Utility of a Reverse Phase Protein Array to Evaluate Multiple Biomarkers in Diffuse Large B-Cell Lymphoma

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Purpose: Diffuse large B-cell lymphoma (DLBCL), the most common non-Hodgkin lymphoma, is a heterogeneous lymphoma with different clinical manifestations and molecular alterations, and several markers are currently being measured routinely for its diagnosis, subtyping, or prognostication by immunohistochemistry (IHC). Here, the utility of a reverse-phase-protein-array (RPPA) as a novel supportive tool to measure multiple biomarkers for DLBCL diagnosis is validated.

Experimental design: The expression of seven markers (CD5, CD10, BCL2, BCL6, MUM1, Ki-67, and C-MYC) is analyzed by RPPA and IHC using 37 DLBCL tissues, and the correlation between the two methods is determined. To normalize tumor content ratio in the tissues, the raw RPPA values of each marker are adjusted by that of CD20 or PAX-5.

Results: The CD20-adjusted data for CD5, MUM1, BCL2, Ki-67, and C-MYC has better correlation with IHC results than PAX-5-adjusted data. Receiver operating characteristic (ROC) analysis reveals that CD5, MUM1, BCL2, and C-MYC exhibit a better sensitivity and specificity >0.750. Furthermore, the CD20-adjusted C-MYC value strongly correlates with that of IHC, and has a particularly high specificity (0.882).

Conclusions and clinical relevance: Although further investigation using a large number of DLBCL specimens needs to be conducted, these results suggest that RPPA could be applicable as a supportive tool for determining lymphoma prognosis.

1. Introduction

Diffuse large B-cell lymphoma (DLBCL) is the most common non-Hodgkin lymphoma in adults, accounting for one-third of cases.\(^1\) DLBCL is regarded as a heterogeneous group of lymphomas with varying clinical manifestations and underlying molecular alterations, and prognostic subtyping is currently based on immunohistochemical (IHC) algorithms, gene expression profiling, and fluorescence in-situ hybridization. Hans et al. developed an IHC algorithm that classified DLBCL into germinal center B cell (GCB) and non-GCB subtypes by analyzing expression patterns of CD10, BCL6, and MUM1,\(^2\) and patients with the GCB subtype have generally favorable outcomes compared with those with the non-GCB subtype.\(^3\)–\(^4\) DLBCL with CD5 expression accounts for approximately 10% of DLBCL,\(^5\) and is characterized by aggressive clinical features and frequent central nervous system relapses.\(^6\)–\(^8\) Previous studies have investigated the protein abundance and/or gene translocation of C-MYC, BCL2, and BCL6, which may also have predictive prognostic potential.\(^9\)–\(^18\) Flow cytometry has been used for the evaluation of cell surface markers to diagnose malignant lymphomas. IHC is especially important for pathological diagnosis and prognostication of DLBCL. However, IHC specimens occasionally show heterogeneous, weak, or nonspecific staining, and such specimens make it difficult to identify markers weakly expressed in some cases.\(^1\)
evaluate protein abundance in a reproducible manner. Moreover, IHC is a semi-quantitative method for measuring protein abundance in vivo, and cannot provide quantitative results. For clinical DLBCL diagnosis, additional quantitative methods are required to improve the false positive rate, sensitivity, and specificity.

In the present study, we focused on reverse phase protein arrays (RPPA), which are a high-throughput clinical proteomics technique, and evaluated whether RPPA can provide quantitative diagnostic information for DLBCL diagnosis. An RPPA analytical system using clinical specimens was previously established by Loiotta’s group. RPPA analysis is a proteomic technology providing quantitative, multiplexed analysis of protein abundance and posttranslational modifications in limited clinical specimens such as cells, tissues, and body fluids. RPPA uses an approach similar to western blotting (antibody-based), but the methodology is different, as there is no separation based on weight. This makes it very important to use highly validated antibodies that do not show any non-specificity upon western blotting. An RPPA microarray can blot more than 1000 protein spots on a single glass slide over a nitrocellulose membrane, allowing for the detection of target proteins with specific antibodies. The greatest advantage of RPPA analysis is that about 10 nL of sample is enough for printing each spot, and a wider range of protein concentrations are acceptable for accurate quantification by RPPA analysis than by western blotting. RPPA analysis is therefore well suited for the detection and quantitation of proteins in clinical samples.

