized by etiology (MTX-LPD vs. *de novo* lymphoma). Among the MTX-LPD cases, in the DLBCL type, 14/24 cases (58.3%) had strong 2+ equivalent expression, and 8/24 (33.3%) had a 1+ expression level. Positive cases (comprising both 1+ and 2+) constituted 91.7%, indicating that most cases exhibited positive expression. Conversely, for the CHL type, 11/24 (45.8%) had positive Notch1 expression. Overall, the DLBCL type had a significantly higher frequency of Notch1 expression ($P < 0.001$). On the other hand, for *de novo* lymphoma, there was no significant difference in the Notch1 positivity rate between DLBCL and CHL.

Table 4 compares Notch1 protein expression in the nuclei of tumor cells between MTX-LPD and *de novo* lymphoma groups, categorized by morphology (DLBCL type vs. CHL type). In the DLBCL morphology group, the Notch1 positivity rate in MTX-LPD was slightly higher compared to that in the *de novo* group, but the difference was not statistically significant ($P = 0.0605$). In the CHL morphology group, there was no significant difference in the positivity rate between MTX-LPD and *de novo* CHL ($P = 0.609$).

We also investigated the potential correlation between EBV infection status and Notch1 protein expression within each MTX-LPD case group (Table 5). Since there was one case each of DLBCL and CHL types in which EBV-ISH could not be performed, we examined 23 cases excluding these cases. There was no direct correlation between the presence or absence of EBV infection and Notch1 protein

Table 2. Comparison of demographics and clinical findings between MTX-LPD and *de novo* lymphoma by morphology

	DLBCL morphology (n = 43)			CHL morphology (n = 39)		
	MTX-LPD (n = 24)	*de novo* (n = 19)	*P*	MTX-LPD (n = 24)	*de novo* (n = 15)	*P*
Age, mean (range)	76.1 (62–87)	69.8 (26–92)	0.239	66.8 (41–82)	49.1 (16–85)	0.032*
Sex (male:female)	3:21	15:4	<0.001**	8:16	11:4	0.017*
Excised or biopsied site n (%)						
Lymph nodes	15 (62.5)	19 (100)	—	23 (95.8)	14 (93.3)	—
Axillary	6 (25.0)	1 (5.3)	—	3 (12.5)	2 (13.3)	—
Cervical	4 (16.7)	13 (68.4)	—	8 (33.3)	8 (53.3)	—
Subclavicular	0	1 (5.3)	—	3 (12.5)	3 (20.0)	—
Mediastinal	0	0	—	1 (4.2)	0	—
Inguinal	3 (12.5)	3 (15.8)	—	4 (16.7)	1 (6.7)	—
Peribronchial	1 (4.2)	0	—	0	0	—
Retroperitoneum	0	0	—	3 (12.5)	0	—
Abdominal	0	1 (5.3)	—	0	0	—
Unknown LN	1 (4.2)	0	—	1 (4.2)	0	—
Soft tissue	2 (8.3)	0	—	0	1 (6.7)	—
Skin	2 (8.3)	0	—	0	0	—
Stomach	2 (8.3)	0	—	0	0	—
Tonsil	1 (4.2)	0	—	0	0	—
Gingiva	1 (4.2)	0	—	0	0	—
Orbit	1 (4.2)	0	—	0	0	—
Liver	0	0	—	1 (4.2)	0	—
Presence of EBV infection†						
Present	15 (62.5)	1 (5.3)	<0.001**	22 (91.7)	5 (33.3)	<0.001**
Absent	8 (33.3)	18 (94.7)		1 (4.2)	10 (66.7)	

†The presence of EBV infection was evaluated by EBER in situ hybridization or LMP-1 immunostaining in proliferating atypical lymphocytes, and 23 cases each of DLBCL type and CHL type in MTX-LPD were evaluated. Significance was calculated using the Mann–Whitney U test. Fisher's exact analysis was used for the statistical analysis of nominal scales. *$P < 0.05$, **$P < 0.001$.

Table 3. Comparison of nuclear expression of Notch1 between DLBCL morphology and CHL morphology by pathogenesis

Notch1 positivity	MTX-LPD			*de novo*		
	DLBCL type (n = 24)	CHL type (n = 24)	*P*	DLBCL (n = 19)	CHL (n = 15)	*P*
2+	14	3		6	4	
1+	8	8		7	3	
0	2	13		6	8	
Positive rate (%)†	91.7	45.8	<0.001**	68.4	46.7	0.1766

† Positive rates were calculated for 1+ and 2+ cases.
Fisher's exact analysis and chi-square test was used for the statistical analysis of nominal scales. $^{**}P < 0.001$.

