

Tumor microenvironmental reaction would diminish incomparable accuracy and swiftness of flow cytometric diagnosis of primary central nervous system lymphoma

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Abstract

Background

Primary central nervous system lymphoma (PCNSL), a relatively rare brain tumor, bears a dire prognosis. On occasion, rapid progression of the tumor makes immediate diagnosis and initiation of therapy imperative. To achieve swift diagnosis, we adopt flow cytometry (FCM) in addition to conventional histopathology. The aim of this study is to reveal utility and drawbacks of FCM diagnosis for PCNSL.

Methods

Patients with suspected PCNSL on neuroradiological findings and received both FCM and histological diagnosis between August 2015 and April 2020 were retrospectively enrolled into the study. Tumor samples were collected by craniotomy with either of endoscope or microscope. The patients' electronic medical records were investigated, and histological findings, results of FCM, and other clinical data were evaluated.

Results

Twenty seven patients met the eligibility criteria. Twenty three patients (11 males and 12 females) were diagnosed with PCNSL by histological confirmation, and 22 cases were B-cell type lymphoma and 1 was T-cell type. Median age at diagnosis was 65. FCM analysis showed lymphoma pattern in 20 cases, but in the other 3 lymphoma cases (FCM discordant: FCM-D) and 4 non-lymphomatous tumor cases, FCM results did not show lymphoma pattern (sensitivity: 86.4%, specificity: 100%). Analysis of FCM-D cases showed infiltration of T lymphocytes or astrocytes into the tumor tissue, indicating tumor microenvironmental reaction, were observed, and it is assumed that those reactions deceived FCM diagnosis.

Conclusions

Despite some disadvantages, diagnosis of PCNSL by FCM is rapid and reliable.

Introduction

Primary central nervous system lymphoma (PCNSL) accounts for 1.9% among all brain tumors in the U.S. [1] and 4.5% in Japan. [2] The prognosis of PCNSL is still poor, with 5-year survival rate is less than 50%. [3, 4] In some cases, acute aggravation of the symptom due to rapid enlargement of tumor is observed, and swift diagnosis for early initiation of treatment is necessary. At this moment, histopathology of paraffin-embedded specimen is the diagnostic modality of choice, and this method usually takes several days for formalin-fixation, preparation, hematoxylin and eosin (HE) staining and immunohistochemistry

for diagnosis. Flow cytometry (FCM) plays a well-accepted role in diagnosis and evaluation of hematopoietic neoplasms including extracranial lymphoid malignancies. [5, 6] FCM takes only a few hours for analysis. However, FCM in the diagnosis of PCNSL have not gained widespread popularity and only a few reports concerning this modality have been published so far. [7, 8, 9] We have experienced cases of PCNSLs in which surgical specimen were provided both to histopathology and FCM, and in most cases early commencement of therapy was succeeded owing to FCM diagnosis by the next day of operation. Yet, in small number of the cases, FCM failed to detect PCNSL. We have investigated the mechanism of FCM deficiency, and revealed effects of tumor microenvironmental reaction such as infiltration of astrocytes or T-lymphocytes. We present the result with review of relevant literatures.

Methods

Patients

This retrospective study protocol was completed following approval from the institutional review board at Kyoto Prefectural University of Medicine. We collected data from consecutive patients with PCNSL and other brain tumors who received FCM analysis of tumor between August 2015 and April 2020. Patients were included if clinical and neuroradiological findings suggested lymphoma. Patients with previous history of malignancy was excluded. Clinical data were collected such as age, sex, previous use of medication including corticosteroids, blood examination including soluble interleukin-2 receptor (sIL-2R), neuroradiological findings, and histological classification of the tumor.