The aim of the present study is to validate the utility of RPPA as a novel complementary diagnostic method for quantitative detection of multiple biomarkers for DLBCL alongside IHC. We analyzed and compared the expression of seven biomarkers by RPPA and IHC using tumor tissue specimens from 37 DLBCL cases, and investigated the correlation between the two methods. We attempted to normalize the tumor cell ratios of the tissue samples used for RPPA. We also determined the specificity and sensitivity of the signal intensities following RPPA analysis. To our knowledge, this is the first comparison of RPPA and IHC using malignant lymphoma tissue samples in the clinical context.

2. Experimental Section

2.1. Case Selection

The ethics committee of the Kanagawa Cancer Center approved the experimental design. The cohort comprised of 37 cases diagnosed as DLBCL from 2007 to 2014 in Kanagawa Cancer Center. Cases were selected for inclusion in the cohort based on the availability of formalin-fixed paraffin-embedded (FFPE) and frozen tissue obtained from biopsy, with DLBCL diagnosed according to 2017 World Health Organization (WHO) classification criteria. The clinical data were retrieved from medical records. Frozen tissue specimens were stored at −80 °C at Kanagawa Cancer Center Biobank. Cases that had received chemotherapy prior to biopsy were excluded.

2.2. Antibodies

Monoclonal antibodies (mAbs) used for IHC and RPPA against CD10 (ab208778), BCL6 (ab172610), MUM1 (ab133590), Ki-67 (ab92742), C-MYC (ab32072), CD20 (ab9475), and PAX5 (ab109443) were obtained from Abcam (Cambridge, UK). Anti-CD5 mAb (MA5-13308) was obtained from Thermo Fisher Scientific (Waltham, MA). Anti-BCL2 mAb (M0887) was obtained from Agilent (Santa Clara, CA). Detailed antibody information is presented in Table 1a.

2.3. Western Blot Analysis

Total cell lysates (15 µg per lane) were separated by SDS-PAGE on a 5–20% gradient gel under reducing conditions. After electrophoresis, the proteins were transferred electrophoretically onto an Immobilon membrane (Millipore). Nonspecific sites were blocked with 5% dry milk in TBS-T (TBS containing 0.05% Tween-20) at 37 °C for 1 h, and the membrane was then incubated overnight at 4 °C with mAbs at the concentrations indicated in Table 1a. After washing with TBS-T, the membrane was incubated for 1 h with peroxidase-conjugated anti-rabbit IgG or antimouse IgG (GE Healthcare UK Ltd., UK) The resulting bands were detected by chemiluminescence according to the manufacturer’s instructions (PerkinElmer, Waltham, MA). An Immunostar LD western blotting detection system (FUJIFILM Wako Pure Chemical Co., Tokyo, Japan) was used for detection.

2.4. Immunohistochemical Analysis

IHC staining was performed on FFPE sections (4 µm thick) using an automated immunostainer (HISTOSTAINER 48A; Nichirei, Tokyo, Japan) with an automated immunostainer (HISTOSTAINER 48A; Nichirei, Tokyo, Japan)
Tokyo, Japan) with primary antibodies listed in Table 1a. The expression of each marker was detected using commercially available detection kits (Histofine Simple Stain MAX-PO kit; Nichirei, Tokyo, Japan). Appropriate positive and negative control tissues were used in each case. Staining for CD5, CD10, BCL2, BCL6, MUM1, PAX5, and CD20 were classified by a score from 1 to 6 based on the proportion of immunopositive tumor cells (1: <1%, 2: 1–9%, 3: 10–29%, 4: 30–49%, 5: 50–79%, 6: 80–100%). Staining for C-MYC and Ki-67 were evaluated in increments of 10%.

### 2.5. Reverse Phase Protein Array

Frozen DLBCL tissue was homogenized using a bead homogenizer (Yasui Kikai, Osaka, Japan). Tumor tissue and cell lysate were extracted from the respective specimens using T-PER protein extraction reagent (Thermo Fisher Scientific, Waltham, MA) supplemented with inhibitors (100 mM NaF, 1 mM Na3VO4, 10 mM NaPPi, 1 mM EDTA, PhosSTOP; Sigma–Aldrich, Missouri, MO) and protease inhibitor cocktail (1.04 mM AEBSF, 800 nM aprotinin, 40 μM bestatin, 14 μM E-64, 20 μM leupeptin, 15 μM pepstatin A; Sigma–Aldrich). The lysates were centrifuged at 15,000 × g at 4 °C for 20 min, and the supernatant was subjected to following analyses. After adjustment of protein concentration to about 1.5 mg mL−1 according to Bradford protein assay, the lysates were manually diluted in twofold serial dilutions with extraction buffer. The diluted lysates were boiled with 2% SDS and 2.5% β-mercaptoethanol, and printed onto nitrocellulose-coated slides in four replicates (Grace Bio-Labs, Bend, OR) using an Aushon Biosystems 2470 arrayer (Burlington, MA). After blocking with an odyssey blocking buffer (LI-COR biosciences, Lincoln, NE) supplemented with 0.1% Tween-20, the blotted slides were probed with validated primary antibodies, followed by secondary antibodies conjugated to infrared dyes, IRDye 680RD and 800CW (LI-COR biosciences). Slides were scanned using an ODYSSEY scanner (LI-COR biosciences). The signal intensity of each spot was quantified using Image Studio (LI-COR biosciences) according to the manufacturer’s instructions.