Table 4. Comparison of nuclear expression of Notch1 between MTX-LPD and *de novo* lymphoma by morphology

Notch1 positivity	DLBCL morphology (n = 43)			CHL morphology (n = 39)		
	MTX-LPD (n = 24)	*de novo* (n = 19)	*P*	MTX-LPD (n = 24)	*de novo* (n = 15)	*P*
2+	14	6		3	4	
1+	8	7		8	3	
0	2	6		13	8	
Positive rate (%)†	91.7	68.4	0.0605	45.8	46.7	0.609

† Positive rates were calculated for 1+ and 2+ cases.
Fisher's exact analysis and chi-square test was used for the statistical analysis of nominal scales.

expression when evaluated by group.

DISCUSSION

In this study, we compared detailed clinical histological data of patients with MTX-LPD and *de novo* lymphoma. By investigating Notch1 expression based on immunohistochemical staining, we sought to determine if *NOTCH1* is involved in the etiology of MTX-LPD.

In the MTX-LPD group, both the DLBCL type and CHL type had a higher proportion of female patients, consistent with previous reports.[33] This likely reflects the higher prevalence of RA among females, who are often treated with MTX. Regarding the morphology, there was no significant age difference between MTX-LPD and *de novo* lymphoma in the DLBCL morphology. However, in the CHL morphology, MTX-LPD patients were significantly older. Although there is no such previous report, this finding could be explained by the fact that *de novo* DLBCL often arises in elderly individuals, and *de novo* CHL exhibits a bimodal distribution in young and old age groups. This might be attributed to an age-related decrease in T-cell repertoire in MTX-LPD, as previously reported.[16]

It is commonly considered that MTX-LPD is more likely to form extranodal lesions.[34,35] Our study confirmed that MTX-LPD had more extranodal lesions than *de novo* lymphoma. However, since our research only collected frozen specimens for *de novo* lymphoma, it is possible that the number of lymph node lesions was artificially increased.

NOTCH1's activating mutations primarily occur in two hot spots: the heterodimerization domain (HD) where S1 and S2 cleavages happen, and the proline/glutamic acid/serine/threonine-rich motif (PEST) domain. HD mutations induce S3 cleavage by γ-secretase, enhancing NICD production, while mutations in the PEST domain inhibit NICD degradation via SCF-FBW7 ubiquitin ligase, thus acting as activating mutations. *NOTCH1* mutations in the PEST domain have been observed in 10% of cases of CLL[23] and mantle cell lymphoma.[24]

Multiple reports exist on the role of *NOTCH1* under immunosuppressive conditions. For instance, post-transplant lymphoproliferative disease (PTLD) arises in patients under immunosuppression post hematopoietic stem cell or solid organ transplantation, with EBV being a key driver in its pathogenesis. Recent reports indicate recurrent *NOTCH1* mutations in early PTLD lesions, specifically in florid follicular hyperplasia (FFH). In one study (30), two out of five *NOTCH1* mutations identified in FFH were in the PEST domain. In a study conducted by Nguyen *et al.*, targeted sequencing was utilized to investigate the distribution of genetic mutations in tumorous cell and infiltrating B cell fractions derived from tumor tissue samples of Peripheral T-cell lymphoma, including angioimmunoblastic T-cell lymphoma (AITL), using laser microdissection.[31] It is known that AITL is associated with cellular immunodeficiency, leading to the onset of B-cell lymphomas. In the study, three out of 19 cases had *NOTCH1* mutations only in CD20-positive B cells. Among them, one was a frameshift mutation in the PEST domain of the Notch1 protein. Based on the findings, it may be plausible that *NOTCH1* mutations in B cells infiltrating AITL tissues might be providing proliferative signals to B cells.[36] However, the specific details and implications of the findings are yet to be fully elucidated. The present study utilized an anti-NICD antibody for immunostaining Notch1 in the nucleus, and showed a significant upregulation of nuclear NICD protein expression in MTX-LPD DLBCL type compared to the CHL type. While it may reflect the signal activation of *NOTCH*, the extent of NICD protein signal expres-