Surgical procedure

All the patients underwent craniotomy for removal of the tumor. Three patients (Cases 5, 7, 8) received small craniotomy for endoscopic biopsy by a 4mm steerable fiberscope (Olympus Optical Company, Tokyo, Japan), and the rest of the patients received craniotomy and tumor removal with operative microscope (M530 OH6, Leica Microsystems GmbH, Wetzlar, Germany) equipped with Leica FL 400 (blue light fluorescence module). Microscopic surgery was assisted by neuronavigation system (Stealth Station S7, Medtronic, Minneapolis, MN), and enhanced lesions were the target. After craniotomy, small corticotomy was made. Then, either of Neuroport (Olympus, Tokyo, Japan) or Viewsite (Vicor Medical, Boca Raton, FL) was inserted. Tumor site was confirmed by both neuronavigation system and direct observation by the surgeon. A few pieces of the lesion were collected with biopsy forceps and were served for intraoperative frozen section. If the results suggested neoplastic features (e.g., increased cellularity, nuclear atypia), then additional tissue samples were taken. In some cases, when brain tissue bulges, bulk removal of the tumor was added to lessen intracranial pressure. Those tumor samples are divided into half, and provided for flow cytometry and formalin-fixation.

When malignant glioma was also suspected prior to surgery, 5-aminolevulinic acid (5-ALA; 20 mg/kg body weight) was administered orally to the patient 3 hours prior to the induction of anesthesia. During the operation, violet-red fluorescence visualization of malignant tumor tissue after excitation with 405 nm

wavelength blue light was inspected. Strength of fluorescence was checked by the surgeon's objective observation. According to the findings "No", "Vague", or "Strong" fluorescent was recorded.

Flow Cytometry and Histopathological Diagnosis

Unfixed tumor tissue samples were disaggregated gently, filtered with a 70- μ m nylon cell strainer (Corning, Corning, NY), then washed and resuspended with phosphate-buffered saline (PBS; Takara, Shiga, Japan) containing 2% bovine albumin (BSA; Nacalai Tesque, Kyoto, Japan) and 0.1% sodium azide (Nacalai Tesque) at a final cell concentration between 0.5–10 x 10³/ μ L. According to the manufacturer's recommendation, 100 μ L of this cell suspension was stained with the combination of the following monoclonal antibodies conjugated with fluorescence isothiocyanate (FITC), phycoerythrin (PE) or PE-cyanin 5 (PC5); CD1a (clone BL6), CD2 (39C1.5), CD3 (UCHT1), CD4 (T4), CD5 (BL1a), CD7 (3A1), CD8 (T8), CD10 (J5), CD19 (B4), CD20 (B9E9), CD33 (D3HL60.251), CD45 (J33), and CD56-PE (NKH-1) and HLA-DR (Immu-357); all of them were purchased from Beckmann-Coulter (Marseille, France). On the other hand, anti-surface immunoglobulin (Sm) kappa light chain-FITC and Sm lambda light chain-PE were purchased from Dako/Agilent (Santa Clara, CA). FITC or PE-conjugated mouse IgG1 (Beckmann-Coulter) were used as negative control. After incubation for 15 minutes at room temperature in complete darkness, samples were washed again and resuspended at a final volume of 0.5 mL. Then, samples were analyzed using NAVIOS flow cytometer and Kaluza software (Beckmann-Coulter). Maximally 5,000 events were gated on forward scatter (FS) and side scatter (SS) plots. When at least 20% of gated cells expressed CD19 and/or CD20 together with restricted Sm light chain expression (kappa/lambda ratio is > 3.0 or < 0.5), samples were diagnosed as B-cell lymphoma. Concerning the diagnosis of T-NHL, aberrant expression of T-cell antigens such as CD2, CD3, CD5 or CD7 was necessary in addition to deviation of CD4/CD8 ratio (> 3.5 or < 1.0).