### 2.6. Cells and Culture Conditions

Human hepatoblastoma, HepG2, pancreatic epithelioid carcinoma, Panc-1, normal embryonic kidney epithelium, HEK293T, endocervical adenocarcinoma, HeLa, astrocytoma, U-251 MG (KO), KHM-10B and Daudi, Burkitt’s lymphoma, and T acute lymphoblastic leukemia, CCRF-CEM cells were purchased from JCRB cell bank (National Institute of Biomedical Innovation, Health and Nutrition, Osaka Japan). Human colorectal carcinoma, HCT-116, and ovarian serous adenocarcinoma, OVCAR-8, were purchased from ATCC as a part of the NCI-60 cancer cell line panel. HepG2, Panc-1, HEK293T, HeLa, and U-251 MG (KO) were cultured in DMEM (Thermo Fisher Scientific) supplemented with 10% FBS at 37 °C and 5% CO2. HCT-116, OVCAR-8, KHM-10B, and CCRF-CEM were cultured in RPMI 1640 medium (Thermo Fisher Scientific) supplemented with 10% FBS. Daudi cells were cultured in RPMI 1640 medium supplemented with 20% FBS at 37 °C and 5% CO2.

### 2.7. Statistical Analysis

The results acquired from immunohistochemical staining and RPPA analysis were compared using the Spearman’s rank correlation method. Receiver operating characteristic (ROC) curves, plotted as true positive fraction against false positive fraction, were used to analyze diagnostic accuracy. Statistical analysis was conducted using SPSS version 23 (IBM, NY, USA).

### 3. Results

#### 3.1. Clinicopathological Features

The clinicopathological features of the 37 cases comprising the present cohort are summarized in Table 1b. The ages of these cases ranged from 19 to 86 years (median 64 years). Nineteen patients were female (51%), and 18 were male (49%). The biopsy site was the cervical lymph node in 16 (43%) cases, the axillary lymph node in three (8%), the inguinal lymph node in three (8%), the abdominal lymph node in three (8%), the retroperitoneal lymph node in two (5%), the subcutaneous tissue in four (11%), the thyroid gland in three (8%), the testis in two (5%), and the mammary gland in one (3%) case. Histologically, all cases showed diffuse proliferation of large neoplastic lymphoid cells with prominent nucleoli. Except for a case of methotrexate-associated DLBCL, 36 cases were designated as DLBCL, not
Table 1b. Clinicopathological features of diffuse large B-cell lymphoma cases.

| Feature                | Value |
|------------------------|-------|
| Total number           | 37    |
| Age (year) Median      | 64    |
| Range 19–86            |       |
| Sex (n)                |       |
| Female                 | 19    |
| Male                   | 18    |
| LDH                    |       |
| Normal                 | 15    |
| Elevated               | 22    |
| PS                     |       |
| 0–1                    | 31    |
| 2–4                    | 6     |
| Ann Arbor stage        |       |
| I                      | 6     |
| II                     | 11    |
| III                    | 7     |
| IV                     | 13    |
| IPI score              |       |
| 0–1                    | 12    |
| 2                      | 8     |
| 3                      | 8     |
| 4–5                    | 8     |
| Unknown                | 1     |

PS, performance status; IPI, International Prognostic Index.

otherwise specified, and two were transformed cases from follicular lymphomas.