Table 5. Investigation of the correlation between the EBV infection status and Notch1 expression rate in the nucleus of tumor cells in each group

	MTX-LPD DLBCL type†				MTX-LPD CHL type‡			
	EBV infection (+) (n = 15)	EBV infection (-) (n = 8)	*P*		EBV infection (+) (n = 22)	EBV infection (-) (n = 1)	*P*
Notch1 (+)	15	6	0.11	Notch1 (+)	11	0	1
Notch1 (-)	0	2		Notch1 (-)	11	1	
	de novo DLBCL				*de novo* CHL		
	EBV infection (+) (n = 1)	EBV infection (-) (n = 18)	*P*		EBV infection (+) (n = 5)	EBV infection (-) (n = 10)	*P*
Notch1 (+)	0	13	0.32	Notch1 (+)	4	3	0.12
Notch1 (-)	1	5		Notch1 (-)	1	7	

† EBER-ISH was not performed in one case. ‡ EBER-ISH was not performed in one case.
Fisher's exact analysis was used for statistical analyses.

sion is influenced by various parameters, including the nuclear translocation rate of NICD, the formation quantity of the complex with transcript factors Su(H) and Mam, post-transcription modification patterns, and the degradation rate of NICD. This complexity could potentially explain why the positivity rate from immunostaining is higher than the reported mutation rate for *NOTCH1*. Future genetic analysis is warranted to identify mutations in the *NOTCH1* PEST domain.

In MTX-LPD, similar to PTLD and AITL, cellular immunity might decrease due to aging or EBV infection, and *NOTCH1* might play a role in the onset of B-cell tumors in these conditions.

The present results showed increased nuclear Notch1 expression in the MTX-LPD DLBCL type compared to the CHL type. This suggests that even within LPDs that arise under the same immunosuppressive conditions, there might be underlying pathophysiological differences. Differences in the expression rate of PD-L1 and prognosis between the two types have been highlighted.[19] Additionally, there are two signaling pathways for *NOTCH1*; Canonical Signaling and non-Canonical signaling. While the exact pathways of non-Canonical signaling (Non-CS) are not fully understood, they are believed to operate independently of NICD nuclear translocation. When mediated through Non-CS, other known active pathways in *de novo* CHL, such as NF-κB signaling or PI3K-AKT signaling, might be activated. This could potentially account for the lower positivity rate of Notch 1 in nuclear staining.

On the other hand, when comparing between MTX-LPD DLBCL type and *de novo* DLBCL, although there were no significant difference between two groups, the extent of Notch1 protein expression tended to be higher in MTX-LPD DLBCL type. Regarding this observation, when comparing MTX-LPD DLBCL type to *de novo* DLBCL, the significantly higher rate of EBV positivity in the MTX-LPD DLBCL type might be related to the underlying pathophysiology. In our study, within each group, no clear correlation was observed between the presence or absence of EBV infection and the positivity rate of Notch1 in the nuclei of tumor cells. However, recent research suggests that activation of the *NOTCH1* signal may repress MHC class II genes and impair T-cell activation, thereby playing a role in tumor immune evasion and virus reactivation due to immunosuppression.[37] It is possible that in MTX-LPD, *NOTCH1* might be involved in the pathogenesis through such mechanisms, which necessitates further investigation.

The present study has several limitations. First, we only evaluated the protein expression of Notch1 in the nucleus and did not investigate whether there are actual mutations in the *NOTCH1* gene in MTX-LPD. It is essential to comprehensively study what kind of genetic mutations exist and whether the accumulation of the *NOTCH1* results from these mutations. Also, the expression of Notch1 was assessed only in tumor cells, but not in surrounding cells. For the activation of the *NOTCH1* signaling pathway, it is fundamentally necessary for cells expressing type I transmembrane proteins, the ligands of the Notch receptor, namely D1/DLL and Ser/JAG, to be adjacent. The nature of such neighboring cells remains a topic for future research. Lastly, while all cases of the MTX-LPD CHL type in our cohort presented with the mixed cellularity type, *de novo* CHL cases had a mixture of the nodular sclerosing type and the mixed cellularity type. It has been reported that *de novo* CHL varies in etiology and EBV positivity rates depending on the histological subtype.[3] Thus, a straightforward comparison between the two groups in our study was deemed challenging.