The tumor samples were formalin fixed immediately after removal during operation. Then embedded in paraffin, and thin sliced at 5 micrometer thickness. Sections were then stained with hematoxylin-eosin using standard protocols. Immunohistochemistry was also performed using standard procedures, with primary antibodies as described below: CD3 (Roche, Diagnostics, Rotkreuz, Switzerland, clone 2GV6), CD10 (Leica Biosystems, Buffalo Grove, IL, clone 56C6), CD20 (Roche Diagnostics, clone L26), CD79a (DAKO, Carpinteria, CA, clone JCB117), bcl-2 (DAKO, clone 124), bcl-6 (Leica Biosystems, clone LN22), MUM-1 (Leica Biosystems, clone EAU32), MIB-1 (DAKO, clone ki67).

The pathologist (Y.S.) and hematologist (T.I.) who evaluated the analysis were blinded for each other's conclusion. Subtype classification of DLBCL, the germinal center B-like (GCB) and activated B-cell (ABC) types were done according to the immunostaining of CD10, BCL6, and MUM-1. [10]

Statistical Analysis

The data were expressed as mean \pm SD. Statistical analysis was performed by using Student's t test (two-tailed), or Fisher's exact test. The criterion for statistical significance was taken as P-values below 0.05.

Statistical analysis was performed using EZR software which is a graphical user interface for R (The R Foundation for Statistical Computing, Vienna, Austria). [11]

Results

We identified 27 consecutive patients who received both FCM analysis and histopathological diagnosis for brain tumor during the observation period. Patient demographics were summarized in Table 1. No patient was positive for human immunodeficiency virus (HIV), Epstein-Barr virus (EBV), or human T-cell leukemia virus type 1 (HTLV-1). None of the patients except for two (case 11, 21) received corticosteroids prior to operation. One (case 11) was given 2 days of betamethasone (8 mg/day) on the day of admission to the referring hospital. The other patient (case 21) was given repeated dose of prednisolone as much as 50 mg/day for 2 months because her disease was at first assumed as neuro-Behçet's disease based on neuroradiological findings. In both cases, operation was done more than 1 week later from the last administration of steroid.

Among the 27 patients 23 had final diagnosis of PCNSL. Of those, FCM analysis detected lymphoma (FCM concordant: FCM-C) in 20 cases (B-NHL or T-lymphoma), and rest 3 were not (FCM discordant: FCM-D) (sensitivity: 86.4%). A typical FCM-C case is shown in Fig. 1A. The 4 patients who were not diagnosed with PCNSL, 3 were glioblastoma, 1 was diffuse midline glioma H3K27M mutated, and for all of them FCM also did not show lymphoma pattern (specificity: 100%, Table 2).

After confirmation of the diagnosis as PCNSL, patients were administered chemotherapeutic medications mainly consisted of high-dose methotrexate, procarbazine, and vincristine. In addition, rituximab was added in all cases when the tumor cells were positive for CD20. The clinical courses of all of those patients are compatible with PCNSL, hence diagnostic accuracy of histopathology was confirmed clinically also. In FCM-C group, median days from surgery to start chemotherapy was 5 days, which were shorter than FCM-D group (24 days), but not reached statistical significance ($p = 0.057$).

Next, to investigate tumor characteristics that would influence FCM concordance, we performed univariate analysis. Univariate analysis revealed only patient age was significantly elder in FCM-D, but there was no correlation between FCM-D and patient sex, previous use of steroid, sIL-2R value, subtype (GCB/ non-GCB) or 5-ALA fluorescence (Table 3). Then we inquired each 3 cases histologically. In case 14, FCM revealed that more than 70% of analyzed cells were T-cells without definite aberrant antigen expression, and B cells were less about 20% of analyzed cells without definite deviation of Sm kappa/lambda ratio ($16.8/7.5 = 2.24$) (Fig. 1B). By histopathological analysis, uniformly proliferating large cells with round-to-oval nuclei indicating lymphomatic change were observed (Fig. 2F). In addition, CD20 positive cells (Fig. 2G) and bcl-6 positive cells (Fig. 2I) made the diagnosis as diffuse large cell lymphoma. In that specimen, T lymphocytes (Fig. 2H) did were observed, but by integrating the findings together, the diagnosis as diffuse large B-cell lymphoma was made. In case 17, histopathological analysis indicated co-existence of high cellularity portion and normo-cellularity portion (Fig. 2K-O). In the high cellularity portion, typical morphology of lymphoma was observed, but in the normocellularity

