Usefulness of immunoglobulin light-chain restriction on immunocytochemical double staining for the cytological diagnosis of B-cell non-Hodgkin's lymphoma

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Abstract

Objective: We examined the usefulness of light-chain restriction on immunocytochemical double staining for cytological diagnosis.

Study Design: We investigated light-chain restriction on immunocytochemical double staining in 40 patients with proliferative lymphatic disorders (B-cell lymphoma: 23 patients, reactive lymphpoid lesions: 13, T-cell lymphoma: 2, and Hodgkin's lymphoma: 2). In addition, the results of flow cytometry (FCM) were also compared in 34 of these patients.

Results: On immunocytochemical double staining, light-chain restriction was detected in 21 (91.3%) of 23 patients with B-cell lymphoma. On FCM, it was detected in 15 (71.4%) of 21 patients with B-cell lymphoma. Neither immunocytochemical double staining nor FCM showed light-chain restriction in any patients with reactive lesions, T-cell lymphoma, or Hodgkin's lymphoma.

Conclusion: Immunocytochemical double staining facilitated the detection of light-chain restriction with a single specimen under morphological observation. The application of this procedure may improve the accuracy of cytological diagnosis.

Key words

Cytology, Immunocytochemical double staining, Immunoglobulin light-chain restriction, Lymph node, B-cell lymphoma, Flow cytometry.

Introduction

Cytological diagnosis with fine needle aspiration is used for diagnosis in patients with lymph node swelling. However, it is difficult to differentiate between malignant lymphoma and benign lymphoid lesion in some patients. In particular, when low-grade B-cell lymphoma is mixed with a large number of reactive lymphocytes, cytological diagnosis is difficult [1-3].

For the differential diagnosis of between malignant lymphoma and benign lymphoid lesion, immunostaining and flow cytometry (FCM) are very important. In particular, in the diagnosis of low-grade B-cell lymphoma without cellular atypia, it is

diagnostically important to confirm immunoglobulin light-chain restriction [3-5]. To verify monoclonal features, a Kappa/Lambda light-chain restriction ratio of more than 3 or Lambda/Kappa light-chain restriction ratio of more than 2 is recommended as a criterion [4,5, 6, 7, 8].

Immunocytochemical double staining (IDS) for cytological diagnosis facilitates the detection of light-chain restriction with a single slide under morphological observation. In this study, we detected light-chain restriction using IDS, compared the results with those of flow cytometry, and examined the usefulness of IDS for cytological diagnosis.

Materials and Methods

Case selection

Samples were selected from records on cytological diagnosis in Kagawa Prefectural Central Hospital between 2009 and 2010.

All data and samples from the patients were collected with their informed consent.

The subjects consisted of 23 patients with B-cell lymphoma group (diffuse large B-cell lymphoma (DLBCL): 9 patients, follicular lymphoma: 8, lymphoplasmacytic lymphoma: 1, plasma cell myeloma: 1, mantle cell lymphoma: 1, Burkitt-like lymphoma: 1, and B-cell lymphoma NOS: 2). The control consisted of 17 patients with negative control group (2 with T-cell lymphoma (peripheral T-cell lymphoma: 1, and lymphoblastic lymphoma: 1), 2 with Hodgkin's lymphoma, and 13 with reactive lesions).

Specimen processing

In 36 patients, cell suspension was prepared from biopsy specimens using fine needle aspiration. In 4, body cavity fluid was used. After all samples were washed in physiological saline, cells were smeared on slide glasses, and fixed in 95% ethanol.

Immunocytochemical double staining

With respect to Kappa + Lambda antibody, reagents, and usage, a Biocare Medical kit (Concord, CA, U.S.A.) was employed. According to its protocol, staining was performed.

The primary antibody consists of an antibody cocktail with mouse (kappa) and rabbit (lambda) light chains antibody.