In conclusion, our research suggests that MTX-LPD has distinct clinical features compared to *de novo* lymphoma. The involvement of *NOTCH1* in the etiology of MTX-LPD requires further study, and potential mutations in the *NOTCH1* gene and also *NOTCH2*, a paralogue of *NOTCH1*, should be examined in future research to identify its pathophysiology.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

REFERENCES

1 Rath T, Rubbert A. Drug combinations with methotrexate to treat rheumatoid arthritis. Clin Exp Rheumatol. 2010; 28: S52-S57.

2 Ellman MH, Hurwitz H, Thomas C, Kozloff M. Lymphoma developing in a patient with rheumatoid arthritis taking low dose weekly methotrexate. J Rheumatol. 1991; 18: 1741-1743.

3 Swerdlow SH, Campo E, Harris NL, *et al.* WHO Classification of Tumours of Haematopoietic and Lymphoid Tissues. 4th ed, Lyon, IARC Press. 2017.

4 Cree IA. The WHO Classification of Haematolymphoid Tumours. Leukemia. 2022; 36: 1701-1702.

5 Ichikawa A, Arakawa F, Kiyasu J, *et al.* Methotrexate/iatrogenic lymphoproliferative disorders in rheumatoid arthritis: histology, E pstein– B arr virus, and clonality are important predictors of disease progression and regression. Eur J Haematol. 2013; 91: 20-28.

6 Kurita D, Miyoshi H, Ichikawa A, *et al.* Methotrexate-associated lymphoproliferative disorders in patients with rheumatoid arthritis: Clinicopathologic features and prognostic factors. Am J Surg Pathol. 2019; 43: 869-884.

7 Satou A, Tabata T, Miyoshi H, *et al.* Methotrexate-associated lymphoproliferative disorders of T-cell phenotype: clinicopathological analysis of 28 cases. Mod Pathol. 2019; 32: 1135-1146.

8 Satou A, Nakamura S. EBV-positive B-cell lymphomas and lymphoproliferative disorders: Review from the perspective of immune escape and immunodeficiency. Cancer Med. 2021; 10: 6777-6785.

9 Gaulard P, Swerdlow S, Harris N, Jaffe E, Sundström C. Other iatrogenic immunodeficiency-associated lymphoproliferative disorders. In: Swerdlow SH, Campo E, Harris NL, *et al.* (eds): World Health Organization Classification of Tumours of Haematopoietic and Lymphoid Tissues. Lyon, IARC Press. 2008; pp. 350-351.

10 Saito S, Takeuchi T. Immune response in LPD during methotrexate administration (MTX-LPD) in rheumatoid arthritis patients. J Clin Exp Hematop. 2019; 59: 145-155.

11 Baecklund E, Iliadou A, Askling J, *et al.* Association of chronic inflammation, not its treatment, with increased lymphoma risk in rheumatoid arthritis. Arthritis Rheum. 2006; 54: 692-701.

12 Ruhland MK, Loza AJ, Capietto AH, *et al.* Stromal senescence establishes an immunosuppressive microenvironment that drives tumorigenesis. Nat Commun. 2016; 7: 11762.

13 Emilie D, Zou W, Fior R, *et al.* Production and roles of IL-6, IL-10, and IL-13 in B-lymphocyte malignancies and in B-lymphocyte hyperactivity of HIV infection and autoimmunity. Methods. 1997; 11: 133-142.

14 Fitzsimmons L, Kelly GL. EBV and apoptosis: The viral master regulator of cell fate? Viruses. 2017; 9: 339.

15 Thorley-Lawson DA, Gross A. Persistence of the Epstein-Barr virus and the origins of associated lymphomas. N Engl J Med. 2004; 350: 1328-1337.

16 Shimoyama Y, Yamamoto K, Asano N, *et al.* Age-related Epstein-Barr virus-associated B-cell lymphoproliferative disorders: special references to lymphomas surrounding this newly recognized clinicopathologic disease. Cancer Sci. 2008; 99: 1085-1091.

17 Gion Y, Iwaki N, Takata K, *et al.* Clinicopathological analysis of methotrexate-associated lymphoproliferative disorders: Comparison of diffuse large B-cell lymphoma and classical Hodgkin lymphoma types. Cancer Sci. 2017; 108: 1271-1280.

18 Ueda Y, Tanaka T, Asakura S, Yano T. A case of classic Hodgkin lymphoma arising after remission of methotrexate-associated follicular lymphoma. J Clin Exp Hematop. 2023; 63: 177-180.