portion infiltration of T cells were detected. The pathological diagnosis was according to the high cellularity portion, so that this case was determined as DLBCL. However, by FCM analysis, mixture of CD3 and CD5 positive T lymphocytes has lead the diagnosis indeterminate. In case 18, FCM was inconclusive because about 15% of analyzed cells were B-lymphocytes without definite deviation of Sm kappa/lambda ratio. Histological findings showed that the same specimen revealed mixture of lymphoma cells and reactive astrocytes (Fig. 2P-T). Compare to those, in FCM-C cases, infiltration of T-lymphocytes or astrocytes were minimal (Fig. 2A-E). In summary, we have determined that invasion of reactive cells such as astrocytes or T lymphocytes, and low volume of tumor cells among tissue were the cause of FCM-D.

Discussion

In this study, we have demonstrated the effectiveness of FCM in the diagnosis of PCNSL. It not only is swift but also has high sensitivity and specificity. Early diagnosis of CNS lymphoma has already reported to prolongs survival. [12] The time interval between operation and beginning of chemotherapy of FCM-C group was shorter than that of FCM-D group, although this difference did not reach statistical significance. By a through literature search, we have found 3 reports mentioning FCM for the diagnosis of PCNSL. [5, 6, 7] According to them, sensitivity and specificity of FCM are 87.8–100%, and 100%, respectively. Our results also support that fact.

In our series, 3 out of 22 cases were FCM-D, but no patient background factors except for age were significantly different between FCM-C and FCM-D groups. We think this was because of small sample size. Instead, by histological analysis, we have disclosed one of the important reasons of discordance. As shown in case 14 and 18, tumor microenvironment (TME) interaction such as infiltration of reactive T-lymphocytes and/or astrocytes were observed. In previously reported cases of FCM diagnosis, 10 out of 77 PCNSL cases were FCM-D. Among those 10 cases, authors guessed the reason as steroid use in 2, patchy infiltrate caused by DLBCL in 1, intravascular DLBCL in 1, and unknown in 6 cases. The “patchy infiltrate caused by DLBCL” seems corresponding to the TME reaction. By recent investigations, role of TME became clear. A certain portion of PCNSL has high amount of tumor infiltrating lymphocytes (TILs), astrocytes, and microglia, [13] and such TME reaction may be related to immune checkpoints. [14] Because those TILs, astrocytes or microglia do not have monoclonality, FCM that analyze sample cells as a whole, cannot detect neoplastic nature in such situation. Histopathological diagnosis is made based on the high-cellularity portion among the specimen by manual search, so that it is not much difficult to distinguish neoplastic portion and infiltrating T-lymphocytes or astrocytes. In summary, stronger reaction of TME would be the noticeable reason of discordance.

As for steroid use, in our cases, it did not cause FCM-D. One reason of this, we presume, is the time interval between final steroid use and surgery was long enough for the tumor cells to re-activate, and the other is employment of open biopsy. Effect of steroid has been shown to necrotize lymphoma cells, [15] while in a recent report, even under the influence of steroid correct lymphoma diagnosis could be made. [16] Histopathologically, in our steroid pretreated cases, some necrotic areas were observed, but area of

viable cells were dominant. Open biopsy can collect tumor tissue by direct visualization. Weaknesses of stereotactic biopsy are mis-sampling and hemorrhagic complications. In one report, 6.2% of the stereotactic biopsy cases were non diagnostic, instead symptomatic hemorrhage was observed in 2.6%, and asymptomatic hemorrhage occurred in as many as 28.8%. [17] In comparison, with open biopsy, surgeon can confirm color and hardness of the tissue. Another advantage is that volume reduction is achievable. Though no benefit of volume reduction in PCNSL was believed until recently, usefulness of craniotomy was reported. [18, 19]