3.2. Validation of Antibodies for IHC and RPPA Analysis

To select mAbs for IHC and RPPA analysis, we checked the specificity of mAbs using cell lysates obtained from normal and cancer cells, including DLBCL cell lines (KHM-10B and Daudi). Cell lysates from ten cell lines were subjected to western blot to select highly specific mAbs against CD10, CD5, BCL6, BCL2, Ki-67, MUM1, BCL2, Ki-67, and C-MYC, whereas the mAb against Ki-67 showed multiple smeared bands with a broad range of molecular weights (Figure 1). We next confirmed positive correlations between western blotting and RPPA. RPPA slides were prepared using the same set of cell lysates and the screened antibodies were used as probes. Comparison of signal intensities obtained from RPPA and western blotting are shown in Figure S1, Supporting Information. RPPA signal intensity obtained with all nine mAbs was positively correlated with the results of western blot analysis, with correlation coefficients ranging from 0.842 to 1.000. These nine mAbs were therefore used for IHC and RPPA analysis using lymphoma tissues.

3.3. Immunohistochemical Features

Lymphoma cells obtained from patient samples were positive for prognostic markers (Table 2a, b and Figure 2). These markers were often expressed in background cells, such as T-cells positive for CD5 and BCL2, and interstitial cells positive for CD10 (Figure S3, Supporting Information). In all cases, lymphoma cells showed diffuse strong positive immunoreactivity for CD20. Almost all cases showed diffuse expression for PAX5, but some cases showed heterogeneous and/or weak expression for PAX5.

3.4. Quantitative Analysis of Nine DLBCL Biomarker Expressions by RPPA

To measure nine kinds of biomarker expressions in 37 DLBCL tissues quantitatively, the RPPA were performed using validated mAbs as shown in Figure 1. The detection images of RPPA obtained by an IR image scanner are shown in Figure S2, Supporting Information. The average expression values are shown in Table S1, Supporting Information and plotted on Figure 3a.

3.5. Correlation of RPPA and IHC Diagnostic Values

The correlation between diagnostic values obtained from RPPA and IHC analysis is shown in Figure 3a. To normalize the tumor cell content in each tissue, the raw RPPA values of CD5, CD10, BCL6, MUM1, BCL2, Ki-67, and C-MYC were adjusted to that of PAX5 or CD20. CD20-adjusted results had correlation coefficients of 0.446, 0.079, 0.037, 0.520, 0.287, 0.329, and 0.718 for CD5, CD10, BCL6, MUM1, BCL2, Ki-67, and C-MYC, respectively. In contrast, PAX5-adjusted results had correlation coefficients of 0.176, 0.147, 0.160, 0.261, 0.184, 0.261, and 0.325 for CD5, CD10, BCL6, MUM1, BCL2, Ki-67, and C-MYC, respectively. CD20-adjusted RPPA values for CD5, MUM1, BCL2, Ki-67, and C-MYC had better correlation with IHC results than raw or PAX5-adjusted RPPA values.

3.6. ROC Analysis

ROC analysis was carried out using RPPA analysis results (Figure 3b and Table 2c). For CD5, CD10, BCL2, BCL6, and MUM1, an IHC score of 1 to 3 was classified as IHC-negative, while a score of 4 to 6 was considered as IHC-positive. ICH cut-off values for Ki-67 and C-MYC were set at 69% and 39%, respectively. The ROC AUC with RPPA raw data for CD5, CD10, BCL6, MUM1, BCL2, Ki-67, and C-MYC was 0.775, 0.543, 0.640, 0.839, 0.866, 0.552, and 0.738, respectively. In contrast, the ROC AUC with CD20-adjusted RPPA data for CD5, CD10, BCL6, MUM1, BCL2, Ki-67, and C-MYC was 0.875, 0.599, 0.563, 0.818, 0.871, 0.748, and 0.874, respectively. The CD20-adjusted AUC values for CD5, CD10, BCL2, Ki-67, and C-MYC were better than those of their raw data. Furthermore, four biomarkers (CD5, MUM1, BCL2, and C-MYC) had both high sensitivity (1.000, 0.783, 0.750, and 0.750, respectively) and specificity (0.788, 0.867, 0.833, and 0.882, respectively).

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Table 2a. Immunohistochemical analysis of CD5, CD10, BCL6, MUM1, BCL2, PAX5, and CD20.

| Markers | Proportion of immunopositive tumor cells |
|---------|----------------------------------------|
|         | Score 1 | Score 2 | Score 3 | Score 4 | Score 5 | Score 6 | Total |
|         | <1%     | 1–9%    | 10–29%  | 30–49%  | 50–79%  | >80%    |       |
| CD5     | 28      | 1       | 3       | 0       | 4       | 1       | 37    |
| CD10    | 20      | 1       | 2       | 0       | 3       | 11      | 37   |
| BCL6    | 5       | 3       | 4       | 4       | 14      | 7       | 37   |
| MUM1    | 4       | 1       | 10      | 5       | 9       | 8       | 37   |
| BCL2    | 4       | 2       | 0       | 2       | 5       | 24      | 37   |
| PAX5    | 0       | 0       | 0       | 0       | 3       | 34      | 37   |
| CD20    | 0       | 0       | 0       | 0       | 0       | 37      | 37   |