A cocktail of conjugated goat anti-rabbit polymer horseradish peroxidase (HRP) and conjugated goat anti-mouse polymer alkaline phosphatase (AP) secondary antibodies react simultaneously with a cocktail of a mouse and a rabbit primary antibody.

The staining method is as follows:

1. Wash in Tris-Buffered Saline (TBS) wash buffer.
2. Apply primary antibody cocktail for 30 minutes.
3. Wash in TBS wash buffer.
4. Apply secondary antibodies for 30 minutes.
5. Wash in TBS wash buffer.
6. Apply DAB for 2 minutes.
7. Rinse in TBS.
8. Apply Vulcan Fast Red for 15 minutes.
9. Wash in deionize water.
10. Apply Mayer's Hematoxylin for 1 minute.
11. Wash in deionize water for 5 minutes.
12. Dehydrate, clear and coverslip.

Count

The smallest lymphocytes were defined as small lymphocytes, and others as medium to large lymphocytes.

We randomly counted 500 lymphocytes at a magnification of 1,000. The cell size varied: small to large. Kappa (red)- and Lambda (brown)-stained cells were determined as a percentage of the above cells (IDS-1). In addition, we counted 200

middle- to large-sized cells appearing in each specimen (IDS-2).

Diagnostic criteria on IDS

The results of measurement were calculated so that the Kappa/Lambda or Lambda/Kappa ratio was 1 or more. As a diagnostic criterion for light-chain restriction (monoclonality), the cut-off value of Kappa/Lambda or Lambda/Kappa ratio was established as >3:1 by comparing our data with previous studies. [4,5,9].

Flow cytometry

In 34 (85%) of the 40 patients, FCM was conducted. The following antibody panels were employed: CD2, CD3, CD4, CD5, CD7, CD8, CD10, CD19, CD20, IgM, CD25, CD30, CD34, CD38, CD45, CD56, and immunoglobulin light chains (Kappa, Lambda).

Statistical analysis

The results were compared between the B-cell lymphoma and light-chain restriction-negative control groups using the Mann-Whitney U test. The data were analyzed with the StatView software (version 5.0; SAS Institute, Inc., San Francisco, IL, U.S.A.).

Results

The results are shown in Table 1. In the negative control group (T-cell lymphoma, Hodgkin's lymphoma, and reactive lymphoid lesions), the light-chain restriction (LCR) ratio was less than 3 in all patients regardless of procedures (range: 1 to 2.1)(Table 2, Figure 1). There was a significant difference in comparison with the B-cell lymphoma group ($P<0.0001$).

Of 21 patients with B-cell lymphoma in whom FCM was performed, light-chain restriction was detected in 15 (71.4%). Of 23 patients in whom IDS-1 was conducted, LCR was detected in 15 (65.2%) (Figure 2). Of 23 patients in whom IDS-2 was conducted, LCR was detected in 21 (91.3%) (Table 3) .

When comparing the LCR detection rate in the 21 patients with B-cell lymphoma, there was no patient in whom LCR was detected on FCM, but not on IDS-1/-2. LCR was detected on both FCM and IDS in 15 patients (71.4%). In 4 (19.0%), LCR was not detected on FCM, but it was detected on IDS. In 2 (9.5%), neither FCM nor IDS showed LCR (Table 4).

The sensitivities of FCM, IDS-1, and IDS-2 were 71.4, 65.2, and 91.3%, respectively. Their specificities were 100% (Table 5).

Discussion

Biopsy is frequently performed in patients with suspected malignant lymphoma. In a portion of each specimen, surface markers are investigated using FCM in many patients. Among these markers, the confirmation of surface immunoglobulin LCR is useful for the diagnosis of B-cell lymphoma. In formalin-fixed paraffin sections, which are usually used, only antigens existing in the cytoplasm can be accurately detected. As immunoglobulins on the cell surface are non-specifically fixed and soluble, it is difficult to verify LCR [10]. For this reason, surface immunoglobulins were examined using FCM. Subsequently, immunostaining is performed to detect immunoglobulins on the cell surface [9-12]. However to our best knowledge there are no reports on IDS. Therefore we examined this method.