Another possible method to diagnose PCNSL is to analyze cerebrospinal fluid (CSF) or using FCM for distinguishing PCNSL from GBM by propidium iodide for staining DNA. The former, in spite of relatively wide acceptance, CSF cytology suffers from its low sensitivity (2–32%). [20] Recently, diagnostic utility of cell free circulating tumor DNA in CSF has been reported. [21] Still, this method is not yet established and time consuming. Besides, collecting CSF in the situation of elevated intracranial pressure is quite dangerous and not recommended. The latter, as reported by Koriyama and colleagues in 2018, distinguishing between PCNSL and GBM by DNA aneuploidy or S phase is also useful, but that method, not checking surface markers, cannot analyze lymphoma subtype. [22] Compare to those, FCM analysis of surface marker can provide useful information to treat lymphoma.

A potential limitation of this study lies in its retrospective nature. However, we believe there does not exist selection bias because we executed FCM in all eligible patients. Another potential limitation of our study is the small sample size. We encountered 23 PCNSL patients, and FCM-D cases were only 3. That made much of statistical analysis no significance, therefore larger study to confirm this result is needed. In addition, we recognize that the equipment performance of flow cytometer seems also important to improve the diagnostic utility for PCNSL. We analyzed the samples using the traditional 2–3 color FCM with 1 laser throughout the course of this study, but we are now in the process of updating to 10-color FCM using 3 lasers.

In conclusion, FCM is a powerful tool for the diagnosis of PCNSL, but there are very few false negative cases: infiltration of reactive immune cells or glia, or necrosis are the possible mechanisms.

Declarations

Funding: None

Conflict of interest: None of the authors have any conflict of interest to declare.

Availability of data and material: Not applicable

Code availability: Not applicable

Ethical approval: This research study was conducted retrospectively from data obtained for clinical purpose. This study was granted IRB approval from Kyoto Prefectural University of Medicine.

Informed consent: Informed consent was obtained from all individuals included in the study, whenever possible, taking into account the retrospective nature of the study.

Contributions

Conception and design: HT. Acquisition of data: HT, TO, ST, TY, YT. Analysis and interpretation of data: IT, YS, TT, SM. Drafting the manuscript: HT. Critically revising the article: IT, YS, EK, JK, NH. Statistical analysis: HT. Reviewed final version of the manuscript and approved it for submission: all authors. Study supervision: NH.

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Tables

Due to technical limitations, table 1, 2, 3 is only available as a download in the Supplemental Files section.