19 Gion Y, Doi M, Nishimura Y, *et al.* PD-L1 expression is associated with the spontaneous regression of patients with methotrexate-associated lymphoproliferative disorders. Cancer Med. 2022; 11: 417-432.

20 Roemer MGM, Advani RH, Ligon AH, *et al.* PD-L1 and PD-L2 genetic alterations define classical Hodgkin lymphoma and predict outcome. J Clin Oncol. 2016; 34: 2690-2697.

21 Takahara T, Sakakibara A, Tsuyuki Y, *et al.* Diagnostic approach for classic Hodgkin lymphoma in small samples with an emphasis on PD-L1 expression and EBV harboring in tumor cells: a brief review from morphology to biology. J Clin Exp Hematop. 2023; 63: 58-64.

22 Kumar R, Juillerat-Jeanneret L, Golshayan D. Notch antagonists: Potential modulators of cancer and inflammatory diseases. J Med Chem. 2016; 59: 7719-7737.

23 Landau DA, Tausch E, Taylor-Weiner AN, *et al.* Mutations driving CLL and their evolution in progression and relapse. Nature. 2015; 526: 525-530.

24 Kridel R, Meissner B, Rogic S, *et al.* Whole transcriptome sequencing reveals recurrent NOTCH1 mutations in mantle cell lymphoma. Blood. 2012; 119: 1963-1971.

25 Kiel MJ, Velusamy T, Betz BL, *et al.* Whole-genome sequencing identifies recurrent somatic NOTCH2 mutations in splenic marginal zone lymphoma. J Exp Med. 2012; 209: 1553-1565.

26 Lee SY, Kumano K, Nakazaki K, *et al.* Gain-of-function mutations and copy number increases of Notch2 in diffuse large B-cell lymphoma. Cancer Sci. 2009; 100: 920-926.

27 Li Z, Yu F, Ye W, *et al.* Clinical features and prognostic significance of NOTCH1 mutations in diffuse large B-cell lymphoma. Front Oncol. 2021; 11: 746577.

28 Lohr JG, Stojanov P, Lawrence MS, *et al.* Discovery and prioritization of somatic mutations in diffuse large B-cell lymphoma (DLBCL) by whole-exome sequencing. Proc Natl Acad Sci USA. 2012; 109: 3879-3884.

29 Karube K, Martínez D, Royo C, *et al.* Recurrent mutations of NOTCH genes in follicular lymphoma identify a distinctive subset of tumours. J Pathol. 2014; 234: 423-430.

30 Butzmann A, Sridhar K, Jangam D, *et al.* Mutations in JAK/STAT and NOTCH1 genes are enriched in post-transplant lymphoproliferative disorders. Front Oncol. 2022; 11: 790481.

31 Nguyen TB, Sakata-Yanagimoto M, Asabe Y, *et al.* Identification of cell-type-specific mutations in nodal T-cell lymphomas. Blood Cancer J. 2017; 7: e516.

32 Jegalian AG, Bodo J, Hsi ED. NOTCH1 intracellular domain immunohistochemistry as a diagnostic tool to distinguish T-lymphoblastic lymphoma from thymoma. Am J Surg Pathol. 2015; 39: 565-572.

33 Shiraiwa S, Kikuti YY, Carreras J, *et al.* Clinicopathological evaluation of methotrexate-associated lymphoproliferative dis-

orders with special focus on Epstein-Barr virus-positive mucocutaneous lesions. J Clin Exp Hematop. 2020; 60: 159-168.
34 Ebeo CT, Girish MR, Byrd RP, Roy TM, Mehta JB. Methotrexate-induced pulmonary lymphoma. Chest. 2003; 123: 2150-2153.
35 Kameda H, Okuyama A, Tamaru J, *et al.* Lymphomatoid granulomatosis and diffuse alveolar damage associated with methotrexate therapy in a patient with rheumatoid arthritis. Clin Rheumatol. 2007; 26: 1585-1589.
36 Sakata-Yanagimoto M. A translational research in angioimmunoblastic T-cell lymphoma. Journal of The Society of Japanese Women Scientists. 2021; 21: 1-12.
37 Mangolini M, Maiques-Diaz A, Charalampopoulou S, *et al.* Viral transduction of primary human lymphoma B cells reveals mechanisms of NOTCH-mediated immune escape. Nat Commun. 2022; 13: 6220.