There are many criteria to establish the cut-off of LCR. Some criteria include a Kappa/Lambda ratio of 3 or more, or a Lambda/Kappa ratio of 2 or more. Others include that of 6 or more [2,3,4,6,8,11,12,13,14,15]. Sneige et al.[5] performed immunostaining using fine-needle aspiration samples, and regarded lesions with an LCR ratio of 6 or more as monoclonal or B-cell lymphoma. Lesions with an LCR ratio of 3 or less were regarded as “reactive lymphoid lesion”. Lesions with intermediate values were evaluated as “atypical lymphoid lesion”. They emphasized the necessity of biopsy or thorough follow-up. In our investigation, all reactive lymphoid lesions showed an LCR ratio of less than 3. Several studies reported that the LCR ratio exceeded 3 in some patients with reactive lymphoid lesions such as toxoplasmosis, collagen disease, and rheumatoid arthritis [6,7]. However, there was no such case in our

series. On IDS, the LCR ratio ranged from 3 and 6 in 4 patients (follicular lymphoma (FL): 1, and DLBCL: 3). Of these, 3 had DLBCL, and tumor presence was morphologically suggested. Using IDS, we could simultaneously count cells in a single specimen while observing of morphological atypia. This may have contributed to the differentiation of lymphoma cells from reactive lymphocytes, with a cut-off of 3.

In 4 patients, LCR was not detected on FCM, but it was detected on IDS. In 2 (Cases 21 and 23) of these, there was no LCR when randomly counting cells on IDS-1. However, when counting middle- to large-sized cells under cellular-morphology observation on IDS-2, LCR could be detected (Figure 3). This was possibly because the number of tumor cells was small, and a large number of reactive lymphocytes appeared as background cells. Crapanzano et al.[8] conducted FCM in 6 patients with marginal zone B-cell lymphoma, and reported that LCR was detected in 3. It was not detected in the other 3 patients, possibly because the number of background cells was large, and that of lymphoma cells was small, as indicated in our study.

In addition, the reasons why LCR could not be detected on FCM include the fragility of large lymphoma cells [14,16,17]. Verstovsek et al.[18] reviewed patients with false-negative reactions on FCM, and indicated that 27% of patients with DLBCL showed false-negative reactions on FCM. Such reactions were related to cellular degeneration in 75%. In Case 32 (Figure 4), marked cellular degeneration occurred in the sample treatment process, and LCR was not detected on FCM. IDS-2 facilitated the detection of LCR through morphological differentiation between cellular degeneration-related enucleated cells/non-specific reactions and degeneration-free tumor cells.

In 1 patient with follicular lymphoma (Case 19), there was no LCR on FCM. However, LCR was noted on IDS with the same sample. The reasons were IDS detected the cytoplasmic immunoglobulin.

Several studies classified the histological type by combining FCM with fine needle aspiration (FNA)[13-19]. In residual samples after cytological diagnosis, FCM analysis was conducted to estimate the histological type.

Conclusion

The LCR detection rate of IDS was similar to or higher than that of FCM. Therefore, the combination of FNA and IDS may improve the accuracy of B-cell lymphoma diagnosis, assisting FCM/ cytological diagnosis.

Conflict of interest statement

We declare that we have no conflict of interest.