Figures

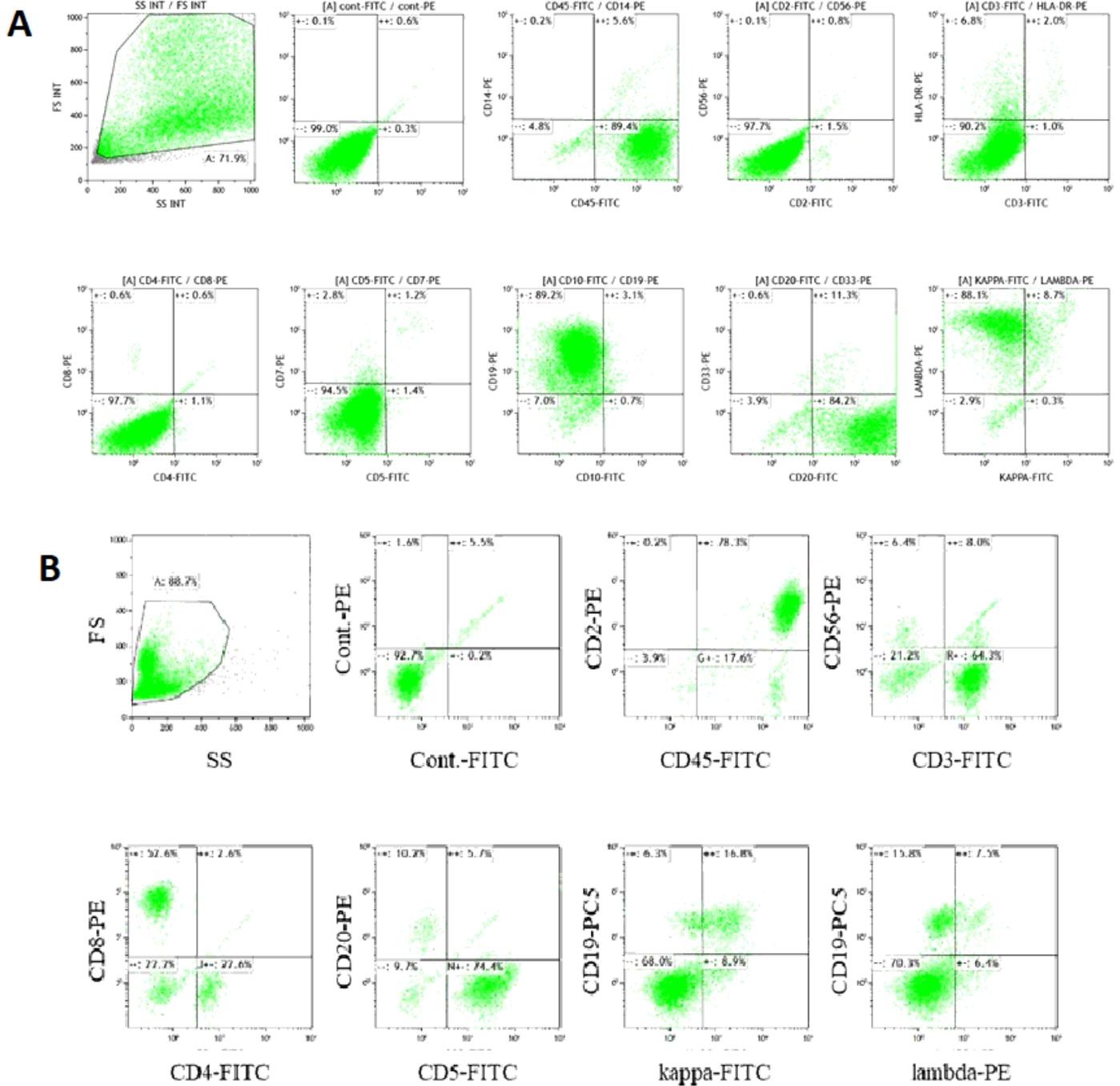


Figure 1

Examples of flow cytometric analysis which resulted in positive (A: Case 12) and negative (B: Case 14) lymphoma pattern, both of which afterwards were histopathologically diagnosed as diffuse large B cell type lymphoma. In A, FCM results showed positive for CD20, but negative for CD3 and CD10. In addition, IgM-kappa, and -lambda was clearly divided. Those were indicating lymphoma. In B, CD45 (leukocyte

common antigen) was positive, but B-cell surface markers (CD19, CD20) were negative. Therefore, FCM analysis was inconclusive.