Table 2b. Immunohistochemical analysis of Ki-67 and C-MYC.

| Markers | Proportion of immunopositive tumor cells |
|---------|----------------------------------------|
|         | 10–19% | 20–29% | 30–39% | 40–49% | 50–59% | 60–69% | 70–79% | 80–89% | >90% | Total |
| Ki-67   | 0       | 0       | 1       | 0       | 3       | 3       | 18      | 7       | 5       | 37    |
| C-MYC   | 3       | 9       | 5       | 1       | 3       | 3       | 8       | 3       | 2       | 37    |

4. Discussion

To examine the utility of RPPA in cancer diagnosis, we compared the correlation between prognostic biomarker expression in DLBCL tissue using both RPPA and conventional IHC analyses. Notably, CD20-adjusted RPPA values were positively correlated with IHC expression in CD5, MUM1, BCL2, Ki-67, and C-MYC. ROC analysis of CD20-adjusted RPPA values showed that CD5, MUM1, BCL2, and C-MYC had high sensitivity (0.750) and specificity (0.882). RPPA C-MYC expression was strongly correlated with IHC expression, and had particularly high specificity. According to IHC analysis, lymphoma cells often had...
heterogeneous C-MYC expression, which may confound the use of C-MYC expression as a prognostic biomarker. In contrast to IHC, RPPA provides highly quantitative, reproducible measurements for protein abundance in clinical tissue, and therefore could be used to determine protein abundance in clinical tissue that is complementary to IHC.\textsuperscript{[21]}

Although DLBCL is a clinicopathologically heterogeneous group of lymphomas, DLBCL with CD5 expression is characterized by aggressive clinical features.\textsuperscript{[5–8]} The prognostic significance of C-MYC expression has been controversial, but some studies suggest that C-MYC expression is associated with poor prognosis in patients with DLBCL.\textsuperscript{[11,14,17]} Co-expression of C-MYC and BCL2 may also contribute to poor prognosis in DLBCL cases.\textsuperscript{[9,10,13]} Our RPPA data for CD5, BCL2, and C-MYC were positively correlated with IHC results, and also showed high sensitivity and specificity. RPPA enables simultaneous quantification of multiple protein markers in a large number of biological samples, and has previously been used for biomarker

Figure 2. Immunohistochemistry analysis of each marker in DLBCL cells. A–G) DLBCL cells were positive for CD5 (A), CD10 (B), BCL6 (C), MUM1 (D), BCL2 (E), Ki-67 (F), and C-MYC (G). H) All cases showed diffuse positive staining for CD20. I) Most cases showed diffuse positive for PAX5, but some cases showed heterogeneous and/or weak PAX5 expression.
Figure 3. a) Correlation between the results of reverse phase protein array (RPPA) and immunohistochemistry (IHC) for each marker. A) For CD5, the correlation coefficient with IHC data was highest in CD20-adjusted data and lowest in PAX5-adjusted data. B) For CD10, the correlation coefficient with IHC data was highest in the raw data and lowest in CD20-adjusted data. C) For BCL6, the correlation coefficient with IHC data was highest in the raw data and lowest in CD20-adjusted data. D) For MUM1, the correlation coefficient with IHC data was highest in CD20-adjusted data and lowest in PAX5-adjusted data. E) For BCL2, the correlation coefficient with IHC data was highest in CD20-adjusted data and lowest in the raw data. F) For Ki-67, the correlation coefficient with IHC data was highest in CD20-adjusted data and lowest in the raw data. G) For C-MYC, the correlation coefficient with IHC data was highest in CD20-adjusted data and lowest in PAX5-adjusted data.