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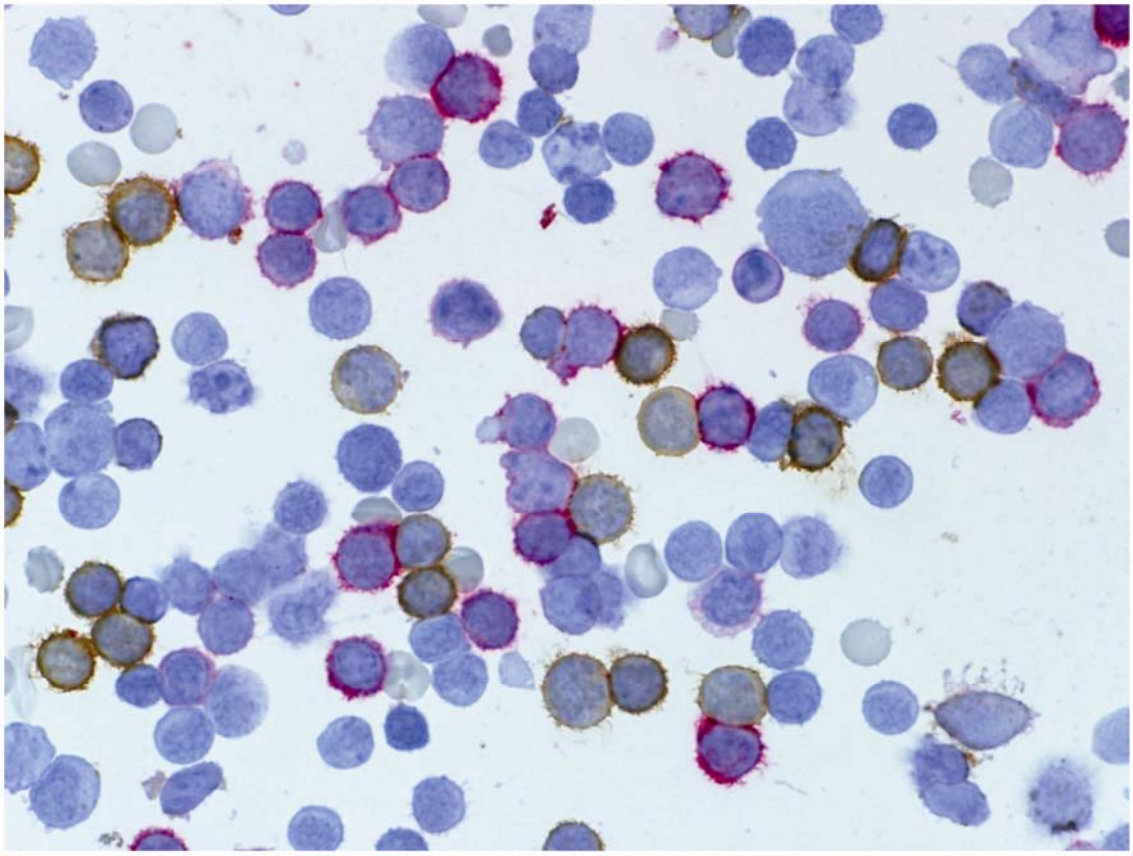

Figure 1. Negative control case; Immunocytochemical double staining(IDS) of Hodgkin lymphoma (case 17) IDS-1:κ/λ rate 1.1

The Kappa (red) and Lambda (brown) reactions of small- to middle-sized lymphocytes were similar. No light-chain restriction was detected.