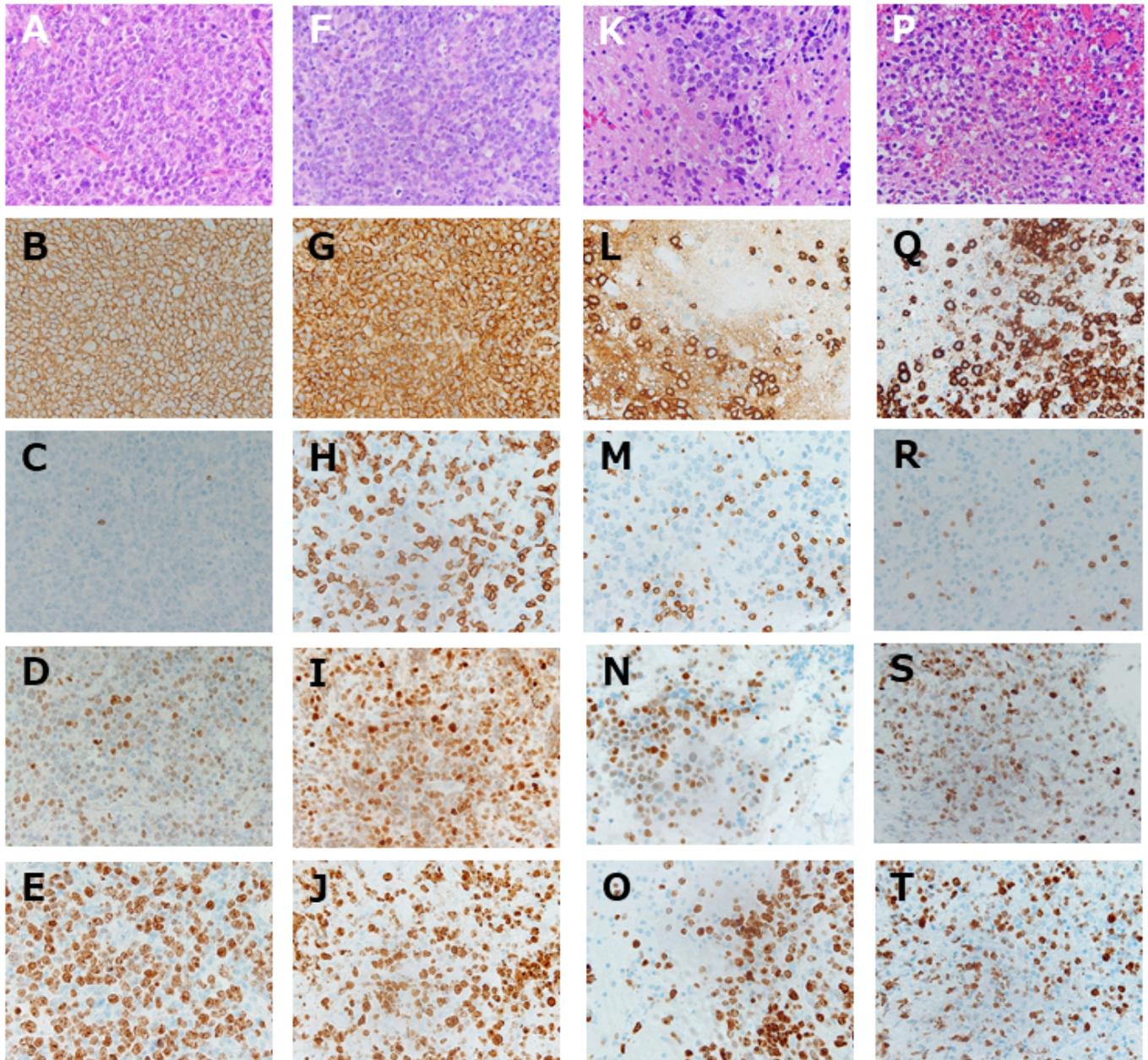


Figure 2

Photomicrography of tissue specimen. A, F, K, P: Hematoxylin-Eosin stain. B, G, L, Q: CD20. C, H, M, R: CD3. D, I, N, S: Bcl-6. E, J, O, T: MIB-1. A-E: Representative FCM-C case (Case 12). Hematoxylin-Eosin (HE) stain shows highly cellular, diffusely growing tumor cells with large round-to-oval nuclei (A). Tumor cells uniformly express pan-B cell marker, CD 20 (B), but T cell marker, CD 3, is negative (C). Bcl-6 is positive (D), and MIB-1 labelling index is about 80% (E). F-J: Case 14. H-E stain reveals high cellularity of large atypical cells with large round, oval nucleoli (F), and the tumor cells seemingly uniformly express CD20 (G), and CD 3, is partially positive, suggesting infiltration of reactive T lymphocytes (H). Expression of Bcl-