As RPPA can be used to analyze many samples at once, it is suitable for use in a research setting but may not be appropriate for clinical use with a few samples. However, it may offer advantages for clinical diagnosis, subtyping, and prognostic classification of lymphoma cases, as it can be used to evaluate

analysis in tumors.[13,21–27] Advantages of using RPPA over conventional biochemical assays, such as western blotting and ELISAs, include RPPA’s ability to provide high-throughput quantification and the requirement of less material for analysis.
Table 2c. Results of ROC analysis.

| Markers | Data                  | AUC  | Sensitivity | Specificity |
|---------|-----------------------|------|-------------|-------------|
| CD5     | Raw data              | 0.775| 0.800       | 0.788       |
|         | PAX5-adjusted data    | 0.612| 0.600       | 0.606       |
|         | CD20-adjusted data    | 0.875| 1.000       | 0.788       |
| CD10    | Raw data              | 0.543| 0.571       | 0.542       |
|         | PAX5-adjusted data    | 0.565| 0.571       | 0.708       |
|         | CD20-adjusted data    | 0.599| 0.929       | 0.417       |
| BCL6    | Raw data              | 0.640| 0.720       | 0.692       |
|         | PAX5-adjusted data    | 0.580| 0.560       | 0.692       |
|         | CD20-adjusted data    | 0.563| 0.520       | 0.615       |
| MUM1    | Raw data              | 0.839| 0.739       | 0.733       |
|         | PAX5-adjusted data    | 0.752| 0.609       | 0.733       |
|         | CD20-adjusted data    | 0.818| 0.783       | 0.867       |
| BCL2    | Raw data              | 0.866| 0.813       | 0.833       |
|         | PAX5-adjusted data    | 0.731| 0.750       | 0.667       |
|         | CD20-adjusted data    | 0.871| 0.750       | 0.833       |
| Ki-67   | Raw data              | 0.552| 0.533       | 0.750       |
|         | PAX5-adjusted data    | 0.667| 0.633       | 0.875       |
|         | CD20-adjusted data    | 0.748| 0.667       | 0.750       |
| C-MYC   | Raw data              | 0.738| 0.700       | 0.647       |
|         | PAX5-adjusted data    | 0.756| 0.750       | 0.647       |
|         | CD20-adjusted data    | 0.874| 0.750       | 0.882       |

To our knowledge, this is the first study that compared the results of RPPA and IHC using tissue samples from malignant lymphoma cases. IHC enables the identification of cells expressing biomarkers for cancer. However, RPPA detects protein abundance in cell lysates, which include both neoplastic and non-neoplastic cells. A previous study quantified multiple biomarkers using formalin-fixed paraffin-embedded breast cancer tissues, and compared the results of RPPA and IHC.

In the present study, the raw RPPA expression value for each marker was normalized to that of CD20 or PAX5 to account for the tumor cell ratios of the tissue samples. The CD20-adjusted RPPA values generally had better correlation with IHC scores than the raw RPPA data. In contrast, the PAX5-adjusted RPPA values had worse correlation with IHC scores. CD20 is therefore suitable for tumor content ratio normalization, and all cases exhibited diffuse strong positive immunoreactivity for CD20.
shown). Therefore, the use of CD20 RPPA values to normalize for tumor content ratio should be restricted to untreated DLBCL cases. Based on these results, it appears that PAX5 is not a suitable marker for determining tumor content, possibly due to heterogeneous and/or weak expression in tumor cells. As PAX-5 is a transcription factor in the nucleus, it may have resistance to solubility of a non-ionic T-PER Lysis buffer in RPPA analysis.

The specificity of each marker for tumor cells may also be an important factor in DLBCL diagnosis. There were only a few background cells with C-MYC expression according to IHC, and this may explain why the CD20-adjusted C-MYC data had the highest correlation coefficient with the IHC data in the present study. In contrast, expression of other markers was immunohistochemically detected in background cells in some cases. Heterogeneous expression of BCL6 was often observed in lymphoma cells. CD10 and BCL6 expression by RPPA did not correlate with that of IHC, and further evaluation of these antibodies is therefore required.

In conclusion, we demonstrated that RPPA analysis using four biomarkers may have clinical utility in diagnosing and classifying DLBCL. CD20-adjusted RPPA expression of C-MYC strongly correlated with IHC data, and had high specificity (0.882). RPPA can analyze and simultaneously quantify multiple proteins in many biological samples, and is therefore suitable for molecular analysis of lymphoma including DLBCL. These findings strongly suggest that combination of quantitative and morphological biomarker information from RPPA and IHC could be a powerful tool for the diagnosis and prognosis of lymphomas. Further investigation into the correlation between RPPA data for relevant biomarkers and the clinical outcomes of each patient is needed to determine the clinical utility of this analysis.

Supporting Information
Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest
The authors declare no conflict of interest.

Keywords
C-MYC, diffuse large B-cell lymphoma (DLBCL), immunohistochemistry (IHC), lymphoma prognosis, reverse-phase-protein-array (RPPA)

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