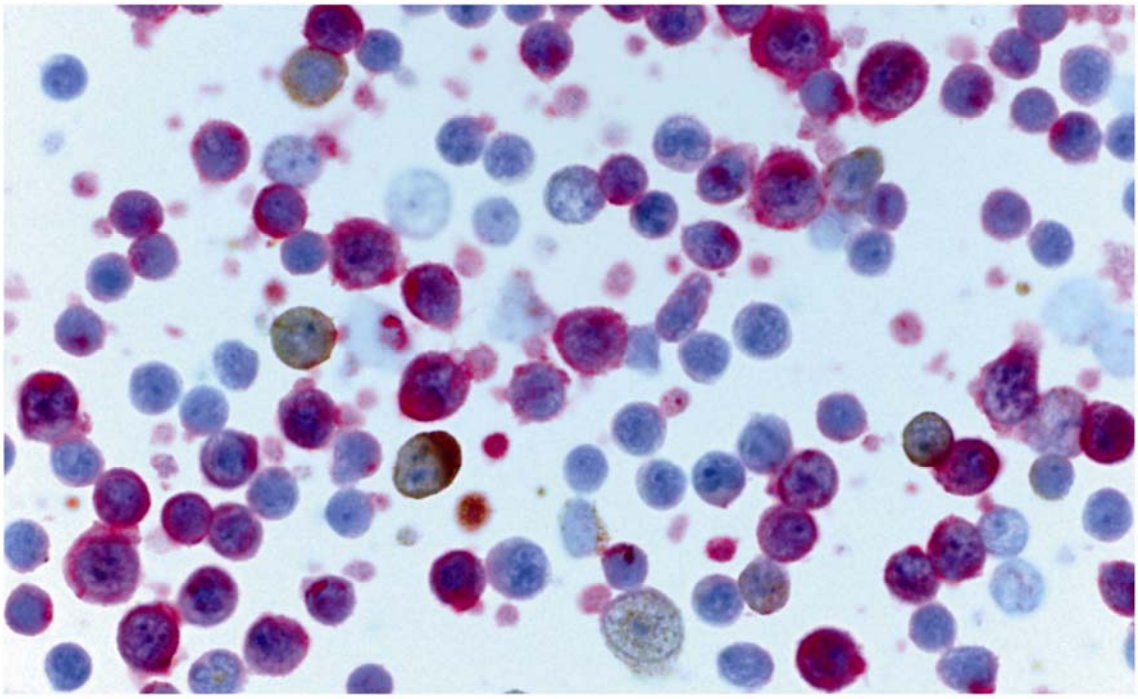

Figure 2. Immunocytochemical double staining of Follicular lymphoma (case 18); IDS-1: κ/λ rate 6.2

Most cells were positive for Kappa, and light-chain restriction was detected.