6 helps the diagnosis of DLBCL. K-O: Case 17. Existence of normo- to hypo-cellular area and some infiltration of T lymphocytes are observed. P-T: Case 18.

Table 1 Characteristics of patients included in this study

Case	Age	Sex	Histological diagnosis	Subtype of B cell lymphoma	FCM	Days between OP and chemo	sIL-2R	5-ALA
1	78	F	DLBCL	non-GCB	B-NHL	3	951	N/A
2	55	F	DLBCL	N/D	B-NHL	8	262	vague
3	69	F	DLBCL	GCB	B-NHL	5	598	(-)
4	53	M	DLBCL	GCB	B-NHL	2	629	N/A
5	80	F	DLBCL	non-GCB	B-NHL	6	519	(-)
6	61	M	DLBCL	N/D	B-NHL	1	554	N/A
7	60	M	DLBCL	GCB	B-NHL	9	327	strong
8	65	F	DLBCL	GCB	B-NHL	8	224	strong
9	60	M	DLBCL	GCB	B-NHL	11	345	N/A
10	65	M	DLBCL	non-GCB	B-NHL	7	306	N/A
11	67	M	DLBCL	non-GCB	B-NHL	6	526	N/A
12	64	F	DLBCL	non-GCB	B-NHL	2	923	N/A
13	79	M	T-cell lymphoma	N/A	T-cell lymphoma	3	303	N/A
14	83	F	DLBCL	non-GCB	(-)	14	984	(-)
15	53	M	DLBCL	GCB	B-NHL	4	589	strong
16	69	F	DLBCL	non-GCB	B-NHL	3	422	N/A
17	71	M	DLBCL	non-GCB	(-)	28	397	(-)
18	83	F	DLBCL	non-GCB	(-)	24	677	vague
19	68	F	DLBCL	GCB	B-NHL	8	389	vague
20	64	F	DLBCL	non-GCB	B-NHL	7	302	strong
21	49	F	DLBCL	non-GCB	B-NHL	4	513	(-)
22	74	F	DLBCL	non-GCB	B-NHL	3	833	vague
23	65	M	DLBCL	non-GCB	B-NHL	3	370	N/A
24	79	F	GBM	N/A	(-)	N/A	276	strong
25	83	M	GBM	N/A	(-)	N/A	325	strong
26	64	M	GBM	N/A	(-)	N/A	259	strong
27	38	M	DMG	N/A	(-)	N/A	154	strong

B-NHL: B-cell non-Hodgkin lymphoma, DLBCL: diffuse large B-cell lymphoma, H3K27M: diffuse midline glioma H3K27M mutated, N/D: no data, N/A: not applicable

Figure 3

Table 2 Diagnostic value of flow cytometry

flow cytometry	histopathology		
	lymphoma	no lymphoma	total
lymphoma	20 ^a	0	20
inconclusive	3 ^b	4	7
total	23	4	27

a: flow cytometry-concordant, b: flow cytometry-discordant

Figure 4

Table 3 Summary of patient background for flow cytometry concordant and discordant

	FCM-C (n=20)	FCM-D (n=3)	p-value
age	65	83	0.047
sex (M:F)	9:10	1:2	0.602
steroid use	3	0	0.644
sIL-2R	422	677	0.356
GCB:non-GCB	7:10	0:3	0.251
5-ALA (No:Vague:Strong)	3:3:4	2:1:0	0.720

Figure 5