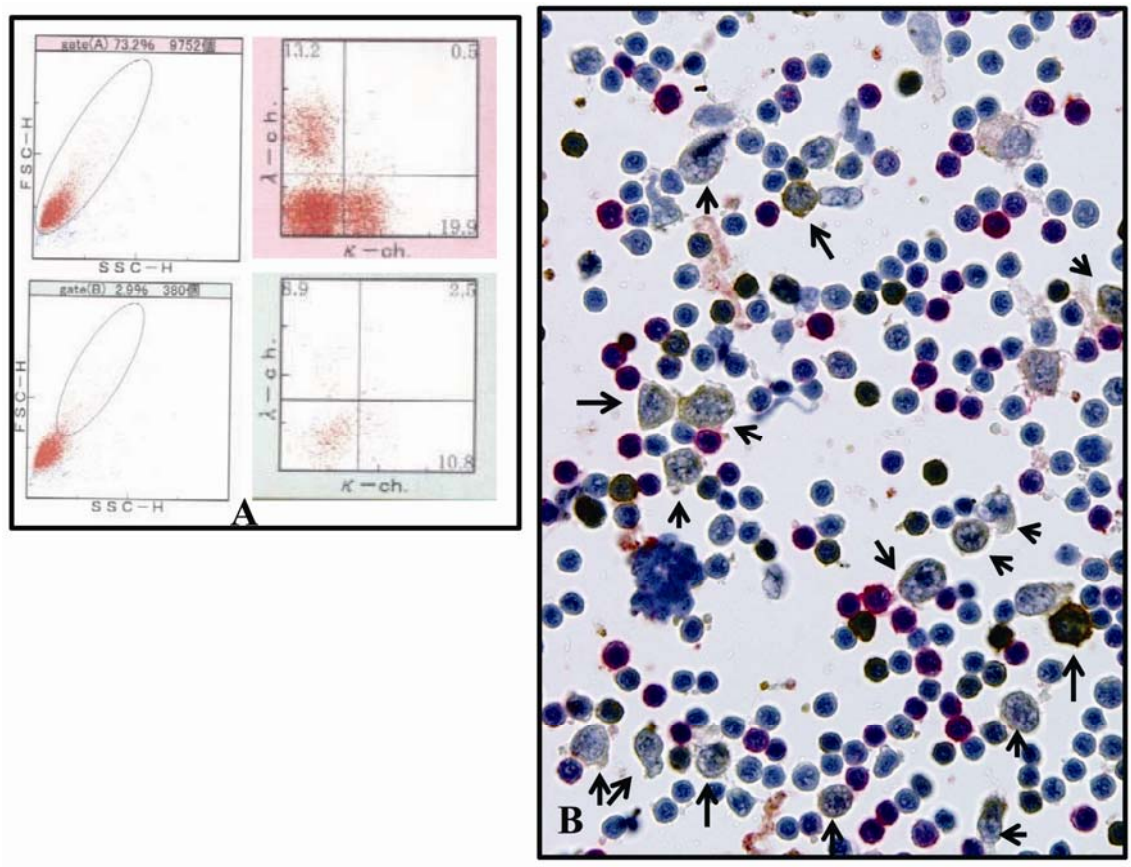

Figure 3. case 23 Follicular lymphoma grade3; κ/λ rate (FCM:1.5/IDS-1:1.6/IDS-2:3.2)
A: There was no light-chain restriction in any FCM gating cell.
B: There was no light-chain restriction in any small lymphocyte. However, large atypical cells were positive for Lambda alone (brown, arrow).

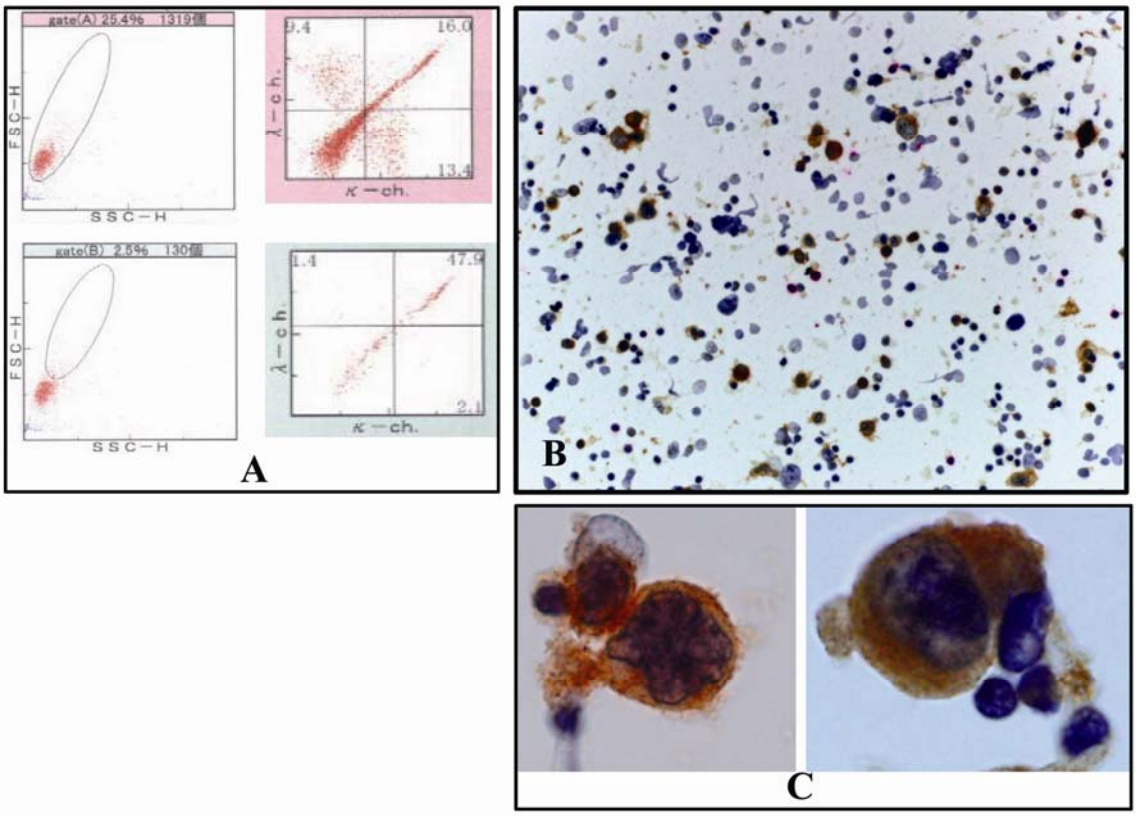

Figure 4. case32 diffuse large B-cell lymphomas; κ/λ rate (FCM:1.2/IDS-1:3.3 /IDS-2:24) A: A large number of degenerative cells were observed in the FCM gating. No light-chain restriction was detected. B: In the background, there were a large number of naked cells.Large, irregular, Lambda (brown)-positive cells were observed. Small lymphocytes were positive for Kappa (red).

C: Large lymphoma cells were positive for Lambda (brown).

Table 1 Percentage of Flow Cytometry and Immunocytochemical double stain

case NO	Histopathology	Flow Cytometry				Immunocytochemical double stain					
						IDS-1(random)			IDS-2(medium-large)		
		CD20	κ	λ	κ/λ or λ / κ	κ	λ	κ/λ or λ / κ	κ	λ	κ/λ or λ / κ
1	Reactive	51.2	17.4	12.1	1.4	23.7	29	1.2	24	44	1.8
2	Reactive		-	-	-	19	13	1.5	36	23	1.5
3	Reactive		-	-	-	20.1	12	1.7	32	22	1.5
4	Reactive	75.3	33.1	28.5	1.2	44.6	25	1.8	25	12	2.1
5	Reactive	63.4	30	26	1.2	21.8	28	1.3	16	19	1.2
6	Reactive		-	-	-	33.4	21	1.6	37	34	1.1
7	Reactive	40.1	14.8	14.9	1	9.6	9.4	1	32	20	1.6
8	Reactive	50.4	24.7	20.3	1.2	5	3.2	1.6	6	3	2
9	Reactive	75.5	36.5	36.7	1	3.8	2.6	1.5	7	6	1.2
10	Reactive	65.1	36.2	28.4	1.3	38.2	33	1.1	33	61	1.9
11	Reactive	33	17.6	14.4	1.2	25.6	25	1	29	31	1.1
12	Reactive	37.4	16.8	16.2	1	9	10	1.1	6	8	1.3
13	Reactive		-	-	-	5.6	2.8	2	15	9	1.6
14	T-LBL	7.5	3.5	4.6	1.3	3.4	4.6	1.4	1	2	2
15	PTCL	28	13	8.6	1.5	15.3	17	1.1	24	44	1.8
16	HL	40.2	19	17	1.1	27.4	20	1.3	44	44	1
17	HL	51.4	29	22	1.4	22.6	20	1.1	33	40	1.2
18	FL	87.9	87.1	3.9	22.3	57.8	9.2	6.2	90.5	9.5	9.5
19	FL	68.9	6.4	4.3	1.5	35	6.2	5.6	32	6	5.3
20	FL	72.4	1	56	56	1	30.3	30	1	23	23
21	FL	82	4.9	3.8	1.3	9.5	9.2	1	10.5	50	4.8
22	FL	83.8	1.4	57	41	5.7	37.7	6.6	7	42	6
23	FL	50.9	20.4	14	1.5	26.2	15.8	1.6	12	38.6	3.2
24	FL	94.3	2.6	1.4	1.9	23	27.6	1.2	40.5	26	1.6
25	FL	72.3	8.1	66	8.1	8.8	77.5	8.8	1	45	45
26	DLBCL	44.1	56	7.8	7.2	9	8.1	1.1	71	11	6.4
27	DLBCL	96	82	2.6	31	53	1	53	88	1	88
28	DLBCL	33.4	2.6	68	26	19	38.8	2	21	64	3

29	DLBCL	98.1	99	1.1	90	88	1	88	92.5	1	93
30	DLBCL	95.7	89.1	8.3	10.7	55	3.2	17	56	8	7
31	DLBCL	29.9	25	27	1.1	6.8	3.4	2	49	26	1.9
32	DLBCL	28.2	29	25	1.2	4	13	3.3	0	24	24
33	DLBCL	92	77	17	4.5	22	12.6	1.7	69.5	15	4.6
34	DLBCL	78.5	3.2	84	26	7	59.2	8.5	14	72	5.1
35	ML		-	-	-	100	1	100	100	1	100
36	LP	24.3	79	1	79	76	4	19	100	1	100
37	PM		-	-	-	1	100	100	1	100	100
38	MCL	93.8	92	1	92	90	2.4	38	90	8.5	11
39	BLL	99.5	99	1	99	100	1	100	100	1	100
40	ML	37.1	1	22.5	22.5	7.2	6.2	1.2	54	4	14

A value of 1 or less was defined as 1.

T-LBL:T-lymphoblastic lymphoma PTCL: peripheral T-cell lymphoma HL: Hodgkin's lymphoma FL:follicular lymphoma DLBCL:diffuse larg B cell lymphoma ML:malignant lymphomaNOS LP:lymphoplasmacytic lymphoma PM: plasma cell myeloma MCL:mantle cell lymphoma BLL: Burkitt-like lymphoma

Table 2　Negative control * (n=17)

			κ:λ ratio	
	LCRr	case	range	median
FCM	<3	17	1-1.5	(1.2)
IDS-1	<3	17	1-2	(1.3)
IDS-2	<3	17	1-2.1	(1.5)

* Negative control : reactive lesion, T cell lymphoma, Hodgkin lymphoma

LCRr : light-chain restriction ratio (κ/λ ratio or λ/κ ratio)

FCM : Flow cytometry

IDS-1 : Immunocytochemical double staining (random)

IDS-2 : Immunocytochemical double staining (medium-large)

Table 3　light-chain restriction ratio of B-cell lymphomas

			κ:λ ratio	
			range	median
FCM (n=21)	<3	6 (28.6%)	1.1-1.9	(1.5)
	>3	15 (71.4%)	4.5 - 99	(22.5)
IDS-1(n=23)	<3	8 (34.7%)	1-2	(1.2)
	>3	15(65.2%)	3.3-100	(24.5)
IDS-2(n=23)	<3	2 (8.6 %)	1.6-1.9	(1.7)
	>3	21 (91.3%)	3-100	(17)

LCRr : light-chain restriction ratio (κ/λ ratio or λ/κ ratio)

FCM : Flow cytometry

IDS-1 : Immunocytochemical double staining (random)

IDS-2 : Immunocytochemical double staining (medium-large)

Table4　light-chain restriction ratio of B-cell lymphomas

Comparison of FCM and IDS (n＝21）

	method			
Method pattern	FCM	IDS-1	IDS-2	case
FCM	detected	-	-	0
FCM ・IDS-1	detected	detected	-	0
FCM ・IDS-1 ・IDS-2	detected	detected	detected	11（52.3%）
FCM ・IDS-2	detected	-	detected	4（19.0%）
IDS-1 ・IDS-2	-	detected	detected	3（14.2%）
IDS-2	-	-	detected	1（4.8%）
negative	-	-	-	2（9.5%）

LCR : light-chain restriction

FCM : Flow cytometry

IDS-1 : Immunocytochemical double staining (random)

IDS-2 : Immunocytochemical double staining (medium-large)

Table 5　Evaluation of LCR in FCM and IDS

	FCM	IDS-1	IDS-2
Sensitivity	71.4%	65.2%	91.3%
Specificity	100%	100%	100%
Positive Predictive Value	100%	100%	100%
Negative Predictive Value	68.4%	68.0%	89.4%

LCR : light-chain restriction

IDS-1 : Immunocytochemical double staining (random)

IDS-2 : Immunocytochemical double staining (medium